127. The method any one of claims 113-126, wherein the second composition is in the form of an intramuscularly injectable composition, intradermally injectable composition, subcutaneously injectable composition, or an intranasally administrable composition.

128. The method of any one of claims 113-127, wherein the second composition is in unit dose form.

129. The method of any one of claims 113-128, wherein the second composition further comprises a chelating agent.

130. The method of claim 129, wherein the chelating agent is ethylenediaminetetraacetic acid (EDTA).

131. The method any one of claims 113-130, wherein the second composition is in a form of a suspension.

132. The method any one of claims 113-131, wherein the second composition is in a form of a homogenous mixture or a heterogeneous mixture.

133. The method any one of claims 113-132, wherein the second composition is in the form of a homogenous mixture.

134. The method any one of claims 113-133, wherein the second composition is contained in a pre-filled syringe.

135. The method any one of claims 113-134, wherein the second composition further comprises a surfactant, an emulsifier, or a combination thereof.

136. The method claim 135, wherein the second composition further comprises the surfactant, wherein the surfactant is polysorbate, polymer glycol, a sorbitan ester, or any combination thereof.

137. The method of claim 136, wherein the second composition comprises the polysorbate, wherein the polysorbate is polysorbate 80.

138. The method of claim 137, wherein the second composition comprises the polymer glycol wherein the polymer glycol is polyethylene glycol.

139. The method any one of claims 113-138, wherein the second composition further comprises a buffering agent.

140. The method of claim 139, wherein the buffering agent is a succinate buffer agent, a tris-acetate- ethylenediaminetetraacetic acid (TAE) buffering agent, a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffering agent, a phosphate buffering agent, a sodium phosphate buffering agent, a potassium phosphate buffering agent, or any combination thereof.

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141. The method any one of claims 113-140, wherein the second composition further comprises sterile water.

142. The method any one of claims 113-141, wherein when the second composition is administered to a plurality of humans, the administration elicits at least one of the following side effects in less than about 10% of the plurality of humans: fatigue, headache, muscle pain, joint pain, decreased appetite, chills, or rash.

143. The method any one of claims 113-142, wherein when the second composition is administered intramuscularly to a human, the pharmaceutical composition elicits an immune response that lasts from about 1 week to about 30 years.

144. The method any one of claims 113-143, wherein when the unique immunogenic saccharide-polypeptide conjugate of the second composition elicits an opsonophagocytic response.

145. The method of any one of claims 113-144, wherein when the second composition is administered intramuscularly, the second composition elicits an immune response that lasts from about 1 week to about 30 years.

146. The method of any one of claims 113-145, wherein second composition retains at least about 80% of its original biological activity when stored in a sealed container for about 24 months at a temperature of about 2°C–8°C and the sealed container is stored in an atmosphere having about 50% relative humidity and 1 atm of atmospheric pressure.

147. The method of any one of claims 113-146, wherein the second composition further comprises a preservative.

148. The method of any one of claims 113-147, wherein the second composition further comprises an anti-fungal compound or a salt thereof.

149. The method of any one of claims 113-148, wherein the second composition further comprises a salt.

150. The method of claims 149, wherein the salt is an inorganic salt.

151. The method of any one of claims 113-150, wherein the immunogenic saccharide-polypeptide conjugate of the second composition is isolated and purified.

152. The method of any one of claims 113-151, wherein the second composition is a vaccine.

153. The method of any one of claims 113-152, which is a method of vaccinating a subject.

154. The method of claim 153, wherein the subject is a human.

155. The method of any one of claims 153-154, wherein the subject or human is a subject or human in need thereof.

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156. The method of any one of claims 113-155, wherein the immunogenic saccharide-polypeptide conjugates of the second composition are individually, respectively, or collectively present in an amount of about: 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, or 100%, by weight, based on the weight of the composition.

157. The method of any one of claims 113-156, which when conducted, does not result in more than about: 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1% suppression of an immunity conveyed by a first vaccine containing an immunogenic saccharide-polypeptide conjugate, as measured by antibody response to the saccharide of the immunogenic saccharide-polypeptide conjugate of the second composition, wherein the antibody response is measured by measuring antibody titers.

158. The method of any one of claims 113-157, wherein the immunogenic saccharide-polypeptide conjugate of the second composition is produced using bacteria.

159. The method of any one of claims 113-158, wherein the immunogenic saccharide-polypeptide conjugate of the second composition is produced using *E. coli*.

160. The method of any one of claims 113-159, wherein the immunogenic saccharide-polypeptide of the second composition comprises the capsular polysaccharide at least partially embedded in the polypeptide.

161. The method of any one of claims 113-160, wherein the immunogenic saccharide-polypeptide of the second composition comprises the capsular polysaccharide chemically cross-linked to the polypeptide.

162. The method of any one of claims 113-161, wherein the immunogenic saccharide-polypeptide of the second composition comprises the capsular polysaccharide at least partially chemically cross-linked to the polypeptide.

163. A method comprising:

administering a second immunogenic saccharide-polypeptide conjugate composition to a subject at least four weeks after a first immunogenic saccharide-polypeptide conjugate composition was administered;

wherein the second immunogenic saccharide-polypeptide conjugate composition comprises a first polypeptide that has at least about 70% length homology, at least about 70% sequence homology, or a combination thereof, to a second polypeptide of the second immunogenic saccharide-polypeptide conjugate composition;

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and wherein the first immunogenic saccharide-polypeptide conjugate composition comprises a first serotype;

wherein the second immunogenic saccharide-polypeptide conjugate composition comprises a second serotype, and wherein the first serotype and the second serotype are different.

164. The method of claim 163, wherein the first immunogenic saccharide-polypeptide conjugate composition comprises a capsular polysaccharide.

165. The method of claim 164, wherein the first immunogenic saccharide-polypeptide conjugate composition comprises a fragment of the capsular polysaccharide.

166. The method of claim 165, wherein the fragment of the capsular polysaccharide comprises a monosaccharide, a disaccharide, a trisaccharide, a tetrasaccharide, a pentasaccharide, or a hexasaccharide.

167. The method of any one of claims 163-166, wherein the first polypeptide is an oligopeptide.

168. The method of any one of claims 163-167, wherein the second polypeptide is an oligopeptide.

169. The method of any one of claims 163-168, wherein the first polypeptide comprises CRM_{197}, tetanus toxoid, a diphtheria toxoid, cholera toxoid, pertussis toxoid, inactivated or mutant pneumococcal pneumolysin, pneumococcal surface protein A, pneumococcal adhesion protein A, pneumococcal lipoprotein PsaA, C5a peptidase group A or group B streptococcus, a non-typable *H. influenzae* P4 protein, a non-typable *H. influenzae* P6 protein, *M. catarrhalis* uspA, keyhole limpet haemocyanin (KLH), OMPC from N. meningitidis, a purified protein derivative of tuberculin(PPD), protein D from *H. influenzae*, any fragment thereof, or any combination thereof .

170. The method of any one of claims 163-169, wherein a toxin activity of the first polypeptide is at least partly mitigated.

171. The method of any one of claims 163-170, wherein the second polypeptide comprises CRM_{197}, tetanus toxoid, a diphtheria toxoid, cholera toxoid, pertussis toxoid, inactivated or mutant pneumococcal pneumolysin, pneumococcal surface protein A, pneumococcal adhesion protein A, pneumococcal lipoprotein PsaA, C5a peptidase group A or group B streptococcus, a non-typable *H. influenzae* P4 protein, a non-typable *H. influenzae* P6 protein, *M. catarrhalis* uspA, keyhole limpet haemocyanin (KLH), OMPC from N. meningitidis, a purified protein derivative of tuberculin(PPD), protein D from *H. influenzae*, PspA, any fragment thereof, or any combination thereof.

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172. The method of any one of claims 163-171, wherein a toxin activity of the second polypeptide is at least partly mitigated.

173. The method of any one of claims 163-172, wherein the first serotype is from a same bacteria as the second serotype.

174. The method of any one of claims 163-173, wherein the first serotype is from a different bacteria than the second serotype.

175. The method of any one of claims 163-174, wherein the first serotype is from *Streptococcus pneumoniae*.

176. The method of any one of claims 163-174, wherein the first serotype is from *Neisseria meningitidis*, *Salmonella enterica*, or *Haemophilus influenzae*.

177. The method of any one of claims 163-176, wherein the second serotype is from *Streptococcus pneumoniae*.

178. The method of any one of claims 163-176, wherein the second bacteria is from *Neisseria meningitidis*, *Salmonella enterica*, or *Haemophilus influenzae*.

179. The method of any one of claims 163-178, wherein the first serotype comprises at least one of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, or 23F from *Streptococcus pneumoniae*, and the second serotype comprises at least eight of 2, 6C, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 20A, 22F, 23A, 23B, 24F, 24B, 31, 33F, 35F, 35B, or 38 from *Streptococcus pneumoniae*.

180. The method of any one of claims 163-178, wherein the first serotype comprises at least one of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, or 23F from *Streptococcus pneumoniae*, and the second serotype comprises at least eight of 2, 6C, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 20A, 20B, 22F, 23A, 23B, 24F, 24B, 31, 33F, 35F, 35B, or 38 from *Streptococcus pneumoniae*.

181. The method of any one of claims 163-178, wherein the first serotype comprises at least eight of 2, 6C, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 20A, 20B, 22F, 23A, 23B, 24F, 24B, 31, 33F, 35F, 35B, and 38 from *Streptococcus pneumoniae*, and the second serotype comprises at least one of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*.

182. The method of any one of claims 163-178, wherein the first serotype comprises at least eight of 2, 6C, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 20A, 22F, 23A, 23B, 24F, 24B, 31, 33F, 35F, 35B, and 38 from *Streptococcus pneumoniae*, and the second serotype comprises at least one of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*.

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183. The method of any one of claims 163-178, wherein the first serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*, and the second serotype comprises 6C, 9N, 15A, 15C, 16F, 23A, 23B, and 33B from *Streptococcus pneumoniae*.

184. The method of any one of claims 163-178, wherein the first serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*, and the second serotype comprises 6C, 15A, 16F, 23A, 23B, and 33B from *Streptococcus pneumoniae*.

185. The method of any one of claims 163-178, wherein the first serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*, and the second serotype comprises 6C, 9N, 15A, 15C, 16F, 17F, 20B, 23A, 23B, 24F, 31, 33B, 34, 35F, and 35B from *Streptococcus pneumoniae*.

186. The method of any one of claims 163-178, wherein the first serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*, and the second serotype comprises 6C, 15A, 15C, 16F, 20B, 23A, 23B, 24F, 31, 33B, 34, 35F, and 35B from *Streptococcus pneumoniae*.

187. The method of any one of claims 163-178, wherein the first serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*, and the second serotype comprises 6C, 8, 9N, 10A, 11A, 15A, 15B, 15C, 16F, 20A, 22F, 23A, 23B, 33F, and 35B from *Streptococcus pneumoniae*.

188. The method of any one of claims 163-178, wherein the first serotype comprises 6C, 9N, 15A, 15C, 16F, 23A, 23B, and 33B from *Streptococcus pneumoniae*, and the second serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*.

189. The method of any one of claims 163-178, wherein the first serotype comprises 6C, 15A, 16F, 23A, 23B, and 33B from *Streptococcus pneumoniae*, and the second serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*.

190. The method of any one of claims 163-178, wherein the first serotype comprises 6C, 9N, 15A, 15C, 16F, 17F, 20B, 23A, 23B, 24F, 31, 33B, 34, 35F, and 35B from *Streptococcus pneumoniae*, and the second serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*.

191. The method of any one of claims 163-178, wherein the first serotype comprises 6C, 15A, 15C, 16F, 20B, 23A, 23B, 24F, 31, 33B, 34, 35F, and 35B from *Streptococcus*

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pneumoniae, and the second serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*.

192. The method of any one of claims 163-178, wherein the first serotype comprises 6C, 8, 9N, 10A, 11A, 15A, 15B, 15C, 16F, 20A, 22F, 23A, 23B, 33F, and 35B from *Streptococcus pneumoniae*, and the second serotype comprises 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*.

193. The method of any one of claims 163-178, wherein the first serotype comprises at least one of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, or 23F from *Streptococcus pneumoniae*, and the second serotype comprises at least eight of 2, 6C, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 22F, 23A, 23B, 24F, 24B, 31, 33F, 35F, 35B, 38, and Serogroup 20 (comprising serotypes 20A and 20B) from *Streptococcus pneumoniae*.

194. The method of any one of claims 163-178, wherein the first serotype comprises at least one of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, or 23F from *Streptococcus pneumoniae*, and the second serotype comprises at least eight of 2, 6C, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 22F, 23A, 23B, 24F, 24B, 31, 33F, 35F, 35B, 38, and Serogroup 20 (comprising serotypes 20A and 20B) from *Streptococcus pneumoniae*.

195. The method of any one of claims 163-178, wherein the first serotype comprises at least eight of 2, 6C, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 22F, 23A, 23B, 24F, 24B, 31, 33F, 35F, 35B, 38, and Serogroup 20 (comprising serotypes 20A and 20B) from *Streptococcus pneumoniae*, and the second serotype comprises at least one of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*.

196. The method of any one of claims 163-178, wherein the first serotype comprises at least eight of 2, 6C, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 22F, 23A, 23B, 24F, 24B, 31, 33F, 35F, 35B, 38, and Serogroup 20 (comprising serotypes 20A and 20B) from *Streptococcus pneumoniae*, and the second serotype comprises at least one of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F from *Streptococcus pneumoniae*.

197. The method of any one of claims 163-196, wherein the first immunogenic saccharide-polypeptide conjugate composition further comprises an adjuvant.

198. The method of claim 197, wherein the adjuvant is an aluminum-cation comprising adjuvant.

199. The method of claim 198, wherein the adjuvant is aluminum phosphate, aluminum sulfate, or aluminum hydroxide.

200. The method of any one of claims 163-199, wherein the first immunogenic saccharide-polypeptide conjugate composition is in the form of an intramuscularly injectable

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composition, intradermally injectable composition, subcutaneously injectable composition, or an intranasally administrable composition.

201. The method of any one of claims 163-200, wherein the first immunogenic saccharide-polypeptide conjugate composition further comprises a chelating agent.

202. The method of claim 201, wherein the chelating agent is ethylenediaminetetraacetic acid (EDTA).

203. The method of any one of claims 163-202, wherein the first immunogenic saccharide-polypeptide conjugate composition is in a form of a suspension.

204. The method of any one of claims 163-203, wherein the first immunogenic saccharide-polypeptide conjugate composition is in a form of a homogenous mixture or a heterogeneous mixture.

205. The method of any one of claims 163-204, wherein the first immunogenic saccharide-polypeptide conjugate composition is in a form of a homogenous mixture.

206. The method of any one of claims 163-205, wherein the first immunogenic saccharide-polypeptide conjugate composition is contained in a pre-filled syringe.

207. The method of any one of claims 163-206, wherein the first immunogenic saccharide-polypeptide conjugate composition further comprises a surfactant or emulsifier, or a combination thereof.

208. The method of claim 207, wherein the first immunogenic saccharide-polypeptide composition comprises the surfactant wherein the surfactant is a polysorbate, a polymer glycol, a sorbitan ester, or any combination thereof.

209. The method of claim 208, wherein the first immunogenic saccharide-polypeptide composition comprises the polysorbate wherein the polysorbate is polysorbate 80.

210. The method claim 208, wherein the first immunogenic saccharide-polypeptide composition comprises the polymer glycol wherein the polymer glycol is polyethylene glycol.

211. The method of any one of claims 163-210, wherein the first immunogenic saccharide-polypeptide conjugate composition further comprises a buffering agent.

212. The method of claim 211, wherein the buffering agent is a succinate buffer agent, a tris-acetate- ethylenediaminetetraacetic acid (TAE) buffering agent, a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffering agent, a phosphate buffering agent, a sodium phosphate buffering agent, a potassium phosphate buffering agent, or any combination thereof.

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213. The method of any one of claims 163-212, wherein the first immunogenic saccharide-polypeptide conjugate composition further comprises sterile water.

214. The method of any one of claims 163-213, wherein when the first immunogenic saccharide-polypeptide conjugate composition is administered to a plurality of humans, the administration elicits at least one of the following side effects in less than about 10% of the plurality of humans: fatigue, headache, muscle pain, joint pain, decreased appetite, chills, or rash.

215. The method of any one of claims 163-214, wherein when first immunogenic saccharide-polypeptide conjugate composition is administered intramuscularly to a human, the first immunogenic saccharide-polypeptide conjugate composition elicits an immune response that lasts from about 1 week to about 30 years.

216. The method any one of claims 163-215, wherein when first immunogenic saccharide-polypeptide conjugate composition elicits an opsonophagocytic response.

217. The method of any one of claims 163-216, wherein the first immunogenic saccharide-polypeptide conjugate composition retains at least about 80% of its original biological activity when stored in a sealed container for about 24 months at a temperature of about 2°C–8°C and the sealed container is stored in an atmosphere having about 50% relative humidity and 1 atm of atmospheric pressure.

218. The method of any one of claims 163-217, wherein the second immunogenic saccharide-polypeptide conjugate composition further comprises an adjuvant.

219. The method of claim 218, wherein the adjuvant is an aluminum-cation comprising adjuvant.

220. The method of claim 218, wherein the adjuvant is aluminum phosphate, aluminum sulfate, or aluminum hydroxide.

221. The method of any one of claims 163-220, wherein the second immunogenic saccharide-polypeptide conjugate composition is in a form of an intramuscularly injectable composition, intradermally injectable composition, subcutaneously injectable composition, or an intranasally administrable composition.

222. The method of any one of claims 163-221, wherein the second immunogenic saccharide-polypeptide conjugate composition further comprises a chelating agent.

223. The method of claim 222, wherein the chelating agent is ethylenediaminetetraacetic acid (EDTA).

224. The method of any one of claims 163-223, wherein the second immunogenic saccharide-polypeptide conjugate composition is in a form of a suspension.

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225. The method of any one of claims 163-224, wherein the second immunogenic saccharide-polypeptide conjugate composition is in a form of a homogenous mixture or a heterogeneous mixture.

226. The method of claim 225, wherein the second immunogenic saccharide-polypeptide conjugate composition is in a form of a homogenous mixture.

227. The method of any one of claims 163-226, wherein the second immunogenic saccharide-polypeptide conjugate composition is contained in a pre-filled syringe.

228. The method of any one of claims 163-227, wherein the second immunogenic saccharide-polypeptide conjugate composition further comprises a surfactant, an emulsifier, or a combination thereof.

229. The method of claim 228, wherein the second immunogenic saccharide-polypeptide conjugate composition comprises the surfactant wherein the surfactant is polysorbate, polymer glycol, a sorbitan ester, or any combination thereof.

230. The method of claim 229, wherein the second immunogenic saccharide-polypeptide conjugate composition comprises the polysorbate wherein the polysorbate is polysorbate 80.

231. The method of claim 230, wherein the second immunogenic saccharide-polypeptide conjugate composition comprises the polymer glycol wherein the polymer glycol is polyethylene glycol.

232. The method of any one of claims 163-231, wherein the second immunogenic saccharide-polypeptide conjugate composition further comprises a buffering agent.

233. The method of claim 232, wherein the buffering agent is a succinate buffer agent, a tris-acetate- ethylenediaminetetraacetic acid (TAE) buffering agent, a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffering agent, a phosphate buffering agent, a sodium phosphate buffering agent, a potassium phosphate buffering agent, or any combination thereof.

234. The method of any one of claims 163-233, wherein the second immunogenic saccharide-polypeptide conjugate composition further comprises sterile water.

235. The method of any one of claims 163-234, wherein when the second immunogenic saccharide-polypeptide conjugate composition is administered to a plurality of humans, the administration elicits at least one of the following side effects in less than about 10% of the plurality of humans: fatigue, headache, muscle pain, joint pain, decreased appetite, chills, or rash.

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236. The method of any one of claims 163-235, wherein when the second immunogenic saccharide-polypeptide conjugate composition is administered intramuscularly to a human, the second immunogenic saccharide-polypeptide conjugate composition elicits an immune response that lasts from about 1 week to about 30 years.

237. The method of any one of claims 163-236, wherein when the second immunogenic saccharide-polypeptide conjugate composition elicits an opsonophagocytic response.

238. The method of any one of claims 163-237, wherein the second immunogenic saccharide-polypeptide conjugate composition retains at least about 80% of its original biological activity when stored in a sealed container for about 24 months at a temperature of about 2°C–8°C and the sealed container is stored in an atmosphere having about 50% relative humidity and 1 atm of atmospheric pressure.

239. The method of any one of claims 163-238, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition is in unit dose form.

240. The method of any one of claims 163-239, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition further comprises a preservative.

241. The method of any one of claims 163-240, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition further comprises an anti-fungal compound or a salt thereof.

242. The method of any one of claims 163-241, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition further comprise a salt.

243. The method of claim 242, wherein the salt is an inorganic salt.

244. The method of any one of claims 163-243, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition is isolated and purified.

245. The method of any one of claims 163-244, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition which is a vaccine.

246. The method of any one of claims 163-245, which is a method of vaccinating a subject.

247. The method of any claim 246, wherein the subject is a human.

248. The method of any one of claims 246-247, wherein the subject or human is a subject or human in need thereof.

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249. The method of any one of claims 163-248, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition comprise immunogenic saccharide-polypeptide conjugate(s) individually, respectively, or collectively present in an amount of about: 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, or 100%, by weight, based on the weight of the composition.

250. The method of any one of claims 163-249, wherein administration of the second immunogenic saccharide-polypeptide conjugate composition does not result in more than about: 50%, 40%, 30%, 20%, 10%, 5%, 4%, 3%, 2%, or 1% suppression of an immunity as measured by antibody response to the saccharide of the immunogenic saccharide-polypeptide conjugate, wherein the antibody response is measured by measuring antibody titers.

251. The method of any one of claims 163-250, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition is produced using a bacteria.

252. The method of any one of claims 163-251, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition is produced using *E. coli*.

253. The method of any one of claims 163-252, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition comprises the capsular polysaccharide at least partially embedded in the polypeptide.

254. The method of any one of claims 163-253, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition comprises the capsular polysaccharide chemically cross-linked to the polypeptide.

255. The method of any one of claims 163-254, wherein the first immunogenic saccharide-polypeptide conjugate composition and/or the second immunogenic saccharide polypeptide composition comprises the capsular polysaccharide at least partially chemically cross-linked to the polypeptide.

256. A method of making a composition comprising:

contacting the immunogenic saccharide-polypeptide conjugate of any one of claims 1-70 with an excipient, an adjuvant, or any combination thereof.

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257. The method of claim 256, wherein the composition is injectable or intranasally administrable.

258. The method claim 256, comprising contacting with the adjuvant, wherein the adjuvant is aluminum phosphate, aluminum sulfate, or aluminum hydroxide.

259. The method of any one of claims 256-258, wherein the composition is in the form of an intramuscularly injectable composition.

260. The method of any one of claims 256-259, further comprising contacting the composition with a chelating agent.

261. The method of claim 260, further comprising contacting the composition with a chelating agent, wherein the chelating agent is ethylenediaminetetraacetic acid (EDTA).

262. The method of any one of claims 256-261, wherein the composition is in a form of a suspension.

263. The method of any one of claims 256-262, wherein the composition is in a form of a homogenous mixture or a heterogeneous mixture.

264. The method of any one of claims 263, wherein the composition is in a form of a homogenous mixture.

265. The method of any one of claims 256-264, wherein the composition is contained in a pre-filled syringe.

266. The method of any one of claims 256-265, further comprising contacting the composition with a surfactant, an emulsifier, or a combination thereof.

267. The method of claim 266, comprising contacting the composition with the surfactant, wherein the surfactant is a polysorbate, a polymer glycol, a sorbitan ester, or any combination thereof.

268. The method of claim 267, comprising contacting the composition with the polysorbate, wherein the polysorbate is polysorbate 80.

269. The method of claim 268, comprising contacting the composition with the polymer glycol, wherein the polymer glycol is polyethylene glycol.

270. The method of any one of claims 256-269, further comprising contacting the composition with a buffering agent.

271. The method of claim 270, wherein the buffering agent is a succinate buffer agent, a tris-acetate- ethylenediaminetetraacetic acid (TAE) buffering agent, a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffering agent, a phosphate buffering agent, a sodium phosphate buffering agent, a potassium phosphate buffering agent, or any combination thereof.

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272. The method of any one of claims 256-271, further comprising contacting the composition with sterile water.

273. The method of any one of claims 256-272, wherein when the composition is administered to a plurality of humans, the administration elicits at least one of the following side effects in less than about 10% of the plurality of humans: fatigue, headache, muscle pain, joint pain, decreased appetite, chills, or rash.

274. The method of any one of claims 256-273, wherein when the composition is administered intramuscularly to a human, the composition elicits an immune response that lasts from about 1 week to about 30 years.

275. The method of any one of claims 256-274, wherein the immunogenic saccharide-polypeptide conjugate elicits an opsonophagocytic response.

276. The method of any one of claims 256-275, wherein the composition retains at least about 80% of its original biological activity when stored in a sealed container for about 24 months at a temperature of about 2°C–8°C and the sealed container is stored in an atmosphere having about 50% relative humidity and 1 atm of atmospheric pressure.

277. The method of any one of claims 256-276, wherein the composition further comprises a preservative.

278. The method of any one of claims 256-277, wherein the composition further comprises an anti-fungal compound or a salt thereof.

279. The method of any one of claims 256-278, wherein the composition further comprises a salt.

280. The method claim 279, wherein the salt is an inorganic salt.

281. The method of any one of claims 256-280, wherein the immunogenic saccharide-polypeptide conjugate of the composition is isolated and purified.

282. The method of any one of claims 256-281, wherein the composition is used as a vaccine.

283. The method of any claims 256-282, wherein the composition is used for vaccinating a subject.

284. The method of claim 283, wherein the subject is a human.

285. The method of any one of claims 267-284, wherein the subject or human is a subject or human in need thereof.

286. The method of any one of claims 256-285, wherein the immunogenic saccharide-polypeptide conjugate(s) of the composition are individually, respectively, or collectively

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present in an amount of about: 0.001, 0.01, 0.1, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95, or 100%, by weight, based on the weight of the composition.

287. The method of any one of claims 256-286, wherein the immunogenic saccharide-polypeptide conjugate of the composition is produced using bacteria.

288. The method of any one of claims 256-287, wherein the immunogenic saccharide-polypeptide conjugate of the composition is produced using E. coli.

289. The method of any one of claims 256-288, wherein the immunogenic saccharide-polypeptide conjugate of the composition comprises the capsular polysaccharide at least partially embedded in the polypeptide.

290. The method of any one of claims 256-289, wherein the immunogenic saccharide-polypeptide conjugate of the composition comprises the capsular polysaccharide chemically cross-linked to the polypeptide.

291. The method of any one of claims 256-290, wherein the immunogenic saccharide-polypeptide conjugate of the composition comprises the capsular polysaccharide at least partially chemically cross-linked to the polypeptide.

292. A kit comprising of a pharmaceutical composition of any of claims 1-111 contained in a container.

293. The kit of claim 292, further comprising instructions for use.

294. The kit of any one of claims 292-293, wherein the container is a syringe.

295. The kit of any one of claims 292-294, wherein the container is a vial comprising a cap, wherein the cap comprises a rubber septum.

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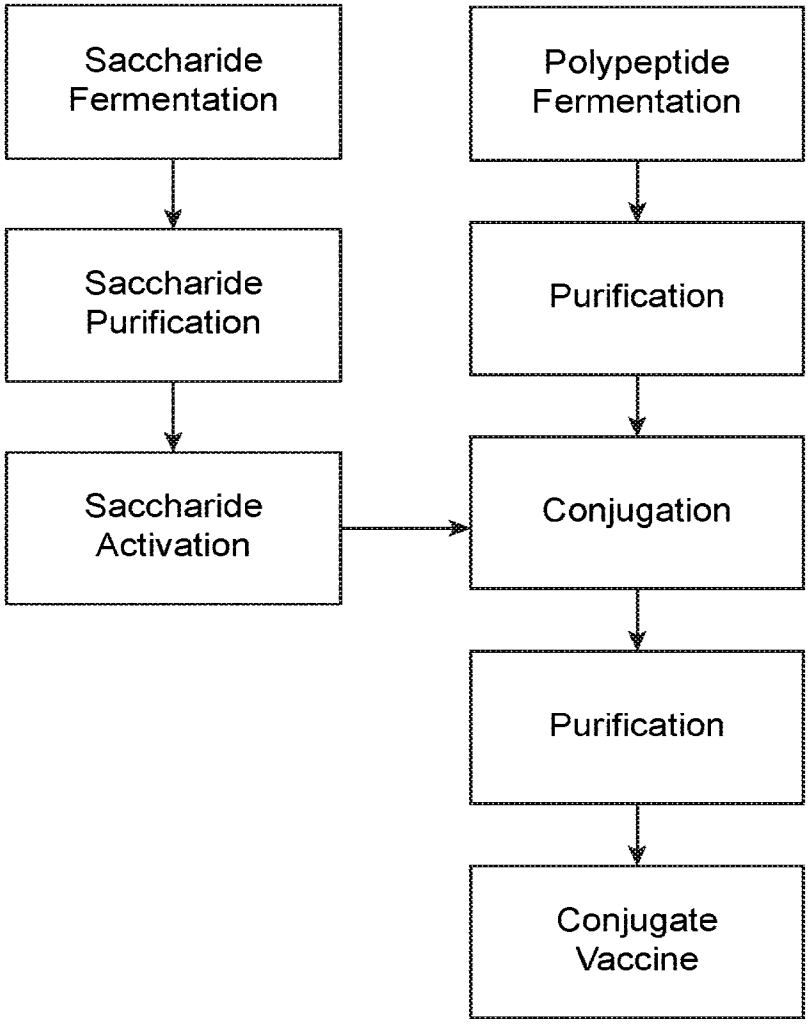


FIG. 1

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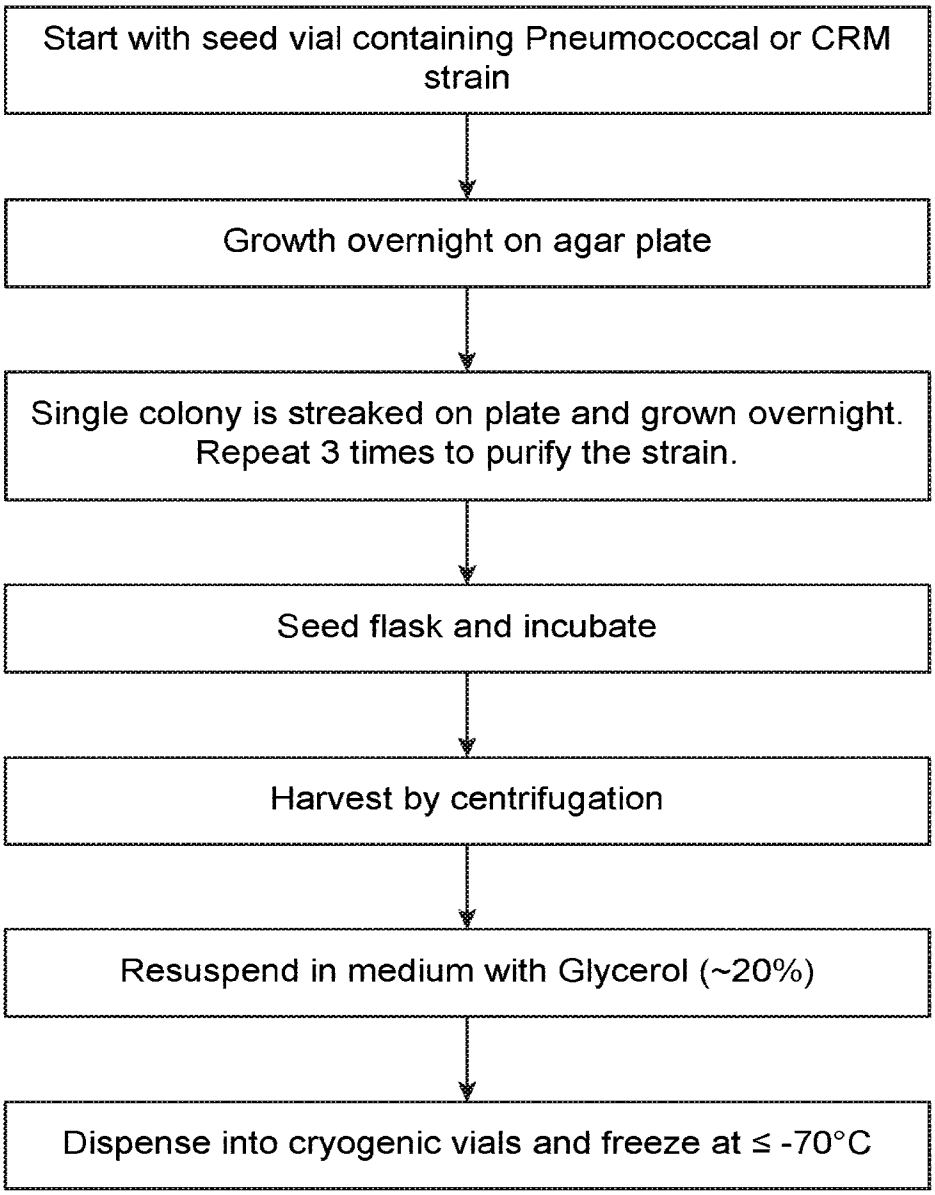


FIG. 2

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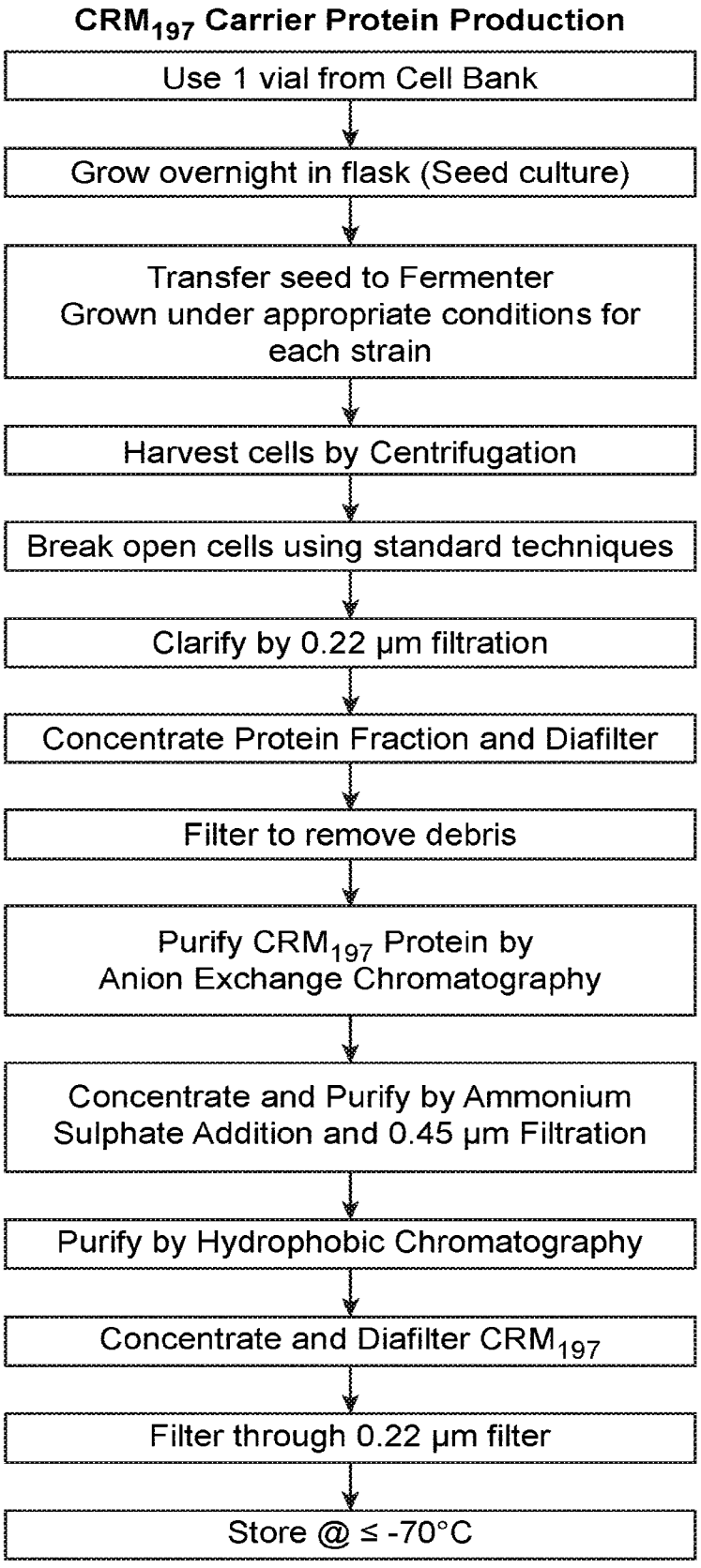


FIG. 3

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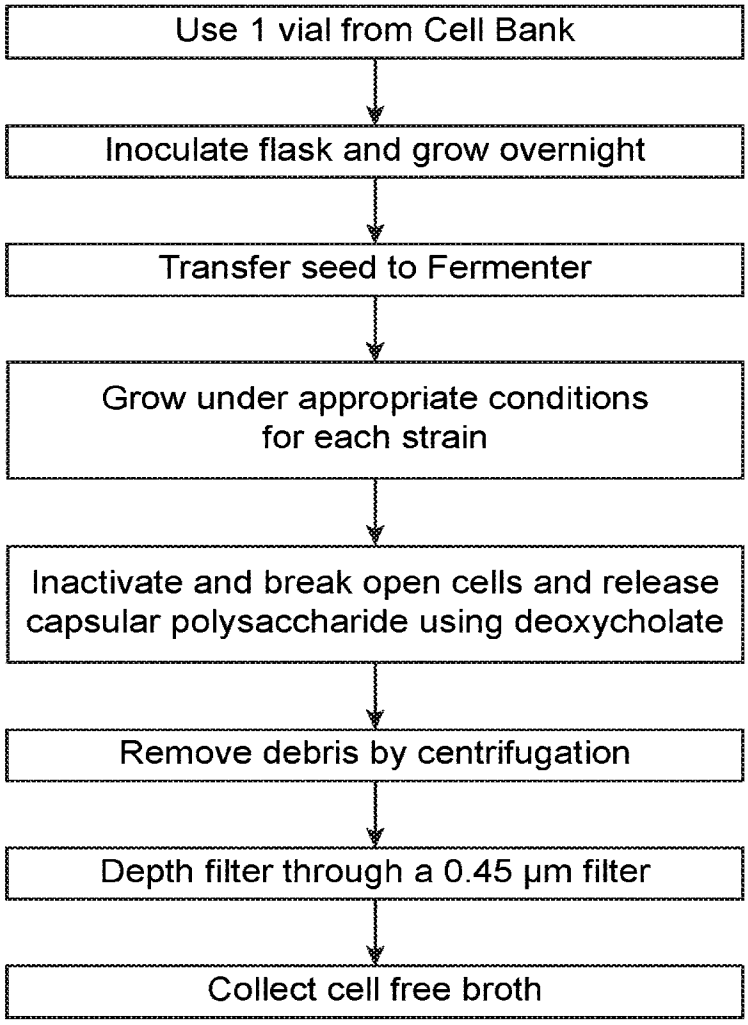


FIG. 4

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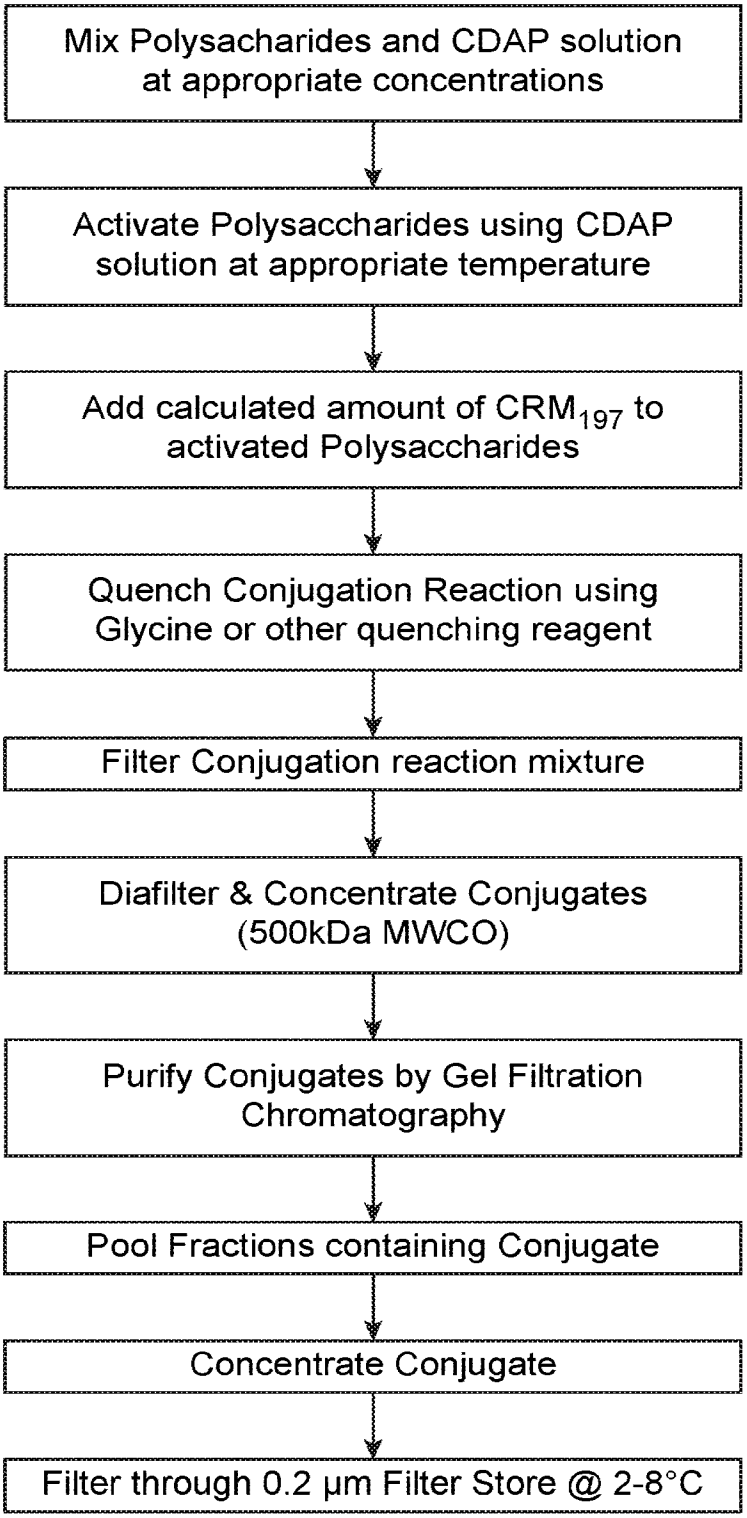


FIG. 5

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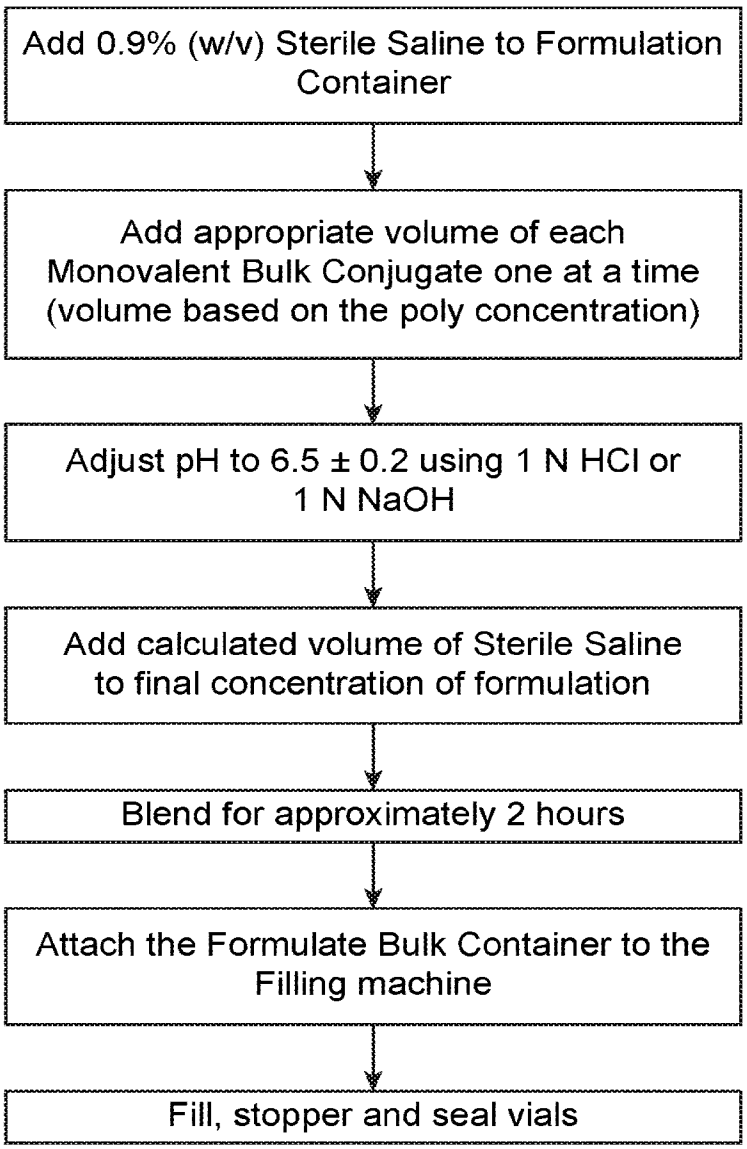


FIG. 6

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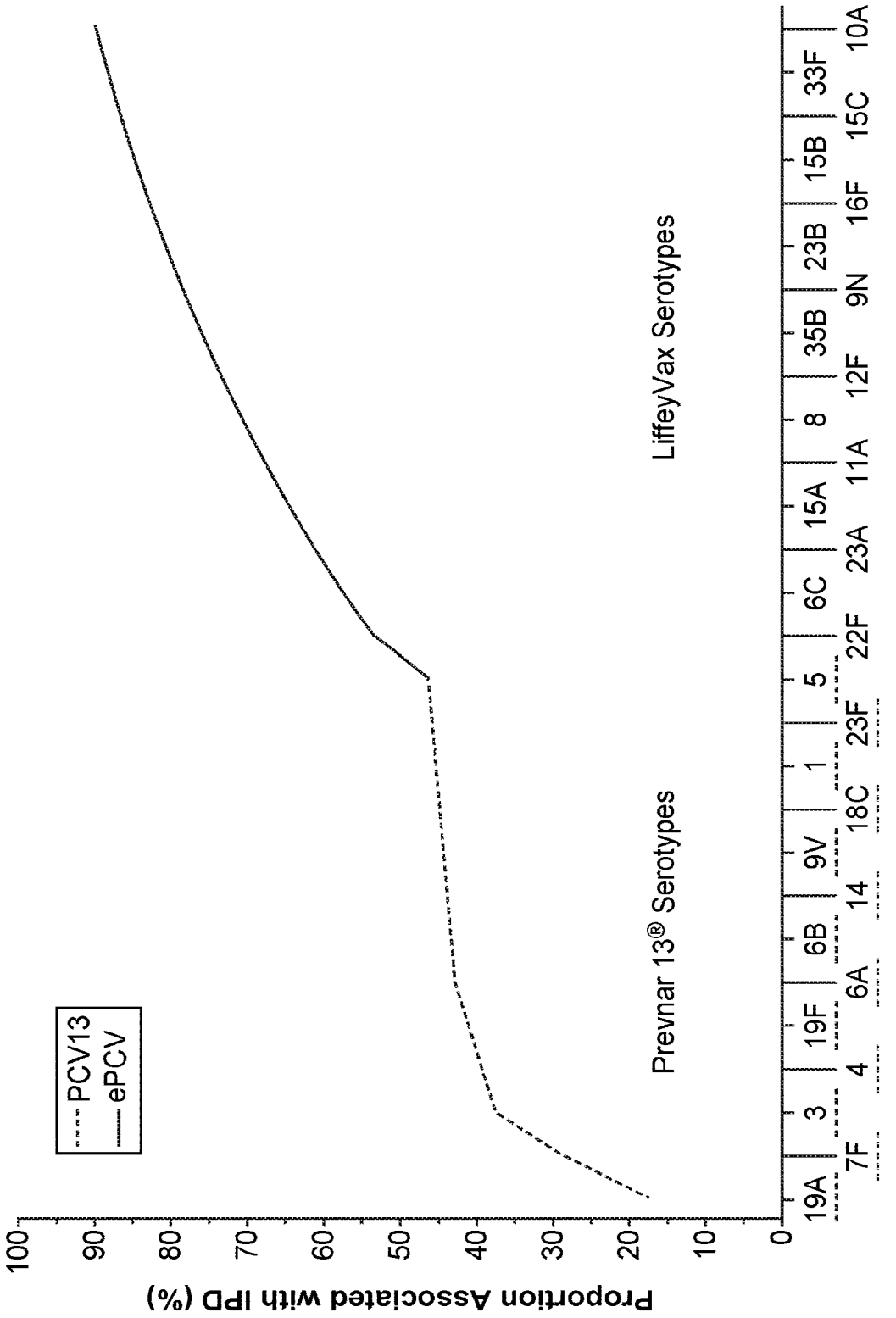


FIG. 7

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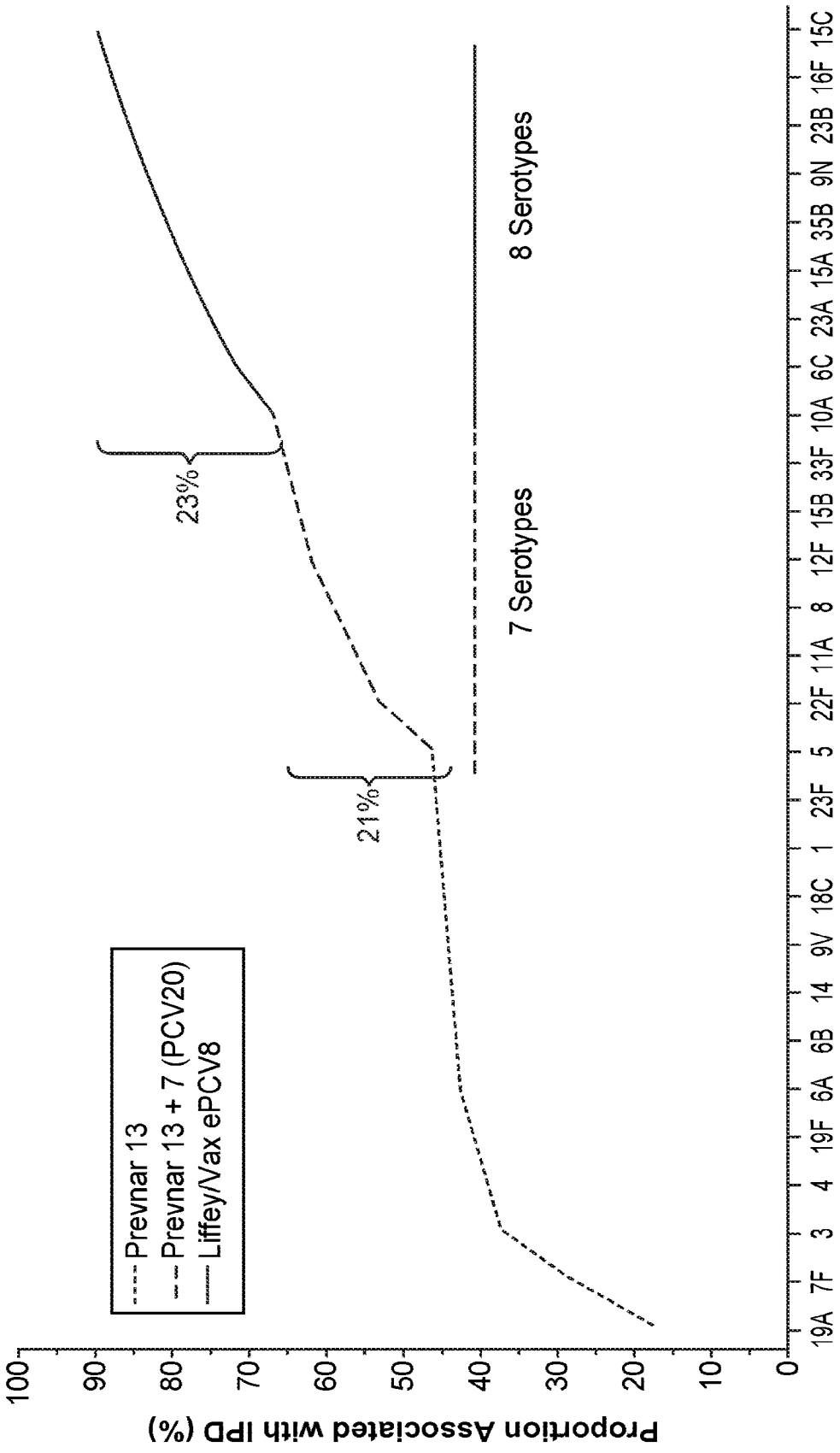


FIG. 8

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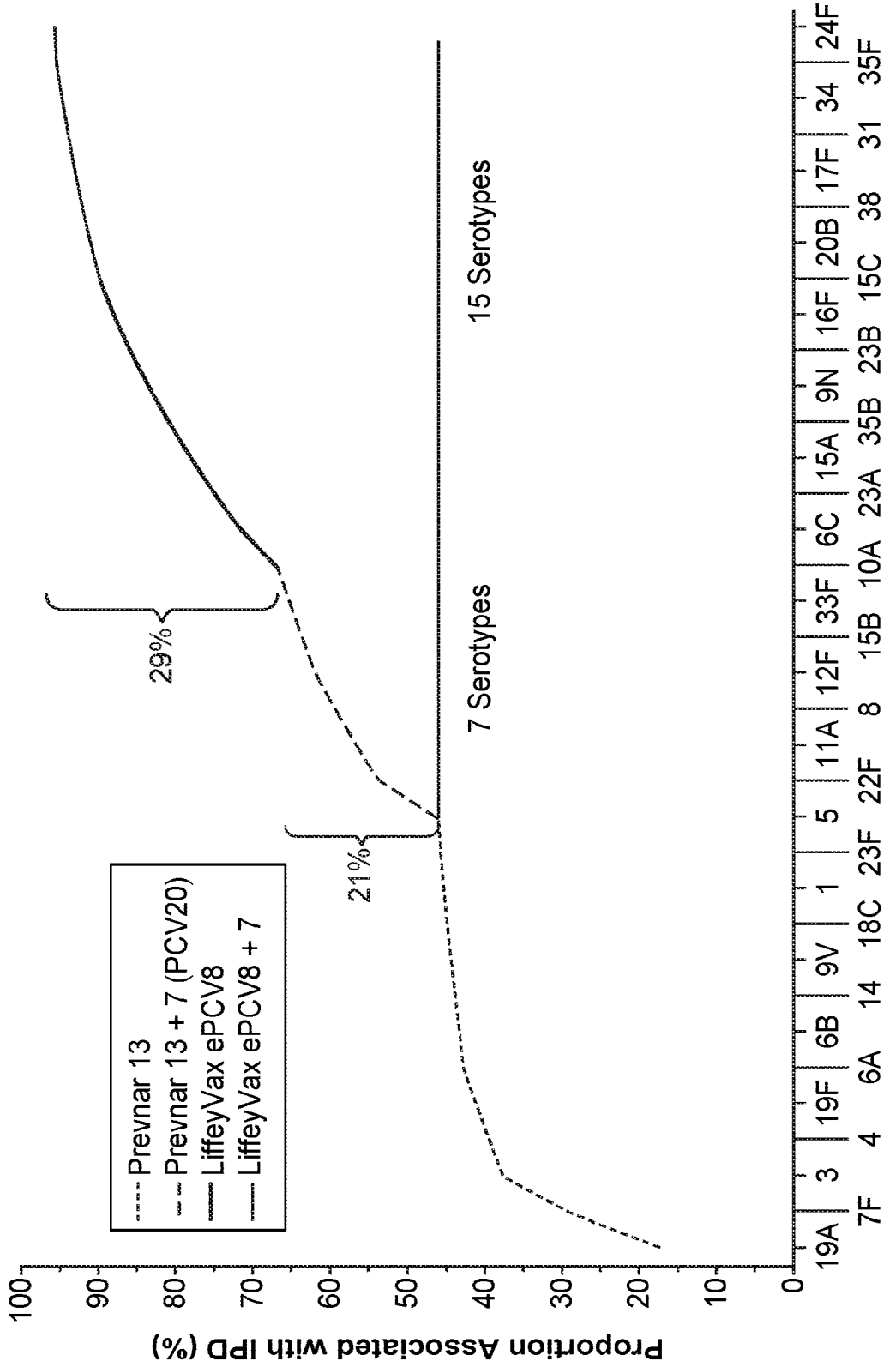


FIG. 9

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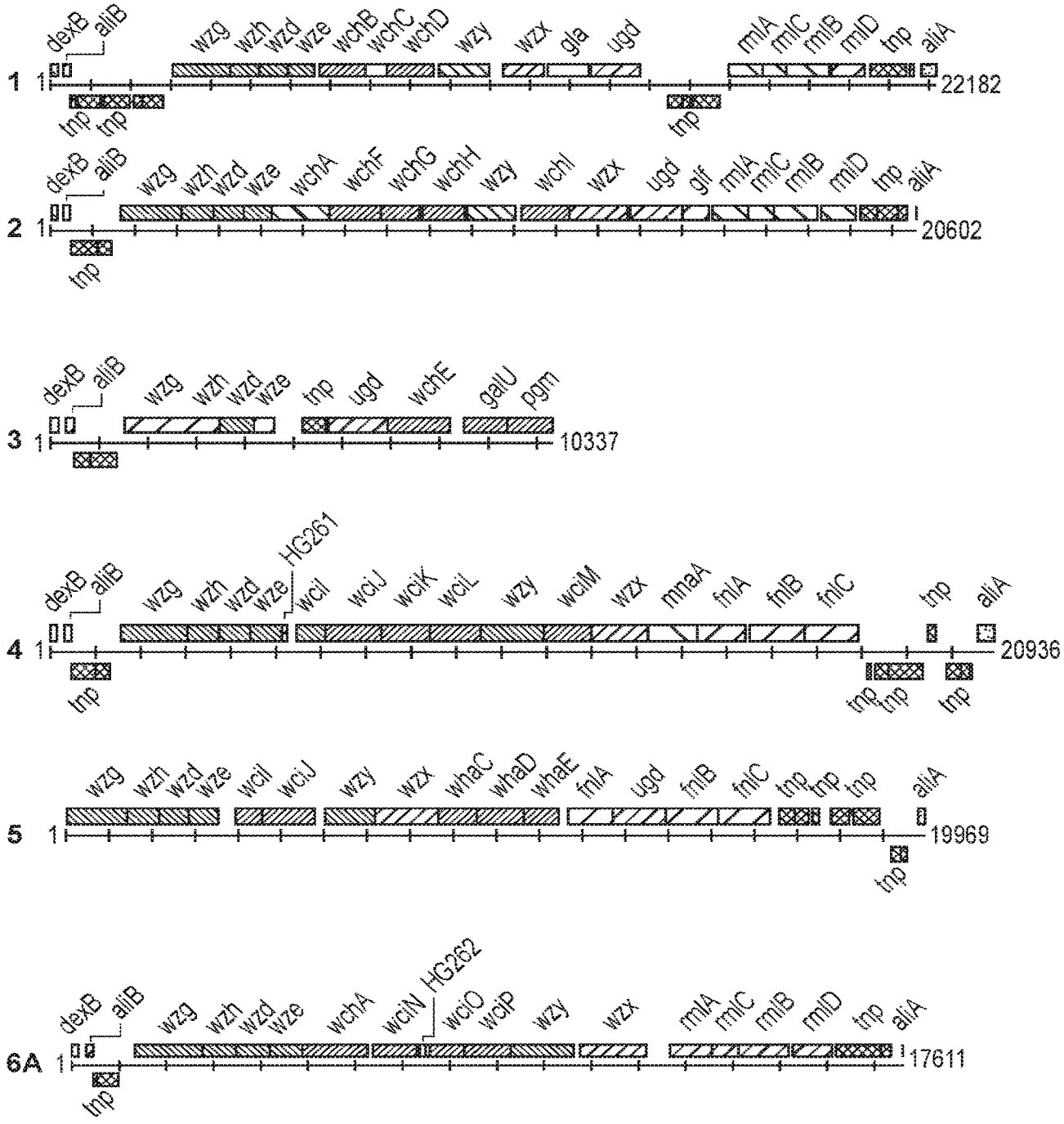


FIG. 10A

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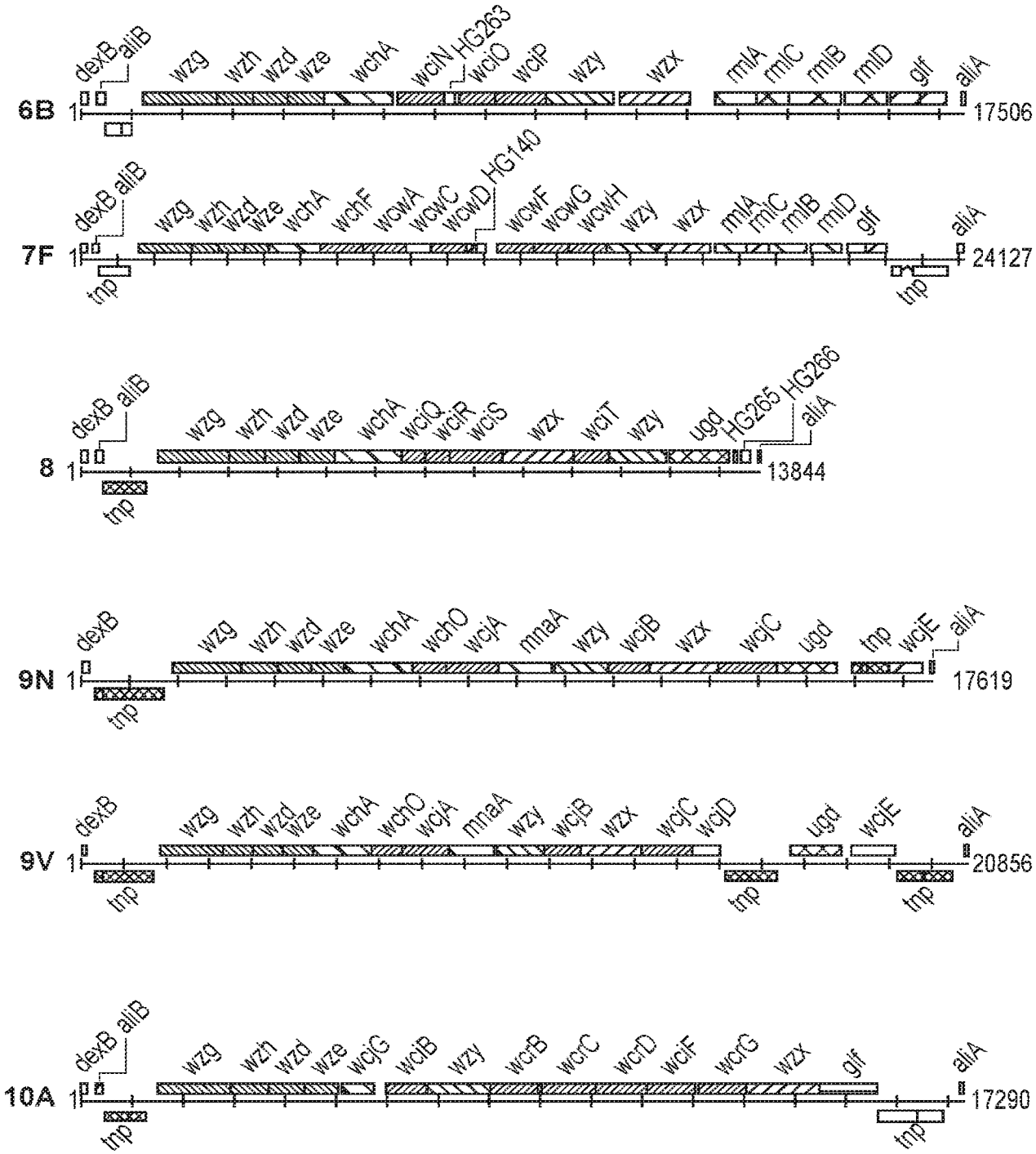


FIG. 10B

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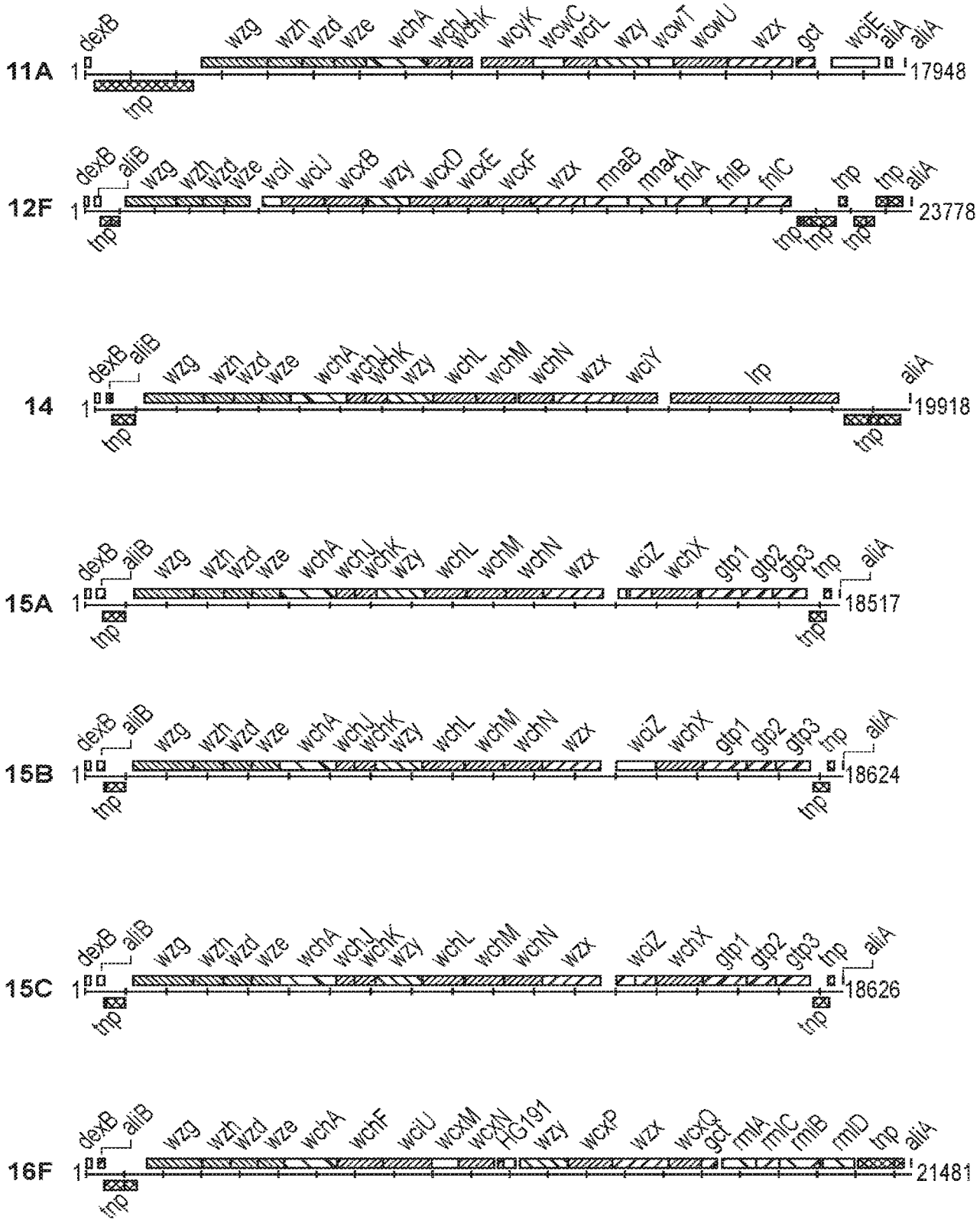


FIG. 10C

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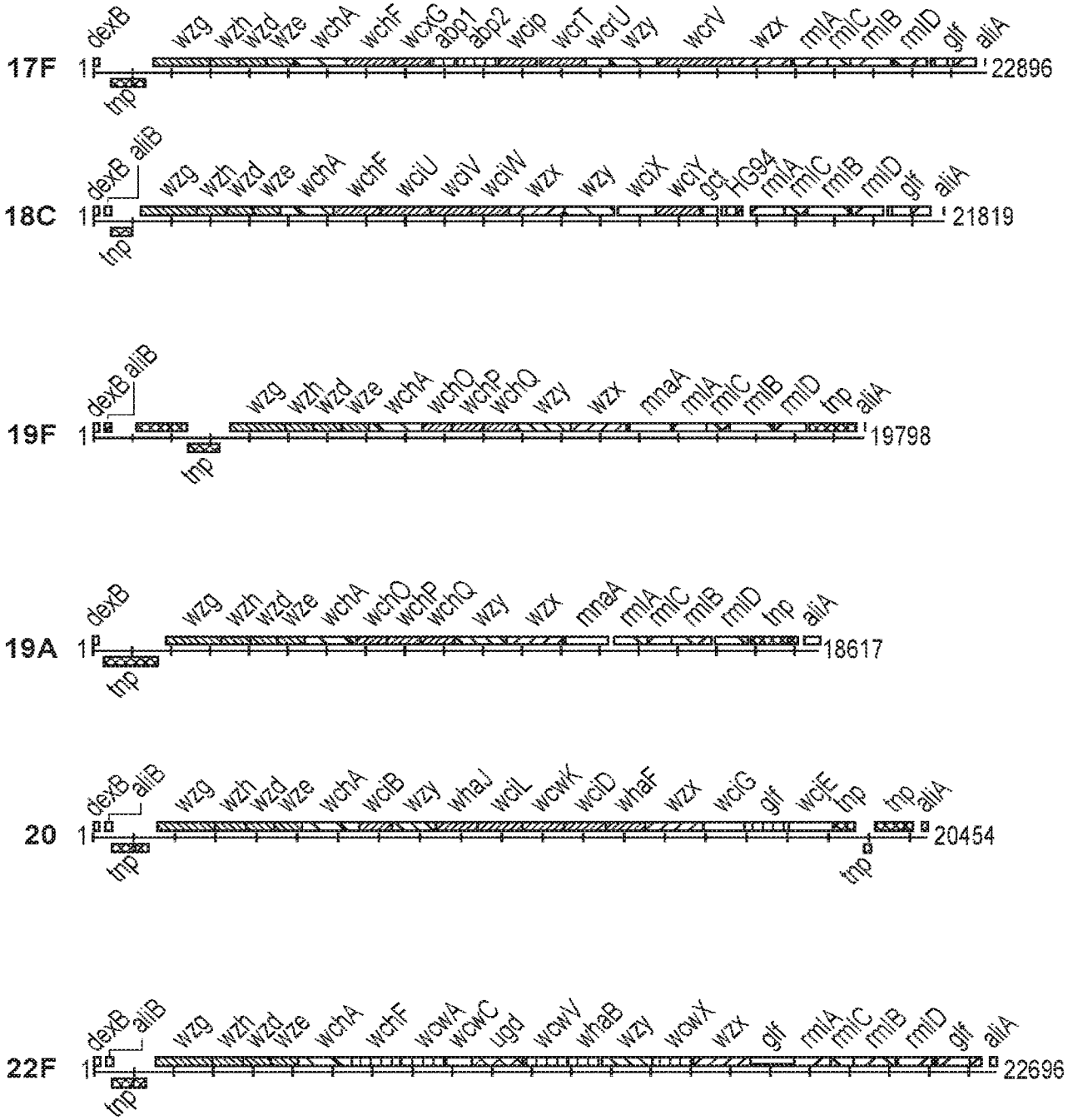


FIG. 10D

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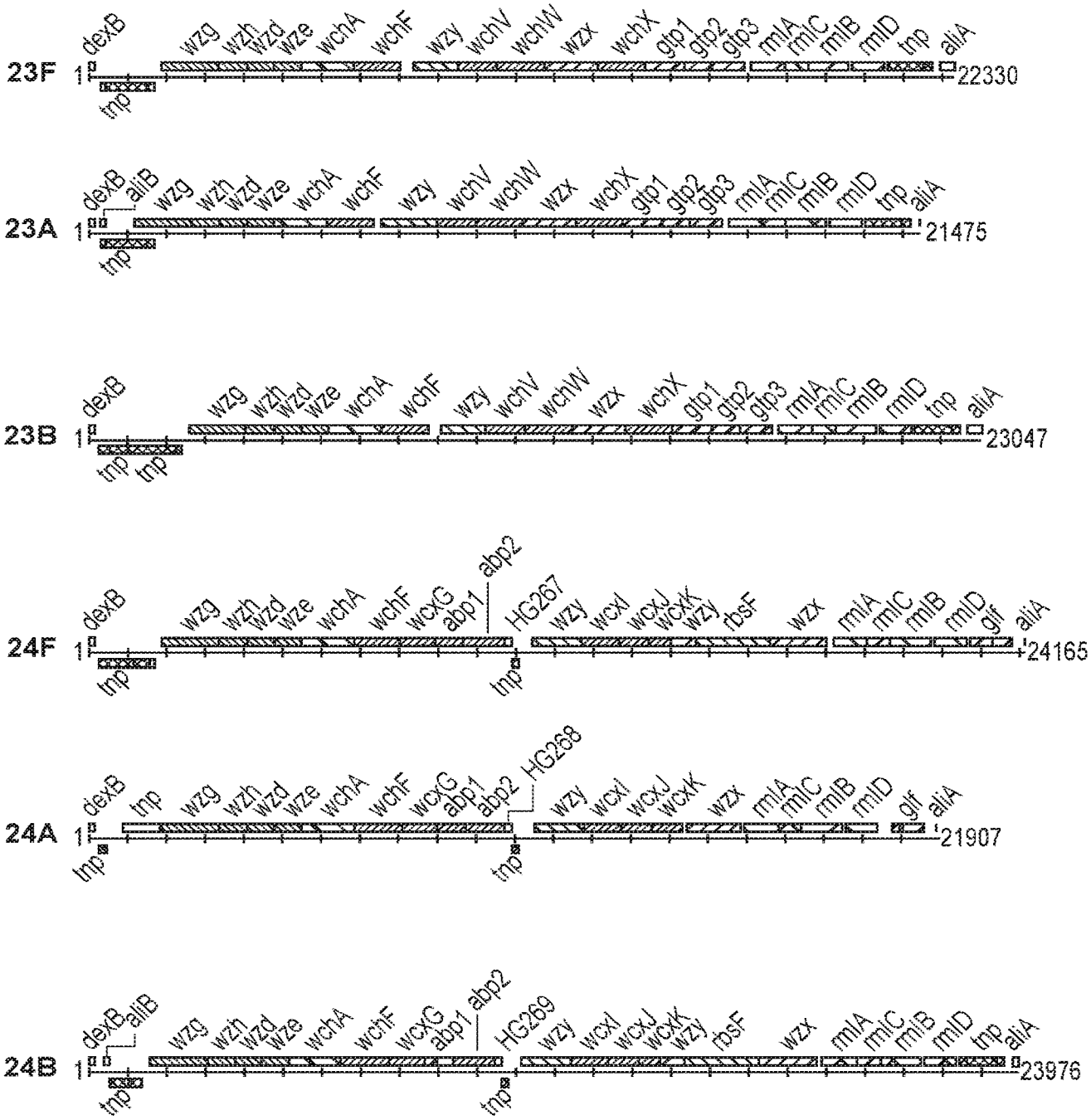


FIG. 10E

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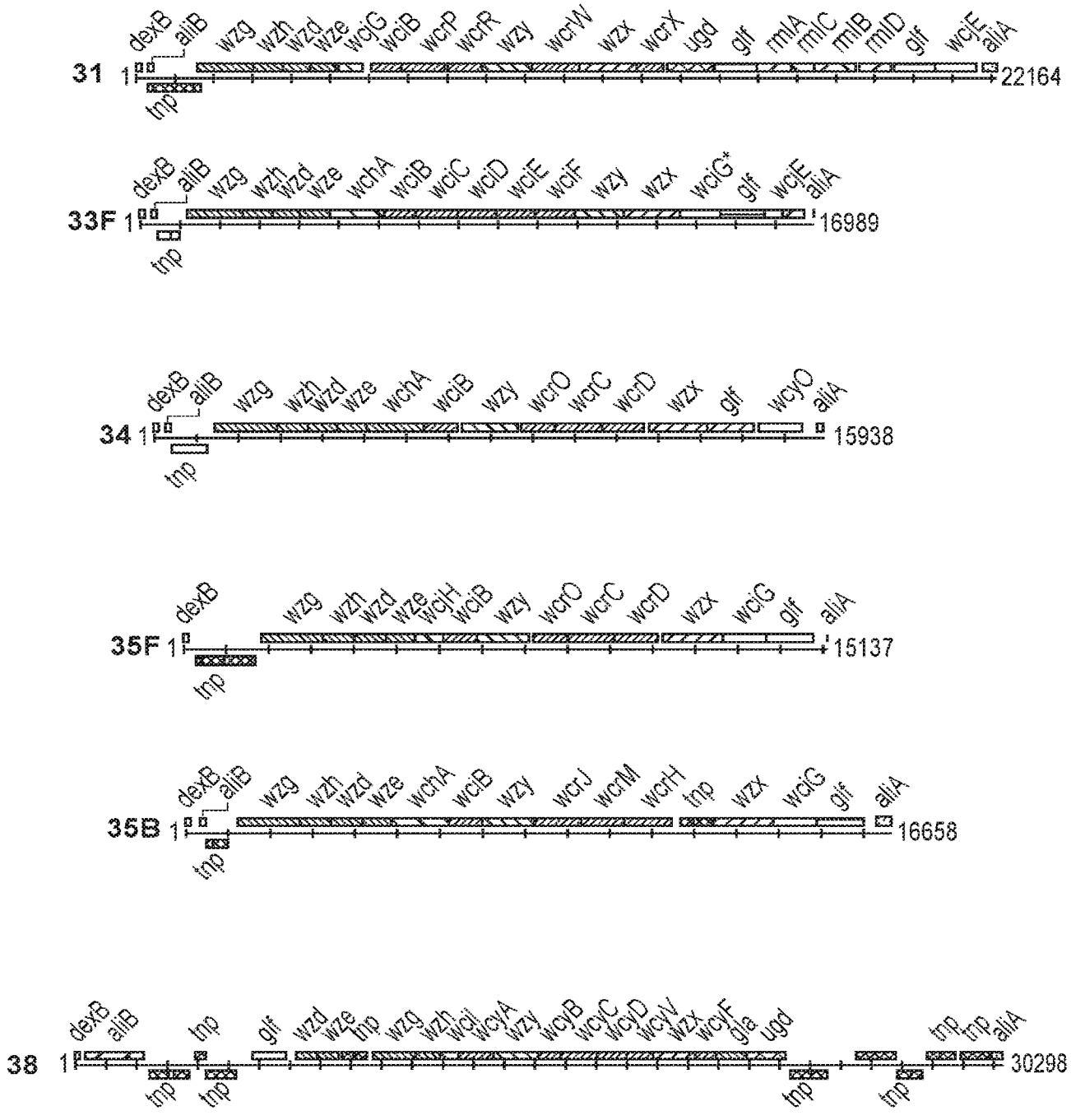


FIG. 10F

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WO 03/051392 A2

(54) Title: VACCINE

(57) Abstract: The present invention provides an optimal formulation of multiple-serotype <I>Streptococcus pneumoniae</I> conjugate vaccines.

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VACCINE

Field of the Invention:

The present invention relates to an improved *Streptococcus pneumonia* vaccine.

Background of the Invention:

Children less than 2 years of age do not mount an immune response to most polysaccharide vaccines, so it has been necessary to render the polysaccharides immunogenic by chemical conjugation to a protein carrier. Coupling the polysaccharide, a T-independent antigen, to a protein, a T-dependent antigen, confers upon the polysaccharide the properties of T dependency including isotype switching, affinity maturation, and memory induction.

However, there can be issues with repeat administration of polysaccharide-protein conjugates, or the combination of polysaccharide-protein conjugates to form multivalent vaccines. For example, it has been reported that a *Haemophilus influenzae* type b polysaccharide (PRP) vaccine using tetanus toxoid (TT) as the protein carrier was tested in a dosage-range with simultaneous immunization with (free) TT and a pneumococcal polysaccharide-TT conjugate vaccine following a standard infant schedule. As the dosage of the pneumococcal vaccine was increased, the immune response to the PRP polysaccharide portion of the Hib conjugate vaccine was decreased, indicating immune interference of the polysaccharide, possibly via the use of the same carrier protein (Dagan et al., Infect Immun. (1998); 66: 2093 - 2098).

The effect of the carrier-protein dosage on the humoral response to the protein itself has also proven to be multifaceted. In human infants as it was reported that increasing the dosage of a tetravalent tetanus toxoid conjugate resulted in a decreased response to the tetanus carrier (Dagan et al., *supra*). Classical analysis of these effects of combination vaccines have been described as carrier induced epitopic suppression, which is not fully understood, but believed to result from an excess amount of carrier protein (Fattom, Vaccine 17: 126 (1999)). This appears to result in competition for Th-cells, by the B-cells to the carrier protein, and B-cells to the polysaccharide. If the B-cells to the carrier protein predominate, there are not enough Th-cells available to provide the necessary help for the B-cells specific to the polysaccharide. However, the observed immunological effects have been inconsistent, with the total amount of carrier protein in some instances increasing the immune response, and in other cases diminishing the immune response.

Hence there remain technical difficulties in combining multiple polysaccharide conjugates into a single, efficacious, vaccine formulation. It is thus an object of the present

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invention to develop an improved formulation of a multiple serotype *Streptococcus pneumoniae* polysaccharide conjugate vaccine.

Summary of the Invention:

In one aspect, the present invention is an improved *Streptococcus pneumonia* vaccine comprising 11 or more polysaccharides from different *S. pneumonia* serotypes conjugated to 2 or more carrier proteins, where the polysaccharides from serotypes 6B, 19F and 23F are conjugated to a first carrier protein and the remaining serotypes are conjugated to 1 or 2 secondary carrier proteins, and where the secondary carrier protein(s) are different from the first carrier protein. Preferably serotypes 6B and 23F are conjugated to the first carrier protein, and more preferably only serotype 6B is conjugated to the first carrier protein. In a preferred embodiment, one of the secondary carrier protein(s) is *H. influenzae* protein D. The present invention may further comprise *S. pneumonia* surface proteins, preferably from the PhtX family, the CbpX family, the CbpX truncate family and Ply.

In a related aspect, the present invention is an improved method to elicit a protective immune response to infants against *S. pneumoniae* by administering the polysaccharide conjugate vaccine of the present invention.

In another related aspect, the present invention is an improved method to elicit a protective immune response, that is, for the prevention or amelioration of pneumococcal infection in the elderly (e.g., pneumonia) and/or in infants (e.g., Otitis media), by administering the polysaccharide conjugated vaccine of the present invention and a *S. pneumonia* surface protein.

Brief Description of the Drawings:

Figure 1 is a graphical representation of the immune response to 12 different pneumococcal polysaccharides as determined by the geometric mean fold increase after polysaccharide immunization.

Figure 2 shows the geometric mean IgG concentration [GMC] (μg/ml) and Opsonic Titres on day 14 (Post II) after immunization of adult rats with 1.0 μg PS-PD alone or combined in a tetravalent, pentavalent, heptavalent or decavalent vaccine.

Figure 3 shows the GMC for 11 serotypes and PD (protein D) versus the dosage of 6B and 23F in one dimension, and the dosage of the 9 others in the second dimension. The trend is always the same for all serotypes and PD. Increasing the dosage of 6B and 23F has a

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dramatic effect on decreasing the immune response to the remaining conjugates, even though the dosage of those conjugates is unchanged.

Figure 4 shows a graph of the IgG GMC in infant rats versus the total amount of PD immunized for 11 serotypes. (i.e., by summing all the PD from each component at each dose). The general trend is that as the dosage of carrier protein increases, there is a decrease in the IgG response to all polysaccharides, and to PD itself. This overall trend is strong evidence of carrier-induced epitopic suppression. However, the fact that the curve is not monotonous is an indication that there is another factor involved which appears to depend on Serotype 6B.

Detailed Description of the Invention:

The present invention provides an optimal formulation of multiple-serotype *Streptococcus pneumoniae* polysaccharide conjugate vaccines, by judicious selection of various polysaccharides conjugated to different, or alternate, carrier proteins. The invention is based on the fact that polysaccharide conjugates of one serotype may influence, or modulate, the immune response observed for other (serotype) polysaccharide conjugates. Thus, an optimal multi-valent polysaccharide conjugate vaccine can be prepared by putting different *S. pneumonia* polysaccharides, *with different immune regulatory properties*, on alternative carrier proteins.

The present invention is based on the combination of several factors: (i) the dosage-response curve to polysaccharides is frequently bell-shaped (Gaussian), with the maximal response at a dosage distinctive for each polysaccharide (i.e., serotype or structure); (ii) the immunogenicity of certain polysaccharides is regulated with age in humans and in animal models; (iii) the combination of *S. pneumoniae* polysaccharide conjugates into multivalent formulations often results in a decrease in imunogenicity of one or more components of the vaccine; (iv) however, certain polysaccharide conjugates result in an enhanced immune response when combined; (v) polysaccharides from serotypes 6B and 23F, and to a lesser degree 19F can regulate the immune response of other polysaccharides (i.e., other serotypes) when they are conjugated to a common carrier protein.

Thus the present invention is based on the complex relationship of all the above and, in contrast to prior studies, concludes that the bell-shaped dosage-response curve (i.e., which denotes peak immunogenicity) of polysaccharide-protein conjugates is heavily influenced by the quantity and nature of other polysaccharides. This immunological effect is referred to as modulation. Moreover, it has been discovered that the modulation of polysaccharide

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conjugates occurs through a common carrier protein. That is, a few polysaccharide conjugates may modulate the immune response to different polysaccharide conjugates, so long as they have a common carrier protein. Thus as noted above, the invention is based on the judicious selection polysaccharides, to determine which polysaccharides should be conjugated to the same or different carrier proteins.

As shown in more detail below: (a) certain S. pneumonia polysaccharides (PS), when conjugated, are strongly regulated with age, in particular serotypes 6B, 14, 19F and 23F. Serotypes 8, 12 and 18C are weakly regulated with age. Serotypes 1, 2, 3, 4, 5, 7F and 9V are not regulated with age (see Figure 1).

In addition (b), polysaccharides 1, 3, 6B, 9V and 23F, when combined into an 11-valent multiformulation, showed an increase in the immune response elicited, as compared to a monovalent polysaccharide conjugate. In contrast, serotype 14 showed a significant decrease in the multivalent formulation (see Figure 2).

Moreover (c), polysaccharides from serotypes 6B and 23F, and to a lesser degree 19F can regulate the immune response of other polysaccharides (i.e., other serotypes) if they are conjugated to a common carrier protein (see Figures 3 and 4).

Thus in one embodiment, the present invention comprises polysaccharides 6B, 19F and 23F conjugated with one (a first) carrier protein, and the remaining polysaccharides are conjugated to an alternative (or secondary) carrier protein(s), with the proviso that the primary and secondary carrier proteins are different. Preferably, polysaccharides 6B and 23F are conjugated with the same carrier protein, and the remaining polysaccharides are conjugated to a secondary carrier protein(s). More preferably, only polysaccharide 6B is conjugated to a primary (first) carrier protein and the remaining polysaccharides are conjugated to a secondary carrier protein(s).

The primary carrier protein need not be limited to a specific embodiment, but may include proteins or fragments thereof of DT (Diphtheria toxoid), TT (Tetanus toxoid), DT crm197 (a DT mutant), other DT point mutants, (e.g.at position Glu-148, see, e.g., U.S. 4,709,017, WO93/25210, WO95/33481), FragC (fragment of TT), Ply (pneumolysin and mutants thereof), PhtA, PhtB, PhtD, PhtE, (Pht A-E are described in more detail below) OmpC (from *N. meningitidis*), PorB (from *N. meningitidis*), etc. Preferably it is DT, TT or crm197. More preferably it is DT.

The secondary carrier protein(s) will also be selected from the group consisting of PD (*Haemophilus influenzae* protein D – see, e.g., EP 0 594 610 B), DT, TT, DT crm197, FragC,

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Ply, PhtA, PhtB, PhtD, PhtE, OmpC, PorB, etc. It is contemplated that 2 different secondary carrier proteins may be used, but preferably, only one secondary carrier protein is to be used in the present invention.

The number of *S. pneumoniae* polysaccharides can range from 11 different serotypes (or "V", valences) to 23 different serotypes (23V). Preferably it is 11, 13 or 16 different serotypes. In another embodiment of the invention, the vaccine may comprise conjugated *S. pneumoniae* polysaccharides and unconjugated *S. pneumoniae* polysaccharides. Preferably, the total number of polysaccharide serotypes is less than or equal to 23. For example, the invention may comprise 11 conjugated serotypes and 12 unconjugated polysaccharides. In a similar manner, the vaccine may comprise 13 or 16 conjugated polysaccharides and 10, or 7 respectively, unconjugated polysaccharides.

Preferably the multivalent pneumococcal vaccine of the invention will be selected from the following serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, although it is appreciated that one or two other serotypes could be substituted depending on the age of the recipient receiving the vaccine and the geographical location where the vaccine will be administered. For example, an 11-valent vaccine may comprise polysaccharides from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. A 13-valent pediatric (infant) vaccine may also include serotypes 6A and 19A, whereas a 13-valent elderly vaccine may include serotypes 8 and 12F.

Preferably, the Streptococcus polysaccharides of the invention are depolymerized (sized) to a final range of 100 – 500 kD. Thus, another feature of the present invention is the ratio of carrier protein to polysaccharide. For the conjugated polysaccharides, the ratio of carrier protein to polysaccharide (P/PS) will be greater than 0.5 (i.e., > 0.5, and up to 1.7) (w/w) for at least seven serotypes. Preferably the ratio is □0.70 to 1.5 (e.g., for at least serotypes 6B, 19F, 23F). More preferably the range will be 0.8 to 1.5 (e.g., for at least serotypes 6B, 19F, 23F). Most preferably still, the ratio of P/PS will at least approach 1 (e.g., 0.9-1.1) for one or more serotypes of the invention (e.g., 4).

A related feature of the present invention is that the level of unconjugated (free) carrier protein is less than 10% of the total amount of carrier protein, and that the level of unconjugated polysaccharide is less than 10% of the total amount of polysaccharide, for each serotype.

The polysaccharides may be linked to the carrier protein(s) by any known method (for example, by Likhite, U.S. Patent 4,372,945 by Armor et al., U.S. Patent 4,474,757, and

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Jennings et al., U.S. Patent 4,356,170). Preferably, CDAP conjugation chemistry is carried out (see WO95/08348).

In CDAP, the cyanylating reagent 1-cyano-dimethylaminopyridinium tetrafluoroborate (CDAP) is preferably used for the synthesis of polysaccharide-protein conjugates. The cyanilation reaction can be performed under relatively mild conditions, which avoids hydrolysis of the alkaline sensitive polysaccharides. This synthesis allows direct coupling to a carrier protein.

The polysaccharide is solubilized in water or a saline solution. CDAP is dissolved in acetonitrile and added immediately to the polysaccharide solution. The CDAP reacts with the hydroxyl groups of the polysaccharide to form a cyanate ester. After the activation step, the carrier protein is added. Amino groups of lysine react with the activated polysaccharide to form an isourea covalent link. After the coupling reaction, a large excess of glycine is then added to quench residual activated functional groups. The product is then passed through a gel permeation column to remove unreacted carrier protein and residual reagents.

In another embodiment, the *S. pneumonia* conjugates may be combined with other polysaccharides, for example, N. meningitidis types A, C, W, Y, *H. influenzae* type B, *S. aureus*, *S. epidermidis*, Group B Streptococcus, Group A Streptococcus, etc. Preferably it is N. meningitidis (types A and/or C are most preferred) and/or *H. influenzae* type B. Alternatively, the *S. pneumonia* conjugates of the invention may be combined with viral antigens, e.g., inactivated polio virus (IPV), influenza (inactivated, split, subunit (e.g., F, G antigens)), etc. In another alternative, the *S. pneumonia* conjugates may be administered concomitantly with DTPa (diphtheria, tetanus, acellular pertussis) vaccines and DTPa combination vaccines (DTPa +/- Hepatitis B +/- IPV +/- *H. influenzae* type B). Preferred DTPa vaccines have 25Lf or less of Diphtheria toxoid. These additional antigens may be in liquid form or lyophilized form.

In yet another embodiment, the present invention is an improved method to elicit a (protective) immune response in infants (0-2 years old) by administering a safe and effective amount of the vaccine of the invention. Further embodiments of the present invention include the provision of the antigenic *S. pneumoniae* conjugate compositions of the invention for use in medicine and the use of the *S. pneumoniae* conjugates of the invention in the manufacture of a medicament for the prevention (or treatment) of pneumococcal disease.

The present invention further provides an improved vaccine for the prevention or amelioration of pneumococcal infection in infants (e.g., Otitis media), by relying on the addition of pneumococcal proteins to *S. pneumoniae* conjugate compositions of the invention.

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Preferably the pneumococcal protein is from the PhtX family (see below) to which may be added further proteins. Such additional pneumococcal proteins may comprise CbpX, CbpX truncates and Ply (see below), with the proviso that the selected *S. pneumoniae* surface proteins are different from the first and secondary carrier proteins. One or more *Moraxella catarrhalis* protein antigens can also be included in the combination vaccine. Thus, the present invention is an improved method to elicit a (protective) immune response against Otitis media in infants.

In yet another embodiment, the present invention is an improved method to elicit a (protective) immune response in the elderly population (in the context of the present invention a patient is considered elderly if they are 50 years or over in age, typically over 55 years and more generally over 60 years) by administering a safe and effective amount of the vaccine of the invention, preferably in conjunction with one, two, or possibly three *S. pneumoniae* surface proteins, with the proviso that the selected *S. pneumoniae* surface proteins are different from the first and secondary carrier proteins. Preferably the pneumococcal protein is from the PhtX family (see below) to which may be added Ply and optionally CbpX or CbpX truncates (see below).

The *Streptococcus pneumoniae* proteins of the invention are either surface exposed, at least during part of the life cycle of the pneumococcus, or are proteins which are secreted or released by the pneumococcus. Preferably the proteins of the invention are selected from the following categories, such as proteins having a Type II Signal sequence motif of LXXC (where X is any amino acid, e.g., the polyhistidine triad family (PhtX)), choline binding proteins (CbpX), proteins having a Type I Signal sequence motif (e.g., Sp101), proteins having a LPXTG motif (where X is any amino acid, e.g., Sp128, Sp130), and toxins (e.g., Ply). Preferred examples within these categories (or motifs) are the following proteins, or immunologically functional equivalents thereof.

Preferably, the immunogenic composition of the invention comprises one or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133. However, if CbpX is PspC, then the second protein is not PspA or PsaA. More preferably, the immunogenic composition comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate

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chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, and Sp128. More preferably still, the immunogenic composition comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, and pneumolysin (Ply).

The Pht (Poly Histidine Triad) family comprises proteins PhtA, PhtB, PhtD, and PhtE. The family is characterized by a lipidation sequence, two domains separated by a proline-rich region and several histidine triads, possibly involved in metal or nucleoside binding or enzymatic activity, (3-5) coiled-coil regions, a conserved N-terminus and a heterogeneous C terminus. It is present in all strains of pneumococci tested. Homologous proteins have also been found in other Streptococci and Neisseria. Preferred members of the family comprise PhtA, PhtB and PhtD. More preferably, it comprises PhtA or PhtD. Most preferably it comprises PhtD. It is understood, however, that the terms Pht A, B, D, and E refer to proteins having sequences disclosed in the citations below as well as naturally-occurring (and man-made) variants thereof that have a sequence homology that is at least 90% identical to the referenced proteins. Preferably it is at least 95% identical and most preferably it is 97% identical.

With regards to the PhtX proteins, PhtA is disclosed in WO 98/18930, and is also referred to Sp36. As noted above, it is a protein from the polyhistidine triad family and has the type II signal motif of LXXC. PhtD is disclosed in WO 00/37105, and is also referred to Sp036D. As noted above, it also is a protein from the polyhistidine triad family and has the type II LXXC signal motif. PhtB is disclosed in WO 00/37105, and is also referred to Sp036B. Another member of the PhtB family is the C3-Degrading Polypeptide, as disclosed in WO 00/17370. This protein also is from the polyhistidine triad family and has the type II LXXC signal motif. A preferred immunologically functional equivalent is the protein Sp42 disclosed in WO 98/18930. A PhtB truncate (approximately 79kD) is disclosed in WO99/15675 which is also considered a member of the PhtX family. PhtE is disclosed in WO00/30299 and is referred to as BVH-3.

Concerning the Choline Binding Protein family (CbpX), members of that family were originally identified as pneumococcal proteins that could be purified by choline-affininty chromatography. All of the choline-binding proteins are non-covalently bound to phosphorylcholine moieties of cell wall teichoic acid and membrane-associated lipoteichoic acid. Structurally, they have several regions in common over the entire family, although the exact nature of the proteins (amino acid sequence, length, etc.) can vary. In general, choline

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binding proteins comprise an N terminal region (N), conserved repeat regions (R1 and/or R2), a proline rich region (P) and a conserved choline binding region (C), made up of multiple repeats, that comprises approximately one half of the protein. As used in this application, the term "Choline Binding Protein family (CbpX)" is selected from the group consisting of Choline Binding Proteins as identified in WO97/41151, PbcA, SpsA, PspC, CbpA, CbpD, and CbpG. CbpA is disclosed in WO97/41151. CbpD and CbpG are disclosed in WO00/29434. PspC is disclosed in WO97/09994. PbcA is disclosed in WO98/21337.SpsA is a Choline binding protein disclosed in WO 98/39450. Preferably the Choline Binding Proteins are selected from the group consisting of CbpA, PbcA, SpsA and PspC.

Another preferred embodiment is CbpX truncates wherein "CbpX" is defined above and "truncates" refers to CbpX proteins lacking 50% or more of the Choline binding region (C). Preferably such proteins lack the entire choline binding region. More preferably, such protein truncates lack (i) the choline binding region and (ii) retain the proline rich region and at least one repeat region (R1 or R2). More preferably still, the truncate has 2 repeat regions (R1 and R2). Examples of such preferred embodiments are NR1xR2, NR1xR2P, R1xR2P and R1xR2 as illustrated in WO99/51266 or WO99/51188, however, other choline binding proteins lacking a similar choline binding region are also contemplated within the scope of this invention.

The LytX family is membrane associated proteins associated with cell lysis. The N-terminal domain comprises choline binding domain(s), however the LytX family does not have all the features found in the CbpA family noted above and thus for the present invention, the LytX family is considered distinct from the CbpX family. In contrast with the CbpX family, the C-terminal domain contains the catalytic domain of the LytX protein family. The family comprises LytA, B and C. With regards to the LytX family, LytA is disclosed in Ronda et al., Eur J Biochem, 164:621-624 (1987). LytB is disclosed in WO 98/18930, and is also referred to as Sp46. LytC is also disclosed in WO 98/18930, and is also referred to as Sp91. A preferred member of that family is LytC.

Another preferred embodiment are LytX truncates wherein "LytX" is defined above and "truncates" refers to LytX proteins lacking 50% or more of the Choline binding region. Preferably such proteins lack the entire choline binding region. Yet another preferred embodiment of this invention are CbpX truncate-LytX truncate chimeric proteins (or fusions). Preferably this comprises NR1xR2 (or R1xR2) of CbpX and the C-terminal portion (Cterm, i.e., lacking the choline binding domains) of LytX (e.g., LytCCterm or Sp91Cterm). More preferably CbpX is selected from the group consisting of CbpA, PbcA, SpsA and PspC. More

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preferably still, it is CbpA. Preferably, LytX is LytC (also referred to as Sp91). Another embodiment of the present invention is a PspA or PsaA truncates lacking the choline binding domain (C) and expressed as a fusion protein with LytX. Preferably, LytX is LytC.

Pneumolysin is a multifunctional toxin with a distinct cytolytic (hemolytic) and complement activation activities (Rubins et al., Am . Respi. Cit Care Med, 153:1339-1346 (1996)). The toxin is not secreted by pneumococci, but it is released upon lysis of pneumococci under the influence of autolysin. Its effects include e.g., the stimulation of the production of inflammatory cytokines by human monocytes, the inhibition of the beating of cilia on human respiratory epithelial, and the decrease of bactericidal activity and migration of neutrophils. The most obvious effect of pneumolysin is in the lysis of red blood cells, which involves binding to cholesterol. Because it is a toxin, it needs to be detoxified (i.e., non-toxic to a human when provided at a dosage suitable for protection) before it can be administered *in vivo*. Expression and cloning of wild-type or native pneumolysin is known in the art. See, for example, Walker et al. (Infect Immun, 55:1184-1189 (1987)), Mitchell et al. (Biochim Biophys Acta, 1007:67-72 (1989) and Mitchell et al (NAR, 18:4010 (1990)). Detoxification of ply can be conducted by chemical means, e.g., subject toGMBS, or formalin or glutarahdehye treatment or a combination of both. Such methods are well known in the art for various toxins. Alternatively, ply can be genetically detoxified. Thus, the invention encompasses derivatives of pneumococcal proteins which may be, for example, mutated proteins. The term "mutated" is used herein to mean a molecule which has undergone deletion, addition or substitution of one or more amino acids using well known techniques for site directed mutagenesis or any other conventional method. For example, as described above, a mutant ply protein may be altered so that it is biologically inactive whilst still maintaining its immunogenic epitopes, see, for example, WO90/06951, Berry et al. (Infect Immun, 67:981-985 (1999)) and WO99/03884. As used herein, it is understood that the term "Ply" refers to mutated or detoxified pneumolysin suitable for medical use (i.e., non toxic).

With regards to PsaA and PspA, both are know in the art. For example, PsaA and transmembrane deletion variants thereof have been described by Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62. PspA and transmembrane deletion variants thereof have been disclosed in, for example, US 5804193, WO 92/14488, and WO 99/53940.

Sp128 and Sp130 are disclosed in WO00/76540. Sp125 is an example of a pneumococcal surface protein with the Cell Wall Anchored motif of LPXTG (where X is any amino acid). Any protein within this class of pneumococcal surface protein with this motif has

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been found to be useful within the context of this invention, and is therefore considered a further protein of the invention. Sp125 itself is disclosed in WO 98/18930, and is also known as ZmpB – a zinc metalloproteinase. Sp101 is disclosed in WO 98/06734 (where it has the reference # y85993). It is characterized by a Type I signal sequence. Sp133 is disclosed in WO 98/06734 (where it has the reference # y85992). It is also characterized by a Type I signal sequence.

Examples of preferred *Moraxella catarrhalis* protein antigens which can be included in a combination vaccine (especially for the prevention of otitis media) are: OMP106 [WO 97/41731 (Antex) & WO 96/34960 (PMC)]; OMP21; LbpA &/or LbpB [WO 98/55606 (PMC)]; TbpA &/or TbpB [WO 97/13785 & WO 97/32980 (PMC)]; CopB [Helminen ME, *et al.* (1993) Infect. Immun. 61:2003-2010]; UspA1 and/or UspA2 [WO 93/03761 (University of Texas)]; OmpCD; HasR (PCT/EP99/03824); PilQ (PCT/EP99/03823); OMP85 (PCT/EP00/01468); lipo06 (GB 9917977.2); lipo10 (GB 9918208.1); lipo11 (GB 9918302.2); lipo18 (GB 9918038.2); P6 (PCT/EP99/03038); D15 (PCT/EP99/03822); OmplA1 (PCT/EP99/06781); Hly3 (PCT/EP99/03257); and OmpE. Examples of non-typeable *Haemophilus influenzae* antigens which can be included in a combination vaccine (especially for the prevention of otitis media) include: Fimbrin protein [(US 5766608 - Ohio State Research Foundation)] and fusions comprising peptides therefrom [eg LB1(f) peptide fusions; US 5843464 (OSU) or WO 99/64067]; OMP26 [WO 97/01638 (Cortecs)]; P6 [EP 281673 (State University of New York)]; TbpA and/or TbpB; Hia; Hsf; Hin47; Hif; Hmw1; Hmw2; Hmw3; Hmw4; Hap; D15 (WO 94/12641); P2; and P5 (WO 94/26304).

As noted above, the proteins of the invention may also be beneficially combined. Preferred combinations include, but are not limited to, PhtD + NR1xR2, PhtD + NR1xR2P, PhtD + NR1xR2-Sp91Cterm chimeric or fusion proteins, PhtD + Ply, PhtD + Sp128, PhtD + PsaA, PhtD + PspA, PhtA + NR1xR2, PhtA + NR1xR2P, PhtA + NR1xR2-Sp91Cterm chimeric or fusion proteins, PhtA + Ply, PhtA + Sp128, PhtA + PsaA, PhtA + PspA, NR1xR2 + LytC, NR1xR2P + PspA, NR1xR2 + PspA, NR1xR2P + PsaA, NR1xR2 + PsaA, NR1xR2 + Sp128, R1xR2 + LytC, R1xR2 + PspA, R1xR2 + PsaA, R1xR2 + Sp128, R1xR2 + PhtD, R1xR2 + PhtA. Preferably, NR1xR2+/-P (or R1xR2+/-P) is from CbpA or PspC. More preferably it is from CbpA. Other combinations include 3 protein combinations such as PhtD + NR1xR2P + Ply, PhtD + NR1xR2 + Ply, PhtA + NR1xR2 + Ply and PhtA + NR1xR2P + Ply.

The vaccines of the present invention are preferably adjuvanted. Suitable adjuvants include an aluminum salt such as aluminum hydroxide gel (alum) or aluminum phosphate, but

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may also be a salt of calcium, magnesium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatized polysaccharides, or polyphosphazenes. When adjuvanted with aluminum salts, the ratio of aluminum salt to polysaccharide is less than 10:1 (w/w). Preferably it is less than 8:1 and more than 2:1.

It is preferred that the adjuvant be selected to be a preferential inducer of a TH1 type of response. Such high levels of Th1-type cytokines tend to favor the induction of cell mediated immune responses to a given antigen, whilst high levels of Th2-type cytokines tend to favor the induction of humoral immune responses to the antigen.

It is important to remember that the distinction of Th1 and Th2-type immune response is not absolute. In reality an individual will support an immune response which is described as being predominantly Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173). Traditionally, Th1-type responses are associated with the production of the INF-γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of IL-4, IL-5, IL-6, IL-10. Suitable adjuvant systems which promote a predominantly Th1 response include: Monophosphoryl lipid A or a derivative thereof, particularly 3-de-O-acylated monophosphoryl lipid A (3D-MPL) (for its preparation see GB 2220211 A); and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with either an aluminum salt (for instance aluminum phosphate or aluminum hydroxide) or an oil-in-water emulsion. In such combinations, antigen and 3D-MPL are contained in the same particulate structures, allowing for more efficient delivery of antigenic and immunostimulatory signals. Studies have shown that 3D-MPL is able to further enhance the immunogenicity of an alum-adsorbed antigen [Thoelen *et al.* Vaccine (1998) 16:708-14; EP 689454-B1].

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative, particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO 95/17210, and is a preferred formulation. Preferably the vaccine additionally comprises a saponin, more

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preferably QS21. The formulation may also comprise an oil in water emulsion and tocopherol (WO 95/17210). The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL. Unmethylated CpG containing oligonucleotides (WO 96/02555) are also preferential inducers of a TH1 response and are suitable for use in the present invention.

The vaccine preparations of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Intranasal administration of vaccines for the treatment of pneumonia or otitis media is preferred (as nasopharyngeal carriage of pneumococci can be more effectively prevented, thus attenuating infection at its earliest stage). Although the vaccine of the invention may be administered as a single dose, components thereof may also be co-administered together at the same time or at different times (for instance pneumococcal polysaccharides could be administered separately at the same time or 1-2 weeks after the administration of the bacterial protein component of the vaccine for optimal coordination of the immune responses with respect to each other). For co-administration, the optional Th1 adjuvant may be present in any or all of the different administrations, however it is preferred if it is present in combination with the bacterial protein component of the vaccine. In addition to a single route of administration, 2 different routes of administration may be used. For example, polysaccharides may be administered IM (or ID) and bacterial proteins may be administered IN (or ID). In addition, the vaccines of the invention may be administered IM for priming doses and IM or IN (without aluminum) for booster doses.

The amount of conjugate antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 0.1-100 μg of polysaccharide, for polysaccharide conjugates 0.1-50 μg of polysaccharide, preferably 1-10 μg, of which 1 to 5 μg is a preferred range and 2-5 μg is a more preferable range. However, for

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serotype 6B, the preferred dosage will comprise 3-10 μg of polysaccharide, more preferably 5-10 μg of polysaccharide conjugate.

The content of protein antigens in the vaccine will typically be in the range 1-100μg, preferably 5-50μg, most typically in the range 5 - 25μg. Following an initial vaccination, subjects may receive one or several booster immunizations adequately spaced.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The vaccines of the present invention may be stored in solution or lyophilized. As a liquid, the vaccine of the invention is typically stored in 0.5ml solution/dose. Preferably the vaccine is adsorbed onto an aluminum salt. If the solution is lyophilized, it is preferably in the presence of a sugar such as sucrose or lactose or trehalose. It is still further preferable that they are lyophilized and extemporaneously reconstituted prior to use. Lyophilizing of *Streptococcus* polysaccharides may result in a more stable composition (vaccine) and may possibly lead to higher antibody titers in the presence of 3D-MPL and in the absence of an aluminum based adjuvant.

Although the vaccines of the present invention may be administered by any route, administration of the described vaccines into the skin (ID) forms one embodiment of the present invention. Human skin comprises an outer "horny" cuticle, called the stratum corneum, which overlays the epidermis. Underneath this epidermis is a layer called the dermis, which in turn overlays the subcutaneous tissue. Researchers have shown that injection of a vaccine into the skin, and in particular the dermis, stimulates an immune response, which may also be associated with a number of additional advantages. Intradermal vaccination with the vaccines described herein forms a preferred feature of the present invention.

The conventional technique of intradermal injection, the "mantoux procedure", comprises steps of cleaning the skin, and then stretching with one hand, and with the bevel of a narrow gauge needle (26-31 gauge) facing upwards the needle is inserted at an angle of between 10-15°. Once the bevel of the needle is inserted, the barrel of the needle is lowered and further advanced whilst providing a slight pressure to elevate it under the skin. The liquid is then injected very slowly thereby forming a bleb or bump on the skin surface, followed by slow withdrawal of the needle.

More recently, devices that are specifically designed to administer liquid agents into or across the skin have been described, for example the devices described in WO 99/34850 and

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EP 1092444, also the jet injection devices described for example in WO 01/13977; US 5,480,381, US 5,599,302, US 5,334,144, US 5,993,412, US 5,649,912, US 5,569,189, US 5,704,911, US 5,383,851, US 5,893,397, US 5,466,220, US 5,339,163, US 5,312,335, US 5,503,627, US 5,064,413, US 5,520, 639, US 4,596,556, US 4,790,824, US 4,941,880, US 4,940,460, WO 97/37705 and WO 97/13537. Alternative methods of intradermal administration of the vaccine preparations may include conventional syringes and needles, or devices designed for ballistic delivery of solid vaccines (WO 99/27961), or transdermal patches (WO 97/48440; WO 98/28037); or applied to the surface of the skin (transdermal or transcutaneous delivery WO 98/20734 ; WO 98/28037).

When the vaccines of the present invention are to be administered to the skin, or more specifically into the dermis, the vaccine is in a low liquid volume, particularly a volume of between about 0.05 ml and 0.2 ml.

The content of antigens in the skin or intradermal vaccines of the present invention may be similar to conventional doses as found in intramuscular vaccines (see above). However, it is a feature of skin or intradermal vaccines that the formulations may be "low dose". Accordingly the protein antigens in "low dose" vaccines are preferably present in as little as 0.1 to 10μg, preferably 0.1 to 5 μg per dose; and the polysaccharide (preferably conjugated) antigens may be present in the range of 0.01-1μg, and preferably between 0.01 to 0.5 μg of polysaccharide per dose.

As used herein, the term "intradermal delivery" means delivery of the vaccine to the region of the dermis in the skin. However, the vaccine will not necessarily be located exclusively in the dermis. The dermis is the layer in the skin located between about 1.0 and about 2.0 mm from the surface in human skin, but there is a certain amount of variation between individuals and in different parts of the body. In general, it can be expected to reach the dermis by going 1.5 mm below the surface of the skin. The dermis is located between the stratum corneum and the epidermis at the surface and the subcutaneous layer below. Depending on the mode of delivery, the vaccine may ultimately be located solely or primarily within the dermis, or it may ultimately be distributed within the epidermis and the dermis.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

Examples:

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Example 1

Determination of the Polysaccharides to which the Immune Response is Regulated with Age

Human antibody titers to both pre-immune and post-immunization (2 weeks to 3 months) polysaccharides (unconjugated) were collected either internally or via external sources. Figure 1 shows the relationship between the immunogenicity of each serotype polysaccharide, as measured by the geometric mean fold-increase in antibody titre (GFI) after polysaccharide immunization, and the mean age of the subjects in the study. The linear correlations of log geometric mean fold increase and age give an indication if the immune response is regulated with age. As shown in Figure 1, serotypes 6, 14, 19 and 23 are significantly correlated with age ($p < 0.001$), whereas serotypes 8, 12 and 18 are less significantly correlated with age ($0.05 < p < 0.2$). Finally, serotypes 1, 2, 3, 4, 5, 7 and 9 are not significantly correlated with age ($p > \text{or} = 0.20$).

Example 2

General Methodology of Determining Antibody Responses in Various Mammals

The sera were tested for IgG antibodies to the pneumococcal polysaccharides by an ELISA based on a consensus assay for human sera proposed by the joint CDC/WHO workshops held between 1994 and 1996 (WHO 1996, Plikatis et al J. Clin. Microbiol 38: 2043 (2000)). Briefly, purified capsular polysaccharides from ATCC (Rockville, Md, 20852) were coated at 25 μg/ml in phosphate buffered saline (PBS) on high binding microtitre plates (Nunc Maxisorp) overnight at 4 C. The plates were blocked with 10% fetal calf serum (FCS), 1 hour at 37 C. Serum samples were pre-incubated with the 20 μg/ml cell-wall polysaccharide (Statens Serum Institute, Copenhagen) and 10% FCS at room temperature for 30 minutes to neutralize antibodies to this antigen. A reference serum 89SF (courtesy of Dr. C Frasch, USFDA) was treated in the same fashion, and included on every plate. The samples were then diluted two-fold on the microplate in 10% FCS in PBS, and equilibrated at room temperature for 1 hour with agitation. After washing, the microplates were equilibrated with peroxidase labelled anti-human IgG Fc monoclonal antibody (HP6043-HRP, Stratech Scientific Ltd) diluted 1:4000 in 10% FCS in PBS for 1 hour at room temperature with agitation. The ELISA was performed to measure rat IgG using Jackson ImmunoLaboratories Inc. peroxidase-conjugated AffiniPure Goat anti-Rat IgG (H+L) (code 112-035-003) at 1:5000. The titration curves were referenced

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to standard sera for each serotype using logistic log comparison by SoftMax Pro. The polysaccharide concentrations used to coat the ELISA plate have been fixed at 10 μg/ml for all serotypes except 6B and 23F, where 20 μg/ml has been used. In addition, 100% fetal calf serum was used as the diluent when testing antisera for serotype 6B, as this serotype was prone to non-specific ELISA responses. Serology for serotype 3 on Rhesus sera used mHSA comix for the coating antigen. The color was developed using 4 mg OPD (Sigma) per 10 ml pH 4.5 0.1M citrate buffer with 14 μl H2O2 for 15 minutes in the dark at room temperature. The reaction was stopped with 50 μl HCl, and the optical density was read at 490 nm relative to 650 nm. IgG concentrations were determined by reference of titration points to the calibration curve modeled using a 4-parameter logistic log equation calculated by SoftMax Pro software.

To obtain absolute antibody concentrations in μg/ml, pooled reference antisera were calibrated by two independent methods. For rat antisera, the method of Zollinger and Boslego (1981) was used for 11 serotypes, and for 4 serotypes this was compared with values obtained by immunoprecipitation. Excellent correspondence was found between the two methods. For murine sera, purified monoclonal IgG1 antibodies were used, and their active concentrations were confirmed by corollary response (PVW 1999). In this case, reasonable correspondence was found. For Rhesus monkey sera, it was demonstrated that the anti-IgG reagents used react equally with human and Rhesus IgG; thus the calibrated human reference sera 89SF (available from the US FDA) was employed to reference the ELISA.

The ELISA to measure the murine and rat IgG to the pneumococcal polysaccharides was similar with the following exceptions. Locally manufactured polysaccharides were used to coat the ELISA plates at 20 μg/ml in PBS for serotypes 6B and 23F, and 10 μg/ml in PBS for serotypes 14 and 19F. Jackson ImmunoLaboratories Inc. Peroxidase-conjugated affiniPure Goat Anti-mouse IgG (H+L) and AffiniPure Goat Anti-rat IgG (H+L) were employed to detect bound IgG. HP6043-HRP reacted equally with human and Rhesus purified IgG, and so this reagent was used for Rhesus antiserum, and the reference serum was using 89SF.

The reference serum for human and Rhesus serology was 89SF, kindly provided by Dr. Carl Frasch. The universally accepted weight-based concentration calibration values for the human reference serum 89SF for IgG, IgA and IgM against 10 pneumococcal serotypes using 2 different methods was published (Salazar et al).

The protein ELISA was performed similarly to the polysaccharide ELISA with the following modifications. The protein was coated overnight at 2.0 μg/ml in PBS. The serum samples were diluted in PBS containing 10% foetal calf serum and 0.1 % polyvinyl alcohol. Bound human

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antibody was detected using Sigma Peroxydase-conjugated goat affinity purified antibody to Human IgG Fc (reference A-2290). To calibrate the protein response in the human and Rhesus monkey serology, Sandoglobulin lot 069, found to contain significant anti-protein D antibody, was used as the reference and given an arbitrary value of 100 ELISA units. For murine and rat serology, the antibody concentrations were quantified by performing corollary response by either direct antigen coating, or by antibody capture.

The sera were also tested for their ability to kill live pneumococci in an *in vitro* opsonophagocytic assay. The opsonophagocytosis assay was adapted from the published protocol (Romero-Steiner et al. 1997), as well as a detailed protocol provided by Sandy Steiner of the CDC as part of a multi-laboratory study.

Two methods were used. In method A, pneumococcal strains provided by the CDC were replaced by SB production strains were used. Secondly, the HL-60 cells were replaced by freshly purified human neutrophils (PMN). The results are expressed as the serum dilution required for 50% bacterial killing.

In method B, the CDC protocol was followed more closely from a published and detailed standardized protocol provided by the CDC as part of a multi-laboratory study (Romero-Steiner 1997, Romero-Steiner 2000).

Briefly, differentiated HL60 cells were centrifuged at 1000 rpm (300 x g) and the culture supernatant was drawn off. The cells were resuspended in the assay buffer consisting of HBSS-BSA. If antibiotics were present in the culture media, this procedure was repeated to ensure complete removal of antibiotics.

Serum samples were pre-diluted in advance for 4 assays to optimize volume measurements. It was demonstrated that samples diluted 1:2 in assay buffer yielded stable opsonic titres for at least 5 days if kept at 4°C. Twenty-five µl of diluted sera was added to 25 µl of assay buffer in a microplate round-bottom well. Twofold serial dilution were performed with 25 µl volume, again to optimize volume measurements.

Baby rabbit complement and pneumococcal cultures were kept at – 70°C until use. A 4:2:1 volume combination of activated HL60 cells, freshly thawed pneumococal culture and freshly thawed baby rabbit complement was mixed with vortexing. Twenty-five µl of this mixture was rapidly distributed to the microplate wells containing diluted sera, yielding a final volume of 50 µl. This gave 1E 5 HL60, 150 pneumococcal CFU and 7.1% complement concentration per well in the final mixture, except for serotype 6B in which two modifications were made; the final complement concentration was 12.5% and 5% FCS was included in the

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assay buffer to equalise growth of pneumococci during incubation. The microplates were incubated for 2 hours at 37°C with 5% CO2 with shaking at 210 rpm.

After incubation, a viable count was made of the pneumococci from a 20 µl aliquot of the wells. Wells containing only assay buffer with no serum were used as the blank wells to determine the exact number of pneumococci added per well. The mean number of CFU in 8 blank wells on each plate was used for subsequent calculations.

The percent killing was calculated relative to the mean of the blank wells. The titre of a serum sample was determined by the maximum reciprocal dilution of serum able to facilitate greater than 50% killing of the pneumococci. The values are reported as discontinuous titres of 8, 16 32 etc. Samples for which there was less than 50% killing are reported with a titre < 8. Samples in which a prozone effect was observed were repeated, and the second result was taken. If a prozone effect was observed again, the result was considered invalid. This occurs in less than 5% of the samples. Samples which had a titre greater than 1024 were repeated starting at a 1:64 dilution.

Example 3

Effects of combination of pneumococcal PS-PD conjugates on immunogenicity in adult rats

It has been observed that the combination of vaccines into multivalent formulations can result in the decrease in immunogenicity of one or more components of the vaccine. This has been especially observed for conjugate vaccines, and has been called carrier-induced epitopic suppression. The underlying mechanism for this suppression is not well understood, but it tends to happen at higher dosages of carrier protein.

An 11-valent pneumococcal conjugate vaccine is an example of combination vaccines. Since the combination of each serotype's conjugate will add to the total amount of protein used to immunize, it is important to determine whether the combination of each conjugate vaccine into a multivalent formulation results in a significant decrease in the immunogenicity of the conjugate.

Protocol:

Adult rats were immunized with pneumococcal-polysaccharide protein D conjugate vaccines (see, WO00/56360) either individually, or combined in a multivalent formulation.

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Groups of 10 rats were immunized twice 28 days apart, and test bleeds were obtained on day 28 and day 42 (14 days after the second dose).

Antibody concentration was measured as described. The opsonic titres were measured according to method A.

Results:

All conjugates induced specific IgG antibodies as measured by ELISA (Figure 2). Opsonic activity (as determined by the reciprocal of the dilution of pooled sera able to kill 50 % of live pneumococci) was also detected in all sera.

Figure 2 also shows the effect of combination of monovalent PS-PD conjugates on their immunogenicity in adult rats, as measured by IgG concentration and opsonic titre at 14 days post II.

Statistical analysis was performed on all samples to determine if differences in IgG concentration upon combination were significant. Only type 14 showed a significant decrease in ELISA titers upon combination. The IgG concentration was reduced to levels that were similar to the other serotypes. All other differences were not significant, but type 7F approached significance ($p = 0.08$).

Serotypes 1, 3, 6B, 9V and 23F actually show increases upon combination.

Example 4

Independent Variation of the Dosage of Serotypes 6B and 23F

Combination of individual conjugate vaccines into a multi-valent formulation results in increases or decreases of the antibody response. The immune regulation of the response is serotype dependent. To characterize the immune response to a combined 11-Valent conjugate vaccine, an experiment was undertaken which combined the 11 valences in two groups, 6B and 23F together, versus the remaining 9 valences.

Protocol:

Infant and Adult rats were immunized with 11-Valent PS-PD pneumococcal conjugate vaccine in a two-tiered dosage, that is, the 6B&23F dosage varied independently from the other 9 valence, as shown in Table 1.

Table 1: The 11-valent PS-PD Two-Way Dosage Formulation

Group	Dosage 6B and 23F (µg)	Dosage 1, 3, 4, 5, 7F, 9V, 14, 18C, 19F (µg)
1	0.01	0.01

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2	0.01	0.1
3	0.01	1
4	0.1	0.01
5	0.1	0.1
6	0.1	1
7	1	0.01
8	1	0.1
9	1	1

Infant OFA rats were randomized to different mothers and were 7 days old when they received the first immunization. Ten rats per group received 3 immunizations on days 0, 14 and 28. Bleeds were performed on day 42 (14 days post III) and 56 (28 days post III).

Results:

3D analysis of the two-tiered dosages indicates immune regulation in infant rats caused by 6B-PD and 23F-PD. Figure 3 shows the GMC for 11 serotypes and PD versus the dosage of 6B and 23F in one dimension, and the dosage of the 9 others in the second dimension. The trend is always the same for all serotypes and PD. Increasing the dosage of 6B and 23F has a dramatic effect on decreasing the antibody response to the remaining conjugates, even though the dosage of those conjugates is unchanged. This effect is very strong in the infant rats, but only slightly observable in the adult rats (not shown).

Figure 4 shows the antibody concentration against each serotype in the conjugate vaccine as a function of total Protein D content. If carrier-induced epitopic suppression was the principle or only cause of reduction in the immune response with increasing vaccine dosage, it is expected that these curves would be monotonically decreasing. Rather, the wave function indicates there is some other factor influencing the antibody response. As noted from Figure 3, when the dosage is divided combining serotypes 6B and 23F, a smooth 3D surface is obtained, indicating that 6B and 23F regulate the immune response to the other serotypes. Because in Figure 4 serotype 6B does show a monotonically decreasing immune response, it may be surmised the dosage of serotype 6B is the dominant factor, as its interaction with itself is always constant, and thus it only shows the effect of carrier-induced epitopic suppression.

Conclusions:

Independent variation of the dosage of 6B&23F and the other 9 serotypes revealed that the dosage of serotypes 6B&23F exerted an influence on the antibody response to the other

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serotypes. The antibody response to each serotype was reduced with increasing total amount of PD immunized, indicating carrier-induced epitopic suppression, but since the relation is not smooth, there is an additional factor. In addition, the IgG response to PD also decreases with increasing dosage, opposite to what is expected from carrier-induced epitopic suppression. Taken together, these imply a heretofore-unknown regulation of the immune response to conjugate vaccines mapped onto the dosage of serotypes 6B and 23F.

Example 5

Demonstration that the immune regulation from serotypes 6B and 23F is transmitted via the protein carrier.

Objective:

It is apparent that the dosage of conjugates 6B and 23F regulate the antibody response to the other conjugates in a multivalent formulation. The following experiment was performed to determine if the immune regulation associated with 6B&23F-PD (conjugates) in infant rats was due to the polysaccharide, or the polysaccharide protein conjugate.

Protocol:

Conjugates 6B&23F-PD or PS (unconjugated) were combined with other serotypes in a multivalent formulation, with the dosage of 6B&23F at 0.01 and 1.0 μg, and with the plain polysaccharide at 1.0 μg (without 6B&23F conjugates).

Infant OFA rats were randomized to different mothers and were 7 days old when they received the first immunization. Ten rats per group received 3 immunizations on days 0, 14 and 28. Bleeds were performed on day 42 (14 days post III).

Results:

As previously observed, an increase in the 6B&23F-PD dosage decreased the response to 19F. When PS replaced the conjugate, a higher response to 19F was observed.

Conclusion:

The presence of a 1 μg dosage of 6B and 23F conjugate vaccine is sufficient to regulate the immune response to serotype 19F in a multivalent conjugate vaccine, however, the same dosage of plain polysaccharide has no effect. Since it has been determined that serotypes 6B and 23F are regulated in their immune response in humans and animals, we may conclude the immune regulation of serotypes 6B and 23F are transmitted to the other serotypes via the common protein carrier.

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Example 6

Modification of the Protein Carrier for Serotype 6B

The seroconversion rate of against 6B PS-conjugate was low in the infant rat at 0.1ug dosage. Other factors that could influence the immunogenicity of the conjugate were examined. These include the ratio of carbohydrate to protein present in the material, the specific method of linkage used, the presence of free polysaccharide, and the specific carrier protein used.

Modification of the coupling chemistry did not increase the immunogenicity of the 6B conjugates in either the infant rat or mouse models. It does appear that the use of the TT carrier increases immunogenicity in the mouse model, but only at a higher dosage. Conjugates were synthesized with an initial carrier protein (protein D)/PS ration of 2.5:1. Other conjugates were synthesized with an initial carrier protein (Protein D)/PS ratio of 1:1.

Example 7

Clinical Evaluations

Several vaccine formulations of the present invention are undergoing clinical evaluation in humans. Table 2 illustrates the composition of such vaccines.

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Table 2 - S. pneumoniae formulations

Strep PS serotype:	N°1	N°2	N°3	N°4
6B (µgPS -carrier)	**10µg -DT**	5µg -DT	**10µg -DT**	5µg -DT
19F (µgPS -carrier)	3µg -DT	3µg -DT	5µg -**PD**	3µg -**PD**
23F (µgPS -carrier)	5µg -DT	5µg -DT	5µg -DT	5µg -DT
1 (µgPS -carrier)	3µg -PD	3µg -PD	5µg -PD	3µg -PD
3 (µgPS -carrier)	3µg -PD	2µg -PD	5µg -PD	3µg -PD
4 (µgPS -carrier)	3µg -PD	2µg -PD	5µg -PD	3µg -PD
5 (µgPS -carrier)	3µg -PD	3µg -PD	5µg -PD	3µg -PD
7F (µgPS -carrier)	3µg -PD	3µg -PD	5µg -PD	3µg -PD
9V (µgPS -carrier)	3µg -PD	2µg -PD	5µg -PD	3µg -PD
14 (µgPS -carrier)	3µg -PD	2µg -PD	5µg -PD	3µg -PD
18C (µgPS -carrier)	3µg -PD	2µg -PD	5µg -PD	3µg -PD
Total PS content:	**42µg PS**	**32µg PS**	**60µg PS**	**37µg PS**
Protein carrier content:	~22µg **PD** ~18µg **DT**	~18µg **PD** ~14µg **DT**	~42µg **PD** ~15µg **DT**	~25µg **PD** ~10µg **DT**
Alum content (Al^{3+}):	~**0.21** mg	~**0.16** mg	~**0.32** mg	~**0.19** mg
+/- Combination with				
$MenC_{ads\ lyo}$: **µg PS**	10µg PSC			10µg PSC
µg TT	~10µg TT			~10µg TT
µg Al^{3+}	0.05mg Al^{3+}			0.05mg Al^{3+}

While the preferred embodiments of the invention are illustrated by the above, it is to be understood that the invention is not limited to the precise instructions herein disclosed and that the right to all modifications coming within the scope of the following claims is reserved.

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What is claimed is:

1. An improved *Streptococcus pneumonia* vaccine comprising
11 or more polysaccharides from different *S. pneumonia* serotypes conjugated to 2 or more carrier proteins wherein,
serotypes 6B, 19F and 23F are conjugated to a first carrier protein and
remaining serotypes are conjugated to 1 or 2 secondary carrier proteins,
and wherein the secondary carrier proteins are different from the first carrier protein.

2. An improved *Streptococcus pneumonia* vaccine comprising
11 or more polysaccharides from different *S. pneumonia* serotypes conjugated to 2 or more carrier proteins wherein,
serotypes 6B, and 23F are conjugated to a first carrier protein and
remaining serotypes are conjugated to 1 or 2 secondary carrier proteins,
and wherein the secondary carrier protein are different from the first carrier protein.

3. An improved *Streptococcus pneumonia* vaccine comprising
11 or more polysaccharides from different *S. pneumonia* serotypes conjugated to 2 or more carrier proteins wherein,
serotype 6B is conjugated to a first carrier protein and
remaining serotypes are conjugated to 1 or 2 secondary carrier proteins,
and wherein the secondary carrier protein are different from the first carrier protein.

4. The vaccine of any preceding claim wherein the first carrier protein is selected from the group consisting of DT, crm197, TT, Fragment C, Ply, PhtA, PhtB, PhtD, PhtE, OmpC and PorB.

5. The vaccine of any preceding claim wherein the secondary carrier protein comprises one or 2 proteins selected from the group consisting of PD, DT, crm197, TT, Fragment C, Ply, PhtA, PhtB, PhtD, PhtE, OmpC and PorB.

6. The vaccine of any preceding claim wherein there is 1 secondary carrier

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protein.

7. The vaccine of any preceding claim wherein polysaccharides of each serotype are present in an amount of 1-10ug.

8. The vaccine of claim 7 wherein one or more serotypes selected from the group consisting of 1, 3, 4, 5, 7F, 9V, 14 and 18C are present in an amount of 2-5ug.

9. The vaccine of any preceding claim wherein the ratio of carrier protein to polysaccharide is 0.5 to 1.7 (w/w).

10. The vaccine of claim 9 wherein the ratio of carrier protein to polysaccharide is from 0.7 to 1.5 for one or more serotypes selected from the group consisting of 6B, 19F and 23F.

11. The vaccine of any preceding claim wherein the secondary carrier protein is *H. influenzae* protein D
(PD).

12. The vaccine of any proceeding claim wherein polysaccharide serotype 6B is conjugated to a first carrier protein selected from the group consisting of DT, crm197 or TT.

13. The vaccine of claim 12 wherein the first carrier protein is DT.

14. The vaccine of any proceeding claim wherein polysaccharide 6B is present in an amount of 5-10ug/dose.

15. The vaccine of any preceding claim further comprising unconjugated *S. pneumonia* polysaccharides of serotypes different from those conjugated, such that the number of conjugated and unconjugated polysaccharides is less than or equal to 23.

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16. A method of eliciting a protective immune response to infants against *S. pneumonia* by administering the vaccine of any preceding claim.

17. A method of eliciting a protective immune response to the elderly against *S. pneumonia* by administering the (i) vaccine of any preceding claim and (ii) *a S. pneumonia* surface protein from the PhtX family

18. A method of eliciting a protective immune response to infants against Otitis media by administering the (i) vaccine of any preceding claim and (ii) *a S. pneumonia* surface protein from the PhtX family.

19. The method of claim 17 or 18 wherein the PhtX family protein is PhtD or PhtB.

20. The method of claim 19 wherein the PhtX family protein is PhtD.

21. The method of claim 17 further comprising a CbpX family protein.

22. The method of claim 21 wherein the CbpX protein is a truncate lacking the choline binding domain.

23. The method of claim 22 wherein the CbpX truncate is choline binding protein A.

24. The method of claim 18 further comprising Ply.

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Figure 1: Changes in Polysaccharide Immunogenicity with Age:

FIGURE 1A

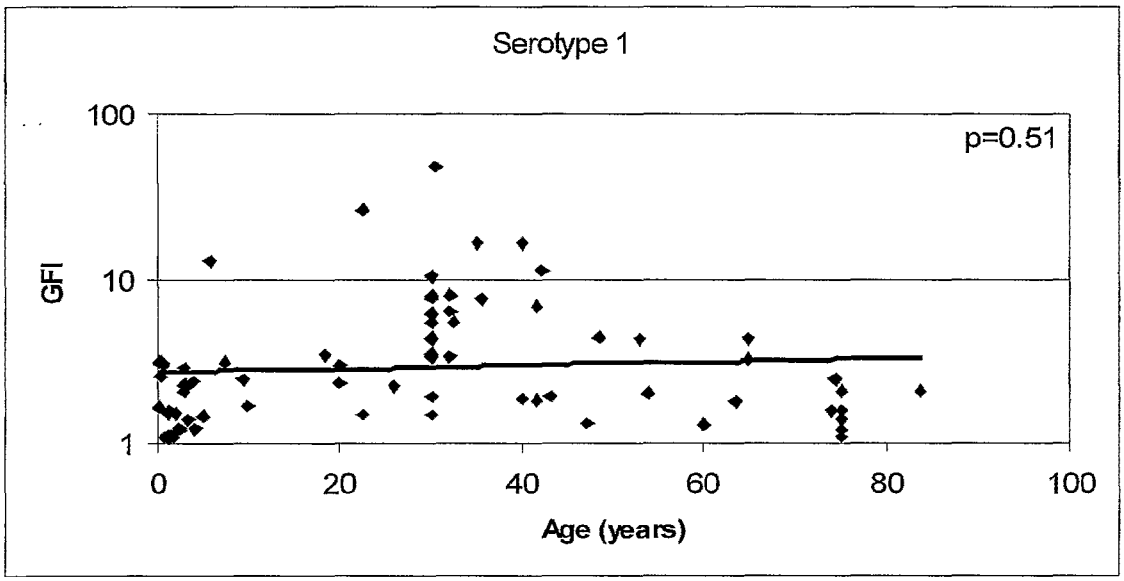
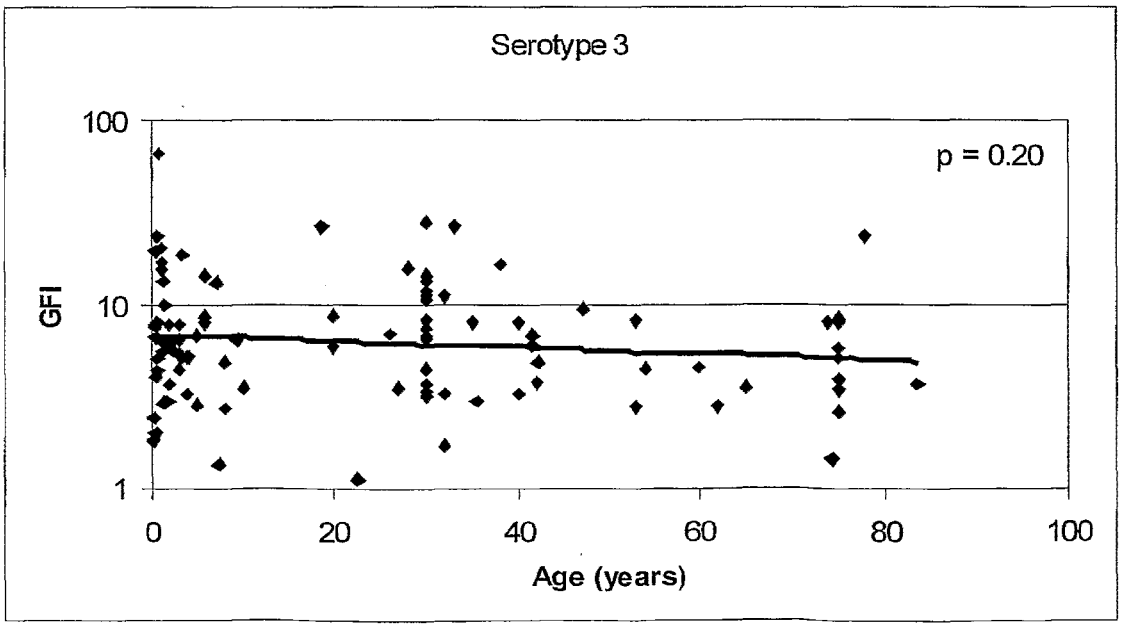


FIGURE 1B



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FIGURE 1C

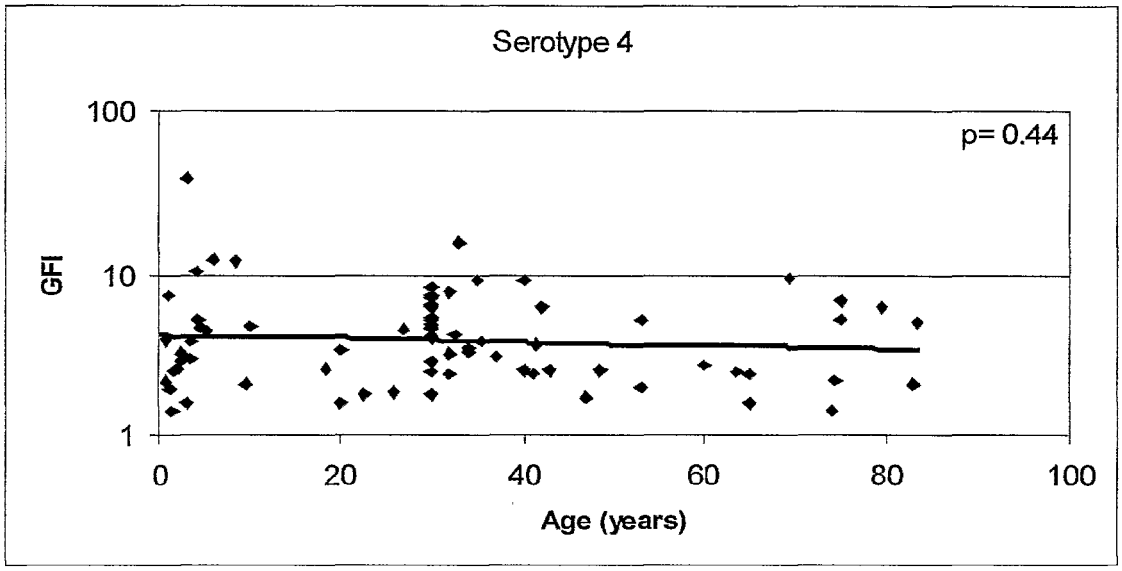
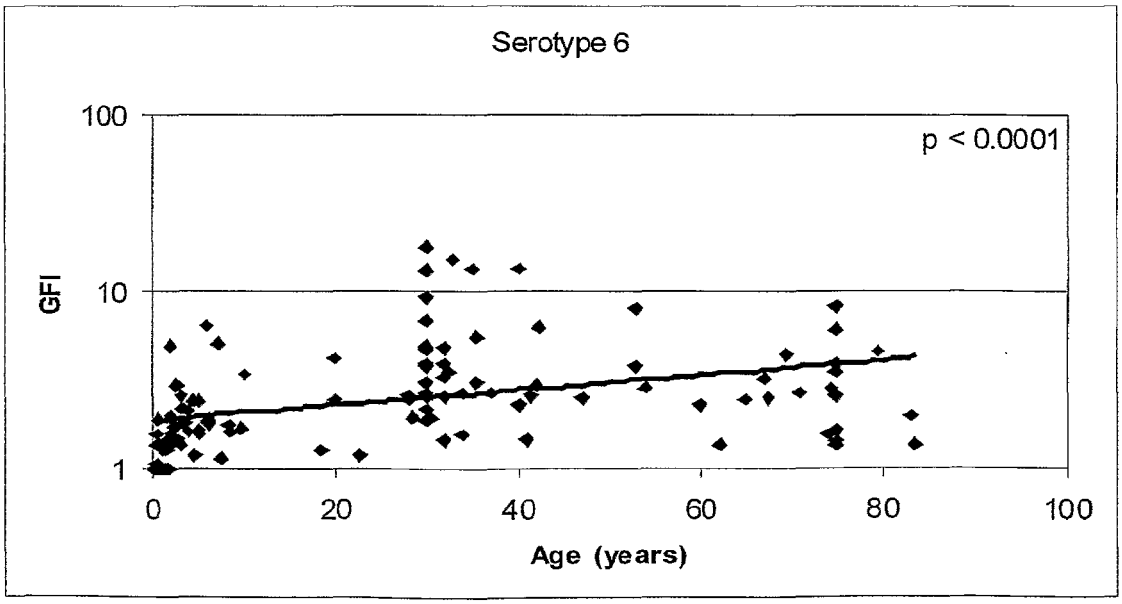


FIGURE 1D



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FIGURE 1E

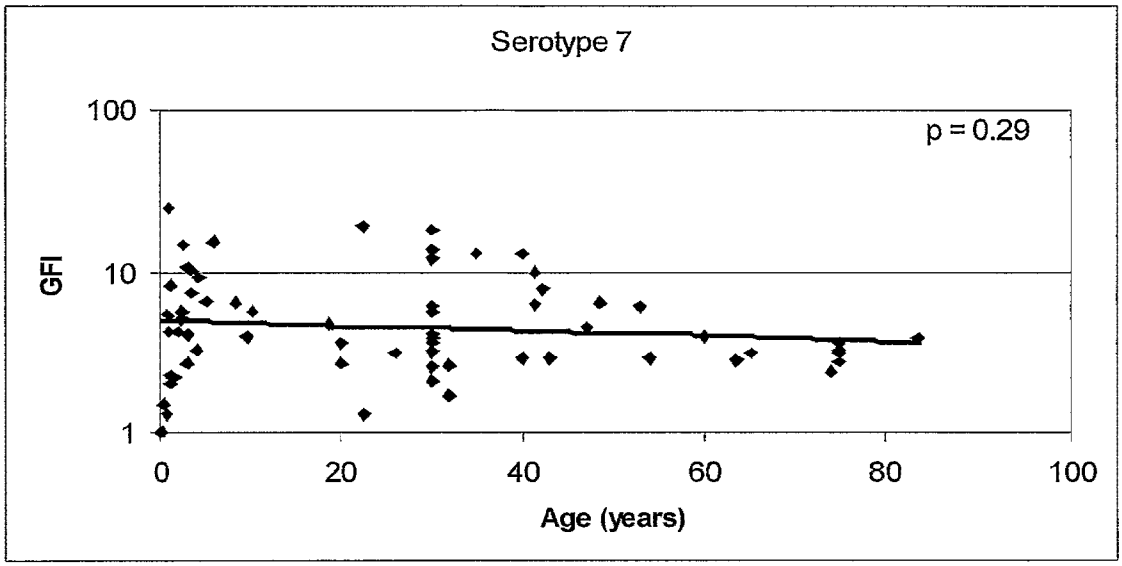
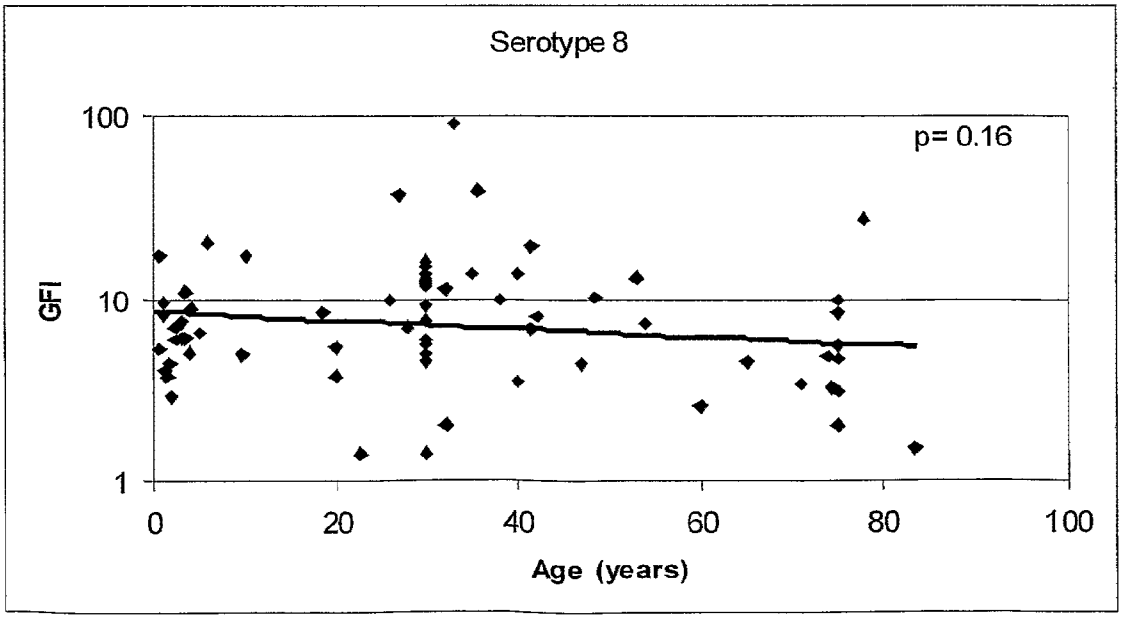


FIGURE 1F



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FIGURE 1G

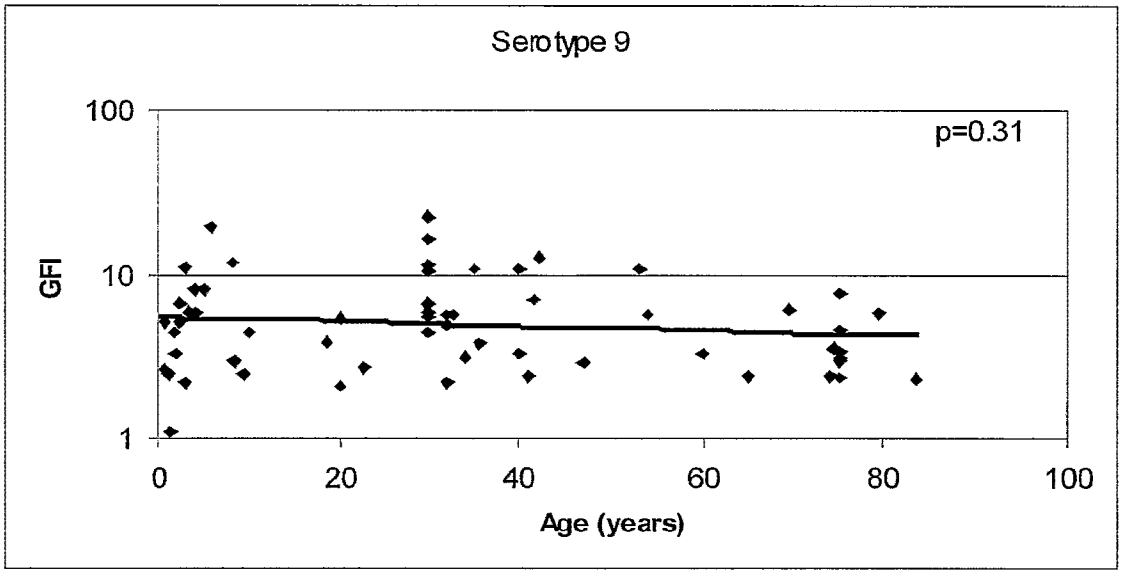
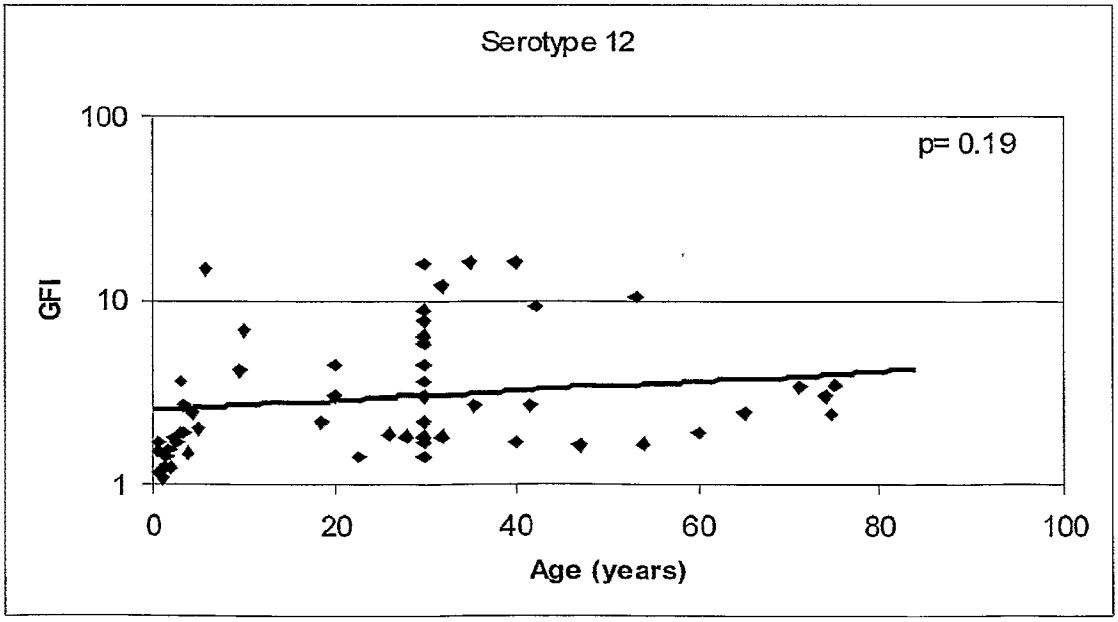


FIGURE 1H



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FIGURE 1I

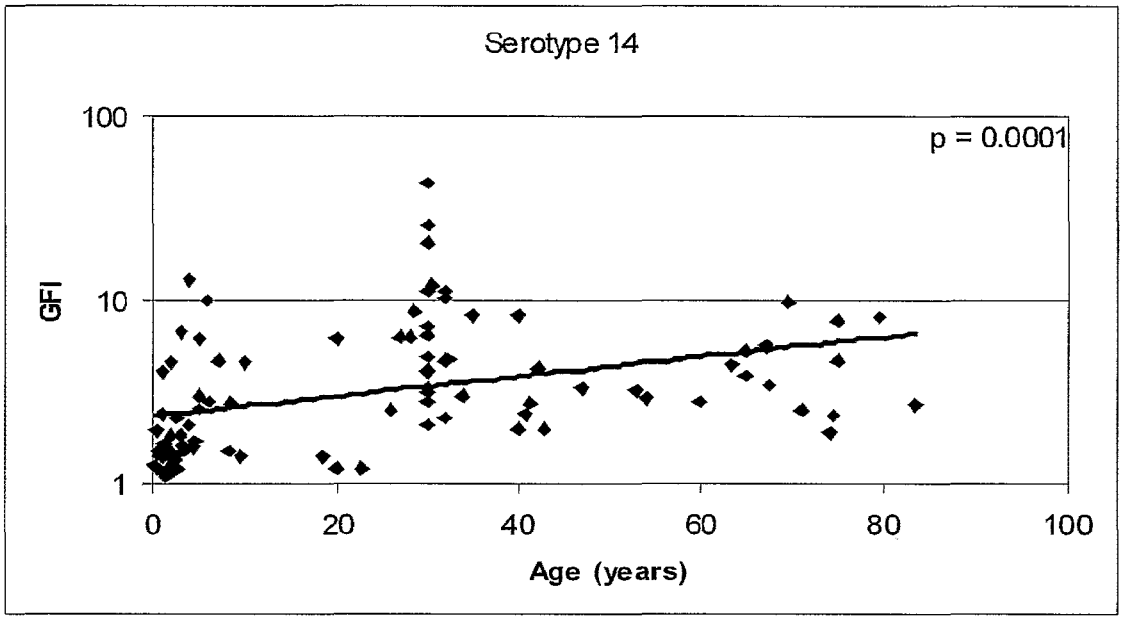
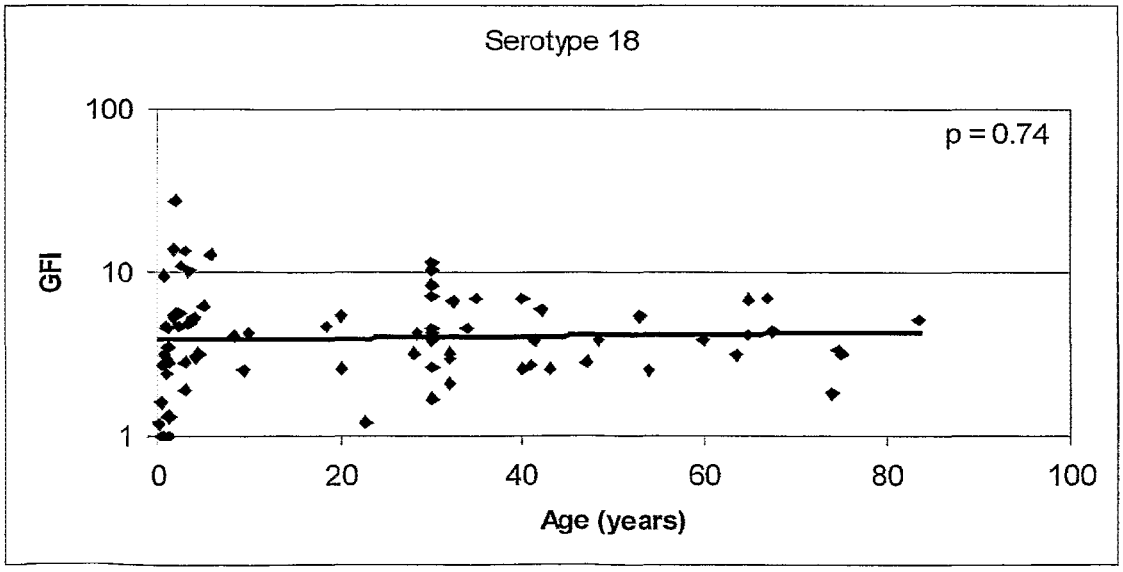


FIGURE 1J



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FIGURE 1K

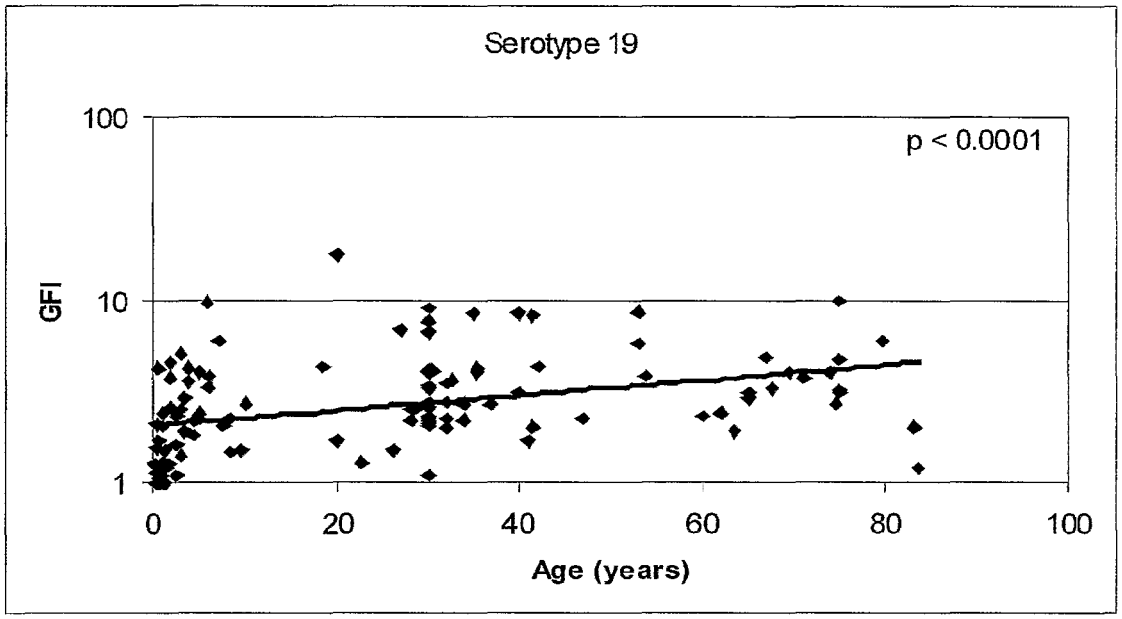
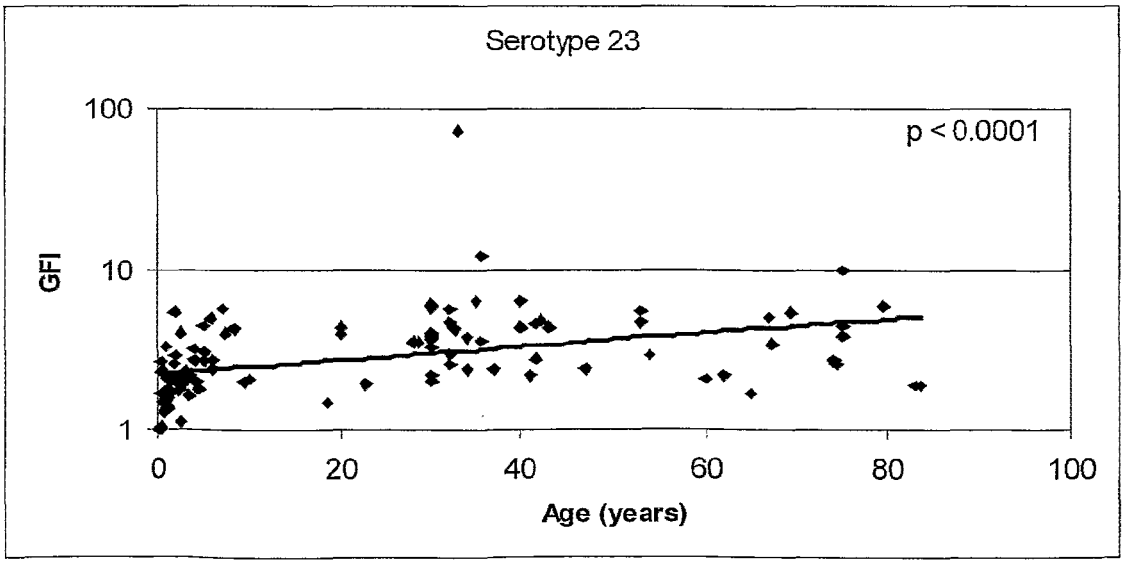


FIGURE 1L



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Figure 2: Geometric mean IgG concentration (μg/ml) and Opsonic Titre on day 14 Post II after immunisation of adult rats with 1.0 μg PS-PD alone or combined in tetravalent, pentavalent, heptavalent or decavalent vaccine:

Serotypes Vaccines		1 D	3 P	4 H	5 D	6B T	7F D	9V H	14 T	18C H	19F T	23F T
Alone	[IgG]	8.3	0.5	9.3	12.6	0.11	15.7	2.8	80*	15	5.2	2.5
	Opsonic Titre	1600	48	1600	1600	<12	1200	800	16000	3000	800	200
Combined	[IgG]	11.6	1.0	4	4.9	0.23	2.2	10	2.2*	3.7	3.7	2.8
	Opsonic Titre	>1600	48	800	800	25	450	950	32000	1000	200	100

T: combined in tetravalent vaccine, 1.0 μg of each PS-PD
P: combined in pentavalent vaccine, 1.0 μg of each PS-PD
H: combined in heptavalent vaccine, 1.0 μg of each PS-PD
D: combined in decavalent vaccine, 1.0 μg of each PS-PD
* $p < 0.001$

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Figure 3: Geometric Mean IgG Concentration Vs. Dosage of PS, infant rats, day 14 PIII:

FIGURE 3A

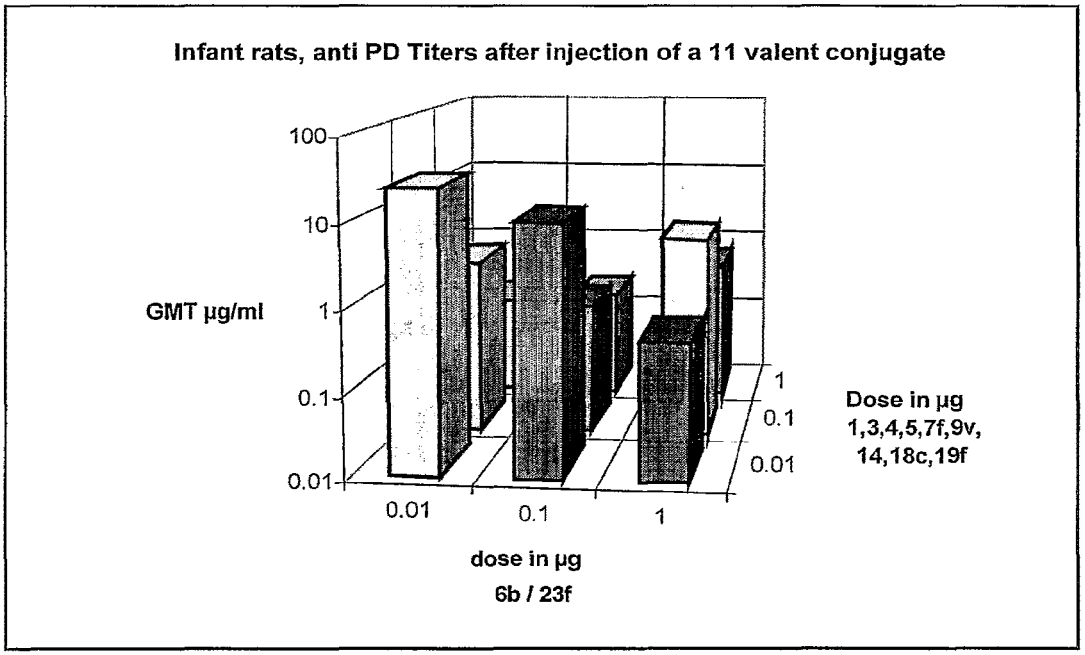
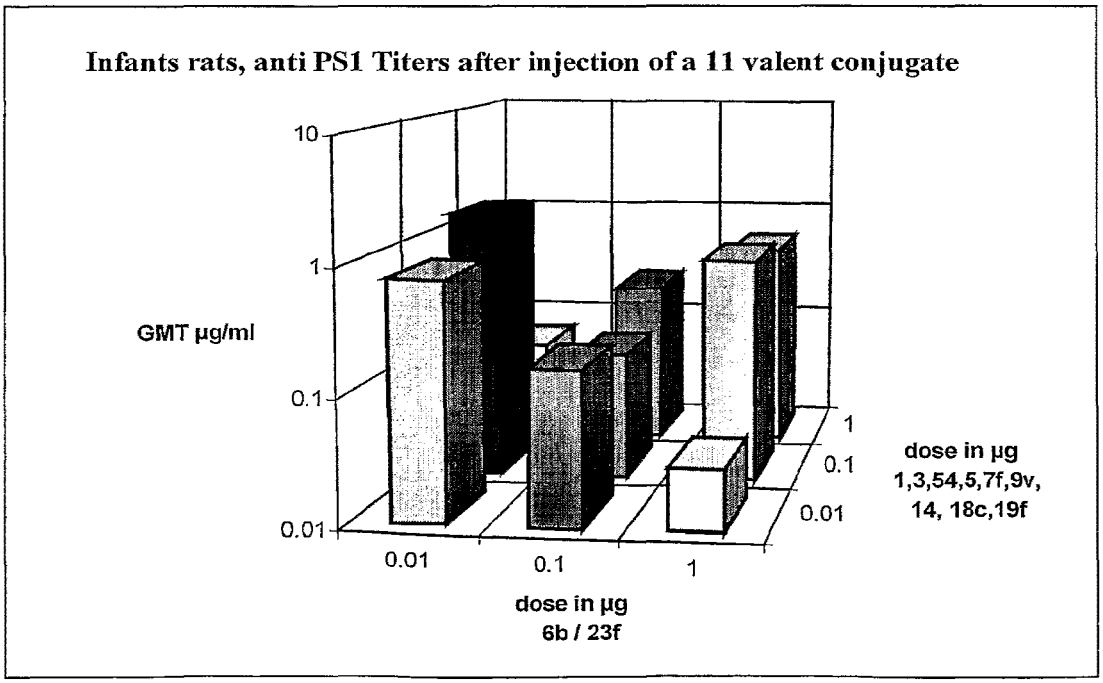


FIGURE 3B



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FIGURE 3C

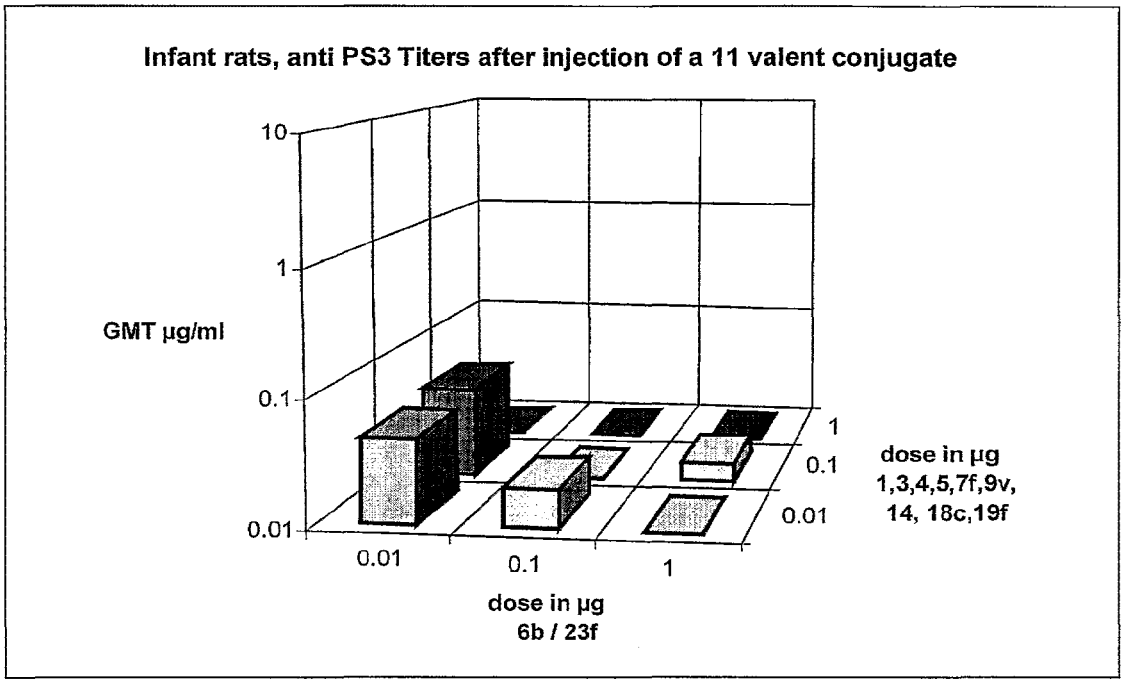
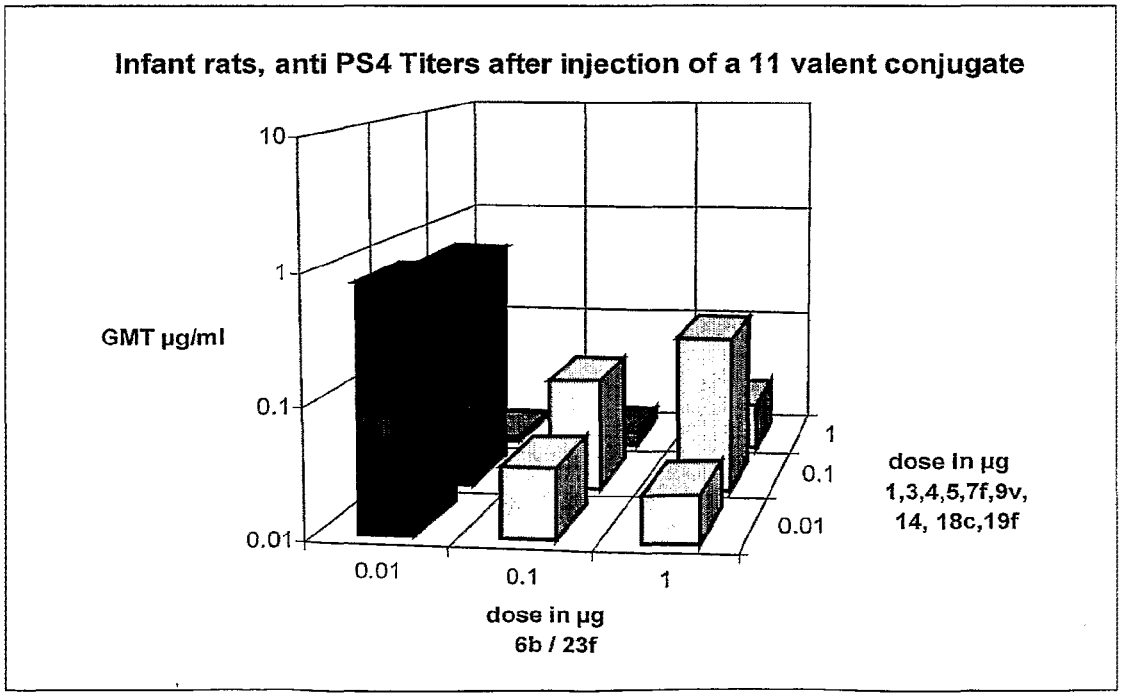


FIGURE 3D



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FIGURE 3E

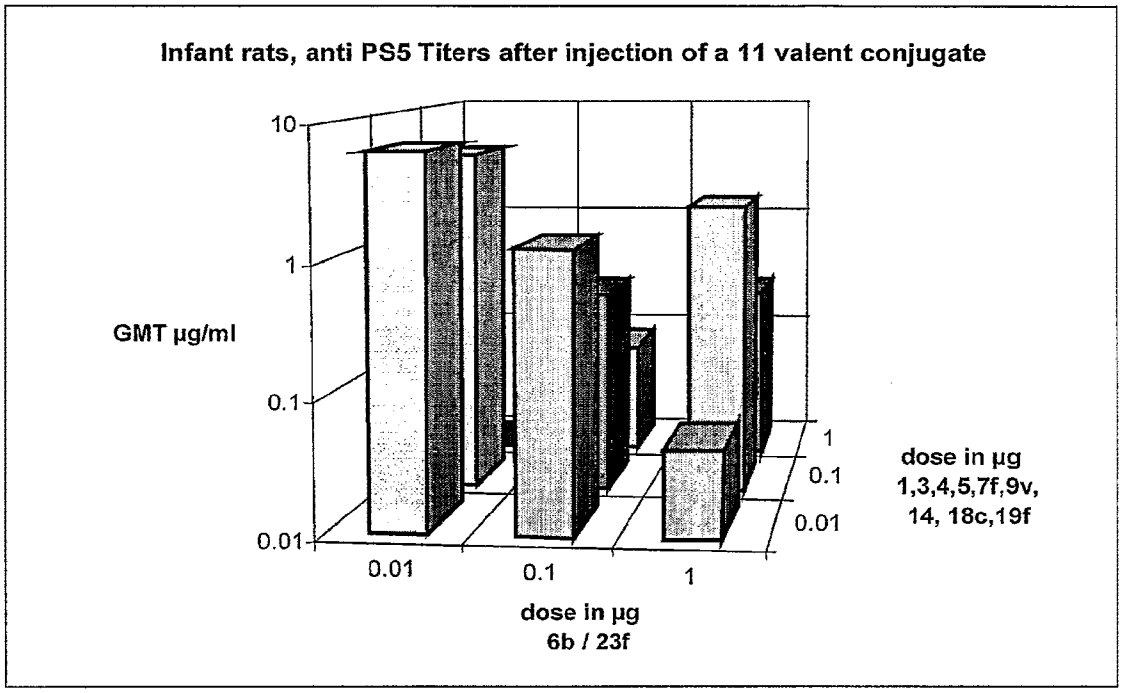
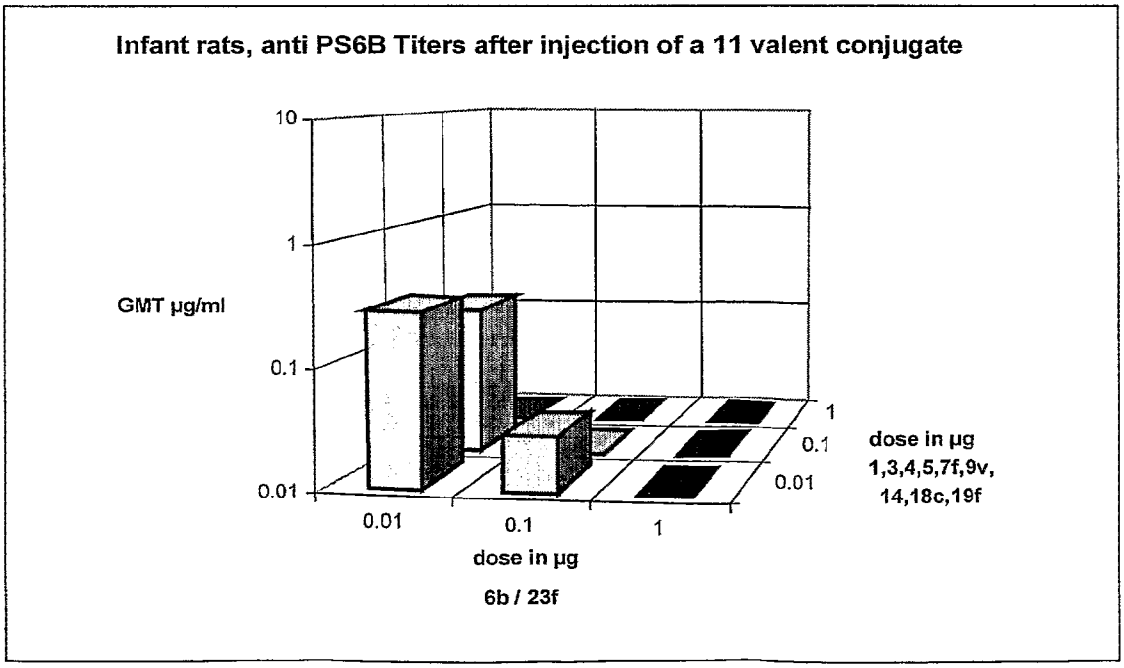


FIGURE 3F



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FIGURE 3G

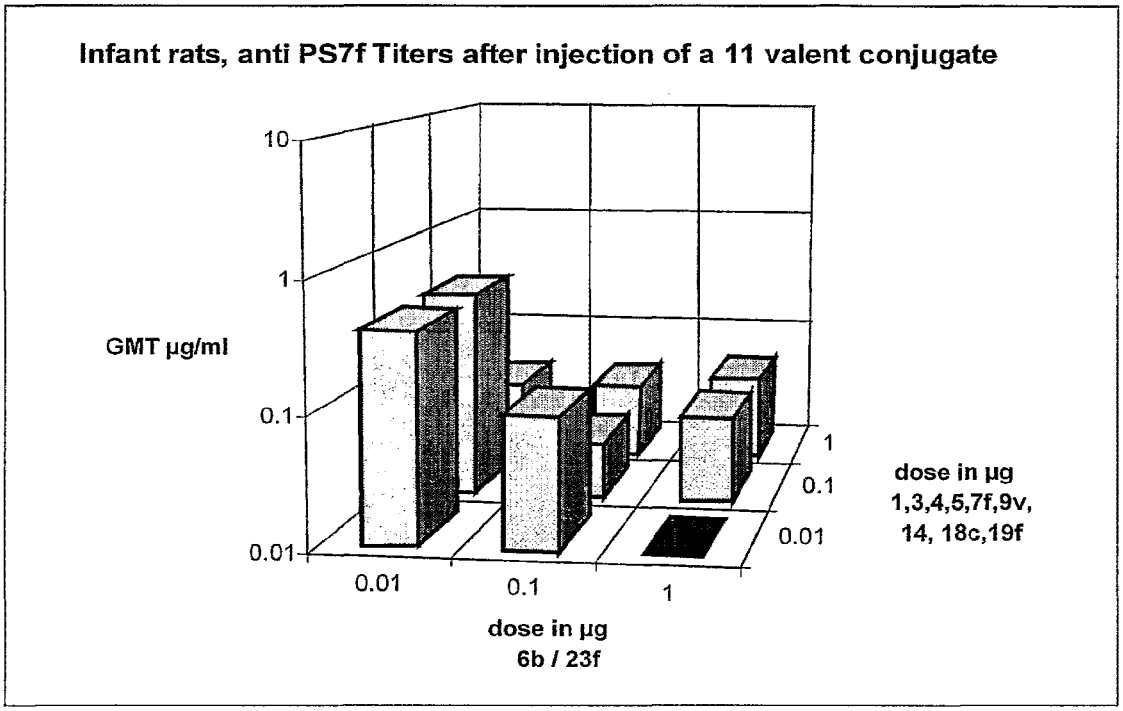
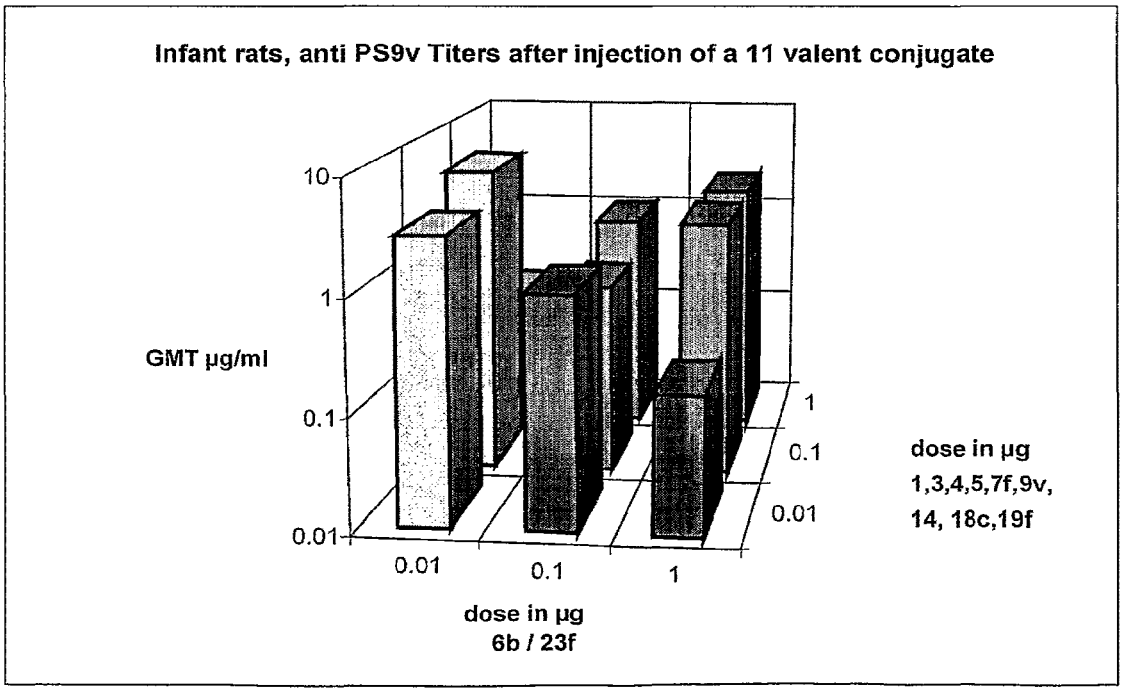


FIGURE 3H



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FIGURE 3I

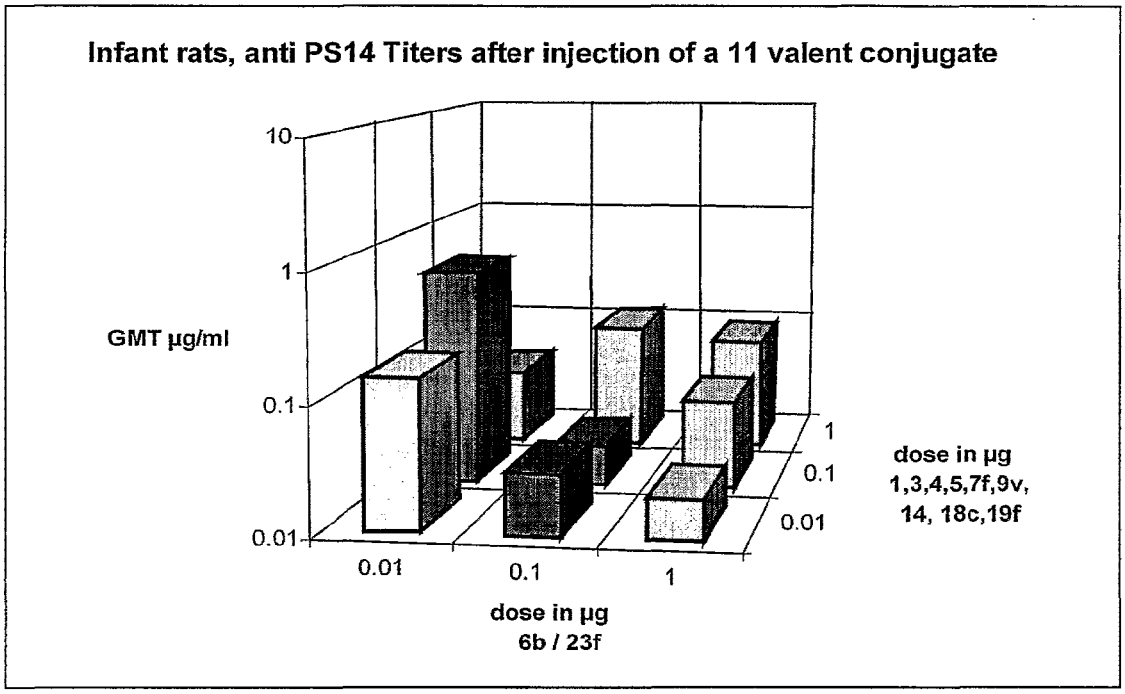
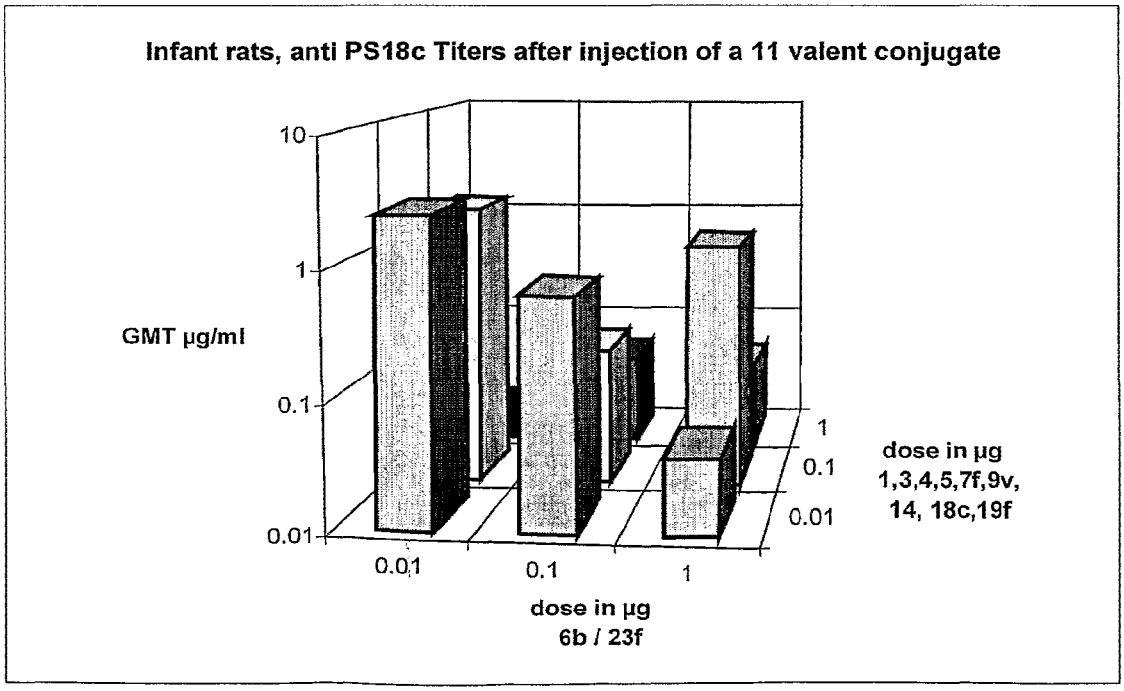


FIGURE 3J



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FIGURE 3K

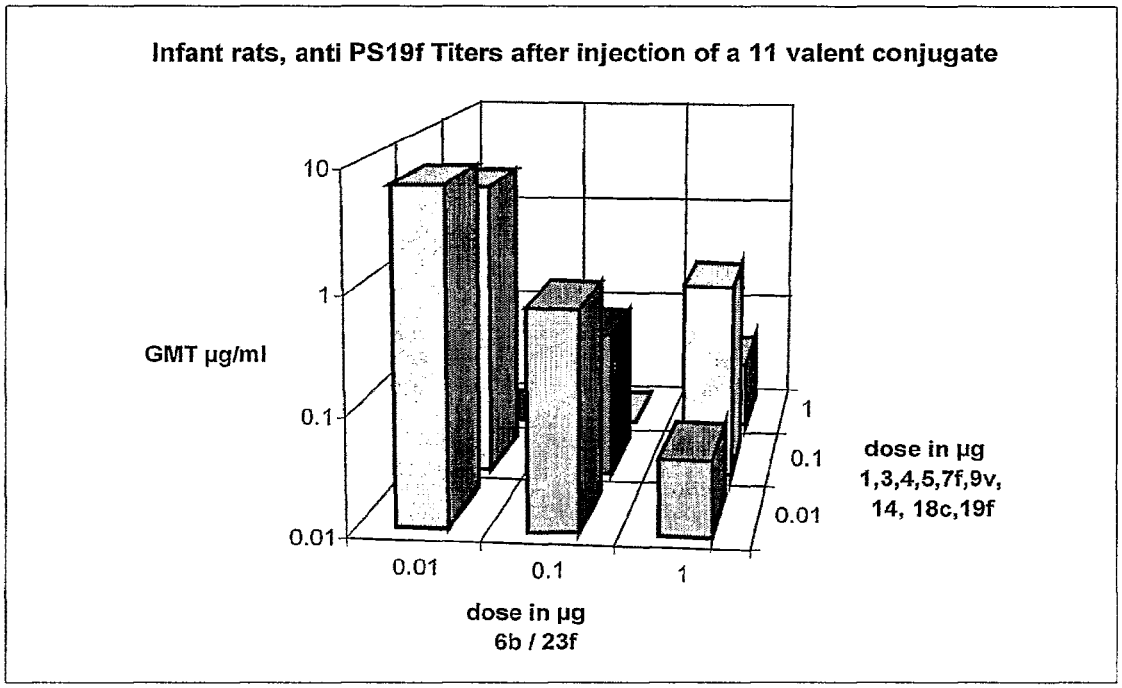
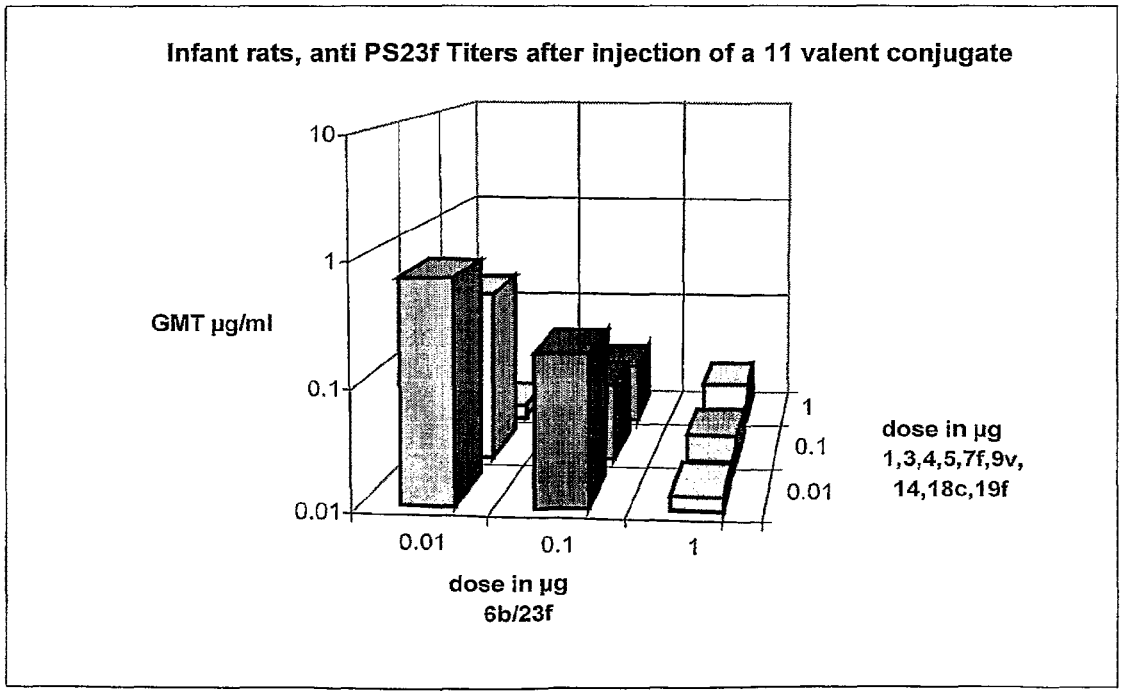


FIGURE 3L



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Figure 4: GM IgG Concentration vs Total PD Dosage in Infant rats, d14 PIII:

FIGURE 4A

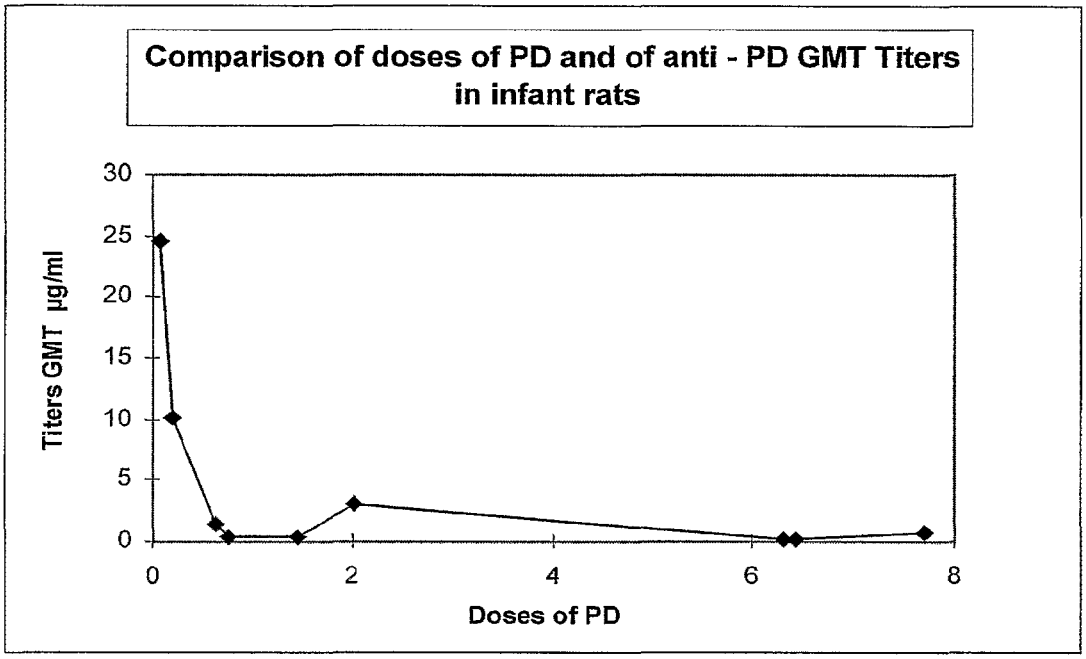
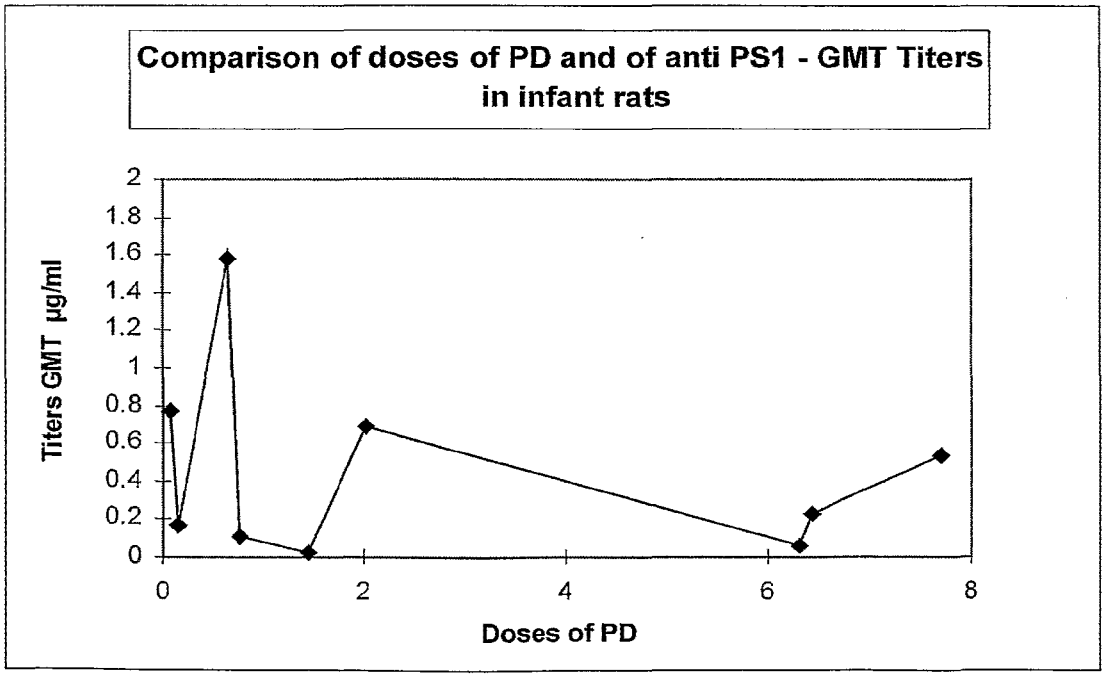


FIGURE 4B



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FIGURE 4C

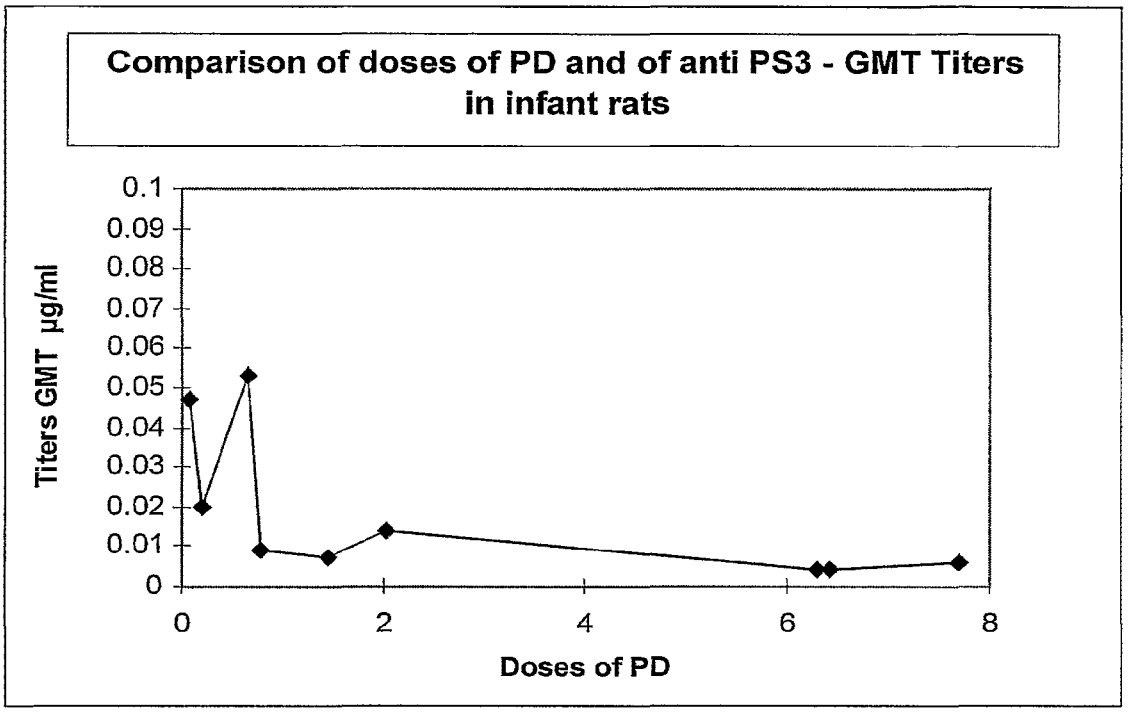
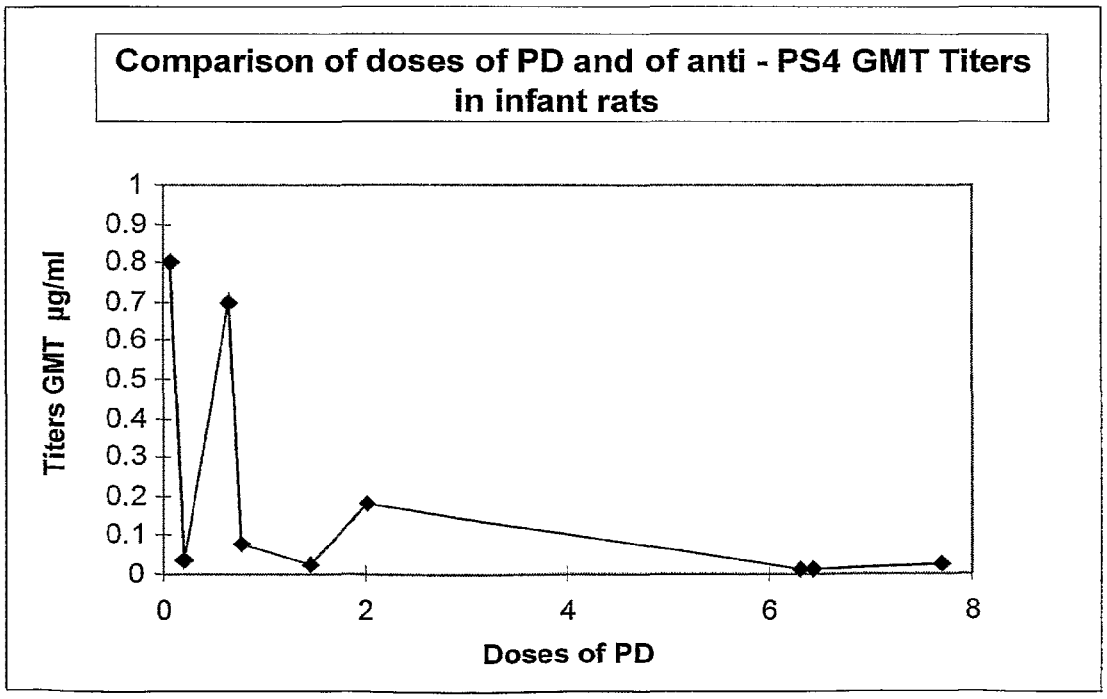


FIGURE 4D



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FIGURE 4E

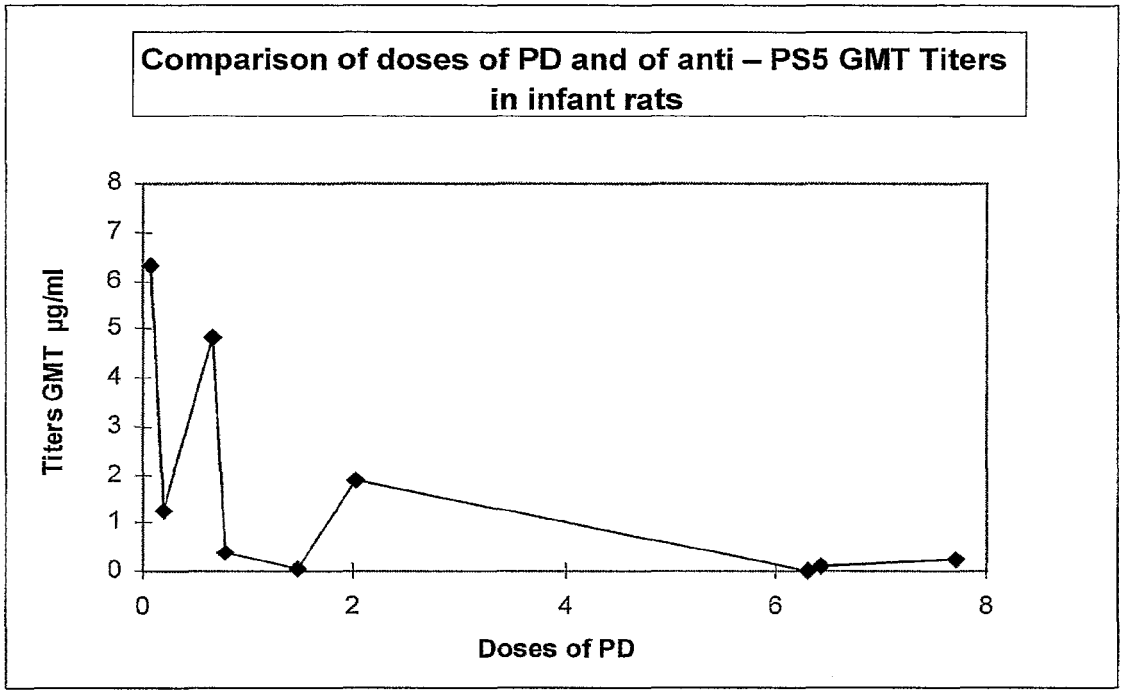
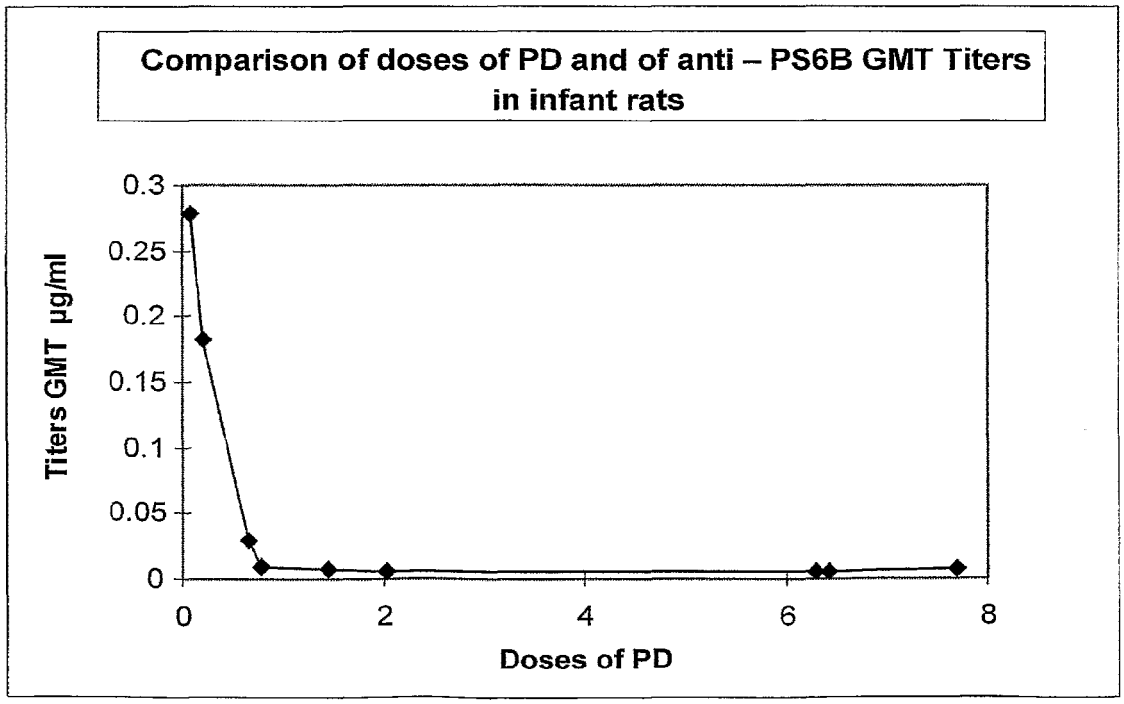


FIGURE 4F



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FIGURE 4G

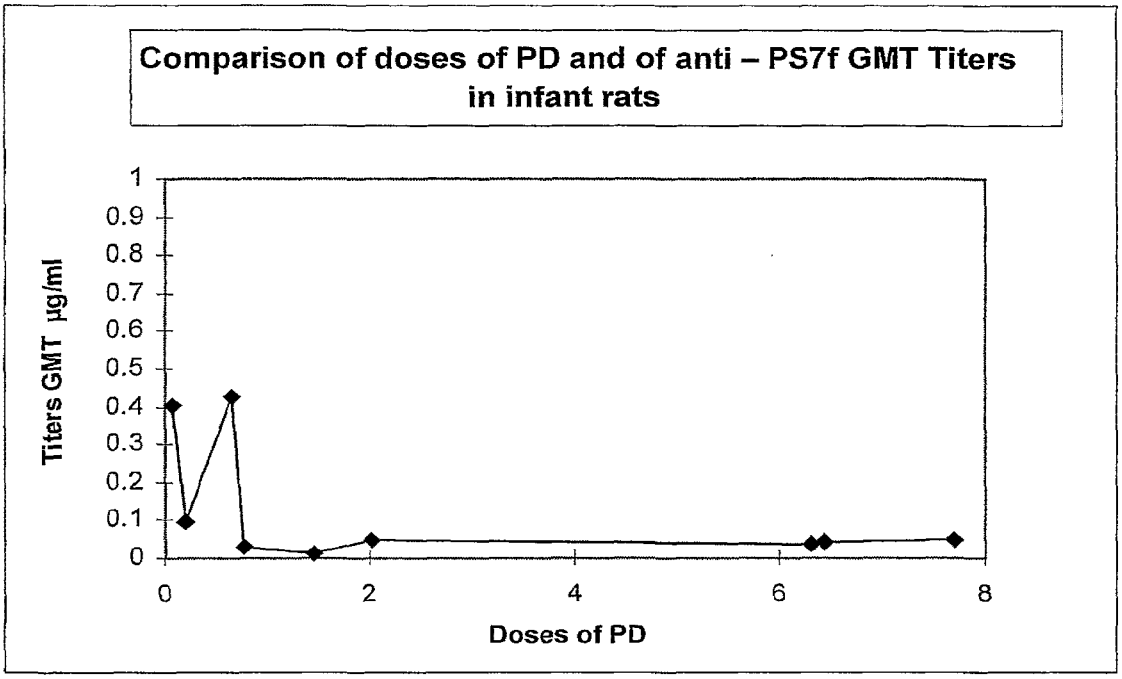
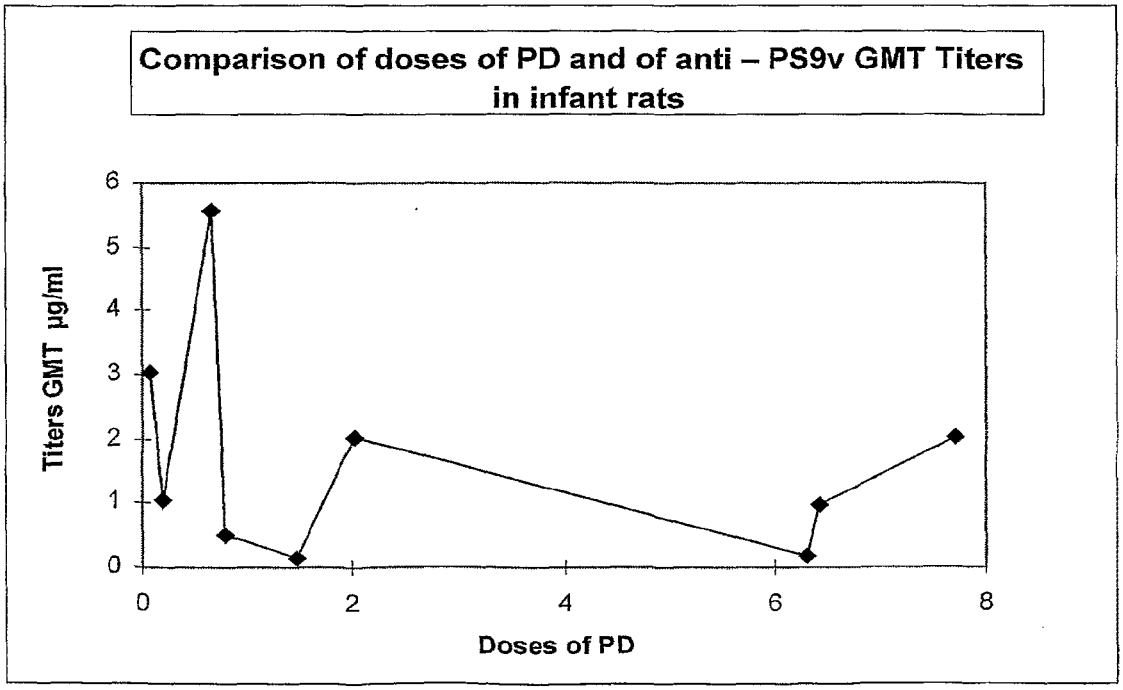


FIGURE 4H



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FIGURE 4I

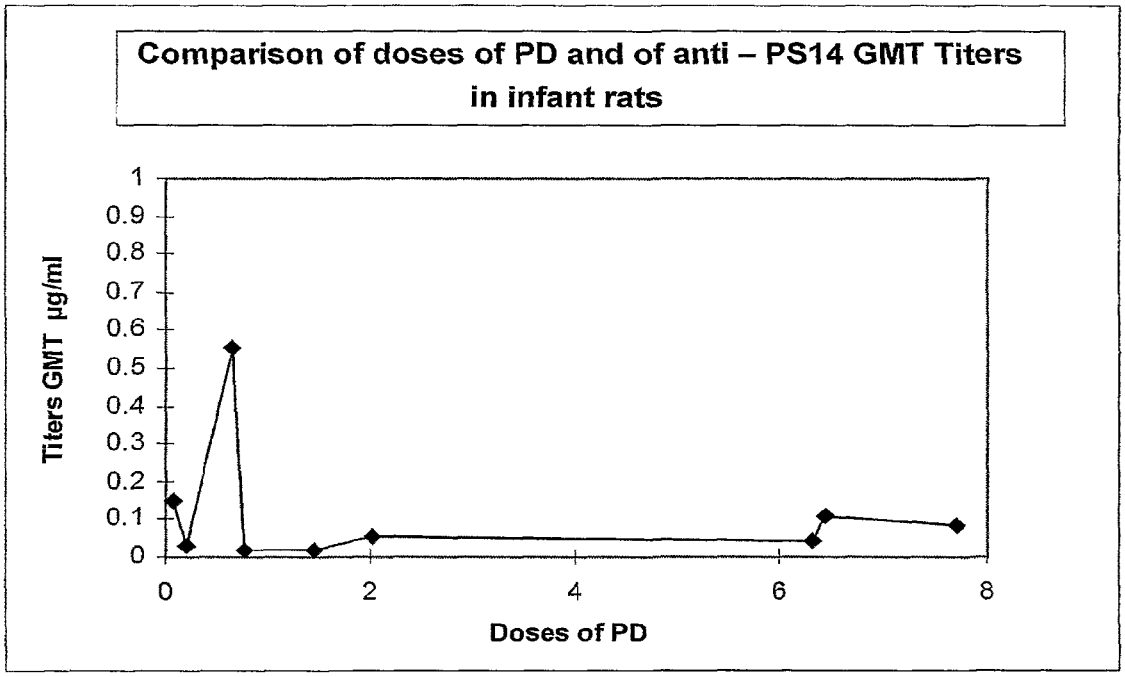
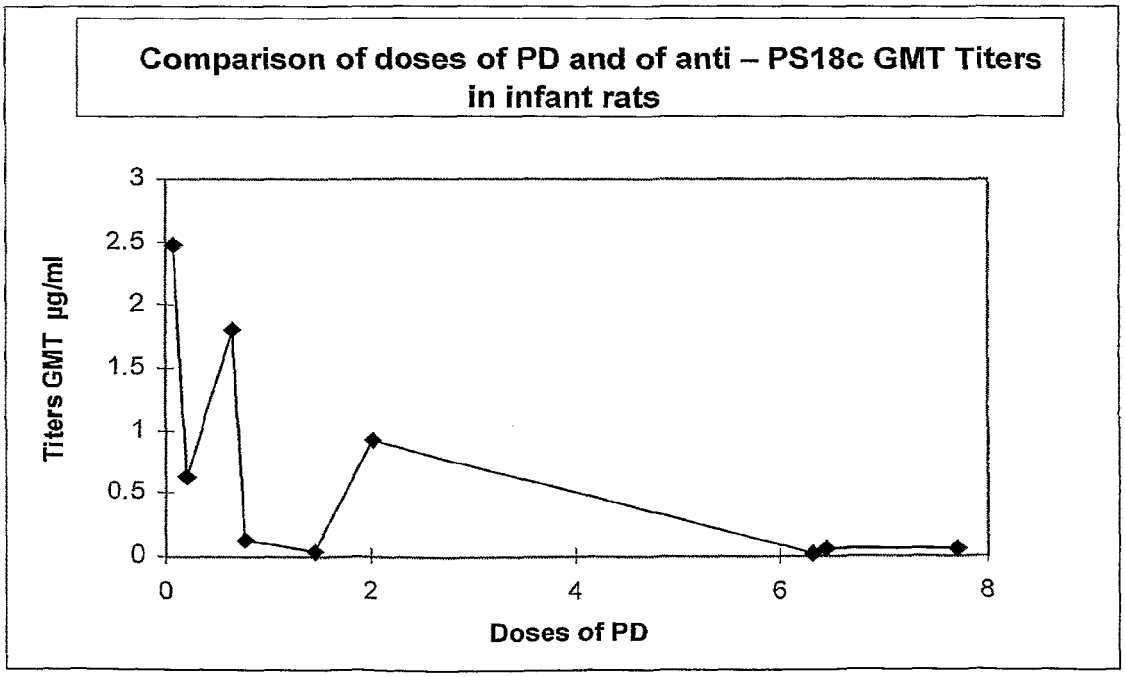


FIGURE 4J



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FIGURE 4K

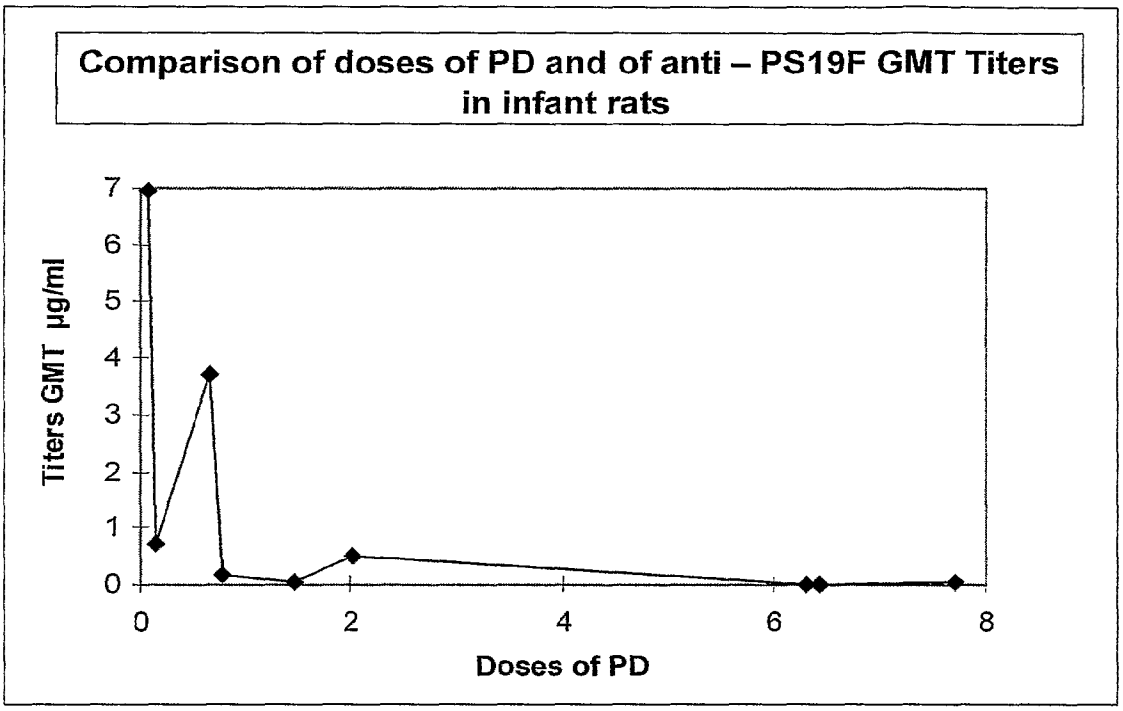
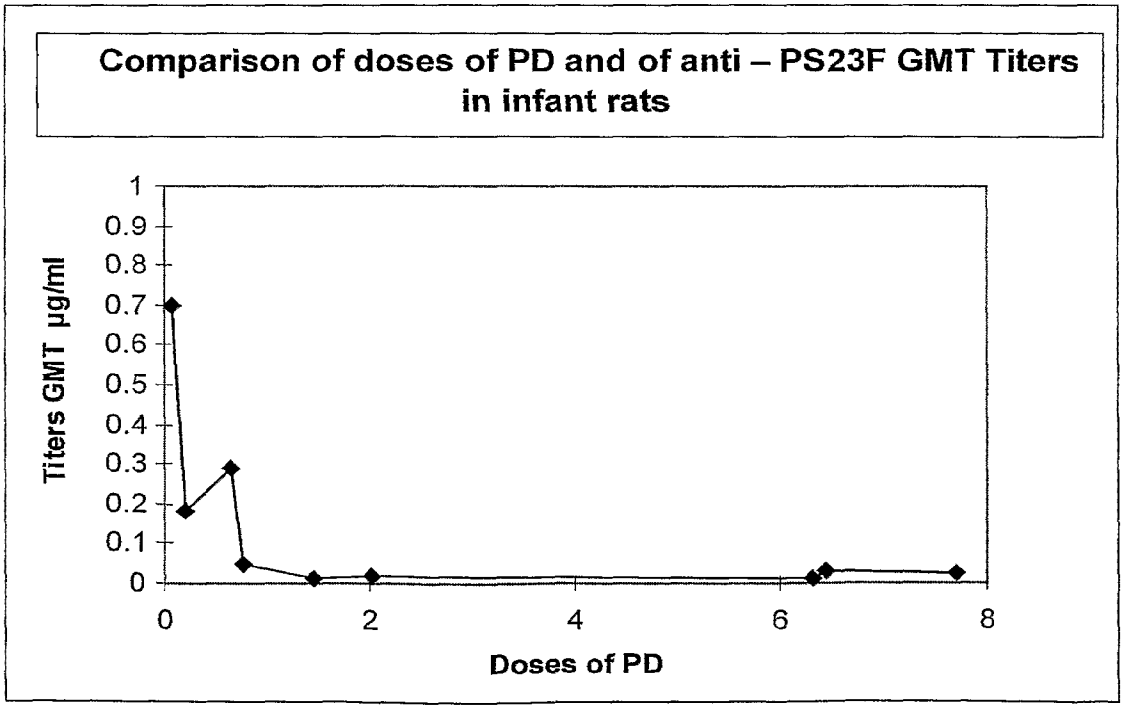


FIGURE 4L



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(54) Title: VACCINE

(57) Abstract: The present invention is in the field of pneumococcal capsular saccharide conjugate vaccines. Specifically, a multivalent Streptococcus *pneumoniae* immunogenic composition is provided with various conjugated capsular saccharides from different *S. pneumoniae* serotypes conjugated to 2 or more different carrier proteins, where the composition comprises serotype 19F capsular saccharide conjugated to diphtheria toxoid (DT) or CRM197, optionally wherein 19F is the only saccharide in the composition conjugated to diphtheria toxoid (DT) or CRM197.

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Field of the Invention

The present invention relates to an improved *Streptococcus pneumoniae* vaccine.

Background of the Invention

Children less than 2 years of age do not mount an immune response to most polysaccharide vaccines, so it has been necessary to render the polysaccharides immunogenic by chemical conjugation to a protein carrier. Coupling the polysaccharide, a T-independent antigen, to a protein, a T-dependent antigen, confers upon the polysaccharide the properties of T dependency including isotype switching, affinity maturation, and memory induction.

However, there can be issues with repeat administration of polysaccharide-protein conjugates, or the combination of polysaccharide-protein conjugates to form multivalent vaccines. For example, it has been reported that a *Haemophilus influenzae* type b polysaccharide (PRP) vaccine using tetanus toxoid (TT) as the protein carrier was tested in a dosage-range with simultaneous immunization with (free) TT and a pneumococcal polysaccharide-TT conjugate vaccine following a standard infant schedule. As the dosage of the pneumococcal vaccine was increased, the immune response to the PRP polysaccharide portion of the Hib conjugate vaccine was decreased, indicating immune interference of the polysaccharide, possibly via the use of the same carrier protein (Dagan *et al.*, Infect Immun. (1998); 66: 2093-2098)

The effect of the carrier-protein dosage on the humoral response to the protein itself has also proven to be multifaceted. In human infants it was reported that increasing the dosage of a tetravalent tetanus toxoid conjugate resulted in a decreased response to the tetanus carrier (Dagan *et al.* supra). Classical analysis of these effects of combination vaccines have been described as carrier induced epitopic suppression, which is not fully understood, but believed to result from an excess amount of carrier protein (Fattom, Vaccine 17: 126 (1999)). This appears to result in competition for Th-cells, by the B-cells to the carrier protein, and B-cells to the polysaccharide. If the B-cells to the carrier protein predominate, there are not enough Th-cells available to provide the necessary help for the B-cells specific to the polysaccharide. However, the observed immunological effects have been inconsistent, with the total amount of carrier protein in some instances increasing the immune response, and in other cases diminishing the immune response.

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Hence there remain technical difficulties in combining multiple polysaccharide conjugates into a single, efficacious, vaccine formulation.

Streptococcus pneumoniae is a Gram-positive bacterium responsible for considerable morbidity and mortality (particularly in the young and aged), causing invasive diseases such as pneumonia, bacteraemia and meningitis, and diseases associated with colonisation, such as acute Otitis media. The rate of pneumococcal pneumonia in the US for persons over 60 years of age is estimated to be 3 to 8 per 100,000. In 20% of cases this leads to bacteraemia, and other manifestations such as meningitis, with a mortality rate close to 30% even with antibiotic treatment.

Pneumococcus is encapsulated with a chemically linked polysaccharide which confers serotype specificity. There are 90 known serotypes of pneumococci, and the capsule is the principle virulence determinant for pneumococci, as the capsule not only protects the inner surface of the bacteria from complement, but is itself poorly immunogenic. Polysaccharides are T-independent antigens, and can not be processed or presented on MHC molecules to interact with T-cells. They can however, stimulate the immune system through an alternate mechanism which involves cross-linking of surface receptors on B cells.

It was shown in several experiments that protection against invasive pneumococci disease is correlated most strongly with antibody specific for the capsule, and the protection is serotype specific.

Streptococcus pneumoniae is the most common cause of invasive bacterial disease and Otitis media in infants and young children. Likewise, the elderly mount poor responses to pneumococcal vaccines [Roghmann *et al.*, (1987), J. Gerontol. 42:265-270], hence the increased incidence of bacterial pneumonia in this population [Verghese and Berk, (1983) Medicine (Baltimore) 62:271-285].

The major clinical syndromes caused by *S. pneumoniae* are widely recognized and discussed in all standard medical textbooks (Fedson DS, Muscher DM. In: Plotkin SA, Orenstein WA, editors. Vaccines. 4rth edition. PhiladelphiaWB Saunders Co, 2004a: 529-588). For instance, Invasive pneumococcal disease (IPD) is defined as any infection in which *S. pneumoniae* is isolated from the blood or another normally sterile site (Musher DM. Streptococcus pneumoniae. In Mandell GL, Bennett JE, Dolin R (eds). Principles and Practice of Infectious diseases (5th ed). New York, Churchill Livingstone, 2001, p2128-2147). Chronic obstructive pulmonary disease (COPD) is recognised as encompassing several conditions (airflow obstruction, chronic bronchitis, bronchiolitis or small airways disease and emphysema) that often coexist. Patients suffer exacerbations of their

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condition that are usually associated with increased breathlessness, and often have increased cough that may be productive of mucus or purulent sputum (Wilson, Eur Respir J 2001 17:995-1007). COPD is defined physiologically by the presence of irreversible or partially reversible airway obstruction in patients with chronic bronchitis and/or emphysema (Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. American Thoracic Society. Am J Respir Crit Care Med. 1995 Nov;152(5 Pt 2):S77-121). Exacerbations of COPD are often caused by bacterial (e.g. pneumococcal) infection (Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clin Microbiol Rev. 2001 Apr;14(2):336-63).

It is thus an object of the present invention to develop an improved formulation of a multiple serotype *Streptococcus pneumoniae* polysaccharide conjugate vaccine.

Brief description of Figures

Figure 1 Bar chart showing 11 valent conjugate immunogenicity in elderly Rhesus monkeys. The lighter bars represent the GMC after two inoculations with 11 valent conjugate in aluminium phosphate adjuvant. The darker bars represent the GMC after two inoculations with 11 valent conjugate in adjuvant C.

Figure 2 Bar chart showing memory B cells for PS3 after inoculation with the 11 valent conjugate in adjuvant C or aluminium phosphate adjuvant.

Figure 3 Bar chart showing anti polysaccharide 19F immunogenicity in Balb/C mice for the 4-valent plain polysaccharides and the 4-valent dPly conjugates.

Figure 4 Bar chart showing anti polysaccharide 22F immunogenicity in Balb/C mice for the 4-valent plain polysaccharides and the 4-valent PhtD conjugates.

Figure 5 Bar chart showing anti-22F IgG response in Balb/c mice

Figure 6 Bar chart showing anti-22F opsono-phagocytosis titres in Balb/c mice.

Figure 7 Bar chart comparing IgG responses induced in young C57B1 mice after immunisation with 13 Valent conjugate vaccine formulated in different adjuvants.

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Figure 8 Bar chart showing the protective efficacy of different vaccine combinations in a monkey pneumonia model.

Figure 9 Bar chart showing anti PhtD IgG response in Balb/c mice after immunisation with 22F-PhtD or 22F-AH-PhtD conjugates.

Figure 10 Protection against type 4 pneumococcal challenge in mice after immunisation with 22F-PhtD or 22F-AH-PhtD.

Description of the Invention

The present invention provides an improved *Streptococcus pneumoniae* vaccine comprising 10 or more (e.g. 11, 12, 13, 14, or 15 or more) capsular saccharides from different *S. pneumoniae* serotypes conjugated to 2 or more carrier proteins, wherein the vaccine comprises serotype 19F capsular saccharide conjugated to diphtheria toxoid or CRM197, and wherein the vaccine optionally further comprises protein D from *Haemophilus influenzae* as free protein or as a further carrier protein or both.

For the purposes of this invention, "immunizing a human host against exacerbations of COPD" or "treatment or prevention of exacerbations of COPD" or "reduction in severity of COPD exacerbations" refers to a reduction in incidence or rate of COPD exacerbations (for instance a reduction in rate of 0.1, 0.5, 1, 2, 5, 10, 20% or more) or a reduction in severity of COPD exacerbations as defined above, for instance within a patient group immunized with the compositions or vaccines of the invention.

Typically the *Streptococcus pneumoniae* vaccine of the present invention will comprise capsular saccharide antigens (preferably conjugated), wherein the saccharides are derived from at least ten serotypes of *S. pneumoniae*. The number of *S. pneumoniae* capsular saccharides can range from 10 different serotypes (or "V", valences) to 23 different serotypes (23V). In one embodiment there are 10, 11, 12, 13, 14 or 15 different serotypes. In another embodiment of the invention, the vaccine may comprise conjugated *S. pneumoniae* saccharides and unconjugated *S. pneumoniae* saccharides. Preferably, the total number of saccharide serotypes is less than or equal to 23. For example, the invention may comprise 10 conjugated serotypes and 13 unconjugated saccharides. In a

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similar manner, the vaccine may comprise 11, 12, 13, 14, 15 or 16 conjugated saccharides and 12, 11, 10, 9, 8 or 7, respectively, unconjugated saccharides.

In one embodiment the multivalent pneumococcal vaccine of the invention will be selected from the following serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F, although it is appreciated that one or two other serotypes could be substituted depending on the age of the recipient receiving the vaccine and the geographical location where the vaccine will be administered, e.g. serotype 6A may be included on the list. For example, an 10-valent vaccine may comprise polysaccharides from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. An 11-valent vaccine may also include saccharides from serotype 3. A 12 or 13-valent paediatric (infant) vaccine may also include the 10 or 11 valent formulation supplemented with serotypes 6A and 19A, or 6A and 22F, or 19A and 22F, or 6A and 15B, or 19A and 15B, or 22F and 15B, whereas a 13-valent elderly vaccine may include the 11 valent formulation supplemented with serotypes 19A and 22F, 8 and 12F, or 8 and 15B, or 8 and 19A, or 8 and 22F, or 12F and 15B, or 12F and 19A, or 12F and 22F, or 15B and 19A, or 15B and 22F. A 14 valent paediatric vaccine may include the 10 valent formulation described above supplemented with serotypes 3, 6A, 19A and 22F; serotypes 6A, 8, 19A and 22F; serotypes 6A, 12F, 19A and 22F; serotypes 6A, 15B, 19A and 22F; serotypes 3, 8, 19A and 22F; serotypes 3, 12F, 19A and 22F; serotypes 3, 15B, 19A and 22F; serotypes 3, 6A, 8 and 22F; serotypes 3, 6A, 12F and 22F; or serotypes 3, 6A, 15B and 22F.

The composition in one embodiment includes capsular saccharides derived from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F (preferably conjugated). In a further embodiment of the invention at least 11 saccharide antigens (preferably conjugated) are included, for example capsular saccharides derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F. In a further embodiment of the invention, at least 12 or 13 saccharide antigens are included, for example a vaccine may comprise capsular saccharides derived from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F or capsular saccharides derived from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F, although further saccharide antigens, for example 23 valent (such as serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F), are also contemplated by the invention.

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The vaccine of the present invention may comprise protein D (PD) from *Haemophilus influenzae* (see e.g. EP 0594610). *Haemophilus influenzae* is a key causative organism of otitis media, and the present inventors have shown that including this protein in a *Streptococcus pneumoniae* vaccine will provide a level of protection against *Haemophilus influenzae* related otitis media (reference POET publication). In one embodiment, the vaccine composition comprises protein D. In one aspect, PD is present as a carrier protein for one or more of the saccharides. In another aspect, protein D could be present in the vaccine composition as a free protein. In a further aspect, protein D is present both as a carrier protein and as free protein. Protein D may be used as a full length protein or as a fragment (WO0056360). In a further aspect, protein D is present as a carrier protein for the majority of the saccharides, for example 6, 7, 8, 9 or more of the saccharides may be conjugated to protein D. In this aspect, protein D may also be present as free protein.

The vaccine of the present invention comprises two or more different types of carrier protein. Each type of carrier protein may act as carrier for more than one saccharide, which saccharides may be the same or different. For example, serotypes 3 and 4 may be conjugated to the same carrier protein, either to the same molecule of carrier protein or to different molecules of the same carrier protein. In one embodiment, two or more different saccharides may be conjugated to the same carrier protein, either to the same molecule of carrier protein or to different molecules of the same carrier protein.

Each *Streptococcus pneumoniae* capsular saccharide may be conjugated to a carrier protein independently selected from the group consisting of TT, DT, CRM197, fragment C of TT, PhtD, PhtDE fusions (particularly those described in WO 01/98334 and WO 03/54007), detoxified pneumolysin and protein D, other than saccharide from serotype 19F which is always conjugated to DT or CRM 197, preferably DT. A more complete list of protein carriers that may be used in the conjugates of the invention is presented below

If the protein carrier is the same for 2 or more saccharides in the composition, the saccharides could be conjugated to the same molecule of the protein carrier (carrier molecules having 2 more different saccharides conjugated to it) [see for instance WO 04/083251]. Alternatively the saccharides may each be separately conjugated to different molecules of the protein carrier (each molecule of protein carrier only having one type of saccharide conjugated to it).

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The carrier protein conjugated to one or more of the *S. pneumoniae* capsular saccharides in the conjugates present in the immunogenic compositions of the invention is optionally a member of the polyhistidine triad family (Pht) proteins, fragments or fusion proteins thereof. The PhtA, PhtB, PhtD or PhtE proteins may have an amino acid sequence sharing 80%, 85%, 90%, 95%, 98%, 99% or 100% identity with a sequence disclosed in WO 00/37105 or WO 00/39299 (e.g. with amino acid sequence 1-838 or 21-838 of SEQ ID NO: 4 of WO 00/37105 for PhtD). For example, fusion proteins are composed of full length or fragments of 2, 3 or 4 of PhtA, PhtB, PhtD, PhtE. Examples of fusion proteins are PhtA/B, PhtA/D, PhtA/E, PhtB/A, PhtB/D, PhtB/E. PhtD/A. PhtD/B, PhtD/E, PhtE/A, PhtE/B and PhtE/D, wherein the proteins are linked with the first mentioned at the N-terminus (see for example WO01/98334).

Where fragments of Pht proteins are used (separately or as part of a fusion protein), each fragment optionally contains one or more histidine triad motif(s) and/or coiled coil regions of such polypeptides. A histidine triad motif is the portion of polypeptide that has the sequence HxxHxH where H is histidine and x is an amino acid other than histidine. A coiled coil region is a region predicted by "Coils" algorithm Lupus, A et al (1991) Science 252; 1162-1164. In an embodiment the or each fragment includes one or more histidine triad motif as well as at least one coiled coil region. In an embodiment, the or each fragment contains exactly or at least 2, 3, 4 or 5 histidine triad motifs (optionally, with native Pht sequence between the 2 or more triads, or intra-triad sequence that is more than 50, 60, 70, 80, 90 or 100 % identical to a native pneumococcal intra-triad Pht sequence – e.g. the intra-triad sequence shown in SEQ ID NO: 4 of WO 00/37105 for PhtD). In an embodiment, the or each fragment contains exactly or at least 2, 3 or 4 coiled coil regions. In an embodiment a Pht protein disclosed herein includes the full length protein with the signal sequence attached, the mature full length protein with the signal peptide (for example 20 amino acids at N-terminus) removed, naturally occurring variants of Pht protein and immunogenic fragments of Pht protein (e.g. fragments as described above or polypeptides comprising at least 15 or 20 contiguous amino acids from an amino acid sequence in WO00/37105 or WO00/39299 wherein said polypeptide is capable of eliciting an immune response specific for said amino acid sequence in WO00/37105 or WO00/39299).

In particular, the term "PhtD" as used herein includes the full length protein with the signal sequence attached, the mature full length protein with the signal peptide (for example 20

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amino acids at N-terminus) removed, naturally occurring variants of PhtD and immunogenic fragments of PhtD (e.g. fragments as described above or polypeptides comprising at least 15 or 20 contiguous amino acids from a PhtD amino acid sequence in WO00/37105 or WO00/39299 wherein said polypeptide is capable of eliciting an immune response specific for said PhtD amino acid sequence in WO00/37105 or WO00/39299 (e.g. SEQ ID NO: 4 of WO 00/37105 for PhtD).

If the protein carrier is the same for 2 or more saccharides in the composition, the saccharides could be conjugated to the same molecule of the protein carrier (carrier molecules having 2 more different saccharides conjugated to it) [see for instance WO 04/083251]. Alternatively the saccharides may each be separately conjugated to different molecules of the protein carrier (each molecule of protein carrier only having one type of saccharide conjugated to it).

Examples of carrier proteins which may be used in the present invention are DT (Diphtheria toxoid), TT (tetanus toxid) or fragment C of TT, DT CRM197 (a DT mutant) other DT point mutants, such as CRM176, CRM228, CRM 45 (Uchida et al J. Biol. Chem. 218; 3838-3844, 1973); CRM 9, CRM 45, CRM102, CRM 103 and CRM107 and other mutations described by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc, 1992; deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala 158 to Gly and other mutations disclosed in US 4709017 or US 4950740; mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in US 5917017 or US 6455673; or fragment disclosed in US 5843711, pneumococcal pneumolysin (Kuo et al (1995) Infect Immun 63; 2706-13) including ply detoxified in some fashion for example dPLY-GMBS (WO 04081515, PCT/EP2005/010258) or dPLY-formol, PhtX, including PhtA, PhtB, PhtD, PhtE and fusions of Pht proteins for example PhtDE fusions, PhtBE fusions (WO 01/98334 and WO 03/54007), (Pht A-E are described in more detail below) OMPC (meningococcal outer membrane protein – usually extracted from *N. meningitidis* serogroup B – EP0372501), PorB (from N. meningitidis), PD (Haemophilus influenzae protein D – see, e.g., EP 0 594 610 B), or immunologically functional equivalents thereof, synthetic peptides (EP0378881, EP0427347), heat shock proteins (WO 93/17712, WO 94/03208), pertussis proteins (WO 98/58668, EP0471177), cytokines, lymphokines, growth factors or hormones (WO 91/01146), artificial proteins comprising multiple human CD4+ T cell epitopes from various pathogen derived antigens (Falugi et al (2001) Eur J Immunol 31; 3816-3824) such as

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N19 protein (Baraldoi et al (2004) Infect Immun 72; 4884-7) pneumococcal surface protein PspA (WO 02/091998), iron uptake proteins (WO 01/72337), toxin A or B of *C. difficile* (WO 00/61761).

Nurkka *et al Pediatric Infectious Disease Journal. 23(11):1008-14, 2004 Nov.* described an 11 valent pneumococcal vaccine with all serotypes conjugated to PD. However, the present inventors have shown that opsonophagocytic activity was improved for antibodies induced with conjugates having 19F conjugated to DT compared with 19F conjugated to PD. In addition, the present inventors have shown that a greater cross reactivity to 19A is seen with 19F conjugated to DT. It is therefore a feature of the composition of the present invention that serotype 19F is conjugated to DT or CRM 197. In one aspect, serotype 19F is conjugated to DT. The remaining saccharide serotypes of the immunogenic composition may all be conjugated to one or more carrier proteins that are not DT (i.e. only 19F is conjugated to DT), or may be split between one or more carrier proteins that are not DT and DT itself. In one embodiment, 19F is conjugated to DT or CRM 197 and all of the remaining serotypes are conjugated to PD. In a further embodiment, 19F is conjugated to DT or CRM 197, and the remaining serotypes are split between PD, and TT or DT or CRM 197. In a further embodiment, 19F is conjugated to DT or CRM 197 and no more than one saccharide is conjugated to TT. In one aspect of this embodiment, said one saccharide is 18C or 12F. In a further embodiment, 19F is conjugated to DT or CRM 197 and no more than two saccharides are conjugated to TT. In a further embodiment, 19F is conjugated to DT or CRM 197, and the remaining serotypes are split between PD, TT and DT or CRM 197. In a further embodiment, 19F is conjugated to DT or CRM 197, and the remaining serotypes are split between PD, TT and pneumolysin. In a further embodiment, 19F is conjugated to DT or CRM 197, and the remaining serotypes are split between PD, TT and CRM 197. In a further embodiment, 19F is conjugated to DT or CRM197 and the remaining serotypes are split between PD, TT, pneumolysin and optionally PhtD or PhtD/E fusion protein. In a further embodiment, 19F is conjugated to DT or CRM197, 19A is conjugated to pneumolysin or TT, one (two or three) further saccharide(s) is conjugated to TT, one further saccharide is conjugated to PhtD or PhtD/E and all further saccharides are conjugated to PD. In a further embodiment 19F is conjugated to DT or CRM197, 19A is conjugated to pneumolysin , one (two or three) further saccharide(s) is conjugated to TT, one further saccharide is conjugated to pneumolysin, 2 further saccharides are conjugated to PhtD or PhtD/E and all further saccharides are conjugated to PD.

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In one embodiment, the immunogenic composition of the invention comprises protein D from *Haemophilus influenzae*. Within this embodiment, If PD is not one of the carrier proteins used to conjugate any saccharides other than 19F, for example 19F is conjugated to DT whilst the other serotypes are conjugated to one or more different carrier proteins which are not PD, then PD will be present in the vaccine composition as free protein. If PD is one of the carrier proteins used to conjugate saccharides other than 19F, then PD may optionally be present in the vaccine composition as free protein.

The term "saccharide" throughout this specification may indicate polysaccharide or oligosaccharide and includes both. Polysaccharides are isolated from bacteria and may be sized to some degree by known methods (see for example EP497524 and EP497525) and preferably by microfluidisation. Polysaccharides can be sized in order to reduce viscosity in polysaccharide samples and/or to improve filterability for conjugated products. Oligosaccharides have a low number of repeat units (typically 5-30 repeat units) and are typically hydrolysed polysaccharides

Capsular polysaccharides of *Streptococcus pneumoniae* comprise repeating oligosaccharide units which may contain up to 8 sugar residues. For a review of the oligosaccharide units for the key *Streptococcus pneumoniae* serotypes see JONES, Christopher. **Vaccines based on the cell surface carbohydrates of pathogenic bacteria**. *An. Acad. Bras. Ciênc.*, June 2005, vol.77, no.2, p.293-324. ISSN 0001-3765. In one embodiment, a capsular saccharide antigen may be a full length polysaccharide, however in others it may be one oligosaccharide unit, or a shorter than native length saccharide chain of repeating oligosaccharide units. In one embodiment, all of the saccharides present in the vaccine are polysaccharides. Full length polysaccharides may be "sized" i.e. their size may be reduced by various methods such as acid hydrolysis treatment, hydrogen peroxide treatment, sizing by emulsiflex® followed by a hydrogen peroxide treatment to generate oligosaccharide fragments or microfluidization.

The inventors have also noted that the focus of the art has been to use oligosaccharides for ease of conjugate production. The inventors have found that by using native or slightly sized polysaccharide conjugates, one or more of the following advantages may be realised: 1) a conjugate having high immunogenicity which is filterable, 2) the ratio of polysaccharide to protein in the conjugate can be altered such that the ratio of

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polysaccharide to protein (w/w) in the conjugate may be increased (which can have an effect on the carrier suppression effect), 3) immunogenic conjugates prone to hydrolysis may be stabilised by the use of larger saccharides for conjugation. The use of larger polysaccharides can result in more cross-linking with the conjugate carrier and may lessen the liberation of free saccharide from the conjugate. The conjugate vaccines described in the prior art tend to depolymerise the polysaccharides prior to conjugation in order to improve conjugation. The present inventors have found that saccharide conjugate vaccines retaining a larger size of saccharide can provide a good immune response against pneumococcal disease.

The immunogenic composition of the invention may thus comprise one or more saccharide conjugates wherein the average size (e.g. weight-average molecular weight; M_w) of each saccharide before conjugation is above 80kDa, 100kDa, 200kDa, 300kDa, 400kDa, 500kDa or 1000kDa. In one embodiment one or more saccharide conjugates of the invention should have an average size of saccharide pre-conjugation of 50-1600, 80-1400, 100-1000, 150-500, or 200-400 kDa (note that where average size is M_w , 'kDa' units should be replaced herein with '$x10^3$'). In one embodiment the conjugate post conjugation should be readily filterable through a 0.2 micron filter such that a yield of more than 50, 60, 70, 80, 90 or 95% is obtained post filtration compared with the pre filtration sample.

For the purposes of the invention, "native polysaccharide" refers to a saccharide that has not been subjected to a process (e.g. post-purification), the purpose of which is to reduce the size of the saccharide. A polysaccharide can become slightly reduced in size during normal purification procedures. Such a saccharide is still native. Only if the polysaccharide has been subjected to sizing techniques would the polysaccharide not be considered native.

For the purposes of the invention, "sized by a factor up to x2" means that the saccharide is subject to a process intended to reduce the size of the saccharide but to retain a size more than half the size of the native polysaccharide. X3, x4 etc. are to be interpreted in the same way i.e. the saccharide is subject to a process intended to reduce the size of the polysaccharide but to retain a size more than a third, a quarter etc. the size of the native polysaccharide.

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In an aspect of the invention, the immunogenic composition comprises *Streptococcus pneumoniae* saccharides from at least 10 serotypes conjugated to a carrier protein, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or each *S. pneumoniae* saccharide is native polysaccharide.

In an aspect of the invention, the immunogenic composition comprises *Streptococcus pneumoniae* saccharides from at least 10 serotypes conjugated to a carrier protein, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or each *S. pneumoniae* saccharide is sized by a factor up to x2, x3, x4, x5, x6, x7, x8, x9 or x10. In one embodiment of this aspect, the majority of the saccharides, for example 6, 7, 8 or more of the saccharides are sized by a factor up to x2, x3, x4, x5, x6, x7, x8, x9 or x 10.

The molecular weight or average molecular weight (or size) of a saccharide herein refers to the weight-average molecular weight (M_w) of the saccharide measured prior to conjugation and is measured by MALLS.

The MALLS technique is well known in the art and is typically carried out as described in example 2. For MALLS analysis of pneumococcal saccharides, two columns (TSKG6000 and 5000PWxl) may be used in combination and the saccharides are eluted in water. Saccharides are detected using a light scattering detector (for instance Wyatt Dawn DSP equipped with a 10mW argon laser at 488nm) and an inferometric refractometer (for instance Wyatt Otilab DSP equipped with a P100 cell and a red filter at 498nm).

In an embodiment the *S. pneumoniae* saccharides are native polysaccharides or native polysaccharides which have been reduced in size during a normal extraction process.

In an embodiment, the *S. pneumoniae* saccharides are sized by mechanical cleavage, for instance by microfluidisation or sonication. Microfluidisation and sonication have the advantage of decreasing the size of the larger native polysaccharides sufficiently to provide a filterable conjugate. Sizing is by a factor of no more than x20, x10, x8, x6, x5, x4, x3 or x2.

In an embodiment, the immunogenic composition comprises *S. pneumoniae* conjugates that are made from a mixture of native polysaccharides and saccharides that are sized by a factor of no more than x20. In one aspect of this embodiment, the majority of the

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saccharides, for example 6, 7, 8 or more of the saccharides are sized by a factor of up to x2, x3, x4, x5 or x6.

In an embodiment, the *Streptococcus pneumoniae* saccharide is conjugated to the carrier protein via a linker, for instance a bifunctional linker. The linker is optionally heterobifunctional or homobifunctional, having for example a reactive amino group and a reactive carboxylic acid group, 2 reactive amino groups or two reactive carboxylic acid groups. The linker has for example between 4 and 20, 4 and 12, 5 and 10 carbon atoms. A possible linker is ADH. Other linkers include B-propionamido (WO 00/10599), nitrophenyl-ethylamine (Gever et al (1979) Med. Microbiol. Immunol. 165; 171-288), haloalkyl halides (US4057685), glycosidic linkages (US4673574, US4808700), hexane diamine and 6-aminocaproic acid (US4459286). In an embodiment, ADH is used as a linker for conjugating saccharide from serotype 18C. In an embodiment, ADH is used as a linker for conjugating saccharide from serotype 22F.

The saccharide conjugates present in the immunogenic compositions of the invention may be prepared by any known coupling technique. The conjugation method may rely on activation of the saccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated saccharide may thus be coupled directly or via a spacer (linker) group to an amino group on the carrier protein. For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using GMBS) or a haloacetylated carrier protein (for example using iodoacetimide [e.g. ethyl iodoacetimide HCl] or N-succinimidyl bromoacetate or SIAB, or SIA, or SBAP). Preferably, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or ADH and the amino-derivatised saccharide is conjugated to the carrier protein using carbodiimide (e.g. EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described in PCT published application WO 93/15760 Uniformed Services University and WO 95/08348 and WO 96/29094

Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S-NHS, EDC, TSTU. Many are described in WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with CDI (Bethell et al J. Biol. Chem. 1979, 254;

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2572-4, Hearn et al J. Chromatogr. 1981. 218; 509-18) followed by reaction of with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group' reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

The conjugates can also be prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508.

A further method involves the coupling of a cyanogen bromide (or CDAP) activated saccharide derivatised with adipic acid dihydrazide (ADH) to the protein carrier by Carbodiimide condensation (Chu C. et al Infect. Immunity, 1983 245 256), for example using EDAC.

In an embodiment, a hydroxyl group (preferably an activated hydroxyl group for example a hydroxyl group activated to make a cyanate ester [e.g. with CDAP]) on a saccharide is linked to an amino or carboxylic group on a protein either directly or indirectly (through a linker). Where a linker is present, a hydroxyl group on a saccharide is preferably linked to an amino group on a linker, for example by using CDAP conjugation. A further amino group in the linker for example ADH) may be conjugated to a carboxylic acid group on a protein, for example by using carbodiimide chemistry, for example by using EDAC. In an embodiment, the pneumococcal capsular saccharide(s) is conjugated to the linker first before the linker is conjugated to the carrier protein. Alternatively the linker may be conjugated to the carrier before conjugation to the saccharide.

A combination of techniques may also be used, with some saccharide-protein conjugates being prepared by CDAP, and some by reductive amination.

In general the following types of chemical groups on a protein carrier can be used for coupling / conjugation:

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A) Carboxyl (for instance via aspartic acid or glutamic acid). In one embodiment this group is linked to amino groups on saccharides directly or to an amino group on a linker with carbodiimide chemistry e.g. with EDAC.

B) Amino group (for instance via lysine). In one embodiment this group is linked to carboxyl groups on saccharides directly or to a carboxyl group on a linker with carbodiimide chemistry e.g. with EDAC. In another embodiment this group is linked to hydroxyl groups activated with CDAP or CNBr on saccharides directly or to such groups on a linker; to saccharides or linkers having an aldehyde group; to saccharides or linkers having a succinimide ester group.

C) Sulphydryl (for instance via cysteine). In one embodiment this group is linked to a bromo or chloro acetylated saccharide or linker with maleimide chemistry. In one embodiment this group is activated/modified with bis diazobenzidine.

D) Hydroxyl group (for instance via tyrosine). In one embodiment this group is activated/modified with bis diazobenzidine.

E) Imidazolyl group (for instance via histidine). In one embodiment this group is activated/modified with bis diazobenzidine.

F) Guanidyl group (for instance via arginine).

G) Indolyl group (for instance via tryptophan).

On a saccharide, in general the following groups can be used for a coupling: OH, COOH or NH2. Aldehyde groups can be generated after different treatments known in the art such as: periodate, acid hydrolysis, hydrogen peroxide, etc.

Direct coupling approaches:

Saccharide-OH + CNBr or CDAP -----> cyanate ester + NH2-Prot ---> conjugate
Saccharide-aldehyde + NH2-Prot ----> Schiff base + NaCNBH3 ----> conjugate
Saccharide-COOH + NH2-Prot + EDAC ----> conjugate
Saccharide-NH2 + COOH-Prot + EDAC ----> conjugate

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Indirect coupling via spacer (linker) approaches:

Saccharide-OH + CNBr or CDAP ---> cyanate ester + NH2----NH2 ----> saccharide----NH2 + COOH-Prot + EDAC -----> conjugate

Saccharide-OH + CNBr or CDAP ----> cyanate ester + NH2-----SH -----> saccharide----SH + SH-Prot (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance) -----> saccharide-S-S-Prot

Saccharide-OH + CNBr or CDAP ---> cyanate ester + NH2----SH -------> saccharide----SH + maleimide-Prot (modification of amino groups) ----> conjugate

Saccharide-OH + CNBr or CDAP ---> cyanate ester + NH2----SH ---> Saccharide-SH + haloacetylated-Prot ----> Conjugate

Saccharide-COOH + EDAC + NH2-----NH2 ---> saccharide------NH2 + EDAC + COOH-Prot ----> conjugate

Saccharide-COOH + EDAC+ NH2----SH -----> saccharide----SH + SH-Prot (native Protein with an exposed cysteine or obtained after modification of amino groups of the protein by SPDP for instance) -----> saccharide-S-S-Prot

Saccharide-COOH + EDAC+ NH2----SH -----> saccharide----SH + maleimide-Prot (modification of amino groups) ----> conjugate

Saccharide-COOH + EDAC + NH2----SH ---> Saccharide-SH + haloacetylated-Prot ----> Conjugate

Saccharide-Aldehyde + NH2-----NH2 ----> saccharide---NH2 + EDAC + COOH-Prot ----> conjugate

Note: instead of EDAC above, any suitable carbodiimide may be used.

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In summary, the types of protein carrier chemical group that may be generally used for coupling with a saccharide are amino groups (for instance on lysine residues), COOH groups (for instance on aspartic and glutamic acid residues) and SH groups (if accessible) (for instance on cysteine residues.

Preferably the ratio of carrier protein to *S. pneumoniae* saccharide is between 1:5 and 5:1; e.g. between 1:0.5-4:1, 1:1-3.5:1, 1.2:1-3:1, 1.5:1-2.5:1; e.g. between 1:2 and 2.5:1; 1:1 and 2:1 (w/w). In an embodiment, the majority of the conjugates, for example 6, 7, 8, 9 or more of the conjugates have a ratio of carrier protein to saccharide that is greater than 1:1, for example 1.1:1, 1.2:1, 1.3:1, 1.4:1, 1.5:1 or 1.6:1.

In an embodiment, at least one *S. pneumoniae* saccharide is conjugated to a carrier protein via a linker using CDAP and EDAC. For example, 18C or 22F may be conjugated to a protein via a linker (for example those with two hydrazino groups at its ends such as ADH) using CDAP and EDAC as described above. When a linker is used, CDAP may be used to conjugate the saccharide to a linker and EDAC may then be used to conjugate the linker to a protein or, alternatively EDAC may be used first to conjugate the linker to the protein, after which CDAP may be used to conjugate the linker to the saccharide.

In general, the immunogenic composition of the invention may comprise a dose of each saccharide conjugate between 0.1 and 20μg, 1 and 10μg or 1 and 3μg of saccharide.

In an embodiment, the immunogenic composition of the invention contains each *S. pneumoniae* capsular saccharide at a dose of between 0.1-20μg; 0.5-10μg; 0,5- 5μg or 1-3μg of saccharide. In an embodiment, capsular saccharides may be present at different dosages, for example some capsular saccharides may be present at a dose of exactly 1μg or some capsular saccharides may be present at a dose of exactly 3μg. In an embodiment, saccharides from serotypes 3, 18C and 19F (or 4, 18C and 19F) are present at a higher dose than other saccharides. In one aspect of this embodiment, serotypes 3, 18C and 19F (or 4, 18C and 19F) are present at a dose of around or exactly 3 μg whilst other saccharides in the immunogenic composition are present at a dose of around or exactly 1μg.

"Around" or "approximately" are defined as within 10% more or less of the given figure for the purposes of the invention.

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In an embodiment, at least one of the *S. pneumoniae* capsular saccharides is directly conjugated to a carrier protein (e.g. using one of the chemistries described above). Preferably the at least one of the *S. pneumoniae* capsular saccharides is directly conjugated by CDAP. In an embodiment, the majority of the capsular saccharides for example 5, 6, 7, 8, 9 or more are directly linked to the carrier protein by CDAP (see WO 95/08348 and WO 96/29094)

The immunogenic composition may comprise *Streptococcus pneumoniae* proteins, herein termed *Streptococcus pneumoniae* proteins of the invention. Such proteins may be used as carrier proteins, or may be present as free proteins, or may be present both as carrier proteins and as free proteins. The *Streptococcus pneumoniae* proteins of the invention are either surface exposed, at least during part of the life cycle of the pneumococcus, or are proteins which are secreted or released by the pneumococcus. Preferably the proteins of the invention are selected from the following categories, such as proteins having a Type II Signal sequence motif of LXXC (where X is any amino acid, e.g., the polyhistidine triad family (PhtX)), choline binding proteins (CbpX), proteins having a Type I Signal sequence motif (e.g., Sp101), proteins having a LPXTG motif (where X is any amino acid, e.g., Sp128, Sp130), and toxins (e.g., Ply). Preferred examples within these categories (or motifs) are the following proteins, or immunologically functional equivalents thereof.

In one embodiment, the immunogenic composition of the invention comprises at least 1 protein selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133. In a further embodiment, the immunogenic composition comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), PspA, PsaA, and Sp128. In one more embodiment, the immunogenic composition comprises 2 or more proteins selected from the group consisting of the Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family, LytX truncates, CbpX truncate-LytX truncate chimeric proteins (or fusions), pneumolysin (Ply), and Sp128.

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The Pht (Poly Histidine Triad) family comprises proteins PhtA, PhtB, PhtD, and PhtE. The family is characterized by a lipidation sequence, two domains separated by a proline-rich region and several histidine triads, possibly involved in metal or nucleoside binding or enzymatic activity, (3-5) coiled-coil regions, a conserved N-terminus and a heterogeneous C terminus. It is present in all strains of pneumococci tested. Homologous proteins have also been found in other Streptococci and Neisseria. In one embodiment of the invention, the Pht protein of the invention is PhtD. It is understood, however, that the terms Pht A, B, D, and E refer to proteins having sequences disclosed in the citations below as well as naturally-occurring (and man-made) variants thereof that have a sequence homology that is at least 90% identical to the referenced proteins. Preferably it is at least 95% identical and most preferably it is 97% identical.

With regards to the PhtX proteins, PhtA is disclosed in WO 98/18930, and is also referred to Sp36. As noted above, it is a protein from the polyhistidine triad family and has the type II signal motif of LXXC. PhtD is disclosed in WO 00/37105, and is also referred to Sp036D. As noted above, it also is a protein from the polyhistidine triad family and has the type II LXXC signal motif. PhtB is disclosed in WO 00/37105, and is also referred to Sp036B. Another member of the PhtB family is the C3-Degrading Polypeptide, as disclosed in WO 00/17370. This protein also is from the polyhistidine triad family and has the type II LXXC signal motif. A preferred immunologically functional equivalent is the protein Sp42 disclosed in WO 98/18930. A PhtB truncate (approximately 79kD) is disclosed in WO99/15675 which is also considered a member of the PhtX family. PhtE is disclosed in WO00/30299 and is referred to as BVH-3. Where any Pht protein is referred to herein, it is meant that immunogenic fragments or fusions thereof of the Pht protein can be used. For example, a reference to PhtX includes immunogenic fragments or fusions thereof from any Pht protein. A reference to PhtD or PhtB is also a reference to PhtDE or PhtBE fusions as found, for example, in WO0198334.

Pneumolysin is a multifunctional toxin with a distinct cytolytic (hemolytic) and complement activation activities (Rubins et al., Am . Respi. Cit Care Med, 153:1339-1346 (1996)). The toxin is not secreted by pneumococci, but it is released upon lysis of pneumococci under the influence of autolysin. Its effects include e.g., the stimulation of the production of inflammatory cytokines by human monocytes, the inhibition of the beating of cilia on human respiratory epithelial, and the decrease of bactericidal activity and migration of

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neutrophils. The most obvious effect of pneumolysin is in the lysis of red blood cells, which involves binding to cholesterol. Because it is a toxin, it needs to be detoxified (i.e., non-toxic to a human when provided at a dosage suitable for protection) before it can be administered *in vivo*. Expression and cloning of wild-type or native pneumolysin is known in the art. See, for example, Walker et al. (Infect Immun, 55:1184-1189 (1987)), Mitchell et al. (Biochim Biophys Acta, 1007:67-72 (1989) and Mitchell et al (NAR, 18:4010 (1990)). Detoxification of ply can be conducted by chemical means, e.g., subject to formalin or glutaraldehyde treatment or a combination of both (WO 04081515, PCT/EP2005/010258). Such methods are well known in the art for various toxins. Alternatively, ply can be genetically detoxified. Thus, the invention encompasses derivatives of pneumococcal proteins which may be, for example, mutated proteins. The term "mutated" is used herein to mean a molecule which has undergone deletion, addition or substitution of one or more amino acids using well known techniques for site directed mutagenesis or any other conventional method. For example, as described above, a mutant ply protein may be altered so that it is biologically inactive whilst still maintaining its immunogenic epitopes, see, for example, WO90/06951, Berry et al. (Infect Immun, 67:981-985 (1999)) and WO99/03884.

As used herein, it is understood that the term "Ply" refers to mutated or detoxified pneumolysin suitable for medical use (i.e., non toxic).

Concerning the Choline Binding Protein family (CbpX), members of that family were originally identified as pneumococcal proteins that could be purified by choline-affininty chromatography. All of the choline-binding proteins are non-covalently bound to phosphorylcholine moieties of cell wall teichoic acid and membrane-associated lipoteichoic acid. Structurally, they have several regions in common over the entire family, although the exact nature of the proteins (amino acid sequence, length, etc.) can vary. In general, choline binding proteins comprise an N terminal region (N), conserved repeat regions (R1 and/or R2), a proline rich region (P) and a conserved choline binding region (C), made up of multiple repeats, that comprises approximately one half of the protein. As used in this application, the term "Choline Binding Protein family (CbpX)" is selected from the group consisting of Choline Binding Proteins as identified in WO97/41151, PbcA, SpsA, PspC, CbpA, CbpD, and CbpG. CbpA is disclosed in WO97/41151. CbpD and CbpG are disclosed in WO00/29434. PspC is disclosed in WO97/09994. PbcA is disclosed in WO98/21337.SpsA is a Choline binding protein disclosed in WO 98/39450.

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Preferably the Choline Binding Proteins are selected from the group consisting of CbpA, PbcA, SpsA and PspC.

Another preferred embodiment is CbpX truncates wherein "CbpX" is defined above and "truncates" refers to CbpX proteins lacking 50% or more of the Choline binding region (C). Preferably such proteins lack the entire choline binding region. More preferably, the such protein truncates lack (i) the choline binding region and (ii) a portion of the N-terminal half of the protein as well, yet retain at least one repeat region (R1 or R2). More preferably still, the truncate has 2 repeat regions (R1 and R2). Examples of such preferred embodiments are NR1xR2 and R1xR2 as illustrated in WO99/51266 or WO99/51188, however, other choline binding proteins lacking a similar choline binding region are also contemplated within the scope of this invention.

The LytX family is membrane associated proteins associated with cell lysis. The N-terminal domain comprises choline binding domain(s), however the LytX family does not have all the features found in the CbpA family noted above and thus for the present invention, the LytX family is considered distinct from the CbpX family. In contrast with the CbpX family, the C-terminal domain contains the catalytic domain of the LytX protein family. The family comprises LytA, B and C. With regards to the LytX family, LytA is disclosed in Ronda et al., Eur J Biochem, 164:621-624 (1987). LytB is disclosed in WO 98/18930, and is also referred to as Sp46. LytC is also disclosed in WO 98/18930, and is also referred to as Sp91. A preferred member of that family is LytC.

Another preferred embodiment are LytX truncates wherein "LytX" is defined above and "truncates" refers to LytX proteins lacking 50% or more of the Choline binding region. Preferably such proteins lack the entire choline binding region. Yet another preferred embodiment of this invention are CbpX truncate-LytX truncate chimeric proteins (or fusions). Preferably this comprises NR1xR2 (or R1xR2) of CbpX and the C-terminal portion (Cterm, i.e., lacking the choline binding domains) of LytX (e.g., LytCCterm or Sp91Cterm). More preferably CbpX is selected from the group consisting of CbpA, PbcA, SpsA and PspC. More preferably still, it is CbpA. Preferably, LytX is LytC (also referred to as Sp91). Another embodiment of the present invention is a PspA or PsaA truncates lacking the choline binding domain (C) and expressed as a fusion protein with LytX. Preferably, LytX is LytC.

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With regards to PsaA and PspA, both are know in the art. For example, PsaA and transmembrane deletion variants thereof have been described by Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62. PspA and transmembrane deletion variants thereof have been disclosed in, for example, US 5804193, WO 92/14488, and WO 99/53940.

Sp128 and Sp130 are disclosed in WO00/76540. Sp125 is an example of a pneumococcal surface protein with the Cell Wall Anchored motif of LPXTG (where X is any amino acid). Any protein within this class of pneumococcal surface protein with this motif has been found to be useful within the context of this invention, and is therefore considered a further protein of the invention. Sp125 itself is disclosed in WO 98/18930, and is also known as ZmpB – a zinc metalloproteinase. Sp101 is disclosed in WO 98/06734 (where it has the reference # y85993). It is characterized by a Type I signal sequence. Sp133 is disclosed in WO 98/06734 (where it has the reference # y85992). It is also characterized by a Type I signal sequence.

Examples of preferred *Moraxella catarrhalis* protein antigens which can be included in a combination vaccine (especially for the prevention of otitis media) are: OMP106 [WO 97/41731 (Antex) & WO 96/34960 (PMC)]; OMP21 or fragments thereof (WO 0018910); LbpA &/or LbpB [WO 98/55606 (PMC)]; TbpA &/or TbpB [WO 97/13785 & WO 97/32980 (PMC)]; CopB [Helminen ME, *et al.* (1993) Infect. Immun. 61:2003-2010]; UspA1 and/or UspA2 [WO 93/03761 (University of Texas)]; OmpCD; HasR (PCT/EP99/03824); PilQ (PCT/EP99/03823); OMP85 (PCT/EP00/01468); lipo06 (GB 9917977.2); lipo10 (GB 9918208.1); lipo11 (GB 9918302.2); lipo18 (GB 9918038.2); P6 (PCT/EP99/03038); D15 (PCT/EP99/03822); OmpIA1 (PCT/EP99/06781); Hly3 (PCT/EP99/03257); and OmpE. Examples of non-typeable *Haemophilus influenzae* antigens or fragments thereof which can be included in a combination vaccine (especially for the prevention of otitis media) include: Fimbrin protein [(US 5766608 - Ohio State Research Foundation)] and fusions comprising peptides therefrom [eg LB1(f) peptide fusions; US 5843464 (OSU) or WO 99/64067]; OMP26 [WO 97/01638 (Cortecs)]; P6 [EP 281673 (State University of New York)]; TbpA and/or TbpB; Hia; Hsf; Hin47; Hif; Hmw1; Hmw2; Hmw3; Hmw4; Hap; D15 (WO 94/12641); P2; and P5 (WO 94/26304).

The proteins of the invention may also be beneficially combined. By combined is meant that the immunogenic composition comprises all of the proteins from within the following combinations, either as carrier proteins or as free proteins or a mixture of the two. For

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example, in a combination of two proteins as set out hereinafter, both proteins may be used as carrier proteins, or both proteins may be present as free proteins, or both may be present as carrier and as free protein, or one may be present as a carrier protein and a free protein whilst the other is present only as a carrier protein or only as a free protein, or one may be present as a carrier protein and the other as a free protein. Where a combination of three proteins is given, similar possibilities exist. Preferred combinations include, but are not limited to, PhtD + NR1xR2, PhtD + NR1xR2-Sp91Cterm chimeric or fusion proteins, PhtD + Ply, PhtD + Sp128, PhtD + PsaA, PhtD + PspA, PhtA + NR1xR2, PhtA + NR1xR2-Sp91Cterm chimeric or fusion proteins, PhtA + Ply, PhtA + Sp128, PhtA + PsaA, PhtA + PspA, NR1xR2 + LytC, NR1xR2 + PspA, NR1xR2 + PsaA, NR1xR2 + Sp128, R1xR2 + LytC, R1xR2 + PspA, R1xR2 + PsaA, R1xR2 + Sp128, R1xR2 + PhtD, R1xR2 + PhtA. Preferably, NR1xR2 (or R1xR2) is from CbpA or PspC. More preferably it is from CbpA. Other combinations include 3 protein combinations such as PhtD + NR1xR2 + Ply, and PhtA + NR1xR2 + PhtD. In one embodiment, the vaccine composition comprises detoxified pneumolysin and PhtD or PhtDE as carrier proteins. In a further embodiment, the vaccine composition comprises detoxified pneumolysin and PhtD or PhtDE as free proteins.

The present invention further provides a vaccine containing the immunogenic compositions of the invention and a pharmaceutically acceptable excipient.

The vaccines of the present invention may be adjuvanted, particularly when intended for use in an elderly population but also for use in infant populations. Suitable adjuvants include an aluminum salt such as aluminum hydroxide gel or aluminum phosphate or alum, but may also be a salt of calcium, magnesium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatized saccharides, or polyphosphazenes.

It is preferred that the adjuvant be selected to be a preferential inducer of a TH1 type of response. Such high levels of Th1-type cytokines tend to favour the induction of cell mediated immune responses to a given antigen, whilst high levels of Th2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

The distinction of Th1 and Th2-type immune response is not absolute. In reality an individual will support an immune response which is described as being predominantly

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Th1 or predominantly Th2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. (Annual Review of Immunology, 7, p145-173). Traditionally, Th1-type responses are associated with the production of the INF-γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of Th1-type immune responses are not produced by T-cells, such as IL-12. In contrast, Th2-type responses are associated with the secretion of Il-4, IL-5, IL-6, IL-10. Suitable adjuvant systems which promote a predominantly Th1 response include: Monophosphoryl lipid A or a derivative thereof, particularly 3-de-O-acylated monophosphoryl lipid A (3D-MPL) (for its preparation see GB 2220211 A); and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with either an aluminum salt (for instance aluminum phosphate or aluminum hydroxide) or an oil-in-water emulsion. In such combinations, antigen and 3D-MPL are contained in the same particulate structures, allowing for more efficient delivery of antigenic and immunostimulatory signals. Studies have shown that 3D-MPL is able to further enhance the immunogenicity of an alum-adsorbed antigen [Thoelen *et al.* Vaccine (1998) 16:708-14; EP 689454-B1].

An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative, particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil in water emulsion is described in WO 95/17210. In one embodiment the immunogenic composition additionally comprises a saponin, which may be QS21. The formulation may also comprise an oil in water emulsion and tocopherol (WO 95/17210). Unmethylated CpG containing oligonucleotides (WO 96/02555) and other immunomodulatory oligonucleotides (WO0226757 and WO03507822) are also preferential inducers of a TH1 response and are suitable for use in the present invention.

Particular adjuvants are those selected from the group of metal Salts, oil in water emulsions, Toll like receptors agonist, (in particular Toll like receptor 2 agonist, Toll like receptor 3 agonist, Toll like receptor 4 agonist, Toll like receptor 7 agonist, Toll like receptor 8 agonist and Toll like receptor 9 agonist), saponins or combinations thereof.

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An adjuvant that can be used with the vaccine compositions of the invention are bleb or outer membrane vesicle preparations from Gram negative bacterial strains such as those taught by WO02/09746 – particularly *N. meningitidis* blebs. Adjuvant properties of blebs can be improved by retaining LOS (lipooligosacccharide) on its surface (e.g. through extraction with low concentrations of detergent [for instanct 0-0.1% deoxycholate]). LOS can be detoxified through the msbB(-) or htrB(-) mutations discussed in WO02/09746. Adjuvant properties can also be improved by retaining PorB (and optionally removing PorA) from meningococcal blebs. Adjuvant properties can also be improved by truncating the outer core saccharide structure of LOS on meningococcal blebs – for instance via the lgtB(-) mutation discussed in WO2004/014417. Alternatively, the aforementioned LOS (e.g. isolated from a msbB(-) and/or lgtB(-) strain) can be purified and used as an adjuvant in the compositions of the invention.

A further adjuvant which may be used with the compositions of the invention may be selected from the group: a saponin, lipid A or a derivative thereof, an immunostimulatory oligonucleotide, an alkyl glucosaminide phosphate, an oil in water emulsion or combinations thereof. A further preferred adjuvant is a metal salt in combination with another adjuvant. It is preferred that the adjuvant is a Toll like receptor agonist in particular an agonist of a Toll like receptor 2, 3, 4, 7, 8 or 9, or a saponin, in particular Qs21. It is further preferred that the adjuvant system comprises two or more adjuvants from the above list. In particular the combinations preferably contain a saponin (in particular Qs21) adjuvant and/or a Toll like receptor 9 agonist such as a CpG containing immunostimulatory oligonucleotide. Other preferred combinations comprise a saponin (in particular QS21) and a Toll like receptor 4 agonist such as monophosphoryl lipid A or its 3 deacylated derivative, 3 D – MPL, or a saponin (in particular QS21) and a Toll like receptor 4 ligand such as an alkyl glucosaminide phosphate.

Particularly preferred adjuvants are combinations of 3D-MPL and QS21 (EP 0 671 948 B1), oil in water emulsions comprising 3D-MPL and QS21 (WO 95/17210, WO 98/56414), or 3D-MPL formulated with other carriers (EP 0 689 454 B1). Other preferred adjuvant systems comprise a combination of 3 D MPL , QS21 and a CpG oligonucleotide as described in US6558670, US6544518.

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In an embodiment the adjuvant is (or comprises) a Toll like receptor (TLR) 4 ligand, preferably an agonist such as a lipid A derivative particularly monophosphoryl lipid A or more particularly 3 Deacylated monophoshoryl lipid A (3 D – MPL).

3 D –MPL is available from GlaxoSmithKline Biologicals North America and primarily promotes CD4+ T cell responses with an IFN-g (Th1) phenotype . It can be produced according to the methods disclosed in GB 2 220 211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 3, 4, 5 or 6 acylated chains. Preferably in the compositions of the present invention small particle 3 D- MPL is used. Small particle 3 D -MPL has a particle size such that it may be sterile-filtered through a 0.22μm filter. Such preparations are described in International Patent Application No. WO 94/21292. Synthetic derivatives of lipid A are known and thought to be TLR 4 agonists including, but not limited to:

OM174 (2-deoxy-6-o-[2-deoxy-2-[(R)-3-dodecanoyloxytetra-decanoylamino]-4-o-phosphono-β-D-glucopyranosyl]-2-[(R)-3-hydroxytetradecanoylamino]-α-D-glucopyranosyldihydrogenphosphate), (WO 95/14026)

OM 294 DP (3S, 9 R) –3--[(R)-dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9(R)-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1,10-bis(dihydrogenophosphate) (WO99 /64301 and WO 00/0462)

OM 197 MP-Ac DP (3S-, 9R) -3-[(R) -dodecanoyloxytetradecanoylamino]-4-oxo-5-aza-9-[(R)-3-hydroxytetradecanoylamino]decan-1,10-diol,1 -dihydrogenophosphate 10-(6-aminohexanoate) (WO 01/46127)

Other TLR4 ligands which may be used are alkyl Glucosaminide phosphates (AGPs) such as those disclosed in WO9850399 or US6303347 (processes for preparation of AGPs are also disclosed), or pharmaceutically acceptable salts of AGPs as disclosed in US6764840. Some AGPs are TLR4 agonists, and some are TLR4 antagonists. Both are thought to be useful as adjuvants.

Another prefered immunostimulant for use in the present invention is Quil A and its derivatives. Quil A is a saponin preparation isolated from the South American tree Quilaja Saponaria Molina and was first described as having adjuvant activity by Dalsgaard et al.

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in 1974 ("Saponin adjuvants", Archiv. für die gesamte Virusforschung, Vol. 44, Springer Verlag, Berlin, p243-254). Purified fragments of Quil A have been isolated by HPLC which retain adjuvant activity without the toxicity associated with Quil A (EP 0 362 278), for example QS7 and QS21 (also known as QA7 and QA21). QS-21 is a natural saponin derived from the bark of Quillaja saponaria Molina which induces CD8+ cytotoxic T cells (CTLs), Th1 cells and a predominant IgG2a antibody response and is a preferred saponin in the context of the present invention.

Particular formulations of QS21 have been described which are particularly preferred, these formulations further comprise a sterol (WO96/33739). The saponins forming part of the present invention may be separate in the form of micelles, mixed micelles (preferentially, but not exclusively with bile salts) or may be in the form of ISCOM matrices (EP 0 109 942 B1) , liposomes or related colloidal structures such as worm-like or ring-like multimeric complexes or lipidic/layered structures and lamellae when formulated with cholesterol and lipid, or in the form of an oil in water emulsion (for example as in WO 95/17210). The saponins may preferably be associated with a metallic salt, such as aluminium hydroxide or aluminium phosphate (WO 98/15287).
Preferably, the saponin is presented in the form of a liposome, ISCOM or an oil in water emulsion.

An enhanced system involves the combination of a monophosphoryl lipid A (or detoxified lipid A) and a saponin derivative, particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739. A particularly potent adjuvant formulation involving tocopherol with or without QS21 and/or 3D-MPL in an oil in water emulsion is described in WO 95/17210. In one embodiment the immunogenic composition additionally comprises a saponin, which may be QS21.

Immunostimulatory oligonucleotides or any other Toll-like receptor (TLR) 9 agonist may also be used.The preferred oligonucleotides for use in adjuvants or vaccines of the present invention are CpG containing oligonucleotides, preferably containing two or more dinucleotide CpG motifs separated by at least three, more preferably at least six or more nucleotides. A CpG motif is a Cytosine nucleotide followed by a Guanine nucleotide. The CpG oligonucleotides of the present invention are typically deoxynucleotides. In a preferred embodiment the internucleotide in the oligonucleotide is phosphorodithioate, or

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more preferably a phosphorothioate bond, although phosphodiester and other internucleotide bonds are within the scope of the invention. Also included within the scope of the invention are oligonucleotides with mixed internucleotide linkages. Methods for producing phosphorothioate oligonucleotides or phosphorodithioate are described in US5,666,153, US5,278,302 and WO95/26204.

Examples of preferred oligonucleotides have the following sequences. The sequences preferably contain phosphorothioate modified internucleotide linkages.
OLIGO 1(SEQ ID NO:1): TCC ATG ACG TTC CTG ACG TT (CpG 1826)
OLIGO 2 (SEQ ID NO:2): TCT CCC AGC GTG CGC CAT (CpG 1758)
OLIGO 3(SEQ ID NO:3): ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG
OLIGO 4 (SEQ ID NO:4): TCG TCG TTT TGT CGT TTT GTC GTT (CpG 2006)
OLIGO 5 (SEQ ID NO:5): TCC ATG ACG TTC CTG ATG CT (CpG 1668)
OLIGO 6 (SEQ ID NO:6): TCG ACG TTT TCG GCG CGC GCC G (CpG 5456)

Alternative CpG oligonucleotides may comprise the preferred sequences above in that they have inconsequential deletions or additions thereto.
The CpG oligonucleotides utilised in the present invention may be synthesized by any method known in the art (for example see EP 468520). Conveniently, such oligonucleotides may be synthesized utilising an automated synthesizer.

The adjuvant may be an oil in water emulsion or may comprise an oil in water emulsion in combination with other adjuvants. The oil phase of the emulsion system preferably comprises a metabolisable oil. The meaning of the term metabolisable oil is well known in the art. Metabolisable can be defined as "being capable of being transformed by metabolism" (Dorland's Illustrated Medical Dictionary, W.B. Sanders Company, 25th edition (1974)). The oil may be any vegetable oil, fish, oil, animal or synthetic oil, which is not toxic to the recipient and is capable of being transformed by metabolism. Nuts, seeds, and grains are common sources of vegetable oils. Synthetic oils are also part of this invention and can include commercially available oils such as NEOBEE® and others. Squalene (2,6,10,15,19, 23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene) is an unsaturated oil which is found in large quantities in shark-liver oil, and in lower quantities in olive oil, wheat germ oil, rice bran oil, and yeast, and is a particularly preferred oil for use in this invention. Squalene is a metabolisable oil by virtue of the fact that it is an intermediate in the biosynthesis of cholesterol (Merck index, 10th Edition, entry no.8619).

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Tocols (e.g. vitamin E) are also often used in oil emulsions adjuvants (EP 0 382 271 B1; US5667784; WO 95/17210). Tocols used in the oil emulsions (preferably oil in water emulsions) of the invention may be formulated as described in EP 0 382 271 B1, in that the tocols may be dispersions of tocol droplets, optionally comprising an emulsifier, of preferably less than 1 micron in diameter. Alternatively, the tocols may be used in combination with another oil, to form the oil phase of an oil emulsion. Examples of oil emulsions which may be used in combination with the tocol are described herein, such as the metabolisable oils described above.

Oil in water emulsion adjuvants per se have been suggested to be useful as adjuvant compositions (EP 0 399 843B), also combinations of oil in water emulsions and other active agents have been described as adjuvants for vaccines (WO 95/17210; WO 98/56414; WO 99/12565; WO 99/11241). Other oil emulsion adjuvants have been described, such as water in oil emulsions (US 5,422,109;EP 0 480 982 B2) and water in oil in water emulsions (US 5,424,067;EP 0 480 981 B). All of which form preferred oil emulsion systems (in particular when incorporating tocols) to form adjuvants and compositions of the present invention.

Most preferably the oil emulsion (for instance oil in water emulsions) further comprises an emulsifier such as TWEEN 80 and/or a sterol such as cholesterol.

A preferred oil emulsion (preferably oil-in-water emulsion) comprises a metabolisible, non-toxic oil, such as squalane, squalene or a tocopherol such as alpha tocopherol (and preferably both squalene and alpha tocopherol) and optionally an emulsifier (or surfactant) such as Tween 80. A sterol (preferably cholesterol) may also be included.

The method of producing oil in water emulsions is well known to the man skilled in the art. Commonly, the method comprises mixing the tocol-containing oil phase with a surfactant such as a PBS/TWEEN80™ solution, followed by homogenisation using a homogenizer, it would be clear to a man skilled in the art that a method comprising passing the mixture twice through a syringe needle would be suitable for homogenising small volumes of liquid. Equally, the emulsification process in microfluidiser (M110S Microfluidics machine, maximum of 50 passes, for a period of 2 minutes at maximum pressure input of 6 bar (output pressure of about 850 bar)) could be adapted by the man skilled in the art to produce smaller or larger volumes of emulsion. The adaptation could be achieved by

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routine experimentation comprising the measurement of the resultant emulsion until a preparation was achieved with oil droplets of the required diameter.

In an oil in water emulsion, the oil and emulsifier should be in an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline.

The size of the oil droplets found within the stable oil in water emulsion are preferably less than 1 micron, may be in the range of substantially 30-600nm, preferably substantially around 30-500nm in diameter, and most preferably substantially 150-500nm in diameter, and in particular about 150 nm in diameter as measured by photon correlation spectroscopy. In this regard, 80% of the oil droplets by number should be within the preferred ranges, more preferably more than 90% and most preferably more than 95% of the oil droplets by number are within the defined size ranges. The amounts of the components present in the oil emulsions of the present invention are conventionally in the range of from 0.5-20% or 2 to 10% oil (of the total dose volume), such as squalene; and when present, from 2 to 10% alpha tocopherol; and from 0.3 to 3% surfactant, such as polyoxyethylene sorbitan monooleate. Preferably the ratio of oil (preferably squalene): tocol (preferably α-tocopherol) is equal or less than 1 as this provides a more stable emulsion. An emulsifier, such as Tween80 or Span 85 may also be present at a level of about 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser.

Examples of preferred emulsion systems are described in WO 95/17210, WO 99/11241 and WO 99/12565 which disclose emulsion adjuvants based on squalene, α-tocopherol, and TWEEN 80, optionally formulated with the immunostimulants QS21 and/or 3D-MPL. Thus in a particularly, preferred embodiment of the present invention, the adjuvant of the invention may additionally comprise further immunostimulants, such as LPS or derivatives thereof, and/or saponins. Examples of further immunostimulants are described herein and in "Vaccine Design – The Subunit and Adjuvant Approach" 1995, Pharmaceutical Biotechnology, Volume 6, Eds. Powell, M.F., and Newman, M.J., Plenum Press, New York and London, ISBN 0-306-44867-X.

In a preferred aspect the adjuvant and immunogenic compositions according to the invention comprise a saponin (preferably QS21) and/or an LPS derivative (preferably 3D-MPL) in an oil emulsion described above, optionally with a sterol (preferably cholesterol). Additionally the oil emulsion (preferably oil in water emulsion) may contain

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span 85 and/or lecithin and/or tricaprylin. Adjuvants comprising an oil-in-water emulsion, a sterol and a saponin are described in WO 99/12565.

Typically for human administration the saponin (preferably QS21) and/or LPS derivative (preferably 3D-MPL) will be present in a human dose of immunogenic composition in the range of 1μg – 200μg, such as 10-100μg, preferably 10μg - 50μg per dose. Typically the oil emulsion (preferably oil in water emulsion) will comprise from 2 to 10% metabolisible oil. Preferably it will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% (preferably 0.4 – 2%) emulsifier (preferably tween 80 [polyoxyethylene sorbitan monooleate]). Where both squalene and alpha tocopherol are present, preferably the ratio of squalene: alpha tocopherol is equal to or less than 1 as this provides a more stable emulsion. Span 85 (Sorbitan trioleate) may also be present at a level of 0.5 to 1% in the emulsions used in the invention. In some cases it may be advantageous that the immunogenic compositions and vaccines of the present invention will further contain a stabiliser, for example other emulsifiers/surfactants, including caprylic acid (merck index 10th Edition, entry no. 1739), of which Tricaprylin is particularly preferred.

Where squalene and a saponin (preferably QS21) are included, it is of benefit to also include a sterol (preferably cholesterol) to the formulation as this allows a reduction in the total level of oil in the emulsion. This leads to a reduced cost of manufacture, improvement of the overall comfort of the vaccination, and also qualitative and quantitative improvements of the resultant immune responses, such as improved IFN-γ production. Accordingly, the adjuvant system of the present invention typically comprises a ratio of metabolisable oil:saponin (w/w) in the range of 200:1 to 300:1, also the present invention can be used in a "low oil" form the preferred range of which is 1:1 to 200:1, preferably 20:1 to 100:1, and most preferably substantially 48:1, this vaccine retains the beneficial adjuvant properties of all of the components, with a much reduced reactogenicity profile. Accordingly, the particularly preferred embodiments have a ratio of squalene:QS21 (w/w) in the range of 1:1 to 250:1, also a preferred range is 20:1 to 200:1, preferably 20:1 to 100:1, and most preferably substantially 48:1. Preferably a sterol (most preferably cholesterol) is also included present at a ratio of saponin:sterol as described herein.

The emulsion systems of the present invention preferably have a small oil droplet size in the sub-micron range. Most preferably the oil droplet sizes will be in the range 120 to 750 nm, and most preferably from 120-600nm in diameter.

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A particularly potent adjuvant formulation (for ultimate combination with AlPO4 in the immunogenic compositions of the invention) involves a saponin (preferably QS21), an LPS derivative (preferably 3D-MPL) and an oil emulsion (preferably squalene and alpha tocopherol in an oil in water emulsion) as described in WO 95/17210 or in WO 99/12565 (in particular adjuvant formulation 11 in Example 2, Table 1).

Examples of a TLR 2 agonist include peptidoglycan or lipoprotein. Imidazoquinolines, such as Imiquimod and Resiquimod are known TLR7 agonists. Single stranded RNA is also a known TLR agonist (TLR8 in humans and TLR7 in mice), whereas double stranded RNA and poly IC (polyinosinic-polycytidylic acid - a commercial synthetic mimetic of viral RNA). are exemplary of TLR 3 agonists. 3D-MPL is an example of a TLR4 agonist whilst CPG is an example of a TLR9 agonist.

The immunogenic composition may comprise an antigen and an immunostimulant adsorbed onto a metal salt. Aluminium based vaccine formulations wherein the antigen and the immunostimulant 3-de-O-acylated monophosphoryl lipid A (3D-MPL), are adsorbed onto the same particle are described in EP 0 576 478 B1, EP 0 689 454 B1, and EP 0 633 784 B1. In these cases then antigen is first adsorbed onto the aluminium salt followed by the adsorption of the immunostimulant 3D-MPL onto the same aluminium salt particles. Such processes first involve the suspension of 3D-MPL by sonication in a water bath until the particles reach a size of between 80 and 500 nm. The antigen is typically adsorbed onto aluminium salt for one hour at room temperature under agitation. The 3D-MPL suspension is then added to the adsorbed antigen and the formulation is incubated at room temperature for 1 hour, and then kept at 4oC until use.

In another process, the immunostimulant and the antigen are on separate metal particles, as described in EP 1126876. The improved process comprises the adsorption of immunostimulant, onto a metallic salt particle, followed by the adsorption of the antigen onto another metallic salt particle, followed by the mixing of the discrete metallic particles to form a vaccine. The adjuvant for use in the present invention may be an adjuvant composition comprising an immunostimulant, adsorbed onto a metallic salt particle, characterised in that the metallic salt particle is substantially free of other antigen. Furthermore, vaccines are provided by the present invention and are characterised in that the immunostimulant is adsorbed onto particles of metallic salt which are substantially free

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from other antigen, and in that the particles of metallic salt which are adsorbed to the antigen are substantially free of other immunostimulant.

Accordingly, the present invention provides an adjuvant formulation comprising immunostimulant which has been adsorbed onto a particle of a metallic salt, characterised in the composition is substantially free of other antigen. Moreover, this adjuvant formulation can be an intermediate which, if such an adjuvant is used, is required for the manufacture of a vaccine. Accordingly there is provided a process for the manufacture of a vaccine comprising admixing an adjuvant composition which is one or more immunostimulants adsorbed onto a metal particle with an antigen. Preferably, the antigen has been pre-adsorbed onto a metallic salt. Said metallic salt may be identical or similar to the metallic salt which is adsorbed onto the immunostimulant. Preferably the metal salt is an aluminium salt, for example Aluminium phosphate or Aluminium hydroxide.

The present invention further provides for a vaccine composition comprising immunostimulant adsorbed onto a first particle of a metallic salt, and antigen adsorbed onto a metallic salt, characterised in that first and second particles of metallic salt are separate particles.

LPS or LOS derivatives or mutations or lipid A derivatives described herein are designed to be less toxic (e.g. 3D-MPL) than native lipopolysaccharides and are interchangeable equivalents with respect to any uses of these moieties described herein. They may be TLR4 ligands as described above. Other such derivatives are described in WO020786737, WO9850399, WO0134617, WO0212258, WO03065806.

In one embodiment the adjuvant used for the compositions of the invention comprises a liposome carrier (made by known techniques from a phospholipids (such as dioleoyl phosphatidyl choline [DOPC]) and optionally a sterol [such as cholesterol]). Such liposome carriers may carry lipid A derivatives [such as 3D-MPL – see above] and/or saponins (such as QS21 – see above). In one embodiment the adjuvant comprises (per 0.5 mL dose) 0.1-10mg, 0.2-7, 0.3-5, 0.4-2, or 0.5-1 mg (e.g. 0.4-0.6, 0.9-1.1, 0.5 or 1 mg) phospholipid (for instance DOPC), 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or 0.125 mg) sterol (for instance cholesterol), 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) lipid A derivative (for instance 3D-MPL), and 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) saponin (for instance QS21).

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This adjuvant is particularly suitable for elderly vaccine formulations. In one embodiment the vaccine composition comprising this adjuvant comprises saccharide conjugates derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F (and may also comprise one or more from serotypes 3, 6A, 19A, and 22F), wherein the GMC antibody titre induced against one or more (or all) the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

In one embodiment the adjuvant used for the compositions of the invention comprises an oil in water emulsion made from a metabolisable oil (such as squalene), an emulsifier (such as Tween 80) and optionally a tocol (such as alpha tocopherol). In one embodiment the adjuvant comprises (per 0.5 mL dose) 0.5-15, 1-13, 2-11, 4-8, or 5-6mg (e.g. 2-3, 5-6, or 10-11 mg) metabolisable oil (such as squalene), 0.1-10, 0.3-8, 0.6-6, 0.9-5, 1-4, or 2-3 mg (e.g. 0.9-1.1, 2-3 or 4-5 mg) emulsifier (such as Tween 80) and optionally 0.5-20, 1-15, 2-12, 4-10, 5-7 mg (e.g. 11-13, 5-6, or 2-3 mg) tocol (such as alpha tocopherol).

This adjuvant may optionally further comprise 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) lipid A derivative (for instance 3D-MPL).

These adjuvants are particularly suitable for infant or elderly vaccine formulations. In one embodiment the vaccine composition comprising this adjuvant comprises saccharide conjugates derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F (and may also comprise one or more from serotypes 3, 6A, 19A, and 22F), wherein the GMC antibody titre induced against one or more (or all) the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

This adjuvant may optionally contain 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or 0.125 mg) sterol (for instance cholesterol), 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) lipid A derivative (for instance 3D-MPL), and 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) saponin (for instance QS21).

This adjuvant is particularly suitable for elderly vaccine formulations. In one embodiment the vaccine composition comprising this adjuvant comprises saccharide conjugates

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derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F (and may also comprise one or more from serotypes 3, 6A, 19A, and 22F), wherein the GMC antibody titre induced against one or more (or all) the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

In one embodiment the adjuvant used for the compositions of the invention comprises aluminium phosphate and a lipid A derivative (such as 3D-MPL). This adjuvant may comprise (per 0.5 mL dose) 100-750, 200-500, or 300-400 μg Al as aluminium phosphate, and 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) lipid A derivative (for instance 3D-MPL).

This adjuvant is particularly suitable for elderly or infant vaccine formulations. In one embodiment the vaccine composition comprising this adjuvant comprises saccharide conjugates derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F (and may also comprise one or more from serotypes 3, 6A, 19A, and 22F), wherein the GMC antibody titre induced against one or more (or all) the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

The vaccine preparations containing immunogenic compositions of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Intranasal administration of vaccines for the treatment of pneumonia or otitis media is preferred (as nasopharyngeal carriage of pneumococci can be more effectively prevented, thus attenuating infection at its earliest stage). Although the vaccine of the invention may be administered as a single dose, components thereof may also be co-administered together at the same time or at different times (for instance pneumococcal saccharide conjugates could be administered separately, at the same time or 1-2 weeks after the administration of the any bacterial protein component of the vaccine for optimal coordination of the immune responses with respect to each other). For co-administration, the optional Th1 adjuvant may be present in any or all of the different administrations. In addition to a single route of administration, 2 different routes of administration may be

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used. For example, saccharides or saccharide conjugates may be administered IM (or ID) and bacterial proteins may be administered IN (or ID). In addition, the vaccines of the invention may be administered IM for priming doses and IN for booster doses.

The content of protein antigens in the vaccine will typically be in the range 1-100μg, preferably 5-50μg, most typically in the range 5 - 25μg. Following an initial vaccination, subjects may receive one or several booster immunizations adequately spaced.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The vaccines of the present invention may be stored in solution or lyophilized. Preferably the solution is lyophilized in the presence of a sugar such as sucrose or lactose. It is still further preferable that they are lyophilized and extemporaneously reconstituted prior to use. Lyophilizing may result in a more stable composition (vaccine) and may possibly lead to higher antibody titers in the presence of 3D-MPL and in the absence of an aluminum based adjuvant.

In one aspect of the invention is provided a vaccine kit, comprising a vial containing an immunogenic composition of the invention, optionally in lyophilised form, and further comprising a vial containing an adjuvant as described herein. It is envisioned that in this aspect of the invention, the adjuvant will be used to reconstitute the lyophilised immunogenic composition.

Although the vaccines of the present invention may be administered by any route, administration of the described vaccines into the skin (ID) forms one embodiment of the present invention. Human skin comprises an outer "horny" cuticle, called the stratum corneum, which overlays the epidermis. Underneath this epidermis is a layer called the dermis, which in turn overlays the subcutaneous tissue. Researchers have shown that injection of a vaccine into the skin, and in particular the dermis, stimulates an immune response, which may also be associated with a number of additional advantages. Intradermal vaccination with the vaccines described herein forms a preferred feature of the present invention.

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The conventional technique of intradermal injection, the "mantoux procedure", comprises steps of cleaning the skin, and then stretching with one hand, and with the bevel of a narrow gauge needle (26-31 gauge) facing upwards the needle is inserted at an angle of between 10-15°. Once the bevel of the needle is inserted, the barrel of the needle is lowered and further advanced whilst providing a slight pressure to elevate it under the skin. The liquid is then injected very slowly thereby forming a bleb or bump on the skin surface, followed by slow withdrawal of the needle.

More recently, devices that are specifically designed to administer liquid agents into or across the skin have been described, for example the devices described in WO 99/34850 and EP 1092444, also the jet injection devices described for example in WO 01/13977; US 5,480,381, US 5,599,302, US 5,334,144, US 5,993,412, US 5,649,912, US 5,569,189, US 5,704,911, US 5,383,851, US 5,893,397, US 5,466,220, US 5,339,163, US 5,312,335, US 5,503,627, US 5,064,413, US 5,520, 639, US 4,596,556, US 4,790,824, US 4,941,880, US 4,940,460, WO 97/37705 and WO 97/13537. Alternative methods of intradermal administration of the vaccine preparations may include conventional syringes and needles, or devices designed for ballistic delivery of solid vaccines (WO 99/27961), or transdermal patches (WO 97/48440; WO 98/28037); or applied to the surface of the skin (transdermal or transcutaneous delivery WO 98/20734 ; WO 98/28037).

When the vaccines of the present invention are to be administered to the skin, or more specifically into the dermis, the vaccine is in a low liquid volume, particularly a volume of between about 0.05 ml and 0.2 ml.

The content of antigens in the skin or intradermal vaccines of the present invention may be similar to conventional doses as found in intramuscular vaccines (see above). However, it is a feature of skin or intradermal vaccines that the formulations may be "low dose". Accordingly the protein antigens in "low dose" vaccines are preferably present in as little as 0.1 to 10μg, preferably 0.1 to 5 μg per dose; and the saccharide (preferably conjugated) antigens may be present in the range of 0.01-1μg, and preferably between 0.01 to 0.5 μg of saccharide per dose.

As used herein, the term "intradermal delivery" means delivery of the vaccine to the region of the dermis in the skin. However, the vaccine will not necessarily be located exclusively in the dermis. The dermis is the layer in the skin located between about 1.0 and about 2.0

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mm from the surface in human skin, but there is a certain amount of variation between individuals and in different parts of the body. In general, it can be expected to reach the dermis by going 1.5 mm below the surface of the skin. The dermis is located between the stratum corneum and the epidermis at the surface and the subcutaneous layer below. Depending on the mode of delivery, the vaccine may ultimately be located solely or primarily within the dermis, or it may ultimately be distributed within the epidermis and the dermis.

The present invention further provides an improved vaccine for the prevention or amelioration of Otitis media caused by *Haemophilus influenzae* by the addition of *Haemophilus influenzae* proteins, for example protein D in free or conjugated form. In addition, the present invention further provides an improved vaccine for the prevention or amelioration of pneumococcal infection in infants (e.g., Otitis media), by relying on the addition of one or two pneumococcal proteins as free or conjugated protein to the *S. pneumoniae* conjugate compositions of the invention. Said pneumococcal free proteins may be the same or different to any *S. pneumoniae* proteins used as carrier proteins. One or more *Moraxella catarrhalis* protein antigens can also be included in the combination vaccine in a free or conjugated form. Thus, the present invention is an improved method to elicit a (protective) immune response against Otitis media in infants.

In another embodiment, the present invention is an improved method to elicit a (protective) immune response in infants (defined as 0-2 years old in the context of the present invention) by administering a safe and effective amount of the vaccine of the invention [a paediatric vaccine]. Further embodiments of the present invention include the provision of the antigenic *S. pneumoniae* conjugate compositions of the invention for use in medicine and the use of the *S. pneumoniae* conjugates of the invention in the manufacture of a medicament for the prevention (or treatment) of pneumococcal disease.

In yet another embodiment, the present invention is an improved method to elicit a (protective) immune response in the elderly population (in the context of the present invention a patient is considered elderly if they are 50 years or over in age, typically over 55 years and more generally over 60 years) by administering a safe and effective amount of the vaccine of the invention, preferably in conjunction with one or two *S. pneumoniae* proteins present as free or conjugated protein, which free *S. pneumoniae* proteins may be the same or different as any *S. pneumoniae* proteins used as carrier proteins.

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A further aspect of the invention is a method of immunising a human host against disease caused by *S. pneumoniae* and optionally *Haemophilus influenzae* infection comprising administering to the host an immunoprotective dose of the immunogenic composition or vaccine or kit of the invention.

A further aspect of the invention is an immunogenic composition of the invention for use in the treatment or prevention of disease caused by *S.pneumoniae* and optionally *Haemophilus influenzae* infection.

A further aspect of the invention is use of the immunogenic composition or vaccine or kit of the invention in the manufacture of a medicament for the treatment or prevention of diseases caused by *S. pneumoniae* and optionally *Haemophilus influenzae* infection.

The terms "comprising", "comprise" and "comprises" herein are intended by the inventors to be optionally substitutable with the terms "consisting of", "consist of" and "consists of", respectively, in every instance.

Embodiments herein relating to "vaccine compositions" of the invention are also applicable to embodiments relating to "immunogenic compositions" of the invention, and vice versa.

All references or patent applications cited within this patent specification are incorporated by reference herein.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

Examples

Example 1: EXPRESSION OF PROTEIN D

***Haemophilus influenzae* protein D**

Genetic construction for protein D expression

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Starting materials

The Protein D encoding DNA

Protein D is highly conserved among *H. influenzae* of all serotypes and non-typeable strains. The vector pHIC348 containing the DNA sequence encoding the entire protein D gene has been obtained from Dr. A. Forsgren, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden. The DNA sequence of protein D has been published by Janson et al. (1991) Infect. Immun. 59: 119-125.

The expression vector pMG1

The expression vector pMG1 is a derivative of pBR322 (Gross *et al.*, 1985) in which bacteriophage λ derived control elements for transcription and translation of foreign inserted genes were introduced (Shatzman *et al.*, 1983). In addition, the Ampicillin resistance gene was exchanged with the Kanamycin resistance gene.

The E. coli strain AR58

The *E. coli* strain AR58 was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::TN10, lambdaKil$^-$ cI857 ΔH1). N99 and SA500 are *E. coli* K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institute of Health.

The expression vector pMG 1

For the production of protein D, the DNA encoding the protein has been cloned into the expression vector pMG 1. This plasmid utilises signals from lambdaphage DNA to drive the transcription and translation of inserted foreign genes. The vector contains the promoter PL, operator OL and two utilisation sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross *et al.*, 1985). Vectors containing the PL promoter, are introduced into an *E. coli* lysogenic host to stabilise the plasmid DNA. Lysogenic host strains contain replication-defective lambdaphage DNA integrated into the genome (Shatzman *et al.*, 1983). The chromosomal lambdaphage DNA directs the synthesis of the cI repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promoter and thereby transcription of the inserted gene. The cI gene of the expression strain AR58 contains a temperature sensitive mutant so that PL directed transcription can be regulated by temperature shift, i.e. an increase in culture temperature inactivates the repressor and

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synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimataka & Rosenberg, 1981).

The E. coli strain AR58

The AR58 lysogenic *E. coli* strain used for the production of the protein D carrier is a derivative of the standard NIH *E. coli* K12 strain N99 (F^- su^- galK2, $lacZ^-$ thr^-). It contains a defective lysogenic lambdaphage (galE::TN10, lambdaKil$^-$ cI857 ΔH1). The Kil^- phenotype prevents the shut off of host macromolecular synthesis. The cI857 mutation confers a temperature sensitive lesion to the cI repressor. The ΔH1 deletion removes the lambdaphage right operon and the hosts bio, uvr3, and chlA loci. The AR58 strain was generated by transduction of N99 with a P1 phage stock previously grown on an SA500 derivative (galE::**TN**10, lambdaKil$^-$ cI857 ΔH1). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a TN10 transposon coding for tetracyclin resistance in the adjacent galE gene.

Construction of vector pMGMDPPrD

The pMG 1 vector which contains the gene encoding the non-structural S1 protein of Influenzae virus (pMGNSI) was used to construct pMGMDPPrD. The protein D gene was amplified by PCR from the pHIC348 vector (Janson *et al.* 1991 Infect. Immun. 59:119-125) with PCR primers containing NcoI and XbaI restriction sites at the 5' and 3' ends, respectively. The NcoI/XbaI fragment was then introduced into pMGNS1 between NcoI and XbaI thus creating a fusion protein containing the N-terminal 81 amino acids of the NS1 protein followed by the PD protein. This vector was labelled pMGNS1PrD.

Based on the construct described above the final construct for protein D expression was generated. A BamHI/BamHI fragment was removed from pMGNS1PrD. This DNA hydrolysis removes the NS1 coding region, except for the first three N-terminal residues. Upon religation of the vector a gene encoding a fusion protein with the following N-terminal amino acid sequence has been generated:

-----MDP SSHSSNMANT-----
NS1 Protein D

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The protein D does not contain a leader peptide or the N-terminal cysteine to which lipid chains are normally attached. The protein is therefore neither excreted into the periplasm nor lipidated and remains in the cytoplasm in a soluble form.

The final construct pMG-MDPPrD was introduced into the AR58 host strain by heat shock at 37 °C. Plasmid containing bacteria were selected in the presence of Kanamycin. Presence of the protein D encoding DNA insert was demonstrated by digestion of isolated plasmid DNA with selected endonucleases. The recombinant *E. coli* strain is referred to as ECD4.

Expression of protein D is under the control of the lambda P_L promoter/ O_L Operator. The host strain AR58 contains a temperature-sensitive cI gene in the genome which blocks expression from lambda P_L at low temperature by binding to O_L. Once the temperature is elevated cI is released from O_L and protein D is expressed.

Small-scale preparation

At the end of the fermentation the cells are concentrated and frozen.

The extraction from harvested cells and the purification of protein D was performed as follows. The frozen cell culture pellet is thawed and resuspended in a cell disruption solution (Citrate buffer pH 6.0) to a final OD_{650} = 60. The suspension is passed twice through a high pressure homogenizer at P = 1000 bar. The cell culture homogenate is clarified by centrifugation and cell debris is removed by filtration. In the first purification step the filtered lysate is applied to a cation exchange chromatography column (SP Sepharose Fast Flow). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer.

In a second purification step impurities are retained on an anionic exchange matrix (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated by ultrafiltration.

The protein D containing ultrafiltration retentate is finally passed through a 0.2 µm membrane.

Large Scale Preparation

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The extraction from harvested cells and the purification of protein D was performed as follows. The harvested broth is cooled and directly passed twice through a high pressure homogenizer at a Pressure of around 800 bars.

In the first purification step the cell culture homogenate is diluted and applied to a cation exchange chromatography column (SP Sepharose Big beads). PD binds to the gel matrix by ionic interaction and is eluted by a step increase of the ionic strength of the elution buffer and filtrated.

In a second purification step impurities are retained on an anionic exchange matrix (Q Sepharose Fast Flow). PD does not bind onto the gel and can be collected in the flow through.

In both column chromatography steps fraction collection is monitored by OD. The flow through of the anionic exchange column chromatography containing the purified protein D is concentrated and diafiltrated by ultrafiltration.

The protein D containing ultrafiltration retentate is finally passed through a 0.2 µm membrane.

Example 1b: EXPRESSION OF PhtD

The PhtD protein is a member of the pneumococcal histidine-triad (Pht) protein family characterized by the presence of histidine-triads (HXXHXH motif). PhtD is a 838 aa-molecule and carries 5 histidine triads (see MedImmune WO00/37105 SEQ ID NO: 4 for amino acid sequence and SEQ ID NO: 5 for DNA sequence). PhtD also contains a proline-rich region in the middle (amino acid position 348-380). PhtD has a 20 aa-N-terminal signal sequence with a LXXC motif.

Genetic construct

The gene sequence of the mature MedImmune PhtD protein (from aa 21 to aa 838) was transferred recombinantly to *E. coli* using the in-house pTCMP14 vector carrying the pλ promoter. The *E. coli* host strain is AR58, which carries the cI857 thermosensitive repressor, allowing heat-induction of the promotor.

Polymerase chain reaction was realized to amplify the *phtD* gene from a MedImmune plasmid (carrying the *phtD* gene from *Streptococcus pneumoniae* strain Norway 4 (serotype 4) – SEQ ID NO: 5 as described in WO 00/37105). Primers, specific for the *phtD* gene only, were used to amplify the *phtD* gene in two fragments. Primers carry either the *Nde*I and *Kpn*I or the *Kpn*I and *Xba*I restriction sites. These primers do not hybridize with any nucleotide from the vector but only with *phtD* specific gene sequences. An artificial ATG start codon was inserted using the first primer carrying the *Nde*I restriction site. The generated PCR products were then inserted into the pGEM-T cloning vector (Promega),

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and the DNA sequence was confirmed. Subcloning of the fragments in the TCMP14 expression vector was then realized using standard techniques and the vector was transformed into *AR58 E. coli*.

PhtD Purification

PhtD purification is achieved as follows:

- Growth of E.coli cells in the presence of Kanamycin: growth 30 hours at 30 °C then induction for 18 hours at 39.5 °C
- Breakage of the E.coli cells from whole culture at OD ±115 in presence of EDTA 5 mM and PMSF 2 mM as protease inhibitors: Rannie, 2 passages, 1000 bars.
- Antigen capture and cells debris removal on expanded bed mode Streamline Q XL chromatography at room temperature (20°C); the column is washed with NaCl 150 mM + Empigen 0.25% pH 6.5 and eluted with NaCl 400 mM + Empigen 0.25% in 25 mM potassium phosphate buffer pH 7.4.
- Filtration on Sartobran 150 cartridge (0.45 + 0.2 µm)
- Antigen binding on Zn^{++} Chelating Sepharose FF IMAC chromatography at pH 7.4 in presence of 5 mM imidazole at 4°C; the column is washed with Imidazole 5 mM and Empigen 1% and eluted with 50 mM imidazole, both in 25 mM potassium phosphate buffer pH 8.0.
- Weak anion exchange chromatography in positive mode on Fractogel EMD DEAE at pH 8.0 (25 mM potassium phosphate) at 4°C; the column is washed with 140 mM NaCl and eluted at 200 mM NaCl while contaminants (proteins and DNA) remain adsorbed on the exchanger.
- Concentration and ultrafiltration with 2 mM Na/K phosphate pH 7.15 on 50 kDa membrane.
- Sterilising filtration of the purified bulk on a Millipak-20 0.2 µm filter cartridge.

Example 1c: EXPRESSION OF PNEUMOLYSIN

Pneumococcal pneumolysin was prepared and detoxified as described in WO2004/081515 and WO2006/032499.

Example 2:

Preparation of conjugates

It is well known in the art how to make purified pneumococcal polysaccharides. For the purposes of these examples the polysaccharides were made essentially as

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described in EP072513 or by closesly-related methods. Before conjugation the polysaccharides may be sized by microfluidisation as described below.

The activation and coupling conditions are specific for each polysaccharide. These are given in Table 1. Sized polysaccharide (except for PS5, 6B and 23F) was dissolved in NaCl 2M, NaCl 0.2M or in water for injection (WFI). The optimal polysaccharide concentration was evaluated for all the serotypes. All serotypes except serotype 18C were conjugated directly to the carrier protein as detailed below. Two alternative serotype 22F conjugates were made; one conjugated directly, one through an ADH linker.

From a 100 mg/ml stock solution in acetonitrile or acetonitrile/water 50%/50% solution, CDAP (CDAP/PS ratio 0.5-1.5 mg/mg PS) was added to the polysaccharide solution. 1.5 minute later, 0.2M-0.3M NaOH was added to obtain the specific activation pH. The activation of the polysaccharide was performed at this pH during 3 minutes at 25 °C. Purified protein (protein D, PhtD, pneumolysin or DT) (the quantity depends on the initial PS/carrier protein ratio) was added to the activated polysaccharide and the coupling reaction was performed at the specific pH for up to 2 hour (depending upon serotype) under pH regulation. In order to quench un-reacted cyanate ester groups, a 2M glycine solution was then added to the mixture. The pH was adjusted to the quenching pH (pH 9.0). The solution was stirred for 30 minutes at 25 °C and then overnight at 2-8 °C with continuous slow stirring.

Preparation of 18C:

18C was linked to the carrier protein via a linker – Adipic acid dihydrazide (ADH) Polysaccharide serotype 18C was microfluidized before conjugation.

Derivatization of tetanus toxoid with EDAC

For derivatization of the tetanus toxoid, purified TT was diluted at 25 mg/ml in 0.2M NaCl and the ADH spacer was added in order to reach a final concentration of 0.2M. When the dissolution of the spacer was complete, the pH was adjusted to 6.2. EDAC (1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide) was then added to reach a final concentration of 0.02M and the mixture was stirred for 1 hour under pH regulation. The reaction of condensation was stopped by increasing pH up to 9.0 for at least 30 minutes at 25°C. Derivatized TT was then diafiltrated (10 kDa CO membrane) in order to remove residual ADH and EDAC reagent.

TT_{AH} bulk was finally sterile filtered until coupling step and stored at -70°C.

Chemical coupling of TT_{AH} to PS 18C

Details of the conjugation parameters can be found in Table 1.

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2 grams of microfluidized PS were diluted at the defined concentration in water and adjusted to 2M NaCl by NaCl powder addition.

CDAP solution (100 mg/ml freshly prepared in 50/50 v/v acetonitrile/WFI) was added to reach the appropriate CDAP/PS ratio.

The pH was raised up to the activation pH 9.0 by the addition of 0.3M NaOH and was stabilised at this pH until addition of TT_{AH}.

After 3 minutes, derivatized TT_{AH} (20 mg/ml in 0.2 M NaCl) was added to reach a ratio TT_{AH} /PS of 2; the pH was regulated to the coupling pH 9.0. The solution was left one hour under pH regulation.

For quenching, a 2M glycine solution, was added to the mixture PS/TT_{AH}/CDAP.

The pH was adjusted to the quenching pH (pH 9.0).

The solution was stirred for 30 min at 25 °C, and then left overnight at 2-8°C with continuous slow stirring.

PS22F$_{AH}$-PhtD conjugate

In a second conjugation method for this saccharide (the first being the direct PS22-PhtD conjugation method shown in Table 1), 22F was linked to the carrier protein via a linker – Adipic acid dihydrazide (ADH). Polysaccharide serotype 22F was microfluidized before conjugation.

PS 22F derivatization

Activation and coupling are performed at 25°C under continuous stirring in a temperature-controlled waterbath.

Microfluidized PS22F was diluted to obtain a final PS concentration of 6 mg/ml in 0.2M NaCl and the solution was adjusted at pH 6.05 ± 0.2 with 0.1N HCl.

CDAP solution (100 mg/ml freshly prepared in acetonitrile/WFI, 50/50) was added to reach the appropriate CDAP/PS ratio (1.5/1 ww).

The pH was raised up to the activation pH 9.00 ±0.05 by the addition of 0.5M NaOH and was stabilised at this pH until addition of ADH.

After 3 minutes, ADH was added to reach the appropriate ADH/PS ratio (8.9/1 w/w); the pH was regulated to coupling pH 9.0. The solution was left for 1 hour under pH regulation.

The PS_{AH} derivative was concentrated and diafiltrated.

Coupling

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PhtD at 10 mg/ml in 0.2M NaCl was added to the $PS22F_{AH}$ derivative in order to reach a PhtD/$PS22F_{AH}$ ratio of 4/1 (w/w). The pH was adjusted to 5.0 ± 0.05 with HCl. The EDAC solution (20 mg/ml in 0.1M Tris-HCl pH 7.5) was added manually in 10 min (250 µl / min) to reach 1 mg EDAC/mg $PS22F_{AH}$. The resulting solution was incubated for 150 min (though 60 mins was also used) at 25°C under stirring and pH regulation. The solution was neutralized by addition of 1M Tris-HCl pH 7.5 (1/10 of the final volume) and let 30 min at 25°C.

Prior to the elution on Sephacryl S400HR, the conjugate was clarified using a 5µm Minisart filter.

The resulting conjugate has a final PhtD/PS ratio of 4.1 (w/w), a free PS content below 1% and an antigenicity (α-PS/α-PS) of 36.3% and anti-PhtD antigenicity of 7.4%.

Purification of the conjugates:

The conjugates were purified by gel filtration using a Sephacryl S400HR gel filtration column equilibrated with 0.15M NaCl (S500HR for 18C) to remove small molecules (including DMAP) and unconjugated PS and protein. Based on the different molecular sizes of the reaction components, PS-PD, PS-TT, PS-PhtD, PS-pneumolysin or PS-DT conjugates are eluted first, followed by free PS, then by free PD or free DT and finally DMAP and other salts (NaCl, glycine).

Fractions containing conjugates are detected by $UV_{280\ nm}$. Fractions are pooled according to their Kd, sterile filtered (0.22µm) and stored at +2-8°C. The PS/Protein ratios in the conjugate preparations were determined.

Specific activation/coupling/quenching conditions of PS S. pneumoniae-Protein D/TT/DT/PhtD/Plyconjugates

Where "**µfluid**" appears in a row header, it indicates that the saccharide was sized by microfluidisation before conjugation. Sizes of saccharides following microfluidisation are given in table 2.

Table 1 **Specific activation/coupling/quenching conditions of PS S. pneumoniae-Protein D/TT/DT/PhtD/Plyconjugates**

Serotype	1 µfluid	4 µfluid	5	6A	6B	7F µfluid
PS conc.(mg/ml)	2.5	2.5	7.1	5.0	5.0	5.0

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PS dissolution	WFI	WFI	WFI	NaCl 2M	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	10.0	10.0	5.0	5.0	5.0	10.0
Initial PD/PS Ratio (w/w)	1.5/1	1.5/1	1/1	1/1	1.1/1	1.2/1
CDAP conc. (mg/mg PS)	0.50	0.50	0.79	0.83	0.83	0.75
$pH_a=pH_c=pH_q$	9.0/9.0/9.0	9.5/9.5/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.5/9.5/9.0	9.5/9.5/9.0

Serotype	**9V µfluid**	**14 µfluid**	**18C µfluid**	**19A µfluid**	**19F µfluid**	**22F µfluid**	**23F**
PS conc.(mg/ml)	5.0	5.0	4.5	15.0	9.0	6.0	2.38
PS dissolution	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 0.2M	NaCl 2M
Carrier protein conc.(mg/ml)	10.0	10.0	20.0 (TT)	10.0 (Ply)	20.0 (DT)	10.0 (PhtD)	5.0
Initial carrier protein/PS Ratio (w/w)	1.2/1	1.2/1	2/1	2.5/1	1.5/1	3/1	1/1
CDAP conc. (mg/mg PS)	0.50	0.75	0.75	1.5	1.5	1.5	0.79
$pH_a=pH_c=pH_q$	9.5/9.5/9.0	9.5/9.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0

Note: pHa,c,q corresponds to the pH for activation, coupling and quenching, respectively

Characterisation:

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Each conjugate was characterised and met the specifications described in Table 2. The polysaccharide content (µg/ml) was measured by the Resorcinol test and the protein content (µg/ml) by the Lowry test. The final PS/PD ratio (w/w) is determined by the ratio of the concentrations.

Free polysaccharide content (%):

The free polysaccharide content of conjugates kept at 4°C or stored 7 days at 37°C was determined on the supernatant obtained after incubation with α-carrier protein antibodies and saturated ammonium sulfate, followed by a centrifugation.

An α-PS/α-PS ELISA was used for the quantification of free polysaccharide in the supernatant. The absence of conjugate was also controlled by an α-carrier protein/α-PS ELISA.

Antigenicity:

The antigenicity on the same conjugates was analyzed in a sandwich-type ELISA wherein the capture and the detection of antibodies were α-PS and α-Protein respectively.

Free protein content (%):

Unconjugated carrier protein can be separated from the conjugate during the purification step. The content of free residual protein was determined using size exclusion chromatography (TSK 5000-PWXL) followed by UV detection (214 nm). The elution conditions allowed separating the free carrier protein and the conjugate. Free protein content in conjugate bulks was then determined versus a calibration curve (from 0 to 50 µg/ml of carrier protein). Free carrier protein in % was obtained as follows: % free carrier = (free carrier (µg/ml)/ (Total concentration of corresponding carrier protein measured by Lowry (µg/ml) * 100%).

Stability:

Molecular weight distribution (K_{av}) and stability was measured on a HPLC-SEC gel filtration (TSK 5000-PWXL) for conjugates kept at 4°C and stored for 7 days at 37°C.

The 10/11/13/14-valent characterization is given in Table 2 (see comment thereunder).

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The protein conjugates can be adsorbed onto aluminium phosphate and pooled to form the final vaccine.

Conclusion:

Immunogenic conjugates have been produced, that have since been shown to be components of a promising vaccine.

TABLE 2 – characteristics of the conjugates

Conjugates	PS size (Dax10^3)	Carrier/PS Ratio	Free PS (Elisa)	Free Carrier	PS Antigenicity (Elisa)	Conj. Size (kDa)
PS1-PD	349-382*	1.5-1.6	1.0%-1.2%	3.9%-4.8%	87%-95%	1499 - 1715
PS4-PD	93-100*	1.5-1.6	4.7-6.5%	3.2%-4.0%	90%-96%	1303 - 1606
PS5-PD***	367-443	0.80	8.7-11.2%	2.2%-3.8%	93%-108%	1998-2352
PS6A-PD	1100-1540	0.61	4.5%	Not done	45.9%	Not done
PS6B-PD***	1069-1391	0.7-0.8	1.3-1.6%	<2.0%	68%-75%	4778-5235
PS7F-PD	255-264*	1.1-1.2	<1%	<1.4%	58%	3907-4452
PS9V-PD	258-280*	1.3-1.5	<1%	<1.3%	67%-69%	9073-9572
PS14-PD	232-241*	1.4	<1%	<1.5%	70%	3430-3779
PS18C-TT	89-97*	2.2-2.4	1.5-2.2%	<4%	46%-56%	5464-6133
PS19A-Ply*	151	3.2	<1%		29%	
PS19F-DT	133-143*	1.4-1.5	4.1%-5.9%	<1.2%-<1.3%	82%-88%	2059-2335
PS22F-PhtD*	159-167	2.17	5.8	Not done	37%	Not done
PS22F-AHPhtD*	159-167	3.66-4.34	<1%	Not done	28-31%	Not done
PS23F-PD***	914-980	0.5	1.4-1.9%	3.7%-4.9%	137%-154%	2933-3152

* PS size following microfluidization of the native PS

A 10 valent vaccine was made by mixing serotype 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F conjugates (e.g. at a dose of 1, 3, 1, 1, 1, 1, 1, 3, 3, 1 μg of saccharide, respectively per human dose). An 11 valent vaccine was made by further adding the serotype 3 conjugate from Table 5 (e.g. at 1 μg of saccharide per human dose). A 13 valent vaccine was made by further adding the serotypes 19A and 22F conjugates above (with 22F either directly linked to PhtD, or alternatively through an ADH linker) [e.g. at a dose of 3 μg each of saccharide per human dose]. A 14 valent vaccine may be made by further

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adding the serotype 6A conjugate above [e.g. at a dose of 1 μg of saccharide per human dose.

Example 3: Evidence that inclusion of *Haemphilus influenzae* protein D in an immunogenic composition of the invention can provide improved protection against acute otitis media (AOM).

Study design.

The study used an 11Pn-PD vaccine – comprising serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F each conjugated to protein D from *H.* influenzae (refer to Table 5 in Example 4). Subjects were randomized into two groups to receive four doses of either the 11Pn-PD vaccine or *Havrix* at approximately 3, 4, 5 and 12-15 months of age. All subjects received GSK Biologicals' *Infanrix- hexa* (DTPa-HBV-IPV/Hib) vaccine concomitantly at 3, 4 and 5 months of age. *Infanrix- hexa* is a combination of *Pediarix* and Hib mixed before administration. Efficacy follow-up for the "According-to-Protocol" analysis started 2 weeks after administration of the third vaccine dose and continued until 24-27 months of age. Nasopharyngeal carriage of *S. pneumoniae* and *H. influenzae* was evaluated in a selected subset of subjects.

Parents were advised to consult the investigator if their child was sick, had ear pain, spontaneous perforation of the tympanic membrane or spontaneous ear discharge. If the investigator suspected an episode of AOM, the child was immediately referred to an Ear, Nose and Throat (ENT) specialist for confirmation of the diagnosis.

A clinical diagnosis of AOM was based on either the visual appearance of the tympanic membrane (i.e. redness, bulging, loss of light reflex) or the presence of middle ear fluid effusion (as demonstrated by simple or pneumatic otoscopy or by microscopy). In addition, at least two of the following signs or symptoms had to be present: ear pain, ear discharge, hearing loss, fever, lethargy, irritability, anorexia, vomiting, or diarrhea. If the ENT specialist confirmed the clinical diagnosis, a specimen of middle ear fluid was collected by tympanocentesis for bacteriological testing.

For subjects with repeated sick visits, a new AOM episode was considered to have started if more than 30 days had elapsed since the beginning of the previous episode. In addition, an AOM episode was considered to be a new bacterial episode if the isolated bacterium/serotype differed from the previous isolate whatever the interval between the two consecutive episodes.

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Trial results

A total of 4968 infants were enrolled, 2489 in the 11Pn-PD group and 2479 in the control group. There were no major differences in the demographic characteristics or risk factors between the two groups.

Clinical episodes and AOM case definition

During the per protocol follow-up period, a total of 333 episodes of clinical AOM were recorded in the 11Pn-PD group and 499 in the control group.

Table 3 presents the protective efficacy of the 11Pn-PD vaccine and both 7-valent vaccines previously tested in Finland (Eskola *et* al N Engl J Med 2001; **344**: 403 – 409 and Kilpi *et al*Clin Infect Dis 2003 **37**:1155-64) against any episode of AOM and AOM caused by different pneumococcal serotypes, *H. influenzae, NTHi* and *M. catarrhalis*. Statistically significant and clinically relevant reduction by 33.6% of the overall AOM disease burden was achieved with 11Pn-PD, irrespective of the etiology (table 3).

The overall efficacy against AOM episodes due to any of the 11 pneumococcal serotypes contained in the 11Pn-PD vaccine was 57.6% (table 3).

Another important finding in the current study is the 35.6% protection provided by the 11Pn-PD vaccine against AOM caused by *H. influenzae* (and specifically 35.3% protection provided by NTHi). This finding is of major clinical significance, given the increased importance of *H. influenzae* as a major cause of AOM in the pneumococcal conjugate vaccine era. In line with the protection provided against AOM, the 11Pn-PD vaccine also reduced nasopharyngeal carriage of *H. influenzae* following the booster dose in the second year of life. These findings are in contrast with previous observations in Finland where, for both 7-valent pneumococcal conjugate vaccines, an increase in AOM episodes due to *H. influenzae* was observed, (Eskola *et al* and Kilpi *et al)* as evidence of etiological replacement.

A clear correlation between protection against AOM episodes due to Hi and antibody levels against the carrier Protein D could not be established, as post-primary anti-PD IgG antibody concentrations in 11Pn-PD vaccinees, that remained Hi AOM episode-free, were essentially the same as post-primary anti-PD IgG antibody levels measured in 11Pn-PD vaccinees that developed at least one Hi AOM episode during the efficacy follow-up period. However, although no correlation could be established between the biological impact of the vaccine and the post-primary IgG anti-PD immunogenicity, it is reasonable

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to assume that the PD carrier protein, which is highly conserved among *H. influenzae* strains, has contributed to a large extent in the induction of the protection against Hi.

The effect on AOM disease was accompanied by an effect on nasopharyngeal carriage that was of similar magnitude for vaccine serotype pneumococci and *H. influenzae* (Figure 1). This reduction of the nasopharyngeal carriage of *H. influenzae* in the PD-conjugate vaccinees supports the hypothesis of a direct protective effect of the PD-conjugate vaccine against *H. influenzae*, even if the protective efficacy could not be correlated to the anti-PD IgG immune responses as measured by ELISA.

In a following experiment a chinchilla otitis media model was used with serum pools from infants immunised with the 11 valent formulation of this example or with the 10 valent vaccine of Example 2 (see also Table 1 and 2 and comments thereunder). Both pools induce a significant reduction of the percentage of animals with otitis media versus the pre-immune serum pool. There is no significant difference beteen the 10 and 11 valent immune pools. This demonstrates that both vaccines have a similar potential to induce protection against otitis media caused by non typeable *H. influenzae* in this model.

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Table 3

	11Pn-PD					Prevnar in FinOM (Eskola et al)					7v-OMP in FinOM (Kilpi et al)				
	n		VE			n		VE			n		VE		
				95%CI					95%CI					95%CI	
Type of AOM episode	11Pn-PD	Control	%	LL	UL	7v-CRM	Control	%	LL	UL	7v-OMP	Control	%	LL	UL
N	2455	2452				786	794				805	794			
Any AOM	333	499	**33.6**	20.8	44.3	1251	1345	**6**	-4	16	1364	1345	**-1**	-12	10
Any AOM with MEF	322	474	**32.4**	19.0	43.6	1177	1267	**7**	-5	17	1279	1267	**0**	-12	10
Culture confirmed pneumococcus	92	189	**51.5**	36.8	62.9	271	414	**34**	21	45	314	414	**25**	11	37
Vaccine pneumococcal serotypes(*)	60	141	**57.6**	41.4	69.3	107	250	**57**	44	67	110	250	**56**	44	66
Other bacterial pathogens															
H. influenzae	44	68	**35.6**	3.8	57.0	315	287	**-11**	-34	8	315	287	**-9**	-32	10
Non-typeable *H. influenzae* (NTHi)	41	63	**35.3**	1.8	57.4	NP	NP	**NP**	NP	NP	NP	NP	**NC**	NP	NP
M. catarrhalis	31	34	**9.4**	-52.5	46.1	379	381	**-1**	-19	15	444	381	**-16**	-36	2

NP = Not published; N = number of subjects in ATP efficacy cohort; n = number of episodes
*Vaccine pneumococcal serotypes: for 11Pn-PD = 11 serotypes, for Prevnar and 7v-OMP = 7 serotypes
MEF = Middle ear fluid

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Example 4:

Selection of carrier protein for serotype 19F

ELISA assay used

The 22F inhibition ELISA method was essentially based on an assay proposed in 2001 by Concepcion and Frasch and was reported by Henckaerts et al., 2006, Clinical and Vaccine Immunology 13:356-360. Briefly, purified pneumococcal polysaccharides were mixed with methylated human serum albumin and adsorbed onto Nunc Maxisorp™ (Roskilde, DK) high binding microtitre plates overnight at 4°C. The plates were blocked with 10% fetal bovine serum (FBS) in PBS for 1 hour at room temperature with agitation. Serum samples were diluted in PBS containing 10% FBS, 10 µg/mL cell-wall polysaccharide (SSI) and 2 µg/mL of pneumococcal polysaccharide of serotype 22F (ATCC), and further diluted on the microtitre plates with the same buffer. An internal reference calibrated against the standard serum 89-SF using the serotype-specific IgG concentrations in 89-SF was treated in the same way and included on every plate. After washing, the bound antibodies were detected using peroxidase-conjugated anti-human IgG monoclonal antibody (Stratech Scientific Ltd., Soham, UK) diluted in 10% FBS (in PBS), and incubated for 1 hour at room temperature with agitation. The color was developed using ready-to-use single component tetramethylbenzidine peroxidase enzyme immunoassay substrate kit (BioRad, Hercules, CA, US) in the dark at room temperature. The reaction was stopped with H_2SO_4 0.18 M, and the optical density was read at 450 nm. Serotype-specific IgG concentrations (in µg/mL) in the samples were calculated by referencing optical density points within defined limits to the internal reference serum curve, which was modelized by a 4-parameter logistic log equation calculated with SoftMax Pro™ (Molecular Devices, Sunnyvale, CA) software. The cut-off for the ELISA was 0.05 µg/mL IgG for all serotypes taking into account the limit of detection and the limit of quantification.

Opsonophagocytosis assay

At the WHO consultation meeting in June 2003, it was recommended to use an OPA assay as set out in Romero-Steiner *et al* Clin Diagn Lab Immunol 2003 **10** (6): pp1019-1024. This protocol was used to test the OPA activity of the serotypes in the following tests.

Preparation of conjugates

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In studies 11Pn-PD&Di-001 and 11Pn-PD&Di-007, three 11-valent vaccine formulations (Table 4) were included in which 3µg of the 19F polysaccharide was conjugated to diphtheria toxoid (19F-DT) instead of 1µg polysaccharide conjugated to protein D (19F-PD). Conjugation parameters for the studies 11Pn-PD, 11 Pn-PD&Di-001 and 11 Pn-PD&Di-007 are disclosed in Tables 5, 6 and 7 respectively.

Anti-pneumococcal antibody responses and OPA activity against serotype 19F one month following primary vaccination with these 19F-DT formulations are shown in Table 8 and 9 respectively.

Table 10 shows 22F-ELISA antibody concentrations and percentages of subjects reaching the 0.2 µg/mL threshold before and after 23-valent plain polysaccharide booster vaccination.

The opsonophagocytic activity was shown to be clearly improved for antibodies induced with these 19F-DT formulations as demonstrated by higher seropositivity rates (opsonophagocytic titers ≥ 1:8) and OPA GMTs one month following primary vaccination (Table 9). One month after 23-valent plain polysaccharide booster vaccination, opsonophagocytic activity of 19F antibodies remained significantly better for children primed with 19F-DT formulations (Table 11).

Table 12 presents immunogenicity data following a 11Pn-PD booster dose in toddlers previously primed with 19F-DT or 19F-PD conjugates compared to a 4th consecutive dose of *Prevnar*®. Given the breakthrough cases reported after the introduction of *Prevnar*® in the US, the improved opsonophagocytic activity against serotype 19F when conjugated to the DT carrier protein may be an advantage for the candidate vaccine.

Table 13 provides ELISA and OPA data for the 19F-DT conjugate with respect to the cross-reactive serotype 19A. It was found that 19F-DT induces low but significant OPA activity against 19A.

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Table4 Pneumococcal conjugate vaccine formulations used in clinical studies.

Formulation	Pneumococcal serotype µg/carrier protein											Al^{3+}
	1	3	4	5	6B	7F	9V	14	18C	19F	23F	mg
11Pn-PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	1/PD	< 0.8
19F-DT Form 1	3/PD	3/PD	3/PD	3/PD	10/DT	3/PD	3/PD	3/PD	3/PD	3/DT	5/DT	≤ 0.35
19F-DT Form 2	3/PD	2/PD	2/PD	3/PD	5/DT	3/PD	2/PD	2/PD	2/PD	3/DT	5/DT	≤ 0.35
19F-DT Form 3	3/PD	3/PD	3/PD	3/PD	3/PD	3/PD	3/PD	3/PD	3/PD	3/DT	3/PD	= 0.5

Table 5 Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D/TT/DTconjugates

Serotype	1 Native	3 µfluid	4 Native	5 Native	6B Native	7F Native
PS conc.(mg/ml)	1.5	2	2.0	7.5	5.5	3.0
PS dissolution	NaCl 150mM	NaCl 2M	WFI	WFI	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/0.7	1/1	1/1	1/1	1/1	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75	0.75
$pH_a=pH_c=pH_q$	9.0/9.0/9.0	9.5/9.5/9.0	8.8/8.8/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.0/9.0/9.0
Coupling time	60 mins	60 mins	45 mins	40 mins	60 mins	60 mins

Serotype	9V Native	14 Native	18C Native	19F Native	23F Native
PS conc.(mg/ml)	1.75	2.5	1.75	4.0	2.5
PS dissolution	NaCl 2M	NaCl 2M	WFI	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5.0
Initial PS/PD Ratio (w/w)	1/0.75	1/0.75	1/1.2	1/1	1/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75
$pH_a=pH_c=pH_q$	8.5/8.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.5/9.5/9.0
Coupling time	60 mins	60 mins	45 mins	30 mins	60 mins

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Table 6 Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D/DTconjugates for the 11 Pn-PD&Di-001 study

Serotype	1 µfluid	3 µfluid	4 µfluid	5 µfluid	6B µfluid	7F Native
PS conc.(mg/ml)	4	2.0	2.5	7.5	10	3.0
PS dissolution	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	10.0	5.0	5.0	5.0 NaCl 2M	20 (DT) NaCl 2M	5.0
Initial PD/PS Ratio (w/w)	1.2/1	1/1	1/1	1/1	1.5/1	1/1
CDAP conc. (mg/mg PS)	1.50	0.75	1.5	2	1.5	0.75
$pH_a=pH_c=pH_q$	9.0/9.0/9.0	9.5/9.5/9.0	9.5/9.5/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9/9/9
Coupling time	60 mins	60 mins	60 mins	60 mins	60 mins	60 mins

Serotype	9V Native	14 Native	18C µfluid	19F µfluid	23F µfluid
PS conc.(mg/ml)	1.75	2.5	5.0	9.0	10
PS dissolution	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M	NaCl 2M
Carrier protein conc.(mg/ml)	5.0	5.0	5.0	20 (DT)	10 (DT)
Initial carrier protein/PS Ratio (w/w)	0.75/1	0.75/1	1.2/1	1.5/1	1.5/1
CDAP conc. (mg/mg PS)	0.75	0.75	1.5	1.5	0.75
$pH_a=pH_c=pH_q$	8.5/8.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0
Coupling time	60 mins	60 mins	30 mins	60 mins	60 mins

Table 7 Specific activation/coupling/quenching conditions of PS S.pneumoniae-Protein D/DTconjugates for the 11 Pn-PD&Di-007 study

Serotype	1 Native	3 µfluid	4 Native	5 Native	6B Native	7F µfluid
PS	1.5	2.0	2	7.5	5.5	5.0

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conc.(mg/ml)						
PS dissolution	NaCl 150 mM	NaCl 2M	WFI	WFI	NaCl 2M	NaCl 2M
PD conc.(mg/ml)	5.0	5.0	5.0	5.0	5	10
Initial PD/PS Ratio (w/w)	0.7/1	1/1	1	1/1	1/1	1.2/1
CDAP conc. (mg/mg PS)	0.75	0.75	0.75	0.75	0.75	0.75
$pH_a=pH_c=pH_q$	9.0/9.0/9.0	9.5/9.5/9.0	8.8/8.8/9.0	9.0/9.0/9.0	9.5/9.5/9.0	9.5./9.5/9
Coupling time	60 mins	60 mins	45 mins	40 mins	60 mins	60 mins

Serotype	9V µfluid	14 µfluid	18C Native	19F µfluid	19F µfluid	23F µfluid
PS conc.(mg/ml)	5.0	5.0	1.75	9.0	10.0	9.5
PS dissolution	NaCl 2M	NaCl 2M	WFI	NaCl 2M	NaCl 2M	NaCl 2M
Carrier protein conc.(mg/ml)	10	10.0	5.0	20 (DT)	5.0 (PD)	10
Initial carrier protein/PS Ratio (w/w)	1.2/1	1.2/1	1.2/1	1.5/1	1.2/1	1/1
CDAP conc. (mg/mg PS)	0.5	0.75	0.75	1.5	0.75	0.75
$pH_a=pH_c=pH_q$	9.5/9.5/9.0	9.5/9.5/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.0/9.0/9.0	9.5/9.5/9.0
Coupling time	60 mins	60 mins	45 mins	120 mins	120 mins	60 mins

Table 8 **Percentage of subjects with 19F antibody concentration ≥ 0.20 µg/mL and 19F antibody Geometric mean antibody concentrations (GMCs with 95% CI; µg/mL) one month following 1µg 19F-PD, 3µg 19F-DT or *Prevnar* (2µg 19F-CRM) primary vaccination (Total cohort)**

Group	11Pn-PD&Di-001 (22F-ELISA)			11Pn-PD&Di-007 (22F-ELISA)		
	N	% ≥ 0.20 µg/mL (95% CI)	GMC (µg/mL) (95% CI)	N	% ≥ 0.20 µg/mL (95% CI)	GMC (µg/mL) (95% CI)

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11Pn-PD	152	**98.7** (95.3-99.8)	**1.93** (1.67-2.22)	50	**100** (92.9-100)	**2.78** (2.31-3.36)
19F-DT Form 1[Γ]	146	**99.3** (96.2-100)	**2.88** (2.45-3.38)	-	-	-
19F-DT Form 2[Γ]	150	**96.0** (91.5-98.5)	**2.43** (2.01-2.94)	-	-	-
19F-DT Form 3[Γ]	-	-	-	50	**96.0** (86.3-99.5)	**3.70** (2.58-5.30)
Prevnar	148	**98.6** (95.2-99.8)	**2.98** (2.60-3.41)	41	**97.6** (87.1-99.9)	**2.91** (2.15-3.94)

[Γ] The composition of the different formulations is provided in table 4.

Table 9 Percentage of subjects with 19F OPA titer ≥ 1:8 and 19F OPA GMTs one month following primary vaccination with 1µg 19F-PD, 3µg 19F-DT or *Prevnar* (2µg 19F-CRM) (Total cohort)

Group	11Pn-PD&Di-001			11Pn-PD&Di-007		
	N	≥ 1:8 (95% CI)	GMT (95% CI)	N	≥ 1:8 (95% CI)	GMT (95% CI)
11Pn-PD	136	**84.6** (77.4-90.2)	**77.8** (58.1-104.4)	46	**95.7** (85.2-99.5)	**167.8** (118.1-238.6)
19F-DT Form 1[Γ]	137	**95.6** (90.7-98.4)	**263.2** (209.4-330.7)	-	-	-
19F-DT Form 2[Γ]	139	**92.1** (86.3-96.0)	**218.9** (166.5-287.9)	-	-	-
19F-DT Form 3[Γ]	-	-	-	49	**91.8** (80.4-97.7)	**403.1** (225.7-719.9)
Prevnar	131	**86.3** (79.2-91.6)	**82.6** (61.1-111.6)	38	**81.6** (65.7-92.3)	**65.0** (37.7-112.2)

[Γ] The composition of the different formulations is provided in Table4.

Table 10 Percentage of subjects with 19F antibody concentration ≥ 0.20 µg/mL and 19F antibody GMCs (µg/mL) prior to and one month following 23-valent plain polysaccharide booster in children primed with 1µg 19F-PD, 3µg 19F-DT or *Prevnar* (2µg 19F-CRM) (Total cohort)

Primary group	11Pn-PD&Di-002 (22F ELISA)					
	Prior to booster vaccination			One month post 23-valent PS booster		
	N	% ≥ 0.20 µg/mL (95% CI)	GMC (µg/ml) (95% CI)	N	% ≥ 0.20 µg/mL (95% CI)	GMC (µg/ml) (95% CI)
11Pn-PD	70	**77.1** (65.6-86.3)	**0.67** (0.45-0.98)	67	**94.0** (85.4-98.3)	**11.50** (7.76-17.03)
19F-DT Form 1[Γ]	68	**91.2** (81.8-96.7)	**0.71** (0.54-0.94)	69	**98.6** (92.2-100)	**14.50** (10.47-20.07)
19F-DT Form 2[Γ]	74	**81.1** (70.3-89.3)	**0.59** (0.43-0.80)	72	**95.8** (88.3-99.1)	**9.90** (6.74-14.54)
Prevnar	65	**64.6** (51.8-76.1)	**0.40** (0.27-0.60)	67	**100** (94.6-100)	**9.40** (6.95-12.71)

[Γ] The composition of the different formulations is provided in Table4.

Table 11 Percentage of subjects with 19F OPA titer ≥ 1:8 and 19F OPA GMTs prior to and one month following 23-valent plain polysaccharide

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booster in children primed with 1µg 19F-PD, 3µg 19F-DT or *Prevnar* (2µg 19F-CRM) (Total cohort)

Primary group	11Pn-PD&Di-002					
	Prior to booster vaccination			One month post 23-valent PS booster		
	N	% ≥ 1:8 (95% CI)	GMT (95% CI)	N	% ≥ 1:8 (95% CI)	GMT (95% CI)
11Pn-PD	29	**27.6** (12.7-47.2)	**10.9** (5.0-23.7)	28	**82.1** (63.1-93.9)	**408.0** (157.3-1058.3)
19F-DT Form 1[Γ]	19	**47.4** (24.4-71.1)	**18.1** (7.2-45.7)	18	**94.4** (72.7-99.9)	**1063.8** (386.6-2927.5)
19F-DT Form 2[Γ]	27	**33.3** (16.5-54.0)	**8.5** (4.7-15.3)	28	**100** (87.7-100)	**957.6** (552.8-1659.0)
Prevnar	24	**12.5** (2.7-32.4)	**8.1** (3.4-19.6)	23	**82.6** (61.2-95.0)	**380.9** (133.2-1089.5)

[Γ] The composition of the different formulations is provided in Table4.

Table 12 Percentage of subjects with antibody concentrations ≥ 0.2 µg/mL, OPA ≥ 1:8 and GMCs/GMTs against 19F pneumococci one month following 11Pn-PD or Prevnar booster in children primed with 1µg 19F-PD, 3µg 19F-DT or *Prevnar* (2µg 19F-CRM) (Total cohort)

Primary group	11Pn-PD&Di-002					
	22F-ELISA assay			OPA assay		
	N	% ≥ 0.20 µg/mL (95% CI)	GMC (µg/ml) (95% CI)	N	% ≥ 1:8 (95% CI)	GMT (95% CI)
11Pn-PD	70	**100** (94.9-100)	**4.52** (3.7-5.5)	21	**100** (83.9-100)	**255.6** (135.5-481.9)
19F-DT Form 1[Γ]	66	**98.5** (91.8-100)	**3.45** (2.8-4.3)	23	**95.7** (78.1-99.9)	**374.0** (192.6-726.2)
19F-DT Form 2[Γ]	70	**98.6** (92.3-100)	**3.80** (2.9-4.9)	29	**96.6** (82.2-99.9)	**249.1** (144.7-428.7)
Prevnar	69	**97.1** (89.9-99.6)	**2.56** (2.0-3.3)	31	**96.8** (83.3-99.9)	**528.7** (319.4-875.2)

[Γ] The composition of the different formulations is provided in Table4.

Table 13 Percentage of subjects with antibody concentrations ≥ 0.2 µg/mL, OPA ≥ 1:8 and GMCs/GMTs against 19A pneumococci one month following primary vaccination with 1µg 19F-PD, 3µg 19F-DT or *Prevnar* (2µg 19F-CRM) (Total cohort)

Group	11Pn-PD&Di-001					
	22F-ELISA assay			OPA assay		
	N	% ≥ 0.20 µg/mL (95% CI)	GMC (µg/mL) (95% CI)	N	% ≥ 1:8 (95% CI)	GMT (95% CI)
11Pn-PD	45	**28.9** (16.4-44.3)	**0.09** (0.07-0.11)	52	**7.7** (2.1-18.5)	**5.2** (4.0-6.8)
19F-DT Form 2[Γ]	51	**29.4** (17.5-43.8)	**0.11** (0.08-0.16)	59	**27.1** (16.4-40.3)	**12.4** (7.6-20.3)
Prevnar	55	**18.2** (9.1-30.9)	**0.10** (0.08-0.12)	61	**3.3** (0.4-11.3)	**4.6** (3.8-5.6)

[Γ] The composition of the different formulations is provided in Table4

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Example 5: Adjuvant experiments in preclinical models: impact on the immunogenicty of pneumococcal 11-valent polysaccharide conjugates in elderly Rhesus monkeys

To optimize the response elicited to conjugate pneumococcal vaccines in the elderly population, GSK formulated an 11-valent polysaccharide (PS) conjugate vaccine with a novel adjuvant Adjuvant C – see below.

Groups of 5 elderly Rhesus monkeys (14 to 28 years-old) were immunized intramuscularly (IM) at days 0 and 28 with 500 µl of either 11-valent PS conjugates adsorbed onto 315 µg of AlPO4 or 11-valent PS conjugates admixed with Adjuvant C.

In both vaccine formulations, the 11-valent PS conjugates were each composed of the following conjugates PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-PD, PS19F-PD, PS23F-DT and PS6B-DT. The vaccine used was 1/5 dose of of the human dose of the vaccine (5 µg of each saccharide per human dose except for 6B [10 µg]) conjugated according to Table 6 conditions (Example 4), except 19F was made according to the following CDAP process conditions: sized saccharide at 9 mg/ml, PD at 5 mg/ml, an initial PD/PS ratio of 1.2/1, a CDAP concentration of 0.75 mg/mg PS, pHa=pHc=pHq 9.0/9.0/9.0 and a coupling time of 60 min.

Anti-PS ELISA IgG levels and opsono-phagocytosis titres were dosed in sera collected at day 42. Anti-PS3 memory B cell frequencies were measured by Elispot from peripheral blood cells collected at day 42.

According to the results shown here below, Adjuvant C significantly improved the immunogenicity of 11-valent PS conjugates versus conjugates with AlPO4 in elderly monkeys. The novel adjuvant enhanced the IgG responses to PS (Figure 1) and the opsono-phagocytosis antibody titres (Table 14). There was also supportive evidence that the frequency of PS3-specific memory B cells is increased by the use of Adjuvant C (Figure 2).

Table 14, Conjugate immunogenicity in elderly Rhesus monkeys (post-II opsono-phagocytosis titres)

		PS1	PS3	PS4	PS5	PS6B	PS7F	PS9V	PS14	PS18C	PS19F	PS23F
11-valent AlPO4	Pre-immune	<8	5	<8	5	<8	16	<8	<8	<8	<8	<8
	day 14 post II	8	181	64	49	64	4096	42	37	169	84	<64
11 valent Adj-C	Pre-immune	5	9	<8	5	8	37	<8	<8	<8	<8	<8
	day 14 post II	776	1351	891	676	6208	16384	111	161	7132	2048	<64

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B Cell Elispot

The principle of the assay relies on the fact that memory B cells mature into plasma cells in vitro following cultivation with CpG for 5 days. In vitro generated antigen-specific plasma cells can be easily detected and therefore be enumerated using the B-cell elispot assay. The number of specific plasma cells mirrors the frequency of memory B cells at the onset of the culture.

Briefly, in vitro generated plasma cells are incubated in culture plates coated with antigen. Antigen-specific plasma cells form antibody/antigen spots, which are detected by conventional immuno-enzymatic procedure and enumerated as memory B cells.
In the present study, Polysaccharides have been used to coat culture plates in order to enumerate respective memory B cells. Results are expressed as a frequency of PS specific memory B cells within a million of memory B cells.

The study shows that Adjuvant C may be able to alleviate the known problem of PS3 boostability (see 5th International Symposium on Pneumococci and Pneumococcal Diseases, April 2-6 2006, Alice Springs, Central Australia.
Specificities of immune responses against a serotype 3 pneumococcal conjugate.
Schuerman L, Prymula R, Poolman J. Abstract book p 245, PO10.06).

Example 6, Effectiveness of detoxified Pneumolysin (dPly) as a protein carrier to enhance the immunogenicity of PS 19F in young Balb/c mice

Groups of 40 female Balb/c mice (4-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 4-valent plain PS or 4-valent dPly-conjugated PS, both admixed with Adjuvant C.
Both vaccine formulations were composed of 0.1 µg (quantity of saccharide) of each of the following PS: PS8, PS12F, PS19F and PS22F.
Anti-PS ELISA IgG levels were dosed in sera collected at day 42.
The anti-PS19F response, shown as an example in Figure 3, was strongly enhanced in mice given 4-valent dPly conjugates compared to mice immunized with the plain PS. The same improvement was observed for the anti-PS8, 12F and 22F IgG responses (data not shown).

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Example 7, Effectiveness of Pneumococcal Histidine Triad Protein D (PhtD) as a protein carrier to enhance the immunogenicity of PS 22F in young Balb/c mice

Groups of 40 female Balb/c mice (4-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 4-valent plain PS or 4-valent PhtD-conjugated PS, both admixed with Adjuvant C.

Both vaccine formulations were composed of 0.1 µg (quantity of saccharide) of each of the following PS: PS8, PS12F, PS19F and PS22F.

Anti-PS ELISA IgG levels were dosed in sera collected at day 42.

The anti-PS22F response, shown as an example in Figure 4, was strongly enhanced in mice given 4-valent PhtD conjugates compared to mice immunized with the plain PS. The same improvement was observed for the anti-PS8, 12F and 19F IgG responses (data not shown).

Example 8, Immunogenicity in elderly C57Bl mice of 13-valent PS conjugates containing 19A-dPly and 22F-PhtD

Groups of 30 old C57Bl mice (>69-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 11-valent PS conjugates or 13-valent PS conjugates, both admixed with Adjuvant C (see below).

The 11-valent vaccine formulation was composed of 0.1 µg saccharide of each of the following conjugates: PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS6B-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-TT, PS19F-DT and PS23F-PD (see Table 1 and comment on 11 valent vaccine discussed under Table 2). The 13-valent vaccine formulation contained in addition 0.1 µg of PS19A-dPly and PS22F-PhtD conjugates (see Table 1 and comment on 13 valent vaccine discussed under Table 2 [using directly-conjugated 22F]). In group 2 and 4 the pneumolysin carrier was detoxified with GMBS treatment, in group 3 and 5 it was done with formaldehyde. In groups 2 and 3 PhtD was used to conjugate PS 22F, in Groups 4 and 5 a PhtD_E fusion (the construct VP147 from WO 03/054007) was used. In group 6 19A was conjugated to diphtheria toxoid and 22F to protein D.

Anti-PS19A and 22F ELISA IgG levels were dosed in individual sera collected at day 42. The ELISA IgG response generated to the other PS was measured in pooled sera.

19A-dPly and 22F-PhtD administered within the 13-valent conjugate vaccine formulation were shown immunogenic in old C57Bl mice (Table 15). The immune response induced against the other PS was not negatively impacted in mice given the 13-valent formulation compared to those immunized with the 11-valent formulation.

Table 15, PS immunogenicity in old C57Bl mice (post-III IgG levels)

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Old C57 Black mice

ELISA		GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
		11V 0.1µg/50µl Adj C	11V 19A-dPly gmbs 22F-PhtD 0.1µg/50µl Adj C	11V 19A-dPly formol 22F-PhtD 0.1µg/50µl Adj C	11V 19A-dPly gmbs 22F-PhtD-E 0.1µg/50µl Adj C	11V 19A-dPly formol 22F-PhtD-E 0.1µg/50µl Adj C	11V 19A-DT 22F-PD 0.1µg/50µl Adj C
1	average Pool	19.30	20.20	24.40	12.80	12.10	13.60
3	average Pool	6.32	4.84	5.21	6.74	2.38	2.54
4	average Pool	60.9	67.1	51.4	47.4	45.5	41.1
5	average Pool	1.34	3.81	3.06	2.75	1.26	1.23
6B	average Pool	4.41	4.12	5.88	1.58	2.31	5.64
7F	average Pool	0.83	0.81	1.65	1.98	0.89	0.99
9V	average Pool	13.8	23.7	20.0	13.1	15.5	9.6
14	average Pool	25.73	42.96	34.12	32.53	23.97	15.60
18C	average Pool	13.4	20.1	11.9	9.1	8.3	8.4
19F	average Pool	57.5	90.0	63.8	36.5	47.0	69.1
23F	average Pool	NR	NR	NR	NR	NR	NR
19A	GMC	0.06	0.09	0.25	0.08	0.23	0.19

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	IC	0.04-0.1	0.05-0.14	0.15-0.41	0.06-0.12	0.14-0.38	0.09-0.3
	%sero	33%	47%	83%	53%	80%	73%
22F	GMC	NR	5.81	3.76	0.54	0.85	2.02
	IC		3.2-10.6	1.8-7.9	0.3-1.1	0.4-1.7	1.2-3.4
	%sero	0%	97%	90%	77%	87%	97%

Example 9, Immunogenicity in young Balb/c mice of 13-valent PS conjugates containing 19A-dPly and 22F-PhtD

Groups of 30 young Balb/c mice (4-weeks old) were immunized IM at days 0, 14 and 28 with 50 µl of either 11-valent PS conjugates or 13-valent PS conjugates, both admixed with Adjuvant C (see below).

The 11-valent vaccine formulation was composed of 0.1 µg saccharide of each of the following conjugates: PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS6B-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-TT, PS19F-DT and PS23F-PD (see Table 1 and comment on 11 valent vaccine discussed under Table 2). The 13-valent vaccine formulation contained in addition 0.1 µg of PS19A-dPly and PS22F-PhtD conjugates (see Table 1 and comment on 13 valent vaccine discussed under Table 2 [using directly-conjugated 22F]). In group 2 and 4 the pneumolysin carrier was detoxified with GMBS treatment, in group 3 and 5 it was done with formaldehyde. In groups 2 and 3 PhtD was used to conjugate PS 22F, in Groups 4 and 5 a PhtD_E fusion (the construct VP147 from WO 03/054007) was used. In group 6 19A was conjugated to diphtheria toxoid and 22F to protein D.

Anti-PS19A and 22F ELISA IgG levels were dosed in individual sera collected at day 42. The ELISA IgG response generated to the other PS was measured in pooled sera.

19A-dPly and 22F-PhtD administered within the 13-valent conjugate vaccine formulation were shown immunogenic in young Balb/c mice (Table 16). The immune response induced against the other PS was not negatively impacted in mice given the 13-valent formulation compared to those immunized with the 11-valent formulation.

Table 16, PS immunogenicity in young Balb/c mice (post-III IgG levels)

BalbC mice							
ELISA		GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
		11V	11V 19A-dPly gmbs 22F-PhtD	11V 19A-dPly formol 22F-PhtD	11V 19A-dPly gmbs 22F-PhtD-E	11V 19A-dPly formol 22F-PhtD-E	11V 19A-DT 22F-PD
		0.1µg/50µl Adj C	0.1µg/50µl Adj C	0.1µg/50µl Adj C	0.1µg/50µl Adj C	0.1µg/50µl Adj C	0.1µg/50µl Adj C
1	average	131.70	101.20	83.00	82.40	67.90	85.50

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	Pool						
3	average Pool	21.85	10.38	12.53	8.83	8.73	14.98
4	average Pool	147.4	127.0	104.4	95.0	113.6	114.2
5	average Pool	21.38	20.29	18.26	18.95	18.02	23.04
6B	average Pool	1.97	4.76	3.72	2.35	1.43	1.05
7F	average Pool	7.69	4.58	4.77	4.24	3.92	3.94
9V	average Pool	30.1	30.7	26.5	21.4	23.4	28.3
14	average Pool	28.78	27.67	26.23	21.54	24.34	13.73
18C	average Pool	53.4	52.37	46.5	57.8	47.8	75.8
19F	average Pool	186.6	157.7	169.3	178.9	181.9	223.2
23F	average Pool	4.98	3.9	5.11	0.57	3.13	4.57
19A	GMC IC %sero	0.4 0.2-0.6 93%	32.8 26.4-40.7 100%	25.1 20.6-30.6 100%	21.6 17.5-26.7 100%	18.9 15.1-23.5 100%	23.5 19.5-28.5 100%
22F	GMC IC %sero	NR 0%	3.99 1.9-8.42 93%	3.76 1.8-8 100%	6.27 3.8-10.4 100%	8.70 5.4-13.9 100%	18.76 15.2-23.1 100%

Example 10, Immunogenicity in Guinea Pigs of 13-valent PS conjugates containing 19A-dPly and 22F-PhtD

Groups of 20 young Guinea Pigs (Hartley Strain; 5 weeks old) were immunized IM at days 0, 14 and 28 with 125 µl of either 11-valent PS conjugates or 13-valent PS conjugates, both admixed with Adjuvant C (see below).

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The 11-valent vaccine formulation was composed of 0.25 µg saccharide of each of the following conjugates: PS1-PD, PS3-PD, PS4-PD, PS5-PD, PS6B-PD, PS7F-PD, PS9V-PD, PS14-PD, PS18C-TT, PS19F-DT and PS23F-PD (see Table 1 and comment on 11 valent vaccine discussed under Table 2). The 13-valent vaccine formulation contained in addition 0.1 µg of PS19A-dPly and PS22F-PhtD conjugates (see Table 1 and comment on 13 valent vaccine discussed under Table 2 [using directly-conjugated 22F]). In group 2 and 4 the pneumolysin carrier was detoxified with GMBS treatment, in group 3 and 5 it was done with formaldehyde. In groups 2 and 3 PhtD was used to conjugate PS 22F, in Groups 4 and 5 a PhtD_E fusion (the construct VP147 from WO 03/054007) was used. In group 6 19A was conjugated to diphtheria toxoid and 22F to protein D.

Anti-PS19A and 22F ELISA IgG levels were dosed in individual sera collected at day 42. The ELISA IgG response generated to the other PS was measured in pooled sera.

Table 17, PS immunogenicity in young Balb/c mice (post-III IgG levels)

Guinea pigs							
ELISA		GROUP 1	GROUP 2	GROUP 3	GROUP 4	GROUP 5	GROUP 6
		11V 0.1µg/50µl Adj C	11V 19A-dPly gmbs 22F-PhtD 0.1µg/50µl Adj C	11V 19A-dPly formol 22F-PhtD 0.1µg/50µl Adj C	11V 19A-dPly gmbs 22F-PhtD-E 0.1µg/50µl Adj C	11V 19A-dPly formol 22F-PhtD-E 0.1µg/50µl Adj C	11V 19A-DT 22F-PD 0.1µg/50µl Adj C
1	average Pool	78.00	77.21	76.15	68.77	68.59	81.04
3	average Pool	7.75	9.31	12.73	7.94	4.75	9.59
4	average Pool	130.7	94.4	132.6	166.8	85.0	101.3
5	average Pool	109.10	117.10	110.70	158.40	74.10	100.40
6B	average Pool	3.14	4.26	14.4	7.63	6.3	7.52
7F	average Pool	154.2	216.0	240.0	181.0	142.0	179.1
9V	average	90.69	105.45	98.20	93.45	54.12	73.05

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	Pool						
14	average Pool	71.19	77.18	46.53	59.67	38.47	53.69
18C	average Pool	109.4	122.3	137.1	79.9	73.7	83.1
19F	average Pool	73.9	102.5	112.2	75.5	62.3	72.1
23F	average Pool	19.19	30.74	29.44	31.52	19.13	24.94
19A	GMC	0.4	25.58	41.49	14.25	27.49	6.74
	IC	0.24-0.68	12-54.5	24.4-70.5	5.9-34.6	16.6-45.4	4-11.3
	%sero	75%	100%	100%	100%	100%	100%
22F	GMC	0.12	2.51	3.67	45.74	30.68	96.38
	IC	0.09-0.16	0.94-6.73	1.59-8.42	29.3-71.4	17-53.3	73.5-126.4
	%sero	10%	95%	95%	100%	100%	100%

Example 11: Formulations being made and tested

a) The following formulations are made (using the 13 valent vaccine from table 1 and serotype 3 from table 5 – see comment on 14 valent vaccine discussed under Table 2 [using directly-conjugated 22F or through an ADH linker]). The saccharides are formulated with aluminium phosphate and 3D-MPL as shown below.

14V 25µg MPL
Sum of BAC Aluminium content -> FF
Per Dose:

PS	carrier	µg PS	µg MPL	ratio PS/AI 1/x	µg AI
1	PD	1		10	10
3	PD	1		10	10
4	PD	3		10	30
5	PD	1		10	10
6A	PD	1		10	10
6B	PD	1		10	10
7F	PD	1		10	10
9V	PD	1		10	10
14	PD	1		10	10
18C	TT_{AH}	3		15	45
19A	dPly	3		10	30
19F	DT	3		10	30
22F	PhtD	3		10	30
23F	PD	1		10	10
BAC MPL 50/200			25	4	100
FF Aluminium content				Sum =	355

14V 10µg MPL
Sum of BAC Aluminium content -> FF
Per Dose:

PS	carrier	µg PS	µg MPL	ratio PS/AI 1/x	µg AI
1	PD	1		10	10
3	PD	1		10	10
4	PD	3		10	30
5	PD	1		10	10
6A	PD	1		10	10
6B	PD	1		10	10
7F	PD	1		10	10
9V	PD	1		10	10
14	PD	1		10	10
18C	TT_{AH}	3		15	45
19A	dPly	3		10	30
19F	DT	3		10	30
22F	PhtD	3		10	30
23F	PD	1		10	10
BAC MPL 50/200			10	4	40
FF Aluminium content				Sum =	295

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b) The same saccharide formulation is adjuvanted with each of the following adjuvants:

- In the table herebelow the concentration of the emulsion components per 500µl dose is shown.

Ingredients	Adjuvant A1 250µl o/w emulsion	Adjuvant A2 125µl o/w emulsion	Adjuvant A3 50µl o/w emulsion
alpha Tocopherol	11.88mg	5.94mg	2.38mg
Squalene	10.7mg	5.35mg	2.14mg
Tween 80	4.85mg	2.43mg	0.97mg

Ingredients	Adjuvant A4 250µl o/w emulsion	Adjuvant A5 250µl o/w emulsion	Adjuvant A6 125µl o/w emulsion	Adjuvant A7 50µl o/w emulsion
alpha Tocopherol	11.88mg	11.88mg	5.94mg	2.38mg
Squalene	10.7mg	10.7mg	5.35mg	2.14mg
Tween 80	4.85mg	4.85mg	2.43mg	0.97mg
3D-MPL	50µg	25µg	25µg	10µg

c) The saccharides are also formulated with two liposome based adjuvants:

Composition of Adjuvant B1
Qualitative Quantitative (per 0.5 mL dose)
Liposomes:
- DOPC 1 mg
- cholesterol 0.25 mg
3DMPL 50 µg
QS21 50 µg
KH_2PO_4 [1] 3.124 mg Buffer
Na_2HPO_4 [1] 0.290 mg Buffer
NaCl 2.922 mg
(100 mM)
WFI q.s. ad 0.5 ml Solvent
pH 6.1
1. Total PO_4 concentration = 50 mM

Composition of Adjuvant B2
Qualitative Quantitative (per 0.5 mL dose)
Liposomes:
- DOPC 0.5 mg
- cholesterol 0.125 mg
3DMPL 25 µg
QS21 25 µg

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KH_2PO_4 1 3.124 mg Buffer
Na_2HPO_4 1 0.290 mg Buffer
NaCl 2.922 mg
(100 mM)
WFI q.s. ad 0.5 ml Solvent
pH 6.1

d) The saccharides are also formulated with Adjuvant C (see above for other compositions where this adjuvant has been used):

Qualitative Quantitative (per 0.5 mL dose)
Oil in water emulsion: 50 µl
- squalene 2.136 mg
- α-tocopherol 2.372 mg
- Tween 80 0.97 mg
- cholesterol 0.1 mg
3DMPL 50 µg
QS21 50 µg
KH_2PO_4 1 0.470 mg Buffer
Na_2HPO_4 1 0.219 mg Buffer
NaCl 4.003 mg
(137 mM)
KCl 0.101 mg
(2.7 mM)
WFI q.s. ad 0.5 ml Solvent
pH 6.8

Example 12, impact of conjugation chemistry on 22F-PhtD conjugate immunogenicity in Balb/c mice

Groups of 30 female Balb/c mice were immunised by the intramuscular (IM) route at days 0, 14 and 28 with 13-valent PS formulations containing PS 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F (dose: 0.3 µg saccharide / PS for PS 4, 18C, 19A, 19F and 22F and 0.1 µg saccharide / PS for the other PS).

PS 18C was conjugated to Tetanus Toxoid, 19F to Diphteria Toxoid, 19A to formol-detoxified Ply, 22F to PhtD and the other PS to PD.

Two formulations, constituted of either 22F-PhtD prepared by direct CDAP chemistry or 22F-AH-PhtD (ADH-derivitized PS), were compared. See Example 2, Table 1 and comment under Table 2 for characteristics of 13 valent vaccine made either with 22F directly conjugated or via an ADH spacer. The vaccine formulations were supplemented with adjuvant C.

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Anti-PS22F ELISA IgG levels and opsono-phagocytosis titres were measured in sera collected at day 42.

22F-AH-PhtD was shown much more immunogenic than 22F-PhtD in terms of both IgG levels (figure 5) and opsono-phagocytic titres (figure 6).

Example 13, impact of new adjuvants on immunogenicity of *Streptoccoccus pneumoniae* capsule PS conjugates

Groups of 40 female Balb/c mice were immunised by the IM route at days 0, 14 and 28 with 13-valent PS formulations containing PS 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F and 23F (dose: 0.3 µg / PS for PS 4, 18C, 19A, 19F and 22F and 0.1 µg / PS for the other PS).

PS 18C was conjugated to Tetanus Toxoid, 19F to Diphteria Toxoid, 19A to formol-detoxified Ply, 22F to PhtD and the other PS to PD. See Example 2, Table 1 and comment under Table 2 for characteristics of 13 valent vaccine made with 22F directly conjugated.

Four formulations, supplemented with either $AlPO_4$, adjuvant A1, adjuvant A4 or adjuvant A5, were compared.

Anti-PS, Ply, PhtD and PD ELISA IgG levels were measured in sera collected at day 42 and pooled per group. The following ratio was calculated for each antigen: IgG level induced with the new adjuvant tested / IgG level induced with $AlPO_4$.

All the new adjuvants tested improved at least 2-fold the immune responses to 13-valent conjugates compared to the classical $AlPO_4$ formulation (figure 7).

Example 14, protective efficacy of a PhtD/detoxified Ply combo in a pneumococcal monkey pneumonia model

Groups of 6 Rhesus monkeys (3 to 8 years-old), selected as those having the lowest pre-existing anti-19F antibody levels, were immunized intramuscularly at days 0 and 28 with either 11-valent PS conjugates (i.e. 1 µg of PS 1, 3, 5, 6B, 7F, 9V, 14 and 23F, and 3 µg of PS 4, 18C and 19F) or PhtD (10 µg) + formol-detoxified Ply (10 µg) or the adjuvant alone.

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PS 18C was conjugated to Tetanus Toxoid, 19F to Diphteria Toxoid and the other PS to PD. See Example 2, Table 1 and comment under Table 2 for characteristics of 11 valent vaccine. All formulations were supplemented with adjuvant C.

Type 19F pneumococci (5.10^6 cfu) were inoculated in the right lung at day 42. Colonies were counted in broncho-alveolar lavages collected at days 1, 3 and 7 post-challenge. The results were expressed as the number of animals per group either dead, lung colonized or cleared at day 7 after challenge.

As shown in figure 8, a good protection close to statistical significance (despite the low number of animals used) was obtained with 11-valent conjugates and the PhtD+dPly combo ($p < 0.12$, Fisher Exact test) compared to the adjuvant alone group.

Example 15, impact of conjugation chemistry on the anti-PhtD antibody response and the protective efficacy against a type 4 challenge induced by 22F-PhtD conjugates

Groups of 20 female OF1 mice were immunised by the intramuscular route at days 0 and 14 with 3 µg of either 22F-PhtD (prepared by direct CDAP chemistry) or 22F-AH-PhtD (ADH-derivitized PS), or the adjuvant alone. Both monovalent 22F conjugates were made by the processes of Example 2 (see also Table 1 and Table 2). Each formulation was supplemented with adjuvant C.

Anti-PhtD ELISA IgG levels were measured in sera collected at day 27.

Mice were challenged intranasally with 5.10^6 cfu of type 4 pneumococci at day 28 (i.e. a pneumococcal serotype not potentially covered by the PS present in the vaccine formulation tested). The mortality induced was monitored until day 8 post-challenge.

22F-AH-PhtD induced a significantly higher anti-PhtD IgG response and better protection against type 4 challenge than 22F-PhtD.

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CLAIMS

1. A *Streptococcus pneumoniae* immunogenic composition comprising 9 or more, 10 or more, 11 or more, 13 or more, or 14 or more capsular saccharides from different *S. pneumoniae* serotypes conjugated to 2 or more different carrier proteins, wherein the composition comprises serotype 19F capsular saccharide conjugated to diphtheria toxoid (DT) or CRM197, optionally wherein 19F is the only saccharide in the composition conjugated to diphtheria toxoid (DT) or CRM197
2. An immunogenic composition according to claim 1 wherein serotype 19F is conjugated to Diphtheria toxoid.
3. An immunogenic composition according to claim 1 or 2 wherein said composition further comprises protein D from *Haemophilus influenzae*.
4. An immunogenic composition according to any preceding claim wherein 19F capsular saccharide is directly conjugated to the carrier protein.
5. An immunogenic composition according to any of claims 1 to 3 wherein 19F capsular saccharide is conjugated to the carrier protein via a linker.
6. An immunogenic composition according to claim 5 wherein the linker is bifunctional.
7. The immunogenic composition of claim 5 or 6 wherein the linker is ADH.
8. The immunogenic composition of claims 5, 6, or 7 wherein the linker is attached to the carrier protein by carbodiimide chemistry, preferably using EDAC.
9. The immunogenic composition of any of claims 5 to 8 wherein the saccharide is conjugated to the linker before the carrier protein is conjugated to the linker.
10. The immunogenic composition of any of claims 5 to 8 wherein the carrier protein is conjugated to the linker before the saccharide is conjugated to the linker.
11. the immunogenic composition of any preceding claim wherein the 19F saccharide is conjugated to the carrier protein or to the linker using CDAP chemistry.
12. The immunogenic composition of any preceding claim wherein the ratio of carrier protein to 19F saccharide is between 5:1 and 1:5, 4:1 and 1:1 or 2:1 and 1:1, or 1.5:1 and 1.4:1 (w/w).
13. The immunogenic composition of any preceding claim wherein the average size (e.g. M_w) of the 19F saccharide is above 100 kDa.
14. The immunogenic composition of claim 13 wherein the average size (e.g. M_w) of the 19F saccharide is between 100-750, 110-500, 120-250, or 125 and 150 kDa.

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15. The immunogenic composition of claim 13 or 14 wherein the 19F saccharide is either a native polysaccharide or is sized by a factor of no more than x5.
16. The immunogenic composition of claim 13, 14 or 15 wherein the 19F saccharide has been sized by microfluidization.
17. The immunogenic composition of any preceding claim wherein the dose of the 19F saccharide conjugate is between 1 and 10 µg, 1 and 5 µg, or 1 and 3 µg of saccharide.
18. An immunogenic composition according to any preceding claim wherein at least 8 of the capsular saccharides are conjugated to the same carrier protein.
19. An immunogenic composition according to claim 18 wherein said carrier protein is not diphtheria toxoid and/or is not CRM197.
20. An immunogenic composition according to any preceding claim comprising 2 different carrier proteins.
21. An immunogenic composition according to any preceding claim comprising 3, 4, 5 or 6 different carrier proteins.
22. An immunogenic composition according to any preceding claim wherein one or more or all carrier proteins is selected from the group consisting of DT, CRM 197, TT, Fragment C, dPly, PhtA, PhyB, PhtD, PhtE, PhtDE OmpC, PorB and *Haemophilus influenzae* Protein D.
23. An immunogenic composition according to claim 22 wherein one carrier protein is protein D.
24. An immunogenic composition according to claim 22 wherein one carrier protein is TT.
25. An immunogenic composition according to any of claims 22, 23 or 24 wherein both TT and protein D are present as carrier proteins.
26. An immunogenic composition according to any of claims 22, 23, 24 or 25 wherein at least 8 of the capsular saccharides are conjugated to protein D.
27. An immunogenic composition according to any preceding claim wherein the composition comprises capsular saccharide 18C conjugated to TT, optionally wherein 18C is the only saccharide in the composition conjugated to tetanus toxoid (TT).
28. An immunogenic composition according to any preceding claim wherein 18C capsular saccharide is directly conjugated to the carrier protein.
29. An immunogenic composition according to any of claims 1 to 27 wherein 18C capsular saccharide is conjugated to the carrier protein via a linker.
30. An immunogenic composition according to claim 29 wherein the linker is bifunctional.
31. The immunogenic composition of claim 29 or 30 wherein the linker is ADH.

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32. The immunogenic composition of claims 29, 30, or 31 wherein the linker is attached to the carrier protein by carbodiimide chemistry, preferably using EDAC.
33. The immunogenic composition of any of claims 29 to 31 wherein the saccharide is conjugated to the linker before the carrier protein is conjugated to the linker.
34. The immunogenic composition of any of claims 29 to 31 wherein the carrier protein is conjugated to the linker before the saccharide is conjugated to the linker.
35. the immunogenic composition of any preceding claim wherein the 18C saccharide is conjugated to the carrier protein or linker using CDAP chemistry.
36. The immunogenic composition of any preceding claim wherein the 18C saccharide is conjugated to the carrier protein or linker using reductive amination.
37. The immunogenic composition of any preceding claim wherein the ratio of carrier protein to 18C saccharide is between 0.5:1-5:1, 1:1-4:1, 1.5:1-3:1, or 2:1 and 2.5:1 (w/w).
38. The immunogenic composition of any preceding claim wherein the average size (e.g. M_w) of the 18C saccharide is above 50 kDa.
39. The immunogenic composition of claim 38 wherein the average size (e.g. M_w) of the 18C saccharide is between 50-500, 60-400, 70-300, 75-200, or 80 and 100 kDa.
40. The immunogenic composition of claim 39 wherein the 18C saccharide is either a native polysaccharide or is sized by a factor of no more than x5.
41. The immunogenic composition of claim 38, 39 or 40 wherein the 18C saccharide has been sized by microfluidization.
42. The immunogenic composition of any preceding claim wherein the dose of the 18C saccharide conjugate is between 1 and 10 µg, 1 and 5 µg, or 1 and 3 µg of saccharide.
43. The immunogenic composition of claim 42 wherein the dose of the 18C saccharide conjugate is 3 µg of saccharide.
44. An immunogenic composition according to any preceding claim wherein a capsular saccharide conjugate of serotype 6B is present, but is not conjugated to DT and/or CRM197.
45. An immunogenic composition according to any preceding claim wherein a capsular saccharide conjugate of serotype 23F is present, but is not conjugated to DT and/or CRM197.
46. An immunogenic composition according to any preceding claim wherein at least serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F are present as conjugated saccharides.

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47. An immunogenic composition according to any preceding claim wherein serotypes 1, 4, 5, 6B, 7F, 9V, 14 and 23F are present and are all conjugated to protein D.
48. An immunogenic composition according to any preceding claim further comprising serotype 3 present as a conjugated saccharide.
49. An immunogenic composition according to claim 48 wherein serotype 3 is conjugated to protein D.
50. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 6A and/or 15B.
51. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 19A.
52. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 22F.
53. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 8.
54. An immunogenic composition according to any preceding claim further comprising (conjugated capsular saccharide of) serotype 12F.
55. An immunogenic composition according to any preceding claim wherein at least one of the capsular saccharides is directly conjugated to the carrier protein.
56. An immunogenic composition according to any preceding claim wherein at least one of the capsular saccharides is conjugated to the carrier protein via a linker.
57. An immunogenic composition according to claim 56 wherein the linker is bi-functional.
58. The immunogenic composition of claim 56 or 57 wherein the linker is ADH.
59. The immunogenic composition of claims 56, 57, or 58 wherein the linker is attached to the carrier protein by carbodiimide chemistry, preferably using EDAC.
60. The immunogenic composition of any of claims 56 to 59 wherein the saccharide is conjugated to the linker before the carrier protein is conjugated to the linker.
61. The immunogenic composition of any of claims 56 to 59 wherein the carrier protein is conjugated to the linker before the saccharide is conjugated to the linker.
62. The immunogenic composition of any preceding claim wherein at least one of the capsular saccharides is conjugated to the carrier protein and/or linker using CDAP chemistry.
63. The immunogenic composition of any preceding claim wherein at least one of the capsular saccharides is conjugated to the carrier protein and/or linker using reductive amination.

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64. The immunogenic composition of claim 63 wherein capsular saccharide 3 is conjugated to the carrier protein and/or linker using reductive amination.

65. The immunogenic composition of claim 63 or 64 wherein capsular saccharide 1 is conjugated to the carrier protein and/or linker using reductive amination.

66. The immunogenic composition of any preceding claim wherein the ratio of carrier protein to saccharides is between 0.5:1-5:1, 1:2 and 2.5:1, 1:1-3.5:1, or 1.3:1-3.0:1.

67. The immunogenic composition of any preceding claim wherein the average size (e.g. M_w) of the saccharides (in the saccharide conjugates) is above 50 kDa, e.g, 50-1600, 80-1400, 100-1000, 150-500, or 200-400 kDa.

68. The immunogenic composition of any preceding claim which comprises serotype 1 (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 100-1000, 200-800, 250-600, or 300 and 400 kDa.

69. The immunogenic composition of any preceding claim which comprises serotype 4 (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 50-500, 60-300, 70-200, or 75 and 125 kDa.

70. The immunogenic composition of any preceding claim which comprises serotype 5 (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 100-1000, 200-700, 300-500, or 350 and 450 kDa.

71. The immunogenic composition of any preceding claim which comprises serotype 6B (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 500-1600, 750-1500, or 1000 and 1400 kDa.

72. The immunogenic composition of any preceding claim which comprises serotype 7F (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 50-1000, 100-750, 150-500, or 200 and 300 kDa.

73. The immunogenic composition of any preceding claim which comprises serotype 9V (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 50-1000, 100-750, 150-500, 200-400, or 250 and 300 kDa.

74. The immunogenic composition of any preceding claim which comprises serotype 14 (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 50-1000, 100-750, 150-500, or 200 and 250 kDa.

75. The immunogenic composition of any preceding claim which comprises serotype 23F (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 500-1500, 700-1300, 800-1100, or 900 and 1000 kDa.

76. The immunogenic composition of any preceding claim which comprises serotype 19A (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 50 and 800kDa, 110 and 700 kDa, 110-300, 120-200, 130-180, or 140-160 kDa.

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77. The immunogenic composition of any preceding claim which comprises serotype 22F (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 50 and 800 kDa, 110 and 700 kDa, 110-300, 120-200, 130-180, or 150-170 kDa.

78. The immunogenic composition of any preceding claim which comprises serotype 6A (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 500 and 1600 kDa, or 1100 and 1540 kDa.

79. The immunogenic composition of any preceding claim which comprises serotype 3 (saccharide conjugate) having an average saccharide size (e.g. M_w) of between 50 and 1000 kDa, 60 and 800, 70 and 600, 80 and 400, 100 and 300, or 150 and 250 kDa.

80. The immunogenic composition of any preceding claim which comprises serotypes 5, 6B and 23F as native saccharides.

81. The immunogenic composition of any preceding claim wherein the dose of the capsular saccharide conjugates is between 1 and 10 μg, 1 and 5 μg, or 1 and 3 μg of saccharide per conjugate.

82. The immunogenic composition of any preceding claim which comprises conjugates of serotypes 4, 18C and 19F at dosages of 3 μg of saccharide per conjugate.

83. The immunogenic composition of any preceding claim which comprises conjugates of serotypes 1, 5, 6B, 7F, 9V, 14 and 23F at dosages of 1 μg of saccharide per conjugate.

84. The immunogenic composition of any preceding claim which comprises conjugates of serotypes 6A and/or 3 at dosages of 1 μg of saccharide per conjugate.

85. The immunogenic composition of any preceding claim which comprises conjugates of serotypes 19A and/or 22F at dosages of 3 μg of saccharide per conjugate.

86. The immunogenic composition of any preceding claim which further comprises unconjugated *S. pneumoniae* saccharides of serotypes different from those conjugated, such that the number of conjugated and unconjugated saccharide serotypes is less than or equal to 23.

87. The immunogenic composition of any preceding claim which further comprises one or more unconjugated or conjugated *S pneumoniae* proteins.

88. The immunogenic composition of claim 87 which comprises one or more unconjugated *S. pneumoniae* proteins.

89. The immunogenic composition of claim 87 or 88 wherein said one or more *S. pneumoniae* proteins are selected from Poly Histidine Triad family (PhtX), Choline Binding Protein family (CbpX), CbpX truncates, LytX family,

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LytX truncates, CbpX truncate-LytX truncate chimeric proteins, detoxified pneumolysin (Ply), PspA, PsaA, Sp128, Sp101, Sp130, Sp125 and Sp133.

90. The immunogenic composition of claims 87, 88 or 89 which comprises pneumolysin.

91. The immunogenic composition of any of claims 87 to 90 which comprises a PhtX protein.

92. An immunogenic composition according to any preceding claim which comprises pneumolysin as free or carrier protein.

93. An immunogenic composition according to any preceding claim which comprises a PhtX protein as free or carrier protein.

94. The immunogenic composition of claim 93 wherein said PhtX protein is PhtD or a PhtBD or PhtDE fusion protein.

95. A immunogenic composition according to any preceding claim which further comprises an adjuvant.

96. An immunogenic composition according to claim 95 wherein the adjuvant is a preferential inducer of a Th1 response.

97. An immunogenic composition according to claim 95 or 96 wherein the adjuvant comprises one or more components selected from QS21, MPL® or an immunostimulatory oligonucleotide.

98. An immunogenic composition according to any of claims 95 to 97 wherein the adjuvant comprises QS21 and MPL.

99. The immunogenic composition of claim 95, wherein the adjuvant comprises a liposome carrier.

100. The immunogenic composition of claim 99, wherein the adjuvant comprises (per 0.5 mL dose) 0.1-10mg, 0.2-7, 0.3-5, 0.4-2, or 0.5-1 mg (e.g. 0.4-0.6, 0.9-1.1, 0.5 or 1 mg) phospholipid (for instance DOPC).

101. The immunogenic composition of claim 99 or 100, wherein the adjuvant comprises (per 0.5 mL dose) 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or 0.125 mg) sterol (for instance cholesterol).

102. The immunogenic composition of claims 99-101, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) lipid A derivative (for instance 3D-MPL).

103. The immunogenic composition of claims 99-102, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) saponin (for instance QS21).

104. The immunogenic composition of claim 95, wherein the adjuvant comprises an oil in water emulsion.

105. The immunogenic composition of claim 104, wherein the adjuvant comprises (per 0.5 mL dose) 0.5-15, 1-13, 2-11, 4-8, or 5-6mg (e.g. 2-3, 5-6, or 10-11 mg) metabolisable oil (such as squalene).

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106. The immunogenic composition of claim 104 or 105, wherein the adjuvant comprises (per 0.5 mL dose) 0.1-10, 0.3-8, 0.6-6, 0.9-5, 1-4, or 2-3 mg (e.g. 0.9-1.1, 2-3 or 4-5 mg) emulsifier (such as Tween 80).

107. The immunogenic composition of claims 104-106, wherein the adjuvant comprises (per 0.5 mL dose) 0.5-20, 1-15, 2-12, 4-10, 5-7 mg (e.g. 11-13, 5-6, or 2-3 mg) tocol (such as alpha tocopherol).

108. The immunogenic composition of claims 104-107, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) lipid A derivative (for instance 3D-MPL).

109. The immunogenic composition of claims 104-108, wherein the adjuvant comprises (per 0.5 mL dose) 0.025-2.5, 0.05-1.5, 0.075-0.75, 0.1-0.3, or 0.125-0.25 mg (e.g. 0.2-0.3, 0.1-0.15, 0.25 or 0.125 mg) sterol (for instance cholesterol).

110. The immunogenic composition of claims 104-109, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) saponin (for instance QS21).

111. The immunogenic composition of claim 95, wherein the adjuvant comprises a metal salt and lipid A derivative.

112. The immunogenic composition of claim 111, wherein the adjuvant comprises (per 0.5 mL dose) 100-750, 200-500, or 300-400 μg Al as aluminium phosphate.

113. The immunogenic composition of claim 111 or 112, wherein the adjuvant comprises (per 0.5 mL dose) 5-60, 10-50, or 20-30 μg (e.g. 5-15, 40-50, 10, 20, 30, 40 or 50 μg) lipid A derivative (for instance 3D-MPL).

114. A vaccine kit comprising an immunogenic composition according to any of claims 1 to 94 and further comprising for concomitant or sequential administration an adjuvant as defined in any of claims 96 to 113.

115. A vaccine comprising the immunogenic composition of any one of claims 1 to 113 and a pharmaceutically acceptable excipient.

116. A process for making the vaccine according to claim 116 which comprises the step of mixing the immunogenic composition of any of claims 1 to 113 with a pharmaceutically acceptable excipient.

117. A method of immunising a human host against disease caused by *Streptococcus pneumoniae* infection comprising administering to the host an immunoprotective dose of the immunogenic composition of any of claims 1 to 113 or vaccine of claim 115.

118. The method of claim 117, wherein the human host is elderly, and the disease is either or both of pneumonia or invasive pneumococcal disease (IPD).

119. The method of claim 117 or 118, wherein the human host is elderly, and the disease is exacerbations of chronic obstructive pulmonary disease (COPD).

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120. The method of claim 117, wherein the human host is infant, and the disease is otitis media.

121. The method of claim 117 or 120, wherein the human host is infant, and the disease is meningitis and/or bacteraemia.

122. The method of claims 117, 120 or 121, wherein the human host is infant, and the disease is pneumonia and/or conjunctivitis.

123. The immunogenic composition of claims 1 to 113 or vaccine of claim 115 for use in the treatment or prevention of disease caused by *Streptococcus pneumoniae* infection.

124. A use of the immunogenic composition of claims 1 to 113 or vaccine of claim 115 in the manufacture of a medicament for the treatment or prevention of diseases caused by *Streptococcus pneumoniae* infection.

125. The use of claim 124, wherein the disease is either or both of pneumonia or invasive pneumococcal disease (IPD) of elderly humans.

126. The use of claim 124 or 125, wherein the disease is exacerbations of chronic obstructive pulmonary disease (COPD) of elderly humans.

127. The use of claim 124, wherein the disease is otitis media of infant humans.

128. The use of claim 124 or 127, wherein the disease is meningitis and/or bacteraemia of infant humans.

129. The use of claims 124, 127 or 128, wherein the disease is pneumonia and/or conjunctivitis of infant humans.

130. A use of the immunogenic composition of claims 1 to 113 or vaccine of claim 115 comprising a capsular saccharide conjugate of serotype 19F but not comprising capsular saccharide from serotype 19A in the manufacture of a medicament for the treatment or prevention of diseases caused by *Streptococcus pneumoniae* infection by serotype 19A strains.

131. A method of immunising a human host against disease caused by *Streptococcus pneumoniae* serotype 19A infection comprising the steps of administering to the host an immunoprotective dose of the immunogenic composition of claims 1 to 113 or vaccine of claim 115 which comprises a capsular saccharide conjugate of serotype 19F but does not comprise capsular saccharide from serotype 19A.

132. A method of eliciting a protective immune response in infants against Otitis media comprising the administration as separate or combined components, sequentially or concomitantly (i) an immunogenic composition or vaccine according to any of claims 1 to 115 and (ii) Protein D from *Haemophilus influenzae* which protein D may be free and/or conjugated.

133. A method of eliciting a protective immune response to infants against *S. pneumonia* by administering the immunogenic composition or vaccine of any preceding claim.

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134. A method of eliciting a protective immune response to the elderly against *S. pneumonia* by administering in combination, sequentially or concomitantly (i) the immunogenic composition or vaccine of any preceding claim (ii) one or more *S. pneumoniae* surface proteins selected from the group consisting of the PhtX family and pneumolysin.

135. A method of eliciting a protective immune response to infants against Otitis media by administering the immunogenic composition or vaccine of any preceding claim.

136. A method of eliciting a protective immune response to the infants against Otitis media by administering as separate or combined components, sequentially or concomitantly (i) the vaccine of any preceding claim (ii) one or more *S. pneumoniae* surface proteins selected from the group consisting of the PhtX family and pneumolysin.

137. The immunogenic composition of claims 1-113 or vaccine of claim 115, which comprises saccharide conjugates derived from at least all the following serotypes: 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 5, 7F wherein the GMC antibody titre induced against one or more of the vaccine components 4, 6B, 9V, 14, 18C, 19F and 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

138. The immunogenic composition of claim 137, wherein the GMC antibody titre induced against serotype 4 is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

139. The immunogenic composition of claim 137 or 138, wherein the GMC antibody titre induced against serotype 6B is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

140. The immunogenic composition of claims 137-139, wherein the GMC antibody titre induced against serotype 9V is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

141. The immunogenic composition of claims 137-140, wherein the GMC antibody titre induced against serotype 14 is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

142. The immunogenic composition of claims 137-141, wherein the GMC antibody titre induced against serotype 18C is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

143. The immunogenic composition of claims 137-142, wherein the GMC antibody titre induced against serotype 19F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

144. The immunogenic composition of claims 137-143, wherein the GMC antibody titre induced against serotype 23F is not significantly inferior to that induced by the Prevnar® vaccine in human vaccinees.

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145. The immunogenic composition of claims 137-144 which comprises a serotype 3 saccharide conjugate.

146. The immunogenic composition of claims 137-145 which comprises a serotype 6A saccharide conjugate.

147. The immunogenic composition of claims 137-146 which comprises a serotype 19A saccharide conjugate.

148. The immunogenic composition of claims 137-147 which comprises a serotype 22F saccharide conjugate.

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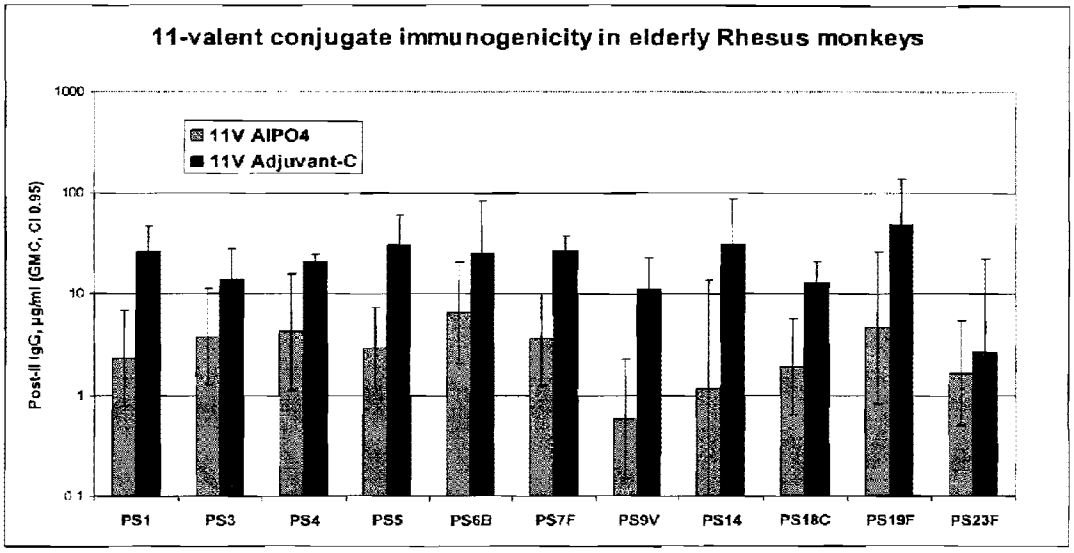


Figure 1, Conjugate immunogenicity in elderly Rhesus monkeys (post-II anti-PS IgG levels)

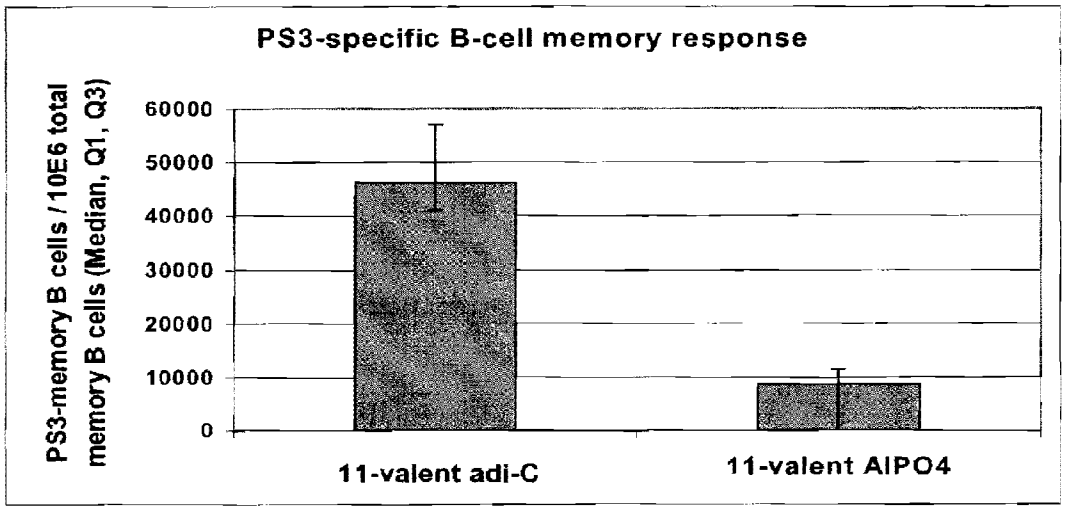


Figure 2, Conjugate immunogenicity in elderly Rhesus monkeys (post-II anti-PS3 memory B cell frequencies)

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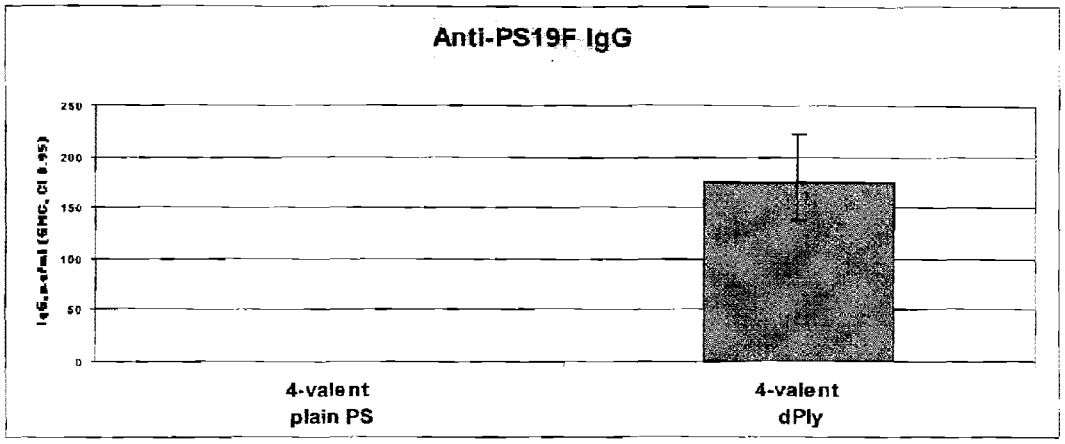


Figure 3, PS19F immunogenicity in Balb/c mice (post-III IgG levels)

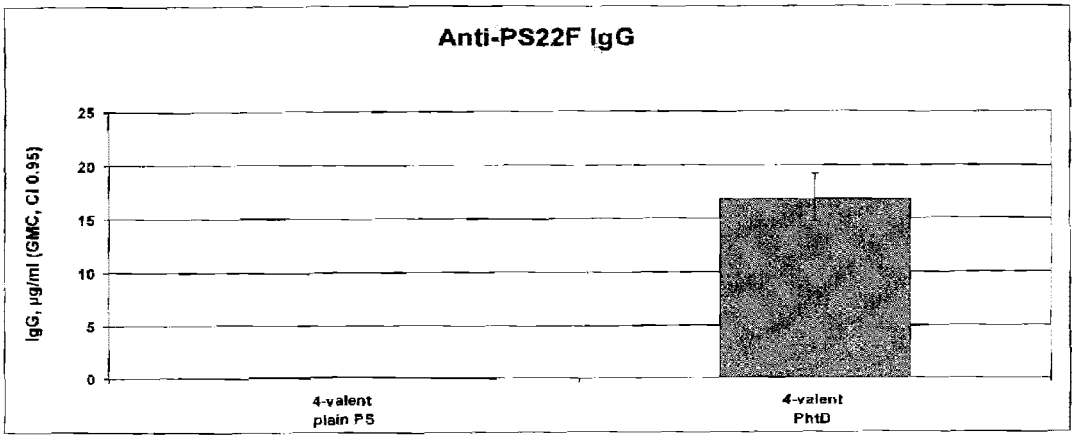


Figure 4, PS22F immunogenicity in Balb/c mice (post-III IgG levels)

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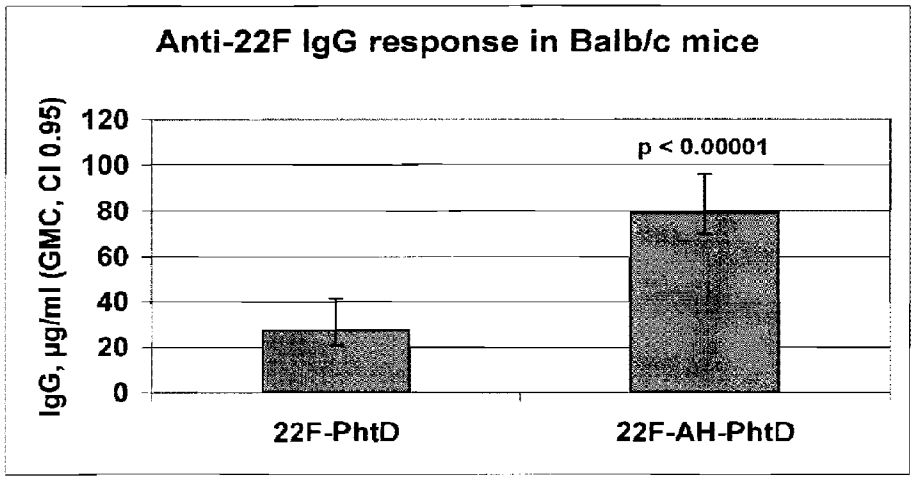


Figure 5, serum anti-PS IgG antibody levels

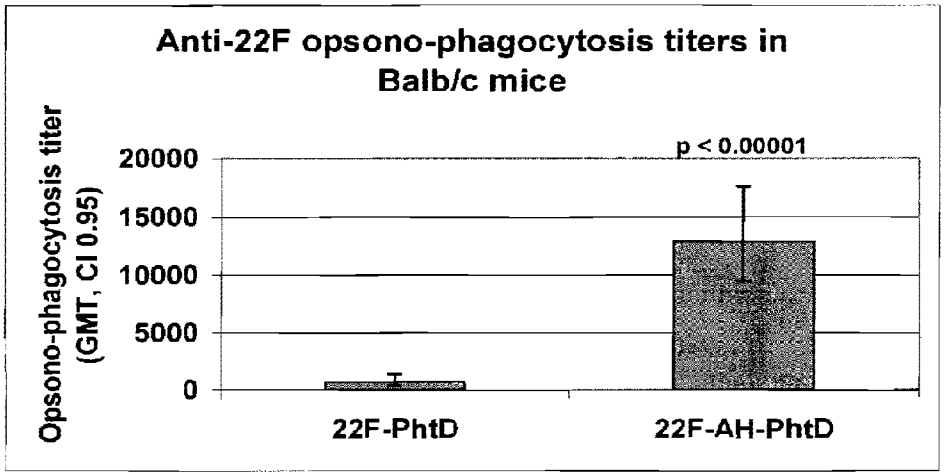


Figure 6, opsono-phagocytosis titres

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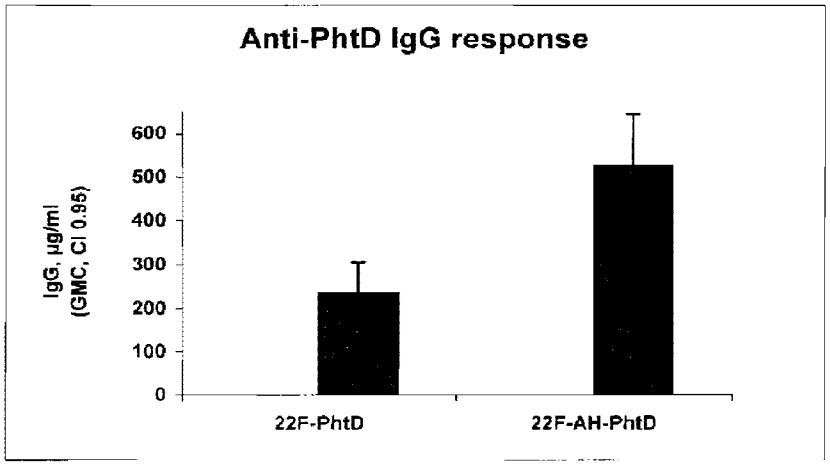


Figure 9 serum anti-PhtD IgG response

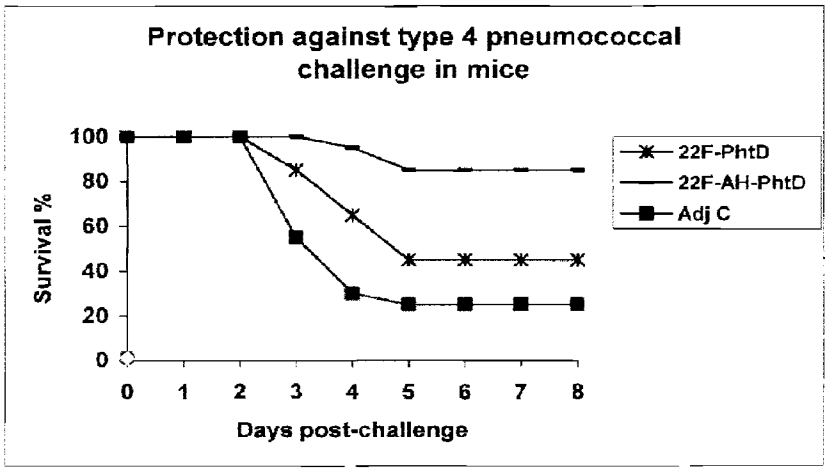


Figure 10
Protection against type 4 pneumococcal challenge in mice

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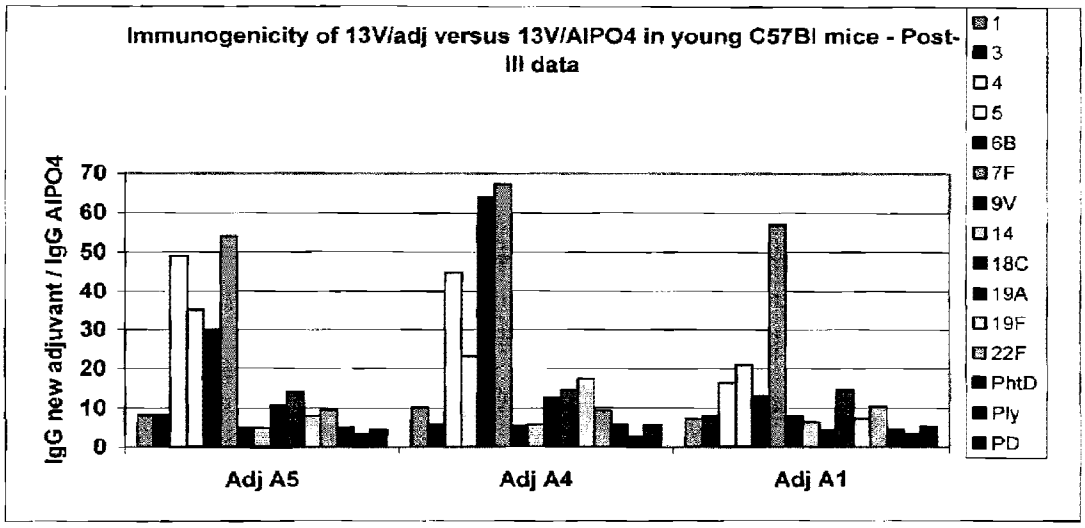


Figure 7, comparison of IgG responses induced with new adjuvants to the response elicited with $AlPO_4$

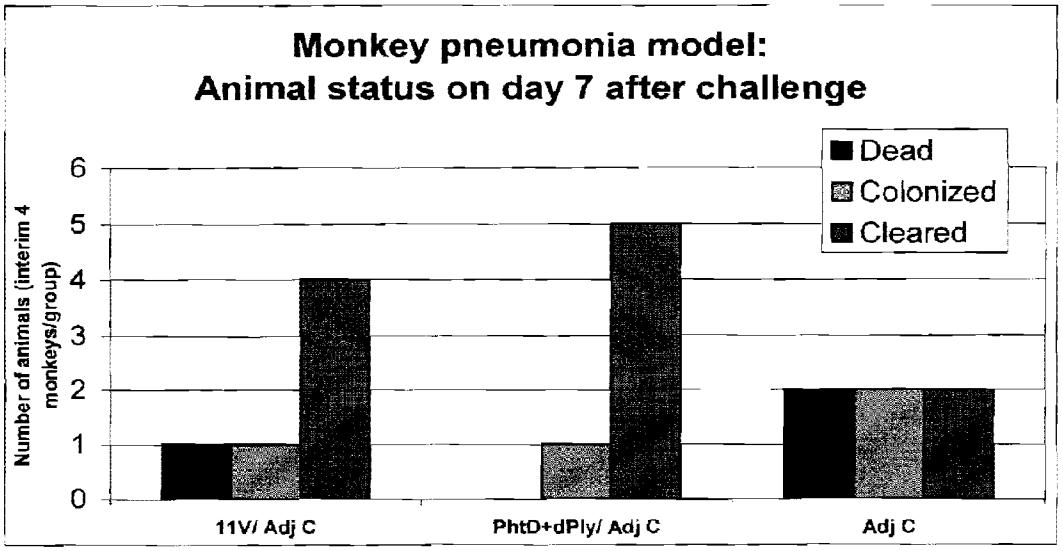


Figure 8, protective efficacy of PhtD + dPly protein combo against type 19F lung colonization in Rhesus monkeys

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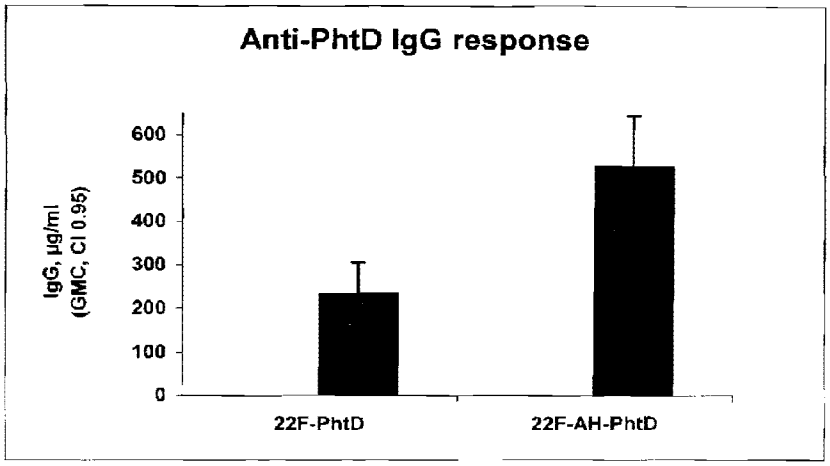


Figure 9 serum anti-PhtD IgG response

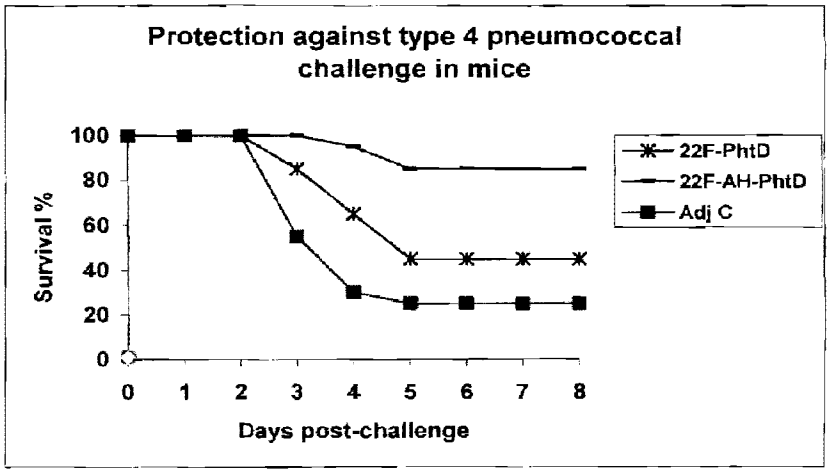


Figure 10
Protection against type 4 pneumococcal challenge in mice

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(54) Title: COMPOSITIONS COMPRISING STREPTOCOCCUS PNEUMONIAE POLYSACCHARIDE-PROTEIN CONJUGATES AND METHODS OF USE THEREOF

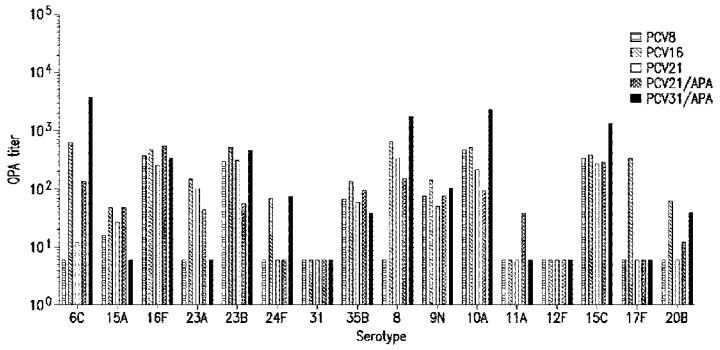


FIG.31A

(57) Abstract: The invention is related to multivalent immunogenic compositions comprising more than one *S. pneumoniae* polysaccharide protein conjugates, wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are as defined herein. In some embodiments, at least one of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent. In further embodiments, each of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent. Also provided are methods for inducing a protective immune response in a human patient comprising administering the multivalent immunogenic compositions of the invention to the patient. The multivalent immunogenic compositions are useful for providing protection against *S. pneumoniae* infection and diseases caused by *S. pneumoniae*. The compositions of the invention are also useful as part of treatment regimens that provide complementary protection for patients that have been vaccinated with a multivalent vaccine indicated for the prevention of pneumococcal disease.

[Continued on next page]

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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

COMPOSITIONS COMPRISING STREPTOCOCCUS PNEUMONIAE POLYSACCHARIDE-PROTEIN CONJUGATES AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

Not Applicable

FIELD OF INVENTION-

The present invention provides multivalent immunogenic compositions having distinct polysaccharide-protein conjugates. Each conjugate consists of a capsular polysaccharide prepared from a different serotype of *Streptococcus pneumoniae* conjugated to a carrier protein, preferably CRM197. The immunogenic compositions provide broad coverage against pneumococcal disease.

BACKGROUND OF THE INVENTION

Streptococcus pneumoniae is a Gram-positive bacterium and the most common cause of invasive bacterial disease (such as pneumonia, bacteraemia, meningitis and Otitis media) in infants and young children. Pneumococcus is encapsulated with a chemically linked polysaccharide which confers serotype specificity. There are over 90 known serotypes of pneumococci, and the capsule is the principle virulence determinant for pneumococci, as the capsule not only protects the inner surface of the bacteria from complement, but is itself poorly immunogenic. Polysaccharides are T-cell independent antigens, and, in most cases, can not be processed or presented on MHC molecules to interact with T-cells. They can however, stimulate the immune system through an alternate mechanism which involves cross-linking of surface receptors on B cells.

The multivalent pneumococcal polysaccharide vaccines that have been licensed for many years have proved valuable in preventing pneumococcal disease in adults, particularly, the elderly and those at high-risk. However, infants and young children respond poorly to unconjugated pneumococcal polysaccharides. The pneumococcal conjugate vaccine, Prevnar®, containing the 7 most frequently isolated serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) causing invasive pneumococcal disease in young children and infants at the time, was first licensed in the United States in February 2000. Following universal use of Prevnar® in the United States, there has been a significant reduction in invasive pneumococcal disease in children due to the serotypes present in Prevnar®. *See* Centers for Disease Control and Prevention, MMWR Morb Mortal Wkly Rep 2005, 54(36):893-7. However, there are limitations in serotype coverage with Prevnar® in certain regions of the world and some evidence of certain emerging serotypes in the United States (for example, 19A and others). See O'Brien *et al.*, 2004, *Am J Epidemiol* 159:634-

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44; Whitney *et al.*, 2003, *N Engl J Med* 348:1737-46; Kyaw *et al.*, 2006, *N Engl J Med* 354:1455-63; Hicks *et al.*, 2007, *J Infect Dis* 196:1346-54; Traore *et al.*, 2009, *Clin Infect Dis* 48:S181-S189.

U.S. Patent Application Publication No. US 2006/0228380 describes a 13-valent pneumococcal polysaccharide-protein conjugate vaccine including serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F. Chinese Patent Application Publication No. CN 101590224 A describes a 14-valent pneumococcal polysaccharide-protein conjugate vaccine including serotypes 1, 2, 4, 5, 6A, 6B, 7F, 9N, 9V, 14, 18C, 19A, 19F and 23F.

Other PCVs have covered 7, 10, 11, or 13 of the serotypes contained in PCV-15 (U.S Pub. No. 2011/0195086), but immune interference has been observed for some serotypes (e.g. lower protection for serotype 3 in GSK's PCV-11) and lower response rates to serotype 6B in Pfizer's PCV-13 (PREVNAR® 13). See Prymula *et al.*, 2006, Lancet 367:740-48 and Kieninger *et al.*, Safety and Immunologic Non-inferiority of 13-valent Pneumococcal Conjugate Vaccine Compared to 7-valent Pneumococcal Conjugate Vaccine Given as a 4-Dose Series in Healthy Infants and Toddlers, presented at the 48th Annual ICAAC/ISDA 46th Annual Meeting, Washington DC, October 25-28, 2008.

The current multivalent pneumococcal vaccines have been effective in reducing the incidence of pneumococcal disease associated with those serotypes present in the vaccines. However, the prevalence of the pneumococci expressing serotypes not present in the currently available vaccines has been increasing. Accordingly, there is a need for additional pneumococcal vaccine compositions which provide protection against different sets of pneumococcal serotypes and which can provide complementary protection against pneumococcal serotypes not present in currently available vaccines.

SUMMARY OF THE INVENTION

The invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are as defined herein.

In particular embodiments of the invention, the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

a) 15A, 16F, 23A, 23B, 24F, 31 and 35B;

b) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20; and

c) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20.

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In further particular embodiments of the invention, the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

a) 15A, 16F, 23A, 23B, 24F, 31 and 35B;

b) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; and

c) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A.

In further particular embodiments of the invention, the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

a) 15A, 16F, 23A, 23B, 24F, 31 and 35B;

b) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B; and

c) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B.

In some embodiments, the set of serotypes of *S. pneumoniae* listed in a), b) or c) further comprises: serotype 6C, serotype 6A, or serotypes 6A and 6B.

In some embodiments, at least one of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent, e.g. dimethylsulfoxide (DMSO). In specific embodiments, each of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent. As shown herein, the use of DMSO as a solvent during reductive amination of polysaccharide-protein conjugates results in unexpectedly superior stability and enhanced immunogenicity for those serotypes relative to the same conjugates prepared under aqueous conditions.

Also provided are methods for inducing a protective immune response in a human patient comprising administering the multivalent immunogenic compositions of the invention to the patient. In some embodiments of the methods of the invention, the patient was previously treated with a multivalent pneumococcal vaccine.

A multivalent immunogenic composition of the invention may be used as part of a treatment regimen with a different, complementary pneumococcal vaccine. Accordingly, the invention provides a method of inducing a protective immune response in a human patient comprising administering a multivalent immunogenic composition of the invention to the patient, further comprising administering a multivalent pneumococcal vaccine to the patient in any order. In particular embodiments, the multivalent pneumococcal vaccine is comprised of multiple *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein. In other embodiments, the multivalent pneumococcal vaccine is comprised of unconjugated capsular polysaccharides.

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Also provided are methods for preparing a serotype 8 *Streptococcus pneumoniae* polysaccharide-protein conjugate utilizing a conjugation reaction in an aprotic solvent, wherein the conjugation reaction does not use cyanoborohydride.

The invention also provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein select serotypes of *S. pneumoniae* provide cross-reactivity to other select serotypes.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the 600 MHz one-dimensional ^{1}H NMR spectrum of the capsular polysaccharide from *S. pneumonia* serotype 6C in deuterium oxide (D_2O) at 50°C. Signals arising from internal standards (DMSO and DSS-d_6) and residual water (HOD) are marked. Minor signals marked by * are due to S. *pneumonia* cell wall residuals such as C-polysaccharide and/or peptidoglycans.

FIGURE 2 depicts the 600 MHz one-dimensional ^{1}H NMR spectrum of the capsular polysaccharide from *S. pneumonia* serotype 15A in deuterium oxide (D_2O) at 50°C. Signals arising from internal standards (DMSO and DSS-d_6) and residual water (HOD) are marked. Minor signals marked by * are due to S. *pneumonia* cell wall residuals such as C-polysaccharide and/or peptidoglycans.

FIGURE 3 depicts the 600 MHz one-dimensional ^{1}H NMR spectrum of the capsular polysaccharide from *S. pneumonia* serotype de-O-acetylated 15B in deuterium oxide (D_2O) at 50°C. Signals arising from internal standards (DMSO and DSS-d_6) and residual water (HOD) are marked. Minor signals marked by * are due to S. *pneumonia* cell wall residuals such as C-polysaccharide and/or peptidoglycans.

FIGURE 4 depicts the 600 MHz one-dimensional ^{1}H NMR spectrum of the capsular polysaccharide from *S. pneumonia* serotype 35B in D_2O at 50°C. Signals arising from internal standards (DMSO and DSS-d_6) and residual water (HOD) are marked. Minor signals marked by * are due to S. *pneumonia* cell wall residuals such as C-polysaccharide and/or peptidoglycans.

FIGURE 5 depicts the ^{1}H NMR identity region useful for serotype identification of *S. pneumonia* serotype 6C. Signal positions of each anomeric proton of the repeating unit from each monosaccharide residue are marked.

FIGURE 6 depicts the ^{1}H NMR identity region useful for serotype identification of *S. pneumonia* serotype 15A. Signal positions of each anomeric proton of the repeating unit from each monosaccharide residue are marked.

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FIGURE 7 depicts the ^{1}H NMR identity region useful for serotype identification of *S. pneumonia* serotype de-O-acetylated 15B. Signal positions of each anomeric proton from each monosaccharide residue are marked.

FIGURE 8 depicts the ^{1}H NMR identity region useful for serotype identification of *S. pneumonia* serotype 35B. Signal positions of each anomeric proton of the repeating unit from each monosaccharide residue are marked.

FIGURES 9A-9C depict the 600 MHz one-dimensional ^{1}H NMR spectrum of native capsular polysaccharide from *S. pneumonia* serotype 15B (FIG. 9A), the de-O-acetylated capsular polysaccharide from *S. pneumonia* serotype 15B (FIG. 9B) and the capsular polysaccharide from *S. pneumonia* serotype 15C (FIG. 9C). Spectra were acquired in deuterium oxide (D_2O) at 50°C. Signals arising from internal standards (DMSO and DSS-d6) and residual water (HOD) are marked. Signals marked by * are due to *S. pneumonia* cell wall residuals such as C-polysaccharide and/or peptidoglycans.

FIGURES 10A-10C show the anomeric region of the 600 MHz one-dimensional ^{1}H NMR spectrum of of native capsular polysaccharide from *S. pneumonia* serotype 15B (FIG. 10A), the de-O-acetylated capsular polysaccharide from *S. pneumonia* serotype 15B (FIG. 10B) and the capsular polysaccharide from *S. pneumonia* serotype 15C (FIG 10C). FIGURES 10D-10F show regions of the spectrum where the O-acetyl and N-acetyl methy signals of native capsular polysaccharide from *S. pneumonia* serotype 15B (FIG. 10D) the de-O-acetylated capsular polysaccharide from *S. pneumonia* serotype 15B (FIG. 10E) and the capsular polysaccharide from *S. pneumonia* serotype 15C (FIG. 10F).

FIGURE 11 shows the impact of time and temperature (up to 12 weeks at 4°C-diamonds, up to 4 weeks at 25°C-squares, up to 4 weeks at 37°C-triangles) on the polysaccharide concentration of a PCV16 (0.128 mg/mL) or PCV21 (0.084 mg/mL or 0.169 mg/mL) drug products using HPSEC-UV/MALS/RI (see EXAMPLE 39).

FIGURE 12 shows the impact of horizontal rotation agitation and temperature (1 week at either 4°C, 25°C, or 37°C) on polysaccharide concentration of a PCV16 (0.128 mg/mL) or PCV21 (0.084 mg/mL or 0.169 mg/mL) drug products dispensed in pre-filled syringes (see EXAMPLE 39).

FIGURE 13 shows the impact of PS concentration over time and temperature (up to 4 weeks at either 4°C, 25°C, or 37°C) on the average molecular weight (Mw and Mn) of PCV16 (0.128 mg/mL) or PCV21 (0.084 mg/mL or 0.169 mg/mL) drug products using HPSEC-UV/MALS/RI.

FIGURES 14A and 14B show the impact of time and temperature (up to 1 wk at 4°C or 37°C) on stability of a Pneumococcal Conjugate vaccine (PCV15 or PCV16) prepared with drug substances conjugated in A) a protic solvent (PCV15 formulated using all aqueous conjugation) or B) an aprotic solvent (PCV16 formulated using all DMSO conjugation at 0.064

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mg/mL PnPs) using intrinsic protein fluorescence spectroscopy with an excitation wavelength at 280 nm (EXAMPLE 40).

FIGURE 15 shows ELISA IgG antibody dilution titers (post-dose 2) for rabbits immunized with *S. pneumoniae* monovalent serotypes conjugated to CRM197 and formulated with aluminum phosphate adjuvant (APA). Symbols indicate the individual titers and error bars represent the 95% confidence intervals (CIs) of the geometric mean titers (GMTs).

FIGURE 16 provides serotype specific OPA dilution titers (post-dose 2) for rabbits immunized with *S. pneumoniae* monovalent serotypes conjugated to CRM197 and formulated with aluminum phosphate adjuvant (APA). Symbols indicate the individual titers and error bars represent the 95% confidence intervals (CIs) of the geometric mean titers (GMTs).

FIGURE 17 provides ELISA IgG antibody dilution titers (post-dose 2) for rabbits immunized with *S. pneumoniae* serotype 15 monovalent conjugates. X-axis indicates the vaccine used to immunize rabbits. Dashed lines separate three separate ELISA assays using separate pneumococcal polysaccharide as a coating antigen. Symbols indicate the individual titers and error bars represent the 95% confidence intervals (CIs) of the geometric mean titers (GMTs).

FIGURE 18 shows serotype specific OPA dilution titers (pre-immune, post-dose 1 (PD1, pooled) and post-dose 2 (PD2)) for rabbits immunized with *S. pneumoniae* serotype 15 monovalent conjugates. X-axis indicates the vaccine used to immunize rabbits. Dashed lines separate three OPA assays using separate *S. pneumoniae* bacterial strains. Symbols indicate the individual titers and error bars represent the 95% confidence intervals (CIs) of the geometric mean titers (GMTs).

FIGURE 19A provides a comparison of PD1 antibody responses in NZWR (5 per group) following vaccination with 4, 2, 1, 0.4, 0.08 or 0.016 μg/dose of PCV21. Symbols indicate PD1 geometric mean titer (GMT) ratios (Group 2 μg/dose vs. other dose groups) with error bars representing the 95% confidence interval (CI). FIGURE 19B provides a PCV21 dose comparison PD1 GMT ratios (95% CI) corresponding to FIG. 19A GMT ratios whose lower 95% confidence bound exceed 1.0 are shaded light gray and GMT ratios whose upper 95% confidence bound are less than 1.0 are shaded dark gray. Serotype 15B data is included to evaluate cross protection.

FIGURE 20A provides a comparison of PD2 antibody responses in NZWR (5 per group) following vaccination with 4, 2, 1, 0.4, 0.08 or 0.016 μg/dose of PCV21. Symbols indicate PD2 GMT ratios (Group 2 μg/dose vs. other dose groups) with error bars representing the 95% CI. FIGURE 20B provides a PCV21 dose comparison PD2 GMT ratios (95% confidence interval) corresponding to FIGURE 20A GMT ratios whose lower 95% confidence bound exceed 1.0 are shaded light gray. Serotype 15B data is included to evaluate cross protection.

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FIGURE 21A shows the serotype specific PD1 OPA dilution titers for rabbits immunized with PCV21 (2μg/PnPs). Symbols indicate the individual titers and error bars represent the 95% confidence intervals (CIs) of the geometric mean titers (GMTs).* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. FIGURE 21B shows serotype specific PD2 OPA dilution titers for rabbits immunized with PCV21 (2μg/PnPs). Symbols indicate the individual titers and error bars represent the 95% confidence intervals (CIs) of the geometric mean titers (GMTs), **** $p<0.0001$. Serotype 15B data is included to evaluate cross protection.

FIGURE 22A-C show the impact of time and temperature (i.e. 7 Days at 4°C, 1 Day at 37°C or 7 Days at 37°C) on stability of three Pneumococcal Conjugate vaccines (PCV1, PCV7, and PCV14), prepared with all drug substances conjugated in either aqueous solvent or DMSO solvent, using intrinsic protein fluorescence spectroscopy with an excitation wavelength at 280 nm. FIGURE 22D shows the impact of time and temperature (i.e. 7 Days at 4°C or 37°C) on stability of a 21-valent Pneumococcal Conjugate vaccine (PCV21 at 0.084 mg/mL PnPs), prepared with all drug substances conjugated in DMSO solvent, using intrinsic protein fluorescence spectroscopy with an excitation wavelength at 280 nm.

FIGURE 23 shows the impact of temperature (7 Days at 4°C or 37°C) and agitation at 4°C on the particle size distribution as analyzed by Nanoparticle Tracking Analysis (NTA) of six (6) PCV21 Pneumococcal Conjugate vaccine drug products formulated with PS-20 (0%, 0.025%, 0.05%, 0.1%, 0.15% or 0.2% ; all w/v PS-20) at 0.084 mg/mL PnPs.

FIGURE 24 shows the impact of temperature (4°C and 37°C) and agitation on the three PCV21 drug product average molecular weight as analyzed by HPSEC/UV/MALS/RI assay. Three PCV21 Pneumococcal Conjugate vaccine drug products were formulated with different concentrations of PS-20 (0.05%, 0.1%, and 0.15% w/v) at 0.084 mg/mL PnPs.

FIGURE 25 shows that PCV21 immunized mice are protected from *S. pneumoniae* 24F intratracheal challenge.

FIGURE 26A-B shows pre (pooled), PD1 (pooled) and PD2 IgG antibody dilution titers as determined by ECL for rabbits immunized with PCV21 unadjuvanted or formulated with APA. Error bars represent the 95% confidence intervals (CI) of the geometric mean titer (GMT). Serotype 15B data is included to evaluate cross protection.

FIGURE 27A-C shows pre (pooled), PD1 (pooled) and PD2 IgG antibody dilution titers as determined by ECL for rabbits immunized with PCV8 unadjuvanted, PCV16 unadjuvanted, and PCV31 with APA. Error bars represent the 95% confidence intervals (CI) of the geometric mean titer (GMT). Serotype 15B data is included to evaluate cross protection.

FIGURE 28 shows the comparison of PD2 ECL antibody responses in NZWR (5 per group) following vaccination with PCV21 with or without APA. Symbols indicate PD2 GMT ratios with error bars representing the 95% CIs. Serotype 15B data is included to evaluate cross protection.

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FIGURE 29 shows the comparison of PD2 ECL antibody responses in NZWR (5 per group) for common serotypes and cross protected serotype 15B following vaccination with PCV21, PCV8 or PCV16. Symbols indicate PD2 ECL GMT ratios with error bars representing the 95% CIs.

FIGURE 30 shows the comparison of PD2 ECL antibody responses in NZWR (5 per group) for common serotypes and cross protected serotype 15B following vaccination with PCV21/APA or PCV31/APA. Symbols indicate PD2 ECL GMT ratios with error bars representing the 95% CIs.

FIGURE 31A-D shows serotype specific Pre (pooled) (A, B) and PD2 (C, D) OPA dilution titers for rabbits immunized with PCVs. Error bars represent the 95% CIs of the GMTs. PCV21 was used as the benchmark for statistical comparisons, * $p<0.05$. Figures A and C show data for 8 common serotypes in all PCVs evaluated (6C, 15A, 16F, 23A, 23B, 24F, 31, 35B) and additional 8 common serotypes in PCV16, PCV21 and PCV31 (8, 9N, 10A, 11A, 12F, 15C, 17F, 20B). Figures B and D show data for additional 16 serotypes not included in Figures A and C. Five of them are also common serotypes in PCV21 and PCV31 (3, 7F, 19A, 22F, 33F), ten serotypes are contained exclusively in PCV31 (1, 4, 5, 6A, 6B, 9V, 14, 18C, 19F, 23F). Serotype 15B data is included to evaluate cross protection.

FIGURE 32 shows pre, PD1, PD2 and PD3 IgG antibody dilution titers as determined by ECL for adult Rhesus macaques (n=8) immunized with PCV21. Error bars represent the 95% CIs of the GMTs. Serotype 15B data is included to evaluate cross protection.

FIGURE 33 shows pre, PD1 (pooled) and PD3 OPA dilution titers of PCV21 immunized adult Rhesus macaques (n=8). Error bars represent the 95% CIs of the GMTs. Serotype 15B data is included to evaluate cross protection.

FIGURE 34 shows pre and PD3 (day 70) OPA titers to four serotypes not contained in the PCV21 vaccine for adult Rhesus macaques immunized with PCV21.

FIGURE 35 shows the comparison of PD1 ECL antibody responses in adult Rhesus macaques (5 per group) following vaccination with PCV21 with or without APA. Symbols indicate PD1 ECL GMT ratios (PCV21 vs. PCV21/APA) with error bars representing the 95% CIs. Serotype 15B data is included to evaluate cross protection.

FIGURE 36 shows PD1 IgG antibody responses to serotype 3, 7F, 19A, 22F, and 33F in adult Rhesus macaques (2-5 per group) following vaccination with PCV21 compared to PCV15 or Prevnar13. Symbols indicate PD1 ECL GMT ratios with error bars representing the 95% CIs.

FIGURE 37 shows ELISA IgG antibody dilution titers to 6A, 6B, and 6C ((pre-immune and PD1, pooled) and PD2) for rabbits immunized with 6A-CRM197 (A) or 6B-CRM197 (B) monovalent drug products. Bars indicate geometric mean titers (GMTs) and error bars represent the 95% confidence intervals (CIs) of the GMTs.

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FIGURE 38 shows serotype specific OPA dilution titers to 6A, 6B, and 6C ((pre-immune and PD1 pooled) and PD2) for rabbits immunized with 6A-CRM197 (A) or 6B-CRM197 (B) monovalent drug products. Bars indicate geometric mean titers (GMTs) and error bars represent the 95% confidence intervals (CIs) of the GMTs.

FIGURE 39 shows serotype specific OPA dilution titers to 20A and 20B (pre-immune (pooled), PD1and PD2) for rabbits immunized with 20A-CRM197/APA (A) or PCV21 (B). Bars indicate geometric mean titers (GMTs) and error bars represent the 95% confidence intervals (CIs) of the GMTs.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides multivalent immunogenic compositions comprising pneumococcal polysaccharide-protein conjugates, wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are as defined herein. In some embodiments, the immunogenic composition comprises a set of pneomococcal serotypes selected from the group consisting of: (a) 15A, 16F, 23A, 23B, 24F, 31 and 35B; (b) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20; and (c) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20. In some embodiments, the immunogenic composition comprises a set of pneomococcal serotypes selected from the group consisting of: (a) 15A, 16F, 23A, 23B, 24F, 31 and 35B; (b) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B; and (c) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B. In other embodiments, the immunogenic composition comprises a set of pneomococcal serotypes selected from the group consisting of: (a) 15A, 16F, 23A, 23B, 24F, 31 and 35B; (b) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; and (c) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A. In further embodiments, the immunogenic composition comprises (i) serotype 6A; (ii) serotypes 6A and 6B; or (iii) serotype 6C. In a particular embodiment, the invention comprises multiple pneumococcal *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise serotypes 3, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 19A, 20A, 22F, 23A, 23B, 24F, 31, 33F and 35B. Said composition was found to be immunogenic in rabbits and generate functional antibody which killed vaccine-type bacterial strains at all doses tested (EXAMPLE 43). In another particular embodiment, the invention comprises multiple pneumococcal *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise serotypes 3, 7F, 19A, 22F, 33F, 8, 9N, 10A,

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11A, 12F, 15C, 17F, 20, 6A, 15A, 15C, 16F, 23A, 23B, 24F, 31 and 35B. In another particular embodiment, the invention comprises multiple pneumococcal *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise serotypes 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20B, 6A, 15A, 15C, 16F, 23A, 23B, 24F, 31 and 35B. In another particular embodiment, the invention comprises multiple pneumococcal *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise serotypes 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6A, 15A, 15C, 16F, 23A, 23B, 24F, 31 and 35B.

The multivalent immunogenic compositions of the invention are useful for immunizing a patient against vaccine-type *S. pneumoniae serotypes* and as part of a treatment regimen with different, complementary pneumococcal vaccine(s). Accordingly, the invention provides a method of inducing a protective immune response in a human patient comprising administering a multivalent immunogenic composition of the invention to the patient, and further comprising administering a multivalent pneumococcal vaccine to the patient, in any order. In other embodiments, the multivalent immunogenic compositions of the invention are administered to a patient who had been previously immunized with a different multivalent pneumococcal vaccine.

In embodiments of the invention, conjugates from at least one pneumococcal serotype are prepared using reductive amination in an aprotic solvent such as DMSO. In further embodiments, the multivalent immunogenic composition comprises pneumococcal conjugates that were each prepared using reductive amination in an aprotic solvent. It is shown herein that PCV1, PCV7, PCV14, PCV16 and PCV21 (as defined, *infra*) drug products comprising drug substances that were each prepared using reductive amination in an aprotic solvent were stable against depolymerization or chemical degradation of the carbohydrate and stable against aggregation of the protein in the drug product formulation (see EXAMPLE 40 and EXAMPLE 45). The use of DMSO solvent enhances the covalent associations of polysaccharide to protein through direct consumption of lysine residues on the surface of the carrier protein. The increased covalent association has a direct benefit to increasing the stability of the polysaccharide protein conjugate of multivalent immunogenic compositions comprising polysaccharide antigens conjugated in DMSO.

I. Definitions and Abbreviations

As used throughout the specification and appended claims, the following abbreviations apply:

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APA	aluminum phosphate adjuvant
APC	antigen presenting cell
CI	confidence interval
DMSO	dimethylsulfoxide
DS	polysaccharide-protein Drug Substance
GMC	geometric mean concentraton
GMT	geometric mean titer
HPSEC	high performance size exclusion chromatography
IM	intra-muscular or intra-muscularly
LOS	lipo-oligosaccharide
LPS	lipopolysaccharide
MALS	multi-angle light scattering
MBC	monovalent bulk conjugate
MOPA	multiplexed opsonophagocytic assays
MW	molecular weight
NMWCO	nominal molecular weight cut off
NZWR	New Zealand White rabbit
OPA	opsonophagocytosis assay
PCV	pneumococcal conjugate vaccine
PD1	post-dose 1
PD2	post-dose 2
PnPs	Pneumoccal Polysaccharide
Ps	polysaccharide
PS-20	polysorbate-20
RI	refractive index
UV	ultraviolet
w/v	weight per volume

So that the invention may be more readily understood, certain technical and scientific terms are specifically defined below. Unless specifically defined elsewhere in this document, all other technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs.

As used throughout the specification and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise.

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Reference to "or" indicates either or both possibilities unless the context clearly dictates one of the indicated possibilities. In some cases, "and/or" was employed to highlight either or both possibilities.

The terms "aqueous solvent" or "aqueous conditions" when used with conjugation, such as reductive amination, refers to use of water as the solvent for the conjugation reaction. The water may contain buffers and other components except that no organic solvent is present.

The terms "aprotic solvent", "DMSO solvent" or "DMSO conditions" when used with conjugation, such as reductive amination, refers to use of an aprotic solvent, or a combination of aprotic solvents, (or DMSO, as applicable) as the solvent for the conjugation reaction. The aprotic solvent may have some water present, for example, up to 1%, 2%, 5%, 10% or 20%.

The term "comprises" when used with the immunogenic composition of the invention refers to the inclusion of any other components, such as adjuvants and excipients, or the addition of one or more polysaccharide-protein conjugates that are not specifically enumerated. The term "consisting of" when used with the multivalent polysaccharide-protein conjugate mixture refers to a mixture having those particular *S. pneumoniae* polysaccharide protein conjugates and no other *S. pneumoniae* polysaccharide protein conjugates from a different serotype. "Consists essentially of" and variations such as "consist essentially of" or "consisting essentially of," indicate the inclusion of any recited elements or group of elements, and the optional inclusion of other elements, of similar or different nature than the recited elements, which do not materially change the basic or novel properties of the specified dosage regimen, method, or composition.

"Effective amount" of a composition of the invention refers to a dose required to elicit antibodies that significantly reduce the likelihood or severity of infectivitiy of a microbe, e.g., *S. pneumonia*, during a subsequent challenge.

As used herein, the phrase "indicated for the prevention of pneumococcal disease" means that a vaccine or immunogenic composition is approved by one or more regulatory authorities, such as the US Food and Drug Administration, for the prophylaxis of one or more diseases caused by any serotype of *S. pneumoniae*, including, but not limited to: pneumococcal disease generally, pneumococcal pneumonia, pneumococcal meningitis, pneumococcal bacteremia, invasive disease caused by *S. pneumoniae*, and otitis media caused by *S. pneumoniae*.

A "multivalent pneumococcal vaccine" is a pharmaceutical preparation comprising more than one active agent (*e.g.*, pneumococcal capsular polysaccharide or pneumococcal polysaccharide protein conjugate) that provides active immunity to disease or pathological condition caused by more than one serotype of *S. pneumoniae*.

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The term "polysaccharide" is meant to include any antigenic saccharide element (or antigenic unit) commonly used in the immunologic and bacterial vaccine arts, including, but not limited to, a "saccharide", an "oligosaccharide", a "polysaccharide", a "liposaccharide", a "lipo-oligosaccharide (LOS)", a "lipopolysaccharide (LPS)", a "glycosylate", a "glycoconjugate" and the like.

"PCV1," as used herein, refers to a 1-valent pneumococcal conjugate vaccine comprising one *S. pneumoniae* polysaccharide protein conjugate, comprising capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotype of *S. pneumoniae* is 3 (as exemplified in Example 38 and 45). In specific embodiments, the carrier protein is CRM197.

"PCV7," as used herein, refers to a 7-valent pneumococcal conjugate vaccine comprising seven *S. pneumoniae* polysaccharide protein conjugates, each comprising capsular polysaccharides from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are: 3, 8, 9N, 11A, 19A, 15A, and 10A. In specific embodiments, the carrier protein of one or more of the *S. pneumoniae* polysaccharide protein conjugates is CRM197. In further embodiments, the carrier protein of each of the *S. pneumoniae* polysaccharide protein conjugates is CRM197.

"PCV8," as used herein, refers to a 8-valent pneumococcal conjugate vaccine comprising eight *S. pneumoniae* polysaccharide protein conjugates, each comprising capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are: 6C, 15A, 16F, 23A, 23B, 24F, 31, and 35B. In specific embodiments, the carrier protein of one or more of the *S. pneumoniae* polysaccharide protein conjugates is CRM197. In further embodiments, the carrier protein of each of the *S. pneumoniae* polysaccharide protein conjugates is CRM197.

"PCV14," as used herein, refers to a 14-valent pneumococcal conjugate vaccine comprising fourteen *S. pneumoniae* polysaccharide protein conjugates, each comprising capsular polysaccharides from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are: 3, 7F, 8, 9N, 10A, 11A, 12F, 15A, 16F, 17F, 19A, 20, 22F and 33F. In specific embodiments, the carrier protein of one or more of the *S. pneumoniae* polysaccharide protein conjugates is CRM197. In further embodiments, the carrier protein of each of the *S. pneumoniae* polysaccharide protein conjugates is CRM197.

"PCV15," as used herein, refers to a 15-valent pneumococcal conjugate vaccine comprising fifteen *S. pneumoniae* polysaccharide protein conjugates, each comprising capsular polysaccharides from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are: 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F. In specific embodiments, the carrier protein of one or more of the *S. pneumoniae* polysaccharide

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protein conjugates is CRM197. In further embodiments, the carrier protein of each of the *S. pneumoniae* polysaccharide protein conjugates is CRM197.

"PCV16," as used herein, refers to a 16-valent pneumococcal conjugate vaccine comprising sixteen *S. pneumoniae* polysaccharide protein conjugates, each comprising capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are: 6C, 8, 9N, 10A, 11A, 12F, 15A, 16F, 17F, 20A, 23A, 23B, 24F, 31, and 35B, and at least one of the following serogroup 15 serotypes: 15B, 15C, or de-O-acetylated-15B. In particular embodiments, the serogroup 15 serotype is serotype 15C or de-O-acetylated 15B. As shown herein (see EXAMPLE 3, *infra*), de-O-Acetyated serotype 15B pneumococcal polysaccharide is equivalent to serotype 15C pneumococcal polysaccharide and has an identical NMR spectra. In specific embodiments, the carrier protein of one or more of the *S. pneumoniae* polysaccharide protein conjugates is CRM197. In further embodiments, the carrier protein of each of the *S. pneumoniae* polysaccharide protein conjugates is CRM197.

"PCV21," as used herein, refers to a 21-valent pneumococcal conjugate vaccine comprising twenty-one *S. pneumoniae* polysaccharide protein conjugates, each comprising capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are: 3, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 16F, 17F, 19A, 20A, 22F, 23A, 23B, 24F, 31, 33F and 35B, and at least one of the following serogroup 15 serotypes: 15B, 15C or de-O-acetylated-15B. In particular embodiments, the serogroup 15 serotype is serotype 15C or de-O-acetylated 15B. In specific embodiments, the carrier protein of one or more of the *S. pneumoniae* polysaccharide protein conjugates is CRM197. In further embodiments, the carrier protein of each of the *S. pneumoniae* polysaccharide protein conjugates is CRM197.

S. pneumoniae serotypes 20A and 20B were identified (Calix, J.J. et al., J. Biol. Chem. (2012) 287(33): 27885-27894) within serogroup 20 in 2012 through genetic analysis. As discussed by Calix J.J., et al., conventional sero-typing methods and commercially available antibodies were not able to distinguish these two serotypes previously and there is limited information today regarding serotyping efforts for serotypes 20A and 20B. Further, the disease prevalence of 20A or 20B within an identified disease caused by serogroup 20 is not well understood. As such, one of ordinary skill in the art might elect to include either or both of these two serotypes (20A and/or 20B) in a *S. pneumoniae* vaccine composition. It is also possible that a *S. pneumoniae* vaccine containing one serotype 20 polysaccharide antigen might cross protect against another (e.g., a vaccine including polysaccharide protein conjugates of *S. pneumoniae* serotype 20A, and not serotype 20B, may cross protect against infection with *S. pneumoniae* serotype 20B). Therefore, "serotype 20" as used herein may refer to a composition containing polysaccharide protein conjugates of serotype 20A and/or serotype 20B.

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"CpG-containing nucleotide," "CpG-containing oligonucleotide," "CpG oligonucleotide," and similar terms refer to a nucleotide molecule of 6-50 nucleotides in length that contains an unmethylated CpG moiety. *See, e.g.*, Wang *et al.*, 2003, *Vaccine* 21:4297. CpG-containing oligonucleotides include modified oligonucleotides using any synthetic internucleoside linkages, modified base and/or modified sugar.

An "adjuvant," as defined herein, is a substance that serves to enhance the immunogenicity of an immunogenic composition of the invention. An immune adjuvant may enhance an immune response to an antigen that is weakly immunogenic when administered alone, e.g., inducing no or weak antibody titers or cell-mediated immune response, increase antibody titers to the antigen, and/or lowers the dose of the antigen effective to achieve an immune response in the individual. Thus, adjuvants are often given to boost the immune response and are well known to the skilled artisan.

A "patient" (alternatively referred to herein as a "subject") refers to a mammal capable of being infected with a *S. pneumoniae*. In preferred embodiments, the patient is a human. A patient can be treated prophylactically or therapeutically. Prophylactic treatment provides sufficient protective immunity to reduce the likelihood or severity of a pneomococcal infection or the effects thereof, e.g., pneumococcal pneumonia. Therapeutic treatment can be performed to reduce the severity or prevent recurrence of a *S. pneumoniae* infection or the clinical effects thereof. Prophylactic treatment can be performed using a multivalent immunogenic composition of the invention, as described herein. The composition of the invention can be administered to the general population or to those persons at an increased risk of pneumococcal infection, e.g. the elderly, or those who live with or care for the elderly.

Those "in need of treatment" include those previously exposed to or infected with *S. pneumoniae*, those who were previously vaccinated against *S. pneumoniae*, as well as those prone to have an infection or any person in which a reduction in the likelihood of infection is desired, e.g., the immunocompromised, the elderly, children, adults, or healthy individuals.

A "stable" multivalent immunogenic composition is a composition which has no significant changes observed at a refrigerated temperature (e.g., 2-8°C or 4°C) for at least 1 month, 2 months, 3 months, 6 months, 12 months and/or 24 months. Additionally, a "stable" composition includes one that exhibits desired features at temperatures including at 25°C and 37°C for periods including 1 month, 3 months, 6 months, 12 months, and/or 24 months. Typical acceptable criteria for stability are as follows: no more than about 5%, about 10%, about 15%, or about 20% variability in one or more of the following: (a) the number average molecular weight (Mn) of the *S. pneumoniae* polysaccharide protein conjugates in the composition, (b) weight average molecular weight (Mw) of the *S. pneumoniae* polysaccharide protein conjugates in the composition, (c) total polysaccharide concentration in the composition, (d) emission maximum of the composition measured using intrinsic protein fluorescence spectroscopy at a particular

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excitation wavelength, e.g. 280 nanometers, and (e) the fluorescence intensity of the composition measured using intrinsic protein fluorescence spectroscopy at a particular excitation wavelength. The term "stable" may also be used to refer to a particular pneumococcal conjugate within a multivalent immunogenic composition. In such use, the term refers to a conjugate that exhibits the desired properties over time, at a particular temperature, and such properties vary no more that about 5%, about 10%, about 15%, or about 20% over the time and temperature noted.

II. Multivalent Immunogenic Compositions

The invention provides multivalent immunogenic compositions comprising multiple *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein. Different aspects and embodiments of the multivalent immunogenic compositions of the invention are described, *infra*.

In one embodiment (Embodiment E1), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates, each comprising capsular polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise, consist, or consist essentially of:

(1) 15A,
(2) 16F,
(3) one or more serogroup 23 serotypes selected from: (a) 23A and 23B, (b) 23A and 23F, (c) 23B and 23F, (d) 23A, (e) 23B, and (f) 23F,
(4) 24F,
(5) 31, and
(6) 35B.

In a sub-embodiment of Embodiment E1, the one or more serogroup 23 serotypes are 23A and and 23B (i.e. and no other serogroup 23 serotypes are present). In a further sub-embodiment of Embodiment E1, the one or more serogroup 23 serotypes are 23A and 23F. In another sub-embodiment of Embodiment E1, the one or more serogroup 23 serotypes are 23B and 23F. In yet another sub-embodiment of Embodiment E1, 23A is the only serogroup 23 serotype. In a further sub-embodiment of Embodiment E1, 23B is the only serogroup 23 serotype. In a still further sub-embodiment of Embodiment E1, 23F is the only serogroup 23 serotype.

In a second embodiment (Embodiment E2), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise the serotypes set forth in

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Embodiment E1, or any sub-embodiment of Embodiment E1, and further comprise serotype: (1) 6C, (2) 6A, or (3) 6A and 6B.

In a sub-embodiment of Embodiment E2, the composition comprises serotype 6C. In a further sub-embodiment of Embodiment E2, the composition comprises serotypes 6A and 6B and does not comprise serotype 6C. In a another sub-embodiment of Embodiment E2, the composition comprises serotype 6A.

In a third embodiment (Embodiment E3), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise the serotypes set forth in Embodiments E1, or any sub-embodiments of Embodiment E1, or Embodiment E2, or any sub-embodiment of Embodiment E2, and further comprise serotypes:

(1) 8,
(2) 9N,
(3) 11A
(4) 12F,
(5) 15B or 15C,
(6) 17F, and
(7) 20A and/or 20B.

In a fourth embodiment (Embodiment E4), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise the serotypes set forth in any of Embodiments E1-E3, or any sub-embodiments thereof, and further comprise serotypes: 10A or 39.

In a fifth embodiment (Embodiment E5), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise, consist, or consist essentially of: 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31, and 35B.

In a sixth embodiment (Embodiment E6), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise, consist, or consist essentially of: 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31, and 35B.

In a seventh embodiment (Embodiment E7), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein

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conjugates as described above, wherein the *S. pneumoniae* serotypes comprise, consist, or consist essentially of: 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31, and 35B.

In an eighth embodiment (Embodiment E8), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise, consist, or consist essentially of: 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31, and 35B.

In a ninth embodiment (Embodiment E9), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise the serotypes set forth in any of Embodiments E1-E8 (or any sub-embodiment thereof), and further comprise serotypes: 3, 7F, and 19A.

In a tenth embodiment (Embodiment E10), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise the serotypes set forth in any of Embodiments E1-E9 (or any sub-embodiment thereof), and further comprise serotype 22F.

In an eleventh embodiment (Embodiment E11), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise the serotypes set forth in any of Embodiments E1-E10 (or any sub-embodiment thereof), and further comprise serotype 33F.

In an twelfth embodiment (Embodiment E12), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise, consist, or consist essentially of a set of *S. pneumoniae* serotypes selected from the group consisting of:

(1) 15A, 16F, 23A, 23B, 24F, 31, and 35B,
(2) 15A, 16F, 23F, 23B, 24F, 31, and 35B,
(3) 15A, 16F, 23A, 23F, 24F, 31, and 35B,
(4) 15A, 16F, 23A, 24F, 31, and 35B,
(5) 15A, 16F, 23B, 24F, 31, and 35B,
(6) 15A, 16F, 23F, 24F, 31, and 35B,
(7) 6C, 15A, 16F, 23A, 23B, 24F, 31, and 35B,
(8) 6C, 15A, 16F, 23F, 23B, 24F, 31, and 35B,
(9) 6C, 15A, 16F, 23A, 23F, 24F, 31, and 35B,
(10) 6C, 15A, 16F, 23A, 24F, 31, and 35B,

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(11) 6C, 15A, 16F, 23B, 24F, 31, and 35B,
(12) 6C, 15A, 16F, 23F, 24F, 31, and 35B,
(13) 6A, 15A, 16F, 23A, 23B, 24F, 31, and 35B,
(14) 6A, 15A, 16F, 23F, 23B, 24F, 31, and 35B,
(15) 6A, 15A, 16F, 23A, 23F, 24F, 31, and 35B,
(16) 6A, 15A, 16F, 23A, 24F, 31, and 35B,
(17) 6A, 15A, 16F, 23B, 24F, 31, and 35B,
(18) 6A, 15A, 16F, 23F, 24F, 31, and 35B,
(19) 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, and 35B,
(20) 6A, 6B, 15A, 16F, 23F, 23B, 24F, 31, and 35B,
(21) 6A, 6B, 15A, 16F, 23A, 23F, 24F, 31, and 35B,
(22) 6A, 6B, 15A, 16F, 23A, 24F, 31, and 35B,
(23) 6A, 6B, 15A, 16F, 23B, 24F, 31, and 35B, and
(24) 6A, 6B, 15A, 16F, 23F, 24F, 31, and 35B.

In an thirteenth embodiment (Embodiment E13), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise, consist, or consist essentially of a set of *S. pneumoniae* serotypes selected from the group consisting of:

(1) 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B,
(2) 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B,
(3) 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B,
(4) 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B,
(5) 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,
(6) 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B,
(7) 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,
(8) 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,
(9) 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

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(10) 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(11) 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(12) 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(13) 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(14) 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(15) 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(16) 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(17) 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23F, 24F, 31 and 35B,

(18) 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23F, 24F, 31 and 35B,

(19) 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23F, 24F, 31 and 35B,

(20) 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23F, 24F, 31 and 35B,

(21) 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 15A, 16F, 23F, 24F, 31 and 35B,

(22) 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6A, 6B, 15A, 16F, 23F, 24F, 31 and 35B,

(23) 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6A, 15A, 16F, 23F, 24F, 31 and 35B,

(24) 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 15A, 16F, 23F, 24F, 31 and 35B,

(25) 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6A, 6B, 15A, 16F, 23F, 24F, 31 and 35B,

(26) 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6A, 15A, 16F, 23F, 24F, 31 and 35B,

(27) 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 15A, 16F, 23F, 24F, 31 and 35B,

(28) 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6A, 6B, 15A, 16F, 23F, 24F, 31 and 35B,

(29) 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6A, 15A, 16F, 23F, 24F, 31 and 35B,

(30) 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 15A, 16F, 23F, 24F, 31 and 35B,

(31) 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6A, 6B, 15A, 16F, 23F, 24F, 31 and 35B, and

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(32) 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6A, 15A, 16F, 23F, 24F, 31 and 35B.

The thirteenth embodiment (Embodiment E13), further comprises the set of *S. pneumoniae* serotypes, rows (1) through (32) wherein serotype 20A in each set is substituted with either serotype 20 or serotype 20B.

In a fourteenth embodiment of the invention, (Embodiment E14), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise, consist, or consist essentially of a set of *S. pneumoniae* serotypes selected from any of the sets of serotypes set forth in Embodiment E13, rows (17) through (32), and further comprise serotypes 23A and/or 23B.

In an fifteenth embodiment (Embodiment E15), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise, consist, or consist essentially of a set of *S. pneumoniae* serotypes selected from the group consisting of:

(1) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(2) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(3) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(4) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(5) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(6) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(7) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(8) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15C, 17F, 20A 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(9) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(10) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(11) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A 15A, 16F, 23A, 23B, 24F, 31 and 35B,

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(12) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(13) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(14) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15B, 17F, 20A 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(15) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(16) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B,

(17) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23F, 24F, 31 and 35B,

(18) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6C, 15A, 16F, 23F, 24F, 31 and 35B,

(19) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23F, 24F, 31 and 35B,

(20) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6C, 15A, 16F, 23F, 24F, 31 and 35B,

(21) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 15A, 16F, 23F, 24F, 31 and 35B,

(22) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6A, 6B, 15A, 16F, 23F, 24F, 31 and 35B,

(23) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15C, 17F, 20A, 6A, 15A, 16F, 23F, 24F, 31 and 35B,

(24) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 15A, 16F, 23F, 24F, 31 and 35B,

(25) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6A, 6B, 15A, 16F, 23F, 24F, 31 and 35B,

(26) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15C, 17F, 20A, 6A, 15A, 16F, 23F, 24F, 31 and 35B,

(27) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 15A, 16F, 23F, 24F, 31 and 35B,

(28) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6A, 6B, 15A, 16F, 23F, 24F, 31 and 35B,

(29) 3, 7F, 19A, 22F, 33F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20A, 6A, 15A, 16F, 23F, 24F, 31 and 35B,

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(30) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 15A, 16F, 23F, 24F, 31 and 35B,

(31) 3, 7F, 19A, 22F, 33F, 8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6A, 6B, 15A, 16F, 23F, 24F, 31 and 35B,

(32) 3, 7F, 19A, 22F, 33F,8, 9N, 39, 11A, 12F, 15B, 17F, 20A, 6A, 15A, 16F, 23F, 24F, 31 and 35B,

(33) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B,

(34) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24B, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A,

(35) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24B, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B,

(36) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B,

(37) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24B, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A,

(38) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24B, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B,

(39) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20,

(40) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24B, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20,

(41) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24B, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20,

(42) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20,

(43) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24B, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20, and

(44) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24B, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20.

In a sixteenth embodiment of the invention, (Embodiment E16), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates as described above, wherein the *S. pneumoniae* serotypes comprise, consist, or consist essentially of a set of *S. pneumoniae* serotypes selected from any of the sets of serotypes set forth in Embodiment E15, rows (17) through (32), and further comprise serotypes 23A and/or 23B.

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In an seventeenth embodiment (Embodiment E17), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

i. 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B,
ii. 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A, and
iii. 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

In sub-embodiments of Embodiment E17, the immunogenic composition does not comprise any further *S. pneumoniae* polysaccharide protein conjugates.

In an eighteenth embodiment (Embodiment E18), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotypes 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B. In sub-embodiments of Embodiment E18, the immunogenic composition does not comprise any further *S. pneumoniae* polysaccharide protein conjugates.

In a nineteenth embodiment (Embodiment E19), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotypes 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A. In sub-embodiments of Embodiment E19, the immunogenic composition does not comprise any further *S. pneumoniae* polysaccharide protein conjugates.

In a twentieth embodiment (Embodiment E20), the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotypes 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A. In sub-embodiments of Embodiment E20, the immunogenic composition does not comprise any further *S. pneumoniae* polysaccharide protein conjugates.

In another embodiment, the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotypes 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A. In sub-embodiments of this embodiment (i.e., 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A), the immunogenic composition does not comprise any further *S. pneumoniae* polysaccharide protein conjugates.

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In another embodiment, the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotypes 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20. In sub-embodiments of this embodiment (i.e., 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20), the immunogenic composition does not comprise any further *S. pneumoniae* polysaccharide protein conjugates.

In another embodiment, the invention provides a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from a *S. pneumoniae* serotypes 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B. In sub-embodiments of this embodiment (i.e., 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B), the immunogenic composition does not comprise any further *S. pneumoniae* polysaccharide protein conjugates.

As shown herein (see EXAMPLE 3, *infra*), de-O-Acetyated serotype 15B pneumococcal polysaccharide is equivalent to serotype 15C pneumococcal polysaccharide and has an identical NMR spectra. De-O-Acetylated serotype 15B pneumococcal polysaccharide, as described and disclosed herein, is equivalent to serotype 15C pneumococcal polysaccharide and both may have an O-Acetyl content per repeating unit in the range of 0-5%, or in the range of 0-4%, or in the range of 0-3%, or in the range of 0-2%, or in the range of 0-1%, or in the range of 0-0.5%, or in the range of 0-0.1%, or no O-Acetyl content. In a report by Spencer B.L., et al., 15C may be slightly O-Acetylated (Spencer, B.L. et al., Clin. Vac. Immuno. (2017) 24(8): 1-13). Thus, in any of the embodiments of the multivalent immunogenic compositions herein, de-O-acetylated serotype 15B can be used in place of serotype 15C. Processes for de-O-acetylation are known in the art, for example as described in Rajam et al., *Clinical and Vaccine Immunology*, 2007, 14(9):1223–1227.

In certain embodiments of any of the multivalent immunogenic compositions of the invention, including Embodiments E1 to E20 and any sub-embodiment thereof, the composition further comprises a pharmaceutically acceptable carrier.

Cross-Reactivity

In an embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 6C, conjugated to a carrier protein, wherein serotype 6C of *S. pneumoniae* provides cross-protection against serotypes 6A and 6B of *S. pneumoniae*.

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In another embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates as selected from Embodiments E1 to E20 wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 6C, conjugated to a carrier protein, wherein serotype 6C of *S. pneumoniae* provides cross-protection against serotypes 6A and 6B of *S. pneumoniae*.

In an embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 19A, conjugated to a carrier protein, wherein serotype 19A of *S. pneumoniae* provides cross-protection against serotype 19F of *S. pneumoniae*.

In another embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates as selected from Embodiments E1 to E20 wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 19A, conjugated to a carrier protein, wherein serotype 19A of *S. pneumoniae* provides cross-protection against serotype 19F of *S. pneumoniae*.

In an embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotypes 23A and/or 23B, conjugated to a carrier protein, wherein serotypes 23A and/or 23B of *S. pneumoniae* provides cross-protection against serotype 23F of *S. pneumoniae*.

In another embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates as selected from Embodiments E1 to E20 wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotypes 23A and/or 23B, conjugated to a carrier protein, wherein serotypes 23A and/or 23B of *S. pneumoniae* provides cross-protection against serotype 23F of *S. pneumoniae*.

In an embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 6A, conjugated to a carrier protein, wherein serotype 6A of *S. pneumoniae* provides cross-protection against serotypes 6B and/or 6C of *S. pneumoniae*.

In another embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates as selected from Embodiments E1 to E20 wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 6A, conjugated to a carrier protein, wherein serotype 6A of *S. pneumoniae* provides cross-protection against serotypes 6B and/or 6C of *S. pneumoniae*.

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In an embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 20A, conjugated to a carrier protein, wherein serotype 20A of *S. pneumoniae* provides cross-protection against serotype 20B of *S. pneumoniae*.

In another embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates as selected from Embodiments E1 to E20 wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 20A, conjugated to a carrier protein, wherein serotype 20A of *S. pneumoniae* provides cross-protection against serotype 20B of *S. pneumoniae*.

In an embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 20B, conjugated to a carrier protein, wherein serotype 20B of *S. pneumoniae* provides cross-protection against serotype 20A of *S. pneumoniae*.

In another embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates as selected from Embodiments E1 to E20 wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 20B, conjugated to a carrier protein, wherein serotype 20B of *S. pneumoniae* provides cross-protection against serotype 20A of *S. pneumoniae*.

In an embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 15C, conjugated to a carrier protein, wherein serotype 15C of *S. pneumoniae* provides cross-protection against serotype 15B of *S. pneumoniae*.

In another embodiment the invention provides multivalent immunogenic compositions comprising *S. pneumoniae* polysaccharide protein conjugates as selected from Embodiments E1 to E20 wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype, including serotype 15C, conjugated to a carrier protein, wherein serotype 15C of *S. pneumoniae* provides cross-protection against serotype 15B of *S. pneumoniae*.

Carrier Protein

In particular embodiments of the present invention, CRM197 is used as the carrier protein. CRM197 is a non-toxic variant (i.e., toxoid) of diphtheria toxin having the following sequence of amino acids: .

GADDVVDSSK SFVMENFSSY HGTKPGYVDS IQKGIQKPKS GTQGNYDDDW
KEFYSTDNKY DAAGYSVDNE NPLSGKAGGV VKVTYPGLTK VLALKVDNAE

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TIKKELGLSL TEPLMEQVGT EEFIKRFGDG ASRVVLSLPF AEGSSSVEYI
NNWEQAKALS VELEINFETR GKRGQDAMYE YMAQACAGNR VRRSVGSSLS
CINLDWDVIR DKTKTKIESL KEHGPIKNKM SESPNKTVSE EKAKQYLEEF
HQTALEHPEL SELKTVTGTN PVFAGANYAA WAVNVAQVID SETADNLEKT
TAALSILPGI GSVMGIADGA VHHNTEEIVA QSIALSSLMV AQAIPLVGEL
VDIGFAAYNF VESIINLFQV VHNSYNRPAY SPGHKTQPFL HDGYAVSWNT
VEDSIIRTGF QGESGHDIKI TAENTPLPIA GVLLPTIPGK LDVNKSKTHI
SVNGRKIRMR CRAIDGDVTF CRPKSPVYVG NGVHANLHVA FHRSSSEKIH
SNEISSDSIG VLGYQKTVDH TKVNSKLSLF FEIKS (SEQ ID NO:1)

In one embodiment, CRM197 is isolated from cultures of *Corynebacterium diphtheria* strain C7 (β197) grown in casamino acids and yeast extract-based medium. In another embodiment, CRM197 is prepared recombinantly in accordance with the methods described in U.S. 5,614,382. Typically, CRM197 is purified through a combination of ultra-filtration, ammonium sulfate precipitation, and ion-exchange chromatography. In some embodiments, CRM197 is prepared in *Pseudomonas fluorescens* using Pfenex Expression Technology™ (Pfenex Inc., San Diego, CA).

Other suitable carrier proteins include additional inactivated bacterial toxins such as DT (Diphtheria toxoid) or fragment B of DT (DTFB), TT (tetanus toxid) or fragment C of TT, pertussis toxoid, cholera toxoid (e.g., as described in WO 2004/083251), *E. coli* LT, *E. coli* ST, and exotoxin A from *Pseudomonas aeruginosa*. Bacterial outer membrane proteins such as outer membrane complex c (OMPC), porins, transferrin binding proteins, pneumococcal surface protein A (PspA; *See* WO 02/091998), pneumococcal adhesin protein (PsaA), C5a peptidase from Group A or Group B streptococcus, or *Haemophilus influenzae* protein D, pneumococcal pneumolysin (Kuo *et al.*, 1995, *Infect Immun* 63; 2706-13) including ply detoxified in some fashion for example dPLY-GMBS (*See* WO 04/081515) or dPLY-formol, PhtX, including PhtA, PhtB, PhtD, PhtE and fusions of Pht proteins for example PhtDE fusions, PhtBE fusions (*See* WO 01/98334 and WO 03/54007), can also be used. Other proteins, such as ovalbumin, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or purified protein derivative of tuberculin (PPD), PorB (from *N. meningitidis*), PD (*Haemophilus influenzae* protein D; *see, e.g.*, EP 0 594 610 B), or immunologically functional equivalents thereof, synthetic peptides (*See* EP0378881 and EP0427347), heat shock proteins (*See* WO 93/17712 and WO 94/03208), pertussis proteins (*See* WO 98/58668 and EP0471177), cytokines, lymphokines, growth factors or hormones (*See* WO 91/01146), artificial proteins comprising multiple human CD4+ T cell epitopes from various pathogen derived antigens (*See* Falugi *et al.*, 2001, Eur J Immunol 31:3816-3824) such as N19 protein (See Baraldoi *et al.*, 2004, *Infect Immun* 72:4884-7), iron uptake proteins (*See* WO 01/72337), toxin A or B of *C. difficile* (*See* WO 00/61761), and flagellin (*See* Ben-Yedidia *et al.*, 1998, *Immunol Lett* 64:9) can also be used as carrier proteins.

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Other DT mutants can be used as the carrier protein, such as CRM_{176}, CRM_{228}, CRM_{45} (Uchida *et al.*, 1973, *J Biol Chem* 218:3838-3844); CRM_9, CRM_{45}, CRM_{102}, CRM_{103} and CRM_{107} and other mutations described by Nicholls and Youle in Genetically Engineered Toxins, Ed: Frankel, Maecel Dekker Inc, 1992; deletion or mutation of Glu-148 to Asp, Gln or Ser and/or Ala 158 to Gly and other mutations disclosed in U.S. 4,709,017 or U.S. 4,950,740; mutation of at least one or more residues Lys 516, Lys 526, Phe 530 and/or Lys 534 and other mutations disclosed in U.S. 5,917,017 or U.S. Pat. No. 6,455,673; or fragment disclosed in U.S. 5,843,711. Such DT mutants can also be used to make DTFB variants where the variants comprise the B fragment contain the epitiope regions.

In certain embodiments, the carrier protein is selected from the group consisting of: Outer Membrane Protein Complex (OMPC), tetanus toxoid, diphtheria toxoid, protein D and CRM197.

In some embodiments of the invention, a second carrier can be used for one or more of the polysaccharide protein conjugates in the multivalent immunogenic composition. The second carrier protein is preferably a protein that is non-toxic and non-reactogenic and obtainable in sufficient amount and purity. The second carrier protein is also conjugated or joined with the *S. pneumoniae* polysaccharide to enhance immunogenicity of the antigen. Carrier proteins should be amenable to standard conjugation procedures. In one embodiment, each capsular polysaccharide not conjugated to the first carrier protein is conjugated to the same second carrier protein (e.g., each capsular polysaccharide molecule being conjugated to a single carrier protein). In another embodiment, the capsular polysaccharides not conjugated to the first carrier protein are conjugated to two or more carrier proteins (each capsular polysaccharide molecule being conjugated to a single carrier protein). In such embodiments, each capsular polysaccharide of the same serotype is typically conjugated to the same carrier protein.

In embodiments of the invention, including any of Embodiments E1-E20 and any sub-embodiments thereof, one or more (including 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, where applicable) of the polysaccharide serotypes is conjugated to CRM197. In further embodiments of the invention, including any of Embodiments E1-E20 and any sub-embodiments thereof, each of the polysaccharide serotypes is conjugated to CRM197.

Formulation of the polysaccharide-protein conjugates of the present invention can be accomplished using art-recognized methods. For instance, individual pneumococcal conjugates can be formulated with a physiologically acceptable vehicle to prepare the composition. Examples of such vehicles include, but are not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions.

In a preferred embodiment, the vaccine composition is formulated in L-histidine buffer with sodium chloride.

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In some embodiments of the invention, the multivalent immunogenic composition comprises multiple *S. pneumoniae* polysaccharide protein conjugates comprising capsular polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein and an adjuvant, wherein the *S. pneumoniae* serotypes are as described herein. Suitable adjuvants to enhance effectiveness of the composition include, but are not limited to:

(1) aluminum salts (alum), such as aluminum hydroxide, aluminum phosphate, aluminum sulfate, etc.;

(2) oil-in-water emulsion formulations (with or without other specific immunostimulating agents such as muramyl peptides (defined below) or bacterial cell wall components), such as, for example, (a) MF59 (International Patent Application Publication No. WO 90/14837), containing 5% Squalene, 0.5% Tween 80, and 0.5% Span 85 (optionally containing various amounts of MTP-PE) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA), (b) SAF, containing 10% Squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, (c) Ribi™ adjuvant system (RAS), (Corixa, Hamilton, MT) containing 2% Squalene, 0.2% Tween 80, and one or more bacterial cell wall components from the group consisting of 3-O-deacylated monophosphorylipid A (MPL™) described in U.S. Pat. No. 4,912,094, trehalose dimycolate (TDM), and cell wall skeleton (CWS), preferably MPL+CWS (Detox™); and (d) a Montanide ISA;

(3) saponin adjuvants, such as Quil A or STIMULON™ QS-21 (Antigenics, Framingham, MA) (see, e.g., U.S. Pat. No. 5,057,540) may be used or particles generated therefrom such as ISCOM (immunostimulating complexes formed by the combination of cholesterol, saponin, phospholipid, and amphipathic proteins) and Iscomatrix® (having essentially the same structure as an ISCOM but without the protein);

(4) bacterial lipopolysaccharides, synthetic lipid A analogs such as aminoalkyl glucosamine phosphate compounds (AGP), or derivatives or analogs thereof, which are available from Corixa, and which are described in U.S. Pat. No. 6,113,918; one such AGP is 2-[(R)-3-tetradecanoyloxytetradecanoylamino]ethyl 2-Deoxy-4-O-phosphono-3-O-[(R)-3-tetradecanoyloxytetradecanoyl]-2-[(R)-3-- tetradecanoyloxytetradecanoylamino]-b-D-glucopyranoside, which is also known as 529 (formerly known as RC529), which is formulated as an aqueous form or as a stable emulsion

(5) synthetic polynucleotides such as oligonucleotides containing CpG motif(s) (U.S. Pat. No. 6,207,646); and

(6) cytokines, such as interleukins (e.g., IL-1, IL-2, IL-4, IL-5, IL-6, IL-7, IL-12, IL-15, IL-18, etc.), interferons (e.g., gamma interferon), granulocyte macrophage colony

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stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), tumor necrosis factor (TNF), costimulatory molecules B7-1 and B7-2, etc; and

(7) complement, such as a trimer of complement component C3d.

In another embodiment, the adjuvant is a mixture of 2, 3, or more of the above adjuvants, e.g.,. SBAS2 (an oil-in-water emulsion also containing 3-deacylated monophosphoryl lipid A and QS21).

Muramyl peptides include, but are not limited to, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanine-2-(1'-2' dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE), etc.

In certain embodiments, the adjuvant is an aluminum salt. The aluminum salt adjuvant may be an alum-precipitated vaccine or an alum-adsorbed vaccine. Aluminum-salt adjuvants are well known in the art and are described, for example, in Harlow, E. and D. Lane (1988; Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory) and Nicklas, W. (1992; Aluminum salts. Research in Immunology 143:489-493). The aluminum salt includes, but is not limited to, hydrated alumina, alumina hydrate, alumina trihydrate (ATH), aluminum hydrate, aluminum trihydrate, alhydrogel, Superfos, Amphogel, aluminum (III) hydroxide, aluminum hydroxyphosphate sulfate, Aluminum Phosphate Adjuvant (APA), amorphous alumina, trihydrated alumina, or trihydroxyaluminum.

APA is an aqueous suspension of aluminum hydroxyphosphate. APA is manufactured by blending aluminum chloride and sodium phosphate in a 1:1 volumetric ratio to precipitate aluminum hydroxyphosphate. After the blending process, the material is size-reduced with a high-shear mixer to achieve a monodisperse particle size distribution. The product is then diafiltered against physiological saline and steam sterilized.

In certain embodiments, a commercially available $Al(OH)_3$ (e.g. Alhydrogel or Superfos of Denmark/Accurate Chemical and Scientific Co., Westbury, NY) is used to adsorb proteins in a ratio of 50 - 200 μg protein/mg aluminum hydroxide. Adsorption of protein is dependent, in another embodiment, on the pI (Isoelectric pH) of the protein and the pH of the medium. A protein with a lower pI adsorbs to the positively charged aluminum ion more strongly than a protein with a higher pI. Aluminum salts may establish a depot of antigen that is released slowly over a period of 2-3 weeks, be involved in nonspecific activation of macrophages and complement activation, and/or stimulate innate immune mechanism (possibly through stimulation of uric acid). See, e.g., Lambrecht *et al.*, 2009, Curr Opin Immunol 21:23.

Monovalent bulk aqueous conjugates are typically blended together and diluted to target 4 μg/mL for all serotypes except 6B, which will be diluted to target 8 μg/mL. Once diluted, the batch will be filter sterilized, and an equal volume of aluminum phosphate adjuvant added aseptically to target a final aluminum concentration of 250 μg/mL. The adjuvanted, formulated batch will be filled into single-use, 0.5 mL/dose vials.

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In certain embodiments, the adjuvant is a CpG-containing nucleotide sequence, for example, a CpG-containing oligonucleotide, in particular, a CpG-containing oligodeoxynucleotide (CpG ODN). In another embodiment, the adjuvant is ODN 1826, which may be acquired from Coley Pharmaceutical Group.

Methods for use of CpG oligonucleotides are well known in the art and are described, for example, in Sur *et al.*, 1999, J Immunol. 162:6284-93; Verthelyi, 2006, Methods Mol Med. 127:139-58; and Yasuda *et al.*, 2006, Crit Rev Ther Drug Carrier Syst. 23:89-110.

In alternative embodiments, the immunogenic composition comprises multiple *S. pneumoniae* polysaccharide protein conjugates as described herein, for example in any of Embodiments E1-E20 or any sub-embodiment thereof, and does not comprise an adjuvant.

Formulations

The composition of the invention can be formulated as single dose vials, multi-dose vials or as pre-filled glass or plastic syringes.

In another embodiment, compositions of the present invention are administered orally, and are thus formulated in a form suitable for oral administration, i.e., as a solid or a liquid preparation. Solid oral formulations include tablets, capsules, pills, granules, pellets and the like. Liquid oral formulations include solutions, suspensions, dispersions, emulsions, oils and the like.

Pharmaceutically acceptable carriers for liquid formulations are aqueous or non-aqueous solutions, suspensions, emulsions or oils. Examples of nonaqueous solvents are propylene glycol, polyethylene glycol, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Examples of oils are those of animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, olive oil, sunflower oil, fish-liver oil, another marine oil, or a lipid from milk or eggs.

The pharmaceutical composition may be isotonic, hypotonic or hypertonic. However it is often preferred that a pharmaceutical composition for infusion or injection is essentially isotonic, when it is administrated. Hence, for storage the pharmaceutical composition may preferably be isotonic or hypertonic. If the pharmaceutical composition is hypertonic for storage, it may be diluted to become an isotonic solution prior to administration.

The isotonic agent may be an ionic isotonic agent such as a salt or a non-ionic isotonic agent such as a carbohydrate. Examples of ionic isotonic agents include but are not limited to NaCl, $CaCl_2$, KCl and $MgCl_2$. Examples of non-ionic isotonic agents include but are not limited to mannitol, sorbitol and glycerol.

It is also preferred that at least one pharmaceutically acceptable additive is a buffer. For some purposes, for example, when the pharmaceutical composition is meant for

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infusion or injection, it is often desirable that the composition comprises a buffer, which is capable of buffering a solution to a pH in the range of 4 to 10, such as 5 to 9, for example 6 to 8.

The buffer may, for example, be selected from the group consisting of TRIS, acetate, glutamate, lactate, maleate, tartrate, phosphate, citrate, carbonate, glycinate, histidine, glycine, succinate and triethanolamine buffer.

The buffer may be selected from USP compatible buffers for parenteral use, in particular, when the pharmaceutical formulation is for parenteral use. For example the buffer may be selected from the group consisting of monobasic acids such as acetic, benzoic, gluconic, glyceric and lactic; dibasic acids such as aconitic, adipic, ascorbic, carbonic, glutamic, malic, succinic and tartaric, polybasic acids such as citric and phosphoric; and bases such as ammonia, diethanolamine, glycine, triethanolamine, and TRIS.

Parenteral vehicles (for subcutaneous, intravenous, intraarterial, or intramuscular injection) include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Examples are sterile liquids such as water and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. In general, water, saline, aqueous dextrose and related sugar solutions, glycols such as propylene glycols or polyethylene glycol, Polysorbate 80 (PS-80), Polysorbate 20 (PS-20), and Poloxamer 188 (P188) are preferred liquid carriers, particularly for injectable solutions. Examples of oils are those of animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, olive oil, sunflower oil, fish-liver oil, another marine oil, or a lipid from milk or eggs.

The formulations of the invention may also contain a surfactant. Preferred surfactants include, but are not limited to: the polyoxyethylene sorbitan esters surfactants (commonly referred to as the Tweens), especially PS-20 and PS-80; copolymers of ethylene oxide (EO), propylene oxide (PO), and/or butylene oxide (BO), sold under the DOWFAX™ tradename, such as linear EO/PO block copolymers; octoxynols, which can vary in the number of repeating ethoxy (oxy-1,2-ethanediyl) groups, with octoxynol-9 (Triton X-100, or t-octylphenoxypolyethoxyethanol) being of particular interest; (octylphenoxy)polyethoxyethanol (IGEPAL CA-630/NP-40); phospholipids such as phosphatidylcholine (lecithin); nonylphenol ethoxylates, such as the Tergitol™ NP series; polyoxyethylene fatty ethers derived from lauryl, cetyl, stearyl and oleyl alcohols (known as Brij surfactants), such as triethyleneglycol monolauryl ether (Brij 30); and sorbitan esters (commonly known as the SPANs), such as sorbitan trioleate (Span 85) and sorbitan monolaurate. A preferred surfactant for including in the emulsion is PS-80.

Mixtures of surfactants can be used, e.g. PS-80/Span 85 mixtures. A combination of a polyoxyethylene sorbitan ester such as polyoxyethylene sorbitan monooleate (PS-80) and an

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octoxynol such as t-octylphenoxypolyethoxyethanol (Triton X-100) is also suitable. Another useful combination comprises laureth 9 plus a polyoxyethylene sorbitan ester and/or an octoxynol.

Preferred amounts of surfactants (% by weight) are: polyoxyethylene sorbitan esters (such as PS-80) 0.01 to 1%, in particular about 0.1 %; octyl- or nonylphenoxy polyoxyethanols (such as Triton X-100, or other detergents in the Triton series) 0.001 to 0.1 %, in particular 0.005 to 0.02%; polyoxyethylene ethers (such as laureth 9) 0.1 to 20 %, preferably 0.1 to 10 % and in particular 0.1 to 1 % or about 0.5%.

In certain embodiments, the composition consists essentially of histidine (20mM), saline (150mM) and 0.2% PS-20 or 0.04% PS-80 at a pH of 5.8 with 250 μg/mL of APA (Aluminum Phosphate Adjuvant). PS-20 can range from 0.005% to 0.3% (w/v) with the presence of PS-20 or PS-80 in formulation controlling aggregation during simulated manufacture and in shipping using primary packaging. In another embodiment, PS-20 can range from 0.025% to 0.8% (w/v). In another embodiment, PS-20 can range from 0.05% to 0.8% (w/v). In another embodiment, PS-20 can range from 0.05% to 0.2% (w/v). Process consists of combining a blend of up to 24 serotypes in histidine, saline, and PS-20 or PS-80, then combining this blended material with APA and saline with or without antimicrobial preservatives.

In particular embodiments, the multivalent immunogenic composition comprises *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* in the polysaccharide protein conjugates comprise any of the sets of serotypes set forth herein, and further comprises 20- 80 mM histidine pH 5.8 and 150 mM NaCl. In some embodiments, the multivalent immunogenic composition further comprises from 0.2% to 0.8% w/v polysorbate 20.

The choice of surfactant may need to be optimized for different drug products and drug substances. For multivalent vaccines having 15 or more serotypes, PS-20 and P188 are preferred. The choice of chemistry used to make conjugate can also play an important role in the stabilization of the formulation. In particular, when the conjugation reactions used to prepare different polysaccharide protein conjugates in a multivalent composition include both aqueous solvent and DMSO solvent, particular surfactant systems provide significant differences in stability. Improved stability of polysachharide protein conjugates was seen with polysorbate 20 alone or with poloxamer 188 in combination with a polyol.

The exact mechanism of how a specific detergent protects a biotherapeutic is poorly understood and cannot be predicted *a priori*. Possible stabilization mechanisms include preferential hydration, preferential exclusion, air/liquid interface competition between biotherapeutic and surface, surface tension, and/or direct association of the detergent with the biotherpeutic to mask hydrophobic patches which serve as seeds for aggregation.

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Poloxamer may also be used in the compositions of the invention. A poloxamer is a nonionic triblock copolymer composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)). Poloxamers are also known by the tradename Pluronic®. Because the lengths of the polymer blocks can be customized, many different poloxamers exist that have slightly different properties. For the generic term "poloxamer", these copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits, the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content (e.g., P407 = Poloxamer with a polyoxypropylene molecular mass of 4,000 g/mol and a 70% polyoxyethylene content). For the Pluronic® tradename, coding of these copolymers starts with a letter to define its physical form at room temperature (L = liquid, P = paste, F = flake (solid)) followed by two or three digits. The first digit (two digits in a three-digit number) in the numerical designation, multiplied by 300, indicates the approximate molecular weight of the hydrophobe; and the last digit x 10 gives the percentage polyoxyethylene content (e.g., L61 = Pluronic® with a polyoxypropylene molecular mass of 1,800 g/mol and a 10% polyoxyethylene content). See U.S. Pat. No. 3740421.

Examples of poloxamers have the general formula:
$HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_aH$, wherein a and b blocks have the following values:

Pluronic®	Poloxamer	A	B	Molecular Weight
L31		2	16	1100 (average)
L35				1900 (average)
L44NF	124	12	20	2090 to 2360
L64				2900 (average)
L81				2800 (average)
L121				4400 (average)
P123		20	70	5750 (average)
F68NF	188	80	27	7680 to 9510
F87NF	237	64	37	6840 to 8830
F108NF	338	141	44	12700 to 17400
F127NF	407	101	56	9840 to 14600

Molecular weight units, as used herein, are in Dalton (Da) or g/mol.

Preferably, the poloxamer generally has a molecular weight in the range from 1100 to 17,400 Da, from 7,500 to 15,000 Da, or from 7,500 to 10,000 Da. The poloxamer can be selected from poloxamer 188 or poloxamer 407. The final concentration of the poloxamer in the formulations is from 0.001% to 5% weight/volume, or 0.025% to 1% weight/volume. In certain aspects, the polyol is propylene glycol and is at final concentration from 1% to 20%

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weight/volume. In certain aspects, the polyol is polyethylene glycol 400 and is at final concentration from 1% to 20% weight/volume.

Suitable polyols for the formulations of the invention are polymeric polyols, particularly polyether diols including, but are not limited to, propylene glycol and polyethylene glycol, Polyethylene glycol monomethyl ethers. Propylene glycol is available in a range of molecular weights of the monomer from ~425 to ~2700. Polyethylene glycol and Polyethylene glycol monomethyl ether is also available in a range of molecular weights ranging from ~200 to ~35000 including but not limited to PEG200, PEG300, PEG400, PEG1000, PEG MME 550, PEG MME 600, PEG MME 2000, PEG MME 3350 and PEG MME 4000. A preferred polyethylene glycol is polyethylene glycol 400. The final concentration of the polyol in the formulations of the invention may be 1% to 20% weight/volume or 6% to 20% weight/volume.

The formulation also contains a pH-buffered saline solution. The buffer may, for example, be selected from the group consisting of TRIS, acetate, glutamate, lactate, maleate, tartrate, phosphate, citrate, carbonate, glycinate, histidine, glycine, succinate, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid), MES (2-(*N*-morpholino)ethanesulfonic acid) and triethanolamine buffer. The buffer is capable of buffering a solution to a pH in the range of 4 to 10, 5.2 to 7.5, or 5.8 to 7.0. In certain aspect of the invention, the buffer is selected from the group consisting of phosphate, succinate, histidine, MES, MOPS, HEPES, acetate or citrate. The buffer may furthermore, for example, be selected from USP compatible buffers for parenteral use, in particular, when the pharmaceutical formulation is for parenteral use. The concentrations of buffer will range from 1 mM to 100 mM. The concentrations of buffer will range from 10 mM to 80 mM. The concentrations of buffer will range from 1 mM to 50 mM or 5 mM to 50 mM. In certain aspects, the buffer is histidine at a final concentration of 5 mM to 50 mM, or succinate at a final concentration of 1 mM to 10 mM. In certain aspects, the histidine is at a final concentration of 20 mM ± 2 mM.

While the saline solution (i.e., a solution containing NaCl) is preferred, other salts suitable for formulation include but are not limited to, $CaCl_2$, KCl and $MgCl_2$ and combinations thereof. Non-ionic isotonic agents including but not limited to sucrose, trehalose, mannitol, sorbitol and glycerol may be used in lieu of a salt. Suitable salt ranges include, but are not limited to 25 mM to 500 mM or 40 mM to 170 mM. In one aspect, the saline is NaCl, optionally present at a concentration from 20 mM to 170 mM.

In a preferred embodiment, the formulations comprise a L-histidine buffer with sodium chloride.

In another embodiment, the pharmaceutical composition is delivered in a controlled release system. For example, the agent can be administered using intravenous infusion, a transdermal patch, liposomes, or other modes of administration. In another embodiment, polymeric materials are used; e.g. in microspheres in or an implant.

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The amount of conjugate in each vaccine dose is selected as an amount that induces an immunoprotective response without significant, adverse effects. Such amount can vary depending upon the pneumococcal serotype. Generally, for polysaccharide-based conjugates, each dose will comprise 0.08 to 100 μg of each polysaccharide. In some embodiments of the invention, the dose of each polysaccharide conjugate is from 0.08 to 10 μg. In further embodiments, the dose is from 1 to 5 μg, from 0.4 to 4 μg, from 0.4 to 3 μg, from 0.4 to 2 μg, or from 0.4 to 1 μg. In some embodiments, the dose of one or more polysaccharide conjugates is 100, 150, 200, 250, 300, 400, 500, or 750 ng or 0.4, 0.5, 0.6, 0.7, 0.75, 0.8, 0.9, 1, 1.5, 2, 3, 4, 5, 6, 7, 7.5, 8, 9, 10, 11, 12, 13, 14, 15, 16, 18, 20, 22, 25, 30, 40, 50, 60, 70, 80, 90, or 100 μg.

In some embodiments of the compositions of the invention, all of the polysaccharide conjugates are present in the composition in the same amount. In further embodiments, the polysaccharide conjugates are present in the composition in different amounts (i.e., at least one polysaccharide conjugate is present in an amount that is different than one or more of the other polysaccharide conjugates of the composition).

Optimal amounts of components for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. For example, in another embodiment, the dosage for human vaccination is determined by extrapolation from animal studies to human data. In another embodiment, the dosage is determined empirically.

In one embodiment, the dose of the aluminum salt is 10, 15, 20, 25, 30, 50, 70, 100, 125, 150, 200, 300, 500, or 700 μg, or 1, 1.2, 1.5, 2, 3, 5 mg or more. In yet another embodiment, the dose of alum salt described above is per μg of recombinant protein.

The compositions of this invention may also include one or more proteins from *S. pneumoniae*. Examples of *S. pneumoniae* proteins suitable for inclusion include those identified in International Patent Application Publication Nos. WO 02/083855 and WO 02/053761.

In certain embodiments, the compositions of the invention are administered to a subject by one or more methods known to a person skilled in the art, such as parenterally, transmucosally, transdermally, intramuscularly, intravenously, intra-dermally, intra-nasally, subcutaneously, intra-peritonealy, and formulated accordingly. In one embodiment, compositions of the present invention are administered via epidermal injection, intramuscular injection, intravenous, intra-arterial, subcutaneous injection, or intra-respiratory mucosal injection of a liquid preparation. Liquid formulations for injection include solutions and the like.

III. Methods of Making

Capsular polysaccharides from *Streptococcus pneumoniae* can be prepared by standard techniques known to those skilled in the art. For example, polysaccharides can be

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isolated from bacteria and may be sized to some degree by known methods (see, e.g., European Patent Nos. EP497524 and EP497525); and preferably by microfluidisation accomplished using a homogenizer or by chemical hydrolysis. In one embodiment, each pneumococcal polysaccharide serotype is grown in a soy-based medium. The individual polysaccharides are then purified through standard steps including centrifugation, precipitation, and ultra-filtration. See, e.g., U.S. Patent Application Publication No. 2008/0286838 and U.S. Pat. No. 5,847,112. Polysaccharides can be sized in order to reduce viscosity in polysaccharide samples and/or to improve filterability for conjugated products using techniques such as mechanical or chemical sizing. Chemical hydrolysis may be conducted using acetic acid. Mechanical sizing may be conducted using High Pressure Homogenization Shearing.

The purified polysaccharides can be chemically activated to make the saccharides capable of reacting with the carrier protein. The purified polysaccharides can be connected to a linker. Once activated or connected to a linker, each capsular polysaccharide is separately conjugated to a carrier protein to form a glycoconjugate. The polysaccharide conjugates may be prepared by known coupling techniques.

The polysaccharide can be coupled to a linker to form a polysaccharide-linker intermediate in which the free terminus of the linker is an ester group. The linker is therefore one in which at least one terminus is an ester group. The other terminus is selected so that it can react with the polysaccharide to form the polysaccharide-linker intermediate.

The polysaccharide can be coupled to a linker using a primary amine group in the polysaccharide. In this case, the linker typically has an ester group at both termini. This allows the coupling to take place by reacting one of the ester groups with the primary amine group in the polysaccharide by nucleophilic acyl substitution. The reaction results in a polysaccharide-linker intermediate in which the polysaccharide is coupled to the linker via an amide linkage. The linker is therefore a bifunctional linker that provides a first ester group for reacting with the primary amine group in the polysaccharide and a second ester group for reacting with the primary amine group in the carrier molecule. A typical linker is adipic acid N-hydroxysuccinimide diester (SIDEA).

The coupling can also take place indirectly, i.e. with an additional linker that is used to derivatise the polysaccharide prior to coupling to the linker.

The polysaccharide is coupled to the additional linker using a carbonyl group at the reducing terminus of the polysaccharide. This coupling comprises two steps: (a1) reacting the carbonyl group with the additional linker; and (a2) reacting the free terminus of the additional linker with the linker. In these embodiments, the additional linker typically has a primary amine group at both termini, thereby allowing step (a1) to take place by reacting one of the primary amine groups with the carbonyl group in the polysaccharide by reductive amination. A primary amine group is used that is reactive with the carbonyl group in the polysaccharide.

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Hydrazide or hydroxylamino groups are suitable. The same primary amine group is typically present at both termini of the additional linker. The reaction results in a polysaccharide-additional linker intermediate in which the polysaccharide is coupled to the additional linker via a C—N linkage.

The polysaccharide can be coupled to the additional linker using a different group in the polysaccharide, particularly a carboxyl group. This coupling comprises two steps: (a1) reacting the group with the additional linker; and (a2) reacting the free terminus of the additional linker with the linker. In this case, the additional linker typically has a primary amine group at both termini, thereby allowing step (a1) to take place by reacting one of the primary amine groups with the carboxyl group in the polysaccharide by EDAC activation. A primary amine group is used that is reactive with the EDAC-activated carboxyl group in the polysaccharide. A hydrazide group is suitable. The same primary amine group is typically present at both termini of the additional linker. The reaction results in a polysaccharide-additional linker intermediate in which the polysaccharide is coupled to the additional linker via an amide linkage.

In one embodiment, the chemical activation of the polysaccharides and subsequent conjugation to the carrier protein by reductive amination can be achieved by means described in U.S. Pat. Nos. 4,365,170, 4,673,574 and 4,902,506, U.S. Patent Application Publication Nos. 2006/0228380, 2007/184072, 2007/0231340 and 2007/0184071, and WO2006/110381, WO2008/079653, and WO2008/143709). The chemistry may include the activation of pneumococcal polysaccharide by reaction with any oxidizing agent which oxidizes a terminal hydroxyl group to an aldehyde, such as periodate (including sodium periodate, potassium periodate, or periodic acid). The reaction leads to a random oxidative cleavage of vicinal hydroxyl groups of the carbohydrates with the formation of reactive aldehyde groups.

Coupling to the carrier protein is by reductive amination via direct amination to the lysyl groups of the protein. For example, conjugation can be carried out by reacting a mixture of the activated polysaccharide and carrier protein with a reducing agent such as sodium cyanoborohydride in the presence of nickel. The conjugation reaction may take place under aqueous solution or in the presence of DMSO. *See, e.g.*, US2015/0231270, US2011/0195086 and EP 0471 177 B1. Unreacted aldehydes are then capped with the addition of a strong reducing agent, such as sodium borohydride.

Reductive amination involves two steps, (1) oxidation of the polysaccharide to form reactive aldehydes, (2) reduction of the imine (Schiff base) formed between activated polysaccharide and a carrier protein to form a stable amine conjugate bond. Before oxidation, the polysaccharide is optionally size reduced. Mechanical methods (e.g. homogenization) or chemical hydrolysis may be employed. Chemical hydrolysis may be conducted using acetic acid. The oxidation step may involve reaction with periodate. For the purpose of the present invention, the term "periodate" includes both periodate and periodic acid; the term also includes

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both metaperiodate (IO_4^-) and orthoperiodate (IO_6^-) and includes the various salts of periodate (e.g. , sodium periodate and potassium periodate). In an embodiment the capsular polysaccharide is oxidized in the presence of metaperiodate, preferably in the presence of sodium periodate ($NaIO_4$). In another embodiment the capsular polysaccharide is oxydized in the presence of orthoperiodate, preferably in the presence of periodic acid.

In one embodiment, the oxidizing agent is a stable nitroxyl or nitroxide radical compound, such as piperidine-N-oxy or pyrrolidine-N-oxy compounds, in the presence of an oxidant to selectively oxidize primary hydroxyls (as described in WO 2014/097099). In said reaction, the actual oxidant is the N-oxoammonium salt, in a catalytic cycle. In an aspect, said stable nitroxyl or nitroxide radical compound are piperidine-N-oxy or pyrrolidine-N-oxy compounds. In an aspect, said stable nitroxyl or nitroxide radical compound bears a TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy) or a PROXYL (2,2,5,5-tetramethyl-1-pyrrolidinyloxy) moiety. In an aspect, said stable nitroxyl radical compound is TEMPO or a derivative thereof. In an aspect, said oxidant is a molecule bearing a N-halo moiety. In an aspect, said oxidant is selected from the group consisting of N-Chlorosuccinimide, N-Bromosuccinimide, N-Iodosuccinimide, Dichloroisocyanuric acid, 1 ,3,5-trichloro-1 ,3,5-triazinane-2,4,6-trione, Dibromoisocyanuric acid, 1 ,3,5-tribromo-1 ,3,5-triazinane-2,4,6-trione, Diiodoisocyanuric acid and 1 ,3,5-triiodo-1,3,5-triazinane-2,4,6-trione. Preferably said oxidant is N- Chlorosuccinimide.

In certain aspects, the oxidizing agent is 2,2,6,6-Tetramethyl-1-piperidinyloxy (TEMPO) free radical and N- Chlorosuccinimide (NCS) as the cooxidant (as described in WO 2014/097099). Therefore in one aspect, the glycoconjugates from *S. pneumoniae* are obtainable by a method comprising the steps of: a) reacting a saccharide with 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) and N-chlorosuccinimide (NCS) in an aqueous solvent to produce an activated saccharide; and b) reacting the activated saccharide with a carrier protein comprising one or more amine groups (said method is designated "TEMPO/NCS-reductive amination" thereafter).

Optionally the oxidation reaction is quenched by addition of a quenching agent. The quenching agent maybe selected from vicinal diols, 1-,2-aminoalcohols, amino acids, glutathione, sulfite, bisulfate, dithionite, metabisulfite, thiosulfate, phosphites, hypophosphites or phosphorous acid (such as glycerol, ethylene glycol, propan-1-,2-diol, butan-1 ,2-diol or butan-2,3-diol, ascorbic acid).

In certain embodiments, the instant invention provides a method for preparing a serotype 8 *Streptococcus pneumoniae* polysaccharide-protein conjugate utilizing a conjugation reaction in an aprotic solvent, wherein the conjugation reaction does not use cyanoborohydride. In further embodiments, the conjugation reaction is a Schiff base reduction or reductive amination. In further embodiments, the protein is tetanus toxoid, diphtheria toxoid, or CRM197. In still further embodiments the protein is CRM197. In further embodiments, the conjugation

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reaction is reductive amination. In further embodiments, the reductive amination is performed in dimethylsulfoxide (DMSO).

In some embodiments, the oxidized polysaccharides before conjugation have a molecular weight of between 30 kDa and 1,000 kDa. Molecular weight can be calculated by size exclusion chromatography (SEC) combined with multiangle light scattering detector (MALS) and refractive index detector (RI). In some embodiments, the polysaccharide has a molecular weight of between 50 kDa and 300 kDa. In some embodiments, the polysaccharide has a molecular weight of between 50 kDa and 1,000 kDa. In additional embodiments, the polysaccharide has a molecular weight of between 70 kDa and 900 kDa. In other embodiments, the polysaccharide has a molecular weight of between 100 kDa and 800 kDa. In other embodiments, the polysaccharide has a molecular weight of between 200 kDa and 600 kDa. In further embodiments, the polysaccharide has a molecular weight of 100 kDa to 1,000 kDa; 100 kDa to 900 kDa; 100 kDa to 800 kDa; 100 kDa to 700 kDa; 100 kDa to 600 kDa; 100 kDa to 500 kDa; 100 kDa to 400 kDa; 100 kDa to 300 kDa; 150 kDa to 1,000 kDa; 150 kDa to 900 kDa; 150 kDa to 800 kDa; 150 kDa to 700 kDa; 150 kDa to 600 kDa; 150 kDa to 500 kDa; 150 kDa to 400 kDa; 150 kDa to 300 kDa; 200 kDa to 1,000 kDa; 200 kDa to 900 kDa; 200 kDa to 800 kDa; 200 kDa to 700 kDa; 200 kDa to 600 kDa; 200 kDa to 500 kDa; 200 kDa to 400 kDa; 200 kDa to 300; 250 kDa to 1,000 kDa; 250 kDa to 900 kDa; 250 kDa to 800 kDa; 250 kDa to 700 kDa; 250 kDa to 600 kDa; 250 kDa to 500 kDa; 250 kDa to 400 kDa; 250 kDa to 350 kDa; 300 kDa to 1 ,000 kDa; 300 kDa to 900 kDa; 300 kDa to 800 kDa; 300 kDa to 700 kDa; 300 kDa to 600 kDa; 300 kDa to 500 kDa; 300 kDa to 400 kDa; 400 kDa to 1,000 kDa; 400 kDa to 900 kDa; 400 kDa to 800 kDa; 400 kDa to 700 kDa; 400 kDa to 600 kDa; 500 kDa to 600 kDa.

The second step of the conjugation process is the reduction of the imine (Schiff base) bond between activated polysaccharide and a carrier protein to form a stable conjugate bond (so-called reductive amination), using a reducing agent. Reducing agents which are suitable include the cyanoborohydrides (such as sodium cyanoborohydride or sodium borohydride). In one embodiment the reducing agent is sodium cyanoborohydride.

In certain embodiments, the reductive amination reaction is carried out in aprotic solvent (or a mixture of aprotic solvents). In one embodiment, the reduction reaction is carried out in DMSO or in DMF (dimethylformamide) solvent. The DMSO or DMF solvent may be used to reconstitute the activated polysaccharide and carrier protein, if lyophilized. In one embodiment, the aprotic solvent is DMSO.

At the end of the reduction reaction, there may be unreacted aldehyde groups remaining in the conjugates, which may be capped using a suitable capping agent. In one embodiment this capping agent is sodium borohydride ($NaBH_4$). Suitable alternatives include sodium triacetoxyborohydride or sodium or zinc borohydride in the presence of Bronsted or Lewis acids), amine boranes such as pyridine borane, 2-Picoline Borane, 2,6-diborane-methanol,

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dimethylamine-borane, t-BuMe'PrN-BH_3, benzylamine-BH_3 or 5-ethyl-2-methylpyridine borane (PEMB) or borohydride exchange resin. Following the conjugation (the reduction reaction and optionally the capping), the glycoconjugates may be purified (enriched with respect to the amount of polysaccharide-protein conjugate) by a variety of techniques known to the skilled person. These techniques include dialysis, concentration/diafiltration operations, tangential flow filtration, precipitation/elution,column chromatography (ion exchange chromatography, multimodal ion exchange chromatography, DEAE, or hydrophobic interaction chromatography), and depth filtration. In an embodiment, the glycoconjugates are purified by diafilitration or ion exchange chromatography or size exclusion chromatography.

Glycoconjugates prepared using reductive amination in an aprotic solvent are generally used in multivalent pneumococcal conjugate vaccines. Thus, in certain embodiments for multivalent compositions where not all the serotypes are prepared in an aprotic solvent, the reduction reaction for the remaining seroytpes is carried out in aqueous solvent (e.g., selected from PBS (phosphate buffered saline), MES (2-(*N*-morpholino)ethanesulfonic acid), HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), Bis-tris, ADA (N-(2-Acetamido)iminodiacetic acid), PIPES (piperazine-N,N′-bis(2-ethanesulfonic acid)), MOPSO (3-Morpholino-2-hydroxypropanesulfonic acid), BES (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid), MOPS (3-(N-morpholino)propanesulfonic acid), DIPSO (3-Bis(2-hydroxyethyl) amino-2-hydroxypropane-1-sulfonic acid), MOBS (4-(N-morpholino)butanesulfonic acid), HEPPSO (N-(2-Hydroxyethyl)piperazine-N-(2-hydroxypropanesulfonic acid)), POPSO (Piperazine-1,4-bis(2-hydroxy-3-propanesulfonic acid)), TEA (triethanolamine), EPPS (4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid), Bicine at a pH between 6.0 and 8.5, 7.0 and 8.0, or 7.0 and 7.5).

S. pneumonia capsular polysaccharide-protein conjugates that can be prepared using reductive amination in an aprotic solvent, include, but are not limited to, serotypes: 3, 6A, 6B, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 19A, 20A, 22F, 23A, 23B, 23F, 24F, 31, 33F, 35B, and 39. *S. pneumonia* capsular polysaccharide-protein conjugates that can be prepared using reductive amination in an aprotic solvent, include, but are not limited to, serotypes: 3, 6A, 6B, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 19A, 20, 22F, 23A, 23B, 23F, 24F, 31, 33F, 35B, and 39. *S. pneumonia* capsular polysaccharide-protein conjugates that can be prepared using reductive amination in an aprotic solvent, include, but are not limited to, serotypes: 3, 6A, 6B, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 19A, 20B, 22F, 23A, 23B, 23F, 24F, 31, 33F, 35B, and 39. The polysaccharides may be used in the form of oligosaccharides. These are conveniently formed by fragmentation of purified polysaccharide (e.g. by hydrolysis), which will usually be followed by purification of the fragments of the desired size.

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In certain embodiments, pneumococcal polysaccharide-protein conjugates of one or more of serotypes 3, 6A, 6B, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 19A, 20A, 22F, 23A, 23B, 23F, 24F, 31, 33F, 35B, and 39 are prepared using reductive amination in an aprotic solvent. In certain embodiments, pneumococcal polysaccharide-protein conjugates of one or more of serotypes 3, 6A, 6B, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 19A, 20, 22F, 23A, 23B, 23F, 24F, 31, 33F, 35B, and 39 are prepared using reductive amination in an aprotic solvent. In certain embodiments, pneumococcal polysaccharide-protein conjugates of one or more of serotypes 3, 6A, 6B, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 19A, 20B, 22F, 23A, 23B, 23F, 24F, 31, 33F, 35B, and 39 are prepared using reductive amination in an aprotic solvent.In certain embodiments, each of the serotypes in the multivalent immunogenic composition is prepared using reductive amination in an aprotic solvent. In certain embodiments, polysaccharides of one or more serotypes in a multivalent composition are conjugated using reductive amination in an aprotic solvent and polysaccharides of one or more serotypes are conjugated using reductive amination in an aqueous solvent. In certain embodiments, polysaccharides of two or more serotypes in a multivalent composition are conjugated using reductive amination in an aprotic solvent. In other embodiments, polysaccharides of three or more, four or more, five or more, six or more, seven or more, eight or more, nine or more, ten or more, eleven or more, twelve or more, thirteen or more, fourteen or more, fifteen or more, sixteen or more, seventeen or more, eighteen or more, nineteen or more, twenty or more, or twenty-one or more serotypes in a multivalent composition are conjugated using reductive amination in an aprotic solvent. In certain embodiments, polysaccharides from one or more serotypes in a multivalent composition are conjugated using other chemistries which may be in an aprotic solvent or in an aqueous solvent.

Thus, the invention relates to a multivalent immunogenic composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates, each comprising capsular polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* are as described herein (i.e. in Section II, "Multivalent Immunogenic Compositions"), wherein the conjugation reaction whereby the polysaccharide from one or more of the polysaccharide protein conjugates is conjugated to the carrier protein is in an aprotic solvent. In certain embodiments, at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100% of the serotypes in a multivalent composition are prepared in an aprotic solvent. The remainder of the serotypes are prepared using an alternative chemistry and/or in an aqueous solvent.

It was determined that the use of DMSO as a solvent during reductive amination of polysaccharide-protein conjugates results in the unexpectedly superior stability and enhanced immunogenicity for those serotypes relative to the same conjugates prepared under aqueous conditions (*See* US Application Serial No's. 62/463,216 and 62/555,444). As shown herein (*see*

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EXAMPLE 40), drug product formulations containing the pneumococcal conjugates of the invention prepared using reductive amination in an aprotic solvent (e.g. DMSO) resulted in superior physical and chemical stability as compared to a vaccine utilizing drug substances prepared using a protic (i.e. aqueous) solvent during reductive amination in the conjugation process. Thus, in some embodiments, all of the pneumococcal polysaccharide conjugates in the multivalent composition are prepared in an aprotic solvent.

In certain embodiments of the invention the total polysaccharide concentration in the composition is from about 0.02 to about 0.175 mg/mL. In certain embodiments of the invention the total polysaccharide concentration in the composition is from about 0.03 to about 0.175 mg/mL. In certain embodiments of the invention the total polysaccharide concentration in the composition is from about 0.04 to about 0.175 mg/mL. In other embodiments, the total polysaccharide concentration in the composition is from about 0.065 to about 0.085 mg/mL, about 0.070 to about 0.080 mg/mL, about 0.065 to about 0.080 mg/mL, about 0.070 to about 0.085 mg/mL, about 0.110 to about 0.128 mg/mL, about 0.110 to about 0.175 mg/mL, about 0.10 to about 0.175 mg/mL, about 0.110 to about 0.170 mg/mL, about 0.115 to about 0.15 mg/mL, about 0.110 to about 0.15 mg/mL, about 0.110 to about 0.125 mg/mL, about 0.150 to about 0.170 mg/mL, about 0.150 to about 0.165 mg/mL, about 0.140 to about 0.170 mg/mL, about 0.130 to about 0.170 mg/mL, about 0.150 to about 0.175 mg/mL, about 0.070 to about 0.170 mg/mL, about 0. 065 to about 0.175 mg/mL, or about 0.065 to about 0.180 mg/mL.

In embodiments of the invention wherein one or more, or all, of the polysaccharide-protein conjugates in the multivalent immunogenic compositions are prepared in an aprotic solvent, the total polysaccharide concentration in the composition is stable for 4 weeks or more at 37°C, 4 weeks or more at 25°C, and 12 weeks or more at 4°C.

In certain embodiments of the invention wherein one or more, or all, of the polysaccharide-protein conjugates in the multivalent immunogenic compositions are prepared in an aprotic solvent, the weight average molecular weight (Mw) of all of the *S. pneumoniae* polysaccharide protein conjugates in the composition (average of all conjugates in the composition) is from about 3,500 to about 4,700 kDa, from about 3,500 to about 4,600 kDa, from about 3,500 to about 4,500 kDa, from about 3,500 to about 4,400 kDa, from about 3,500 to about 4,300 kDa, from about 3,500 to about 4,200 kDa, from about 3,600 to about 4,700 kDa, from about 3,600 to about 4,600 kDa, from about 3,600 to about 4,500 kDa, from about 3,600 to about 4,400 kDa, from about 3,600 to about 4,300 kDa, from about 3,600 to about 4,200 kDa, from about 3,700 to about 4,700 kDa, from about 3,700 to about 4,600 kDa, from about 3,700 to about 4,500 kDa, from about 3,700 to about 4,400 kDa, from about 3,700 to about 4,300 kDa, from about 3,700 to about 4,200 kDa, from about 3,800 to about 4,700 kDa, from about 3,800 to about 4,600 kDa, from about 3,800 to about 4,500 kDa, from about 3,800 to about 4,400 kDa, from about 3,800 to about 4,300 kDa, from about 3,800 to about 4,200 kDa, from about 3,900 to

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about 4,700 kDa, from about 3,900 to about 4,600 kDa, from about 3,900 to about 4,500 kDa, from about 3,900 to about 4,400 kDa, from about 3,900 to about 4,300 kDa, or from about 3,900 to about 4,200 kDa.

In certain embodiments of the invention wherein the polysaccharide-protein conjugates in the multivalent immunogenic compositions are prepared in an aprotic solvent, the Mw of each of the *S. pneumoniae* polysaccharide protein conjugates in the composition (for a single serotype) is from about 1,000 to about 10,000 kDa, from about 1,500 to about 5,500 kDa, from about 1,500 to about 5,600 kDa, from about 1,500 to about 5,700 kDa, from about 1,500 to about 5,800 kDa, from about 1,500 to about 5,900 kDa, from about 1,500 to about 6,000 kDa, from about 1,000 to about 5,500 kDa, from about 1,000 to about 5,000 kDa, from about 1,000 to about 4,000 kDa, from about 1,000 to about 4,500 kDa, from about 1,000 to about 4,000 kDa, or from about 1,000 to about 3,500 kDa. In other embodiments, the Mw of a conjugate from a single serotype within the composition is about 1,000 kDa, about 1,100 kDa, about 1,200 kDa, about 1,300 kDa, about 1,400 kDa, about 1,500 kDa, about 1,600 kDa, about 1,700 kDa, about 1,800 kDa, about 1,900 kDa, about 2,000 kDa, about 2,100 kDa, about 2,200 kDa, about 2,300 kDa, about 2,400 kDa, about 2,500 kDa, about 2,600 kDa, about 2,700 kDa, about 2,800 kDa, about 2,900 kDa, about 3,000 kDa, about 3,100 kDa, about 3,200 kDa, about 3,300 kDa, about 3,400 kDa, about 3,500 kDa, about 3,600 kDa, about 3,700 kDa, about 3,800 kDa, about 3,900 kDa, about 4,000 kDa, about 4,100 kDa, about 4,200 kDa, about4,300 kDa, about 4,400 kDa, about 4,500 kDa, about 4,600 kDa, about 4,700 kDa, about 4,800 kDa, about 4,900 kDa, about 5,000 kDa, about 5,100 kDa, about 5,200 kDa, about 5,300 kDa, about 5,400 kDa, or about 5,500 kDa.

In certain embodiments of the invention the polysaccharide-protein conjugates in the multivalent immunogenic compositions are prepared in an aprotic solvent. Compositions containing higher percentages of *S. pneumoniae* polysaccharides conjugated to a carrier protein in an aprotic solvent (as opposed to being prepared in a protic solvent) may be preferred. In certain embodiments, the percentage (as calculated by the number of polysaccharide serotypes prepared in an aprotic solvent divided by the total number of polysaccharide serotypes, where total number includes those prepared in an aprotic solvent or a protic solvent) of *S. pneumoniae* serotype specific conjugates prepared in an aprotic solvent may be greater than 50%, or greater than 60%, or greater than 70%, or greater than 80%, or greater than 90% or are 100%.

In certain embodiments of the invention, the serotype 3 polysaccharide-protein conjugate in the composition is prepared in an aprotic solvent and the Mw of said conjugate is from about 1,000 to about 5,000 kDa, or from about 1,000 to about 4,000 kDa, or from about 1,000 to about 3,000 kDa, or from about 1,000 to about 2,500 kDa, or from about 1,000 to about 2,000 kDa.

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In certain embodiments of the invention wherein one or more, or all, of the polysaccharide-protein conjugates in the multivalent immunogenic compositions are prepared in an aprotic solvent, the number average molecular weight (Mn) of the *S. pneumoniae* polysaccharide protein conjugates in the composition (average of all conjugates in the composition) is from about 900 to about 3,000 kDa, from about 1,000 to about 3,000 kDa, from about 1,000 to about 2,500 kDa, from about 1,500 to about 2,500 kDa, from about 1,800 to about 2,500 kDa, from about 1,900 to about 2,500 kDa, or from about 2,000 to about 2,500 kDa.

In certain embodiments of the invention wherein one or more, or all, of the polysaccharide-protein conjugates in the multivalent immunogenic compositions are prepared in an aprotic solvent, the Mn of each of the *S. pneumoniae* polysaccharide protein conjugates in the composition (for a single serotype) is from about 700 to about 7,000 kDa, from about 1,000 to about 6,000 kDa, from about 1,000 to about 5,000 kDa, from about 1,000 to about 4,000 kDa, from about 1,000 to about 3,000 kDa, from about 900 to about 5,500 kDa, from about 900 to about 5,000 kDa, from about 900 to about 4,500 kDa, from about 900 to about 4,000 kDa, from about 900 to about 3,500 kDa, or from about 900 to about 3,000 kDa.

In embodiments of the invention, the Mw and/or Mn of the *S. pneumoniae* polysaccharide protein conjugates in the composition is stable for 4 weeks or more at 37°C, 4 weeks or more at 25°C, and/or 12 weeks or more at 4°C.

In embodiments of the invention, the polysaccharide concentration, Mw, and/or Mn are determined using HPSEC UV/MALS/RI.

In some embodiment of the invention, wherein one or more, or all, of the polysaccharide-protein conjugates in the multivalent immunogenic compositions are prepared in an aprotic solvent, the emission maximum of the composition measured using intrinsic protein fluorescence spectroscopy with an excitation wavelength at 280 nanometers (nm) is from about 335 nm to about 342 nm. In some embodiments, the emission maximum remains from about 335 nm to about 342 nm and the fluorescence intensity is stable for at least 1 week at 37°C. In some embodiments, the emission maximum remains from about 335 nm to about 342 nm and the fluorescence intensity is stable for 1 week at 37°C.

In some embodiments, all of the pneumococcal polysaccharide conjugates in the multivalent composition are prepared using reductive amination in DMSO. In certain sub-embodiments, the multivalent composition comprising polysaccharide conjugates which were all prepared using DMSO does not comprise an adjuvant.

Without being bound by any theory, one possible mechanism for the enhanced immunogenicity observed with glycoconjugates prepared in DMSO include an increased number of linkages between the carbohydrate (capsular polysaccharide) and lysine residues on the surface of the carrier protein which would result in additional attachment points between the protein and polysaccharide to impart stability and counter chemical depolymerization or

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breakdown of the peptide carbohydrate bond. *See, e.g.*, Hsieh, Characterization of Saccharide-CRM197 Conjugate Vaccines in Brown F, Corbel M, Griffiths E (eds): Physico-Chemical Procedures for the Characterization of Vaccines. Dev. Biol. Basel, Karger, 2000, vol 103, pp. 93-104. An additional benefit of the increased polysaccharide-protein linkages that are created during conjugation in the DMSO solvent could be additional opportunities for successful presentation of peptide-carbohydrate to T-cells. It can be appreciated that due to the genetic variability in the human population resulting in varying abilities and sensitivity of loading or associating with specific peptide sequences conjugated to carbohydrate antigens, that additional points of attachment on the carrier protein would allow for increased chances for successful antigen presentation at the surface of an antigen presenting cell (APC) to allow for a T-cell dependent response to an otherwise T-cell independent antigen. Another possible mecahnsim of enhanced immunogenicity observed by conjugation in the DMSO solvent could be due to the denaturation of CRM197 in organic solvent, which exposes additional lysines for polysaccharide linkages giving increased chances for glycopeptide presentation at the surface of an APC for T-cell dependent response to different peptide epitopes. See Avci *et al.*, 2011, Nature Medicine 17: 1602-1610.

Yet another benefit of conjugation in an organic solvent generating denatured CRM197 in the conjugates could be reduced immunological interference of antibodies against native CRM197 epitopes. A further benefit of the increased polysaccharide-protein linkages that are created during conjugation in the DMSO solvent could be the formation of larger sized polysaccharide protein conjugates resulting in enhanced immunogenicity. The compositions of the invention are believed to provide significant advantages in eliciting a human response.

In certain embodiments, the conjugation reaction is performed by reductive amination wherein nickel is used for greater conjugation reaction efficiency and to aid in free cyanide removal. Transition metals are known to form stable complexes with cyanide and are known to improve reductive methylation of protein amino groups and formaldehyde with sodium cyanoborohydride (S Gidley *et al.*, *Biochem J.* 1982, 203: 331-334; Jentoft *et al. Anal Biochem.* 1980, 106: 186-190). By complexing residual, inhibitory cyanide, the addition of nickel increases the consumption of protein during the conjugation and leads to formation of larger, potentially more immunogenic conjugates.

Differences in starting cyanide levels in sodium cyanoborohydride reagent lots also lead to inconsistent conjugation performance, resulting in variable product attributes, such as conjugate size and conjugate Ps-to-CRM197 ratio. The addition of nickel reduced conjugation inconsistency by complexing cyanide, eliminating differences in sodium cyanoborohydride lots.

Suitable alternative chemistries include the activation of the saccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The

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activated saccharide may thus be coupled directly or via a spacer (linker) group to an amino group on the carrier protein. For example, the spacer could be cystamine or cysteamine to give a thiolated polysaccharide which could be coupled to the carrier via a thioether linkage obtained after reaction with a maleimide-activated carrier protein (for example using GMBS) or a haloacetylated carrier protein (for example using iodoacetimide [e.g. ethyl iodoacetimide HCl] or N-succinimidyl bromoacetate or SIAB, or SIA, or SBAP). Preferably, the cyanate ester (optionally made by CDAP chemistry) is coupled with hexane diamine or adipic acid dihydrazide (ADH) and the amino-derivatised saccharide is conjugated to the carrier protein using carbodiimide (e.g. EDAC or EDC) chemistry via a carboxyl group on the protein carrier. Such conjugates are described in International Patent Application Publication Nos. WO 93/15760, WO 95/08348 and WO 96/29094; and Chu *et al.*, 1983, Infect. Immunity 40:245-256.

Other suitable techniques use carbodiimides, hydrazides, active esters, norborane, p-nitrobenzoic acid, N-hydroxysuccinimide, S--NHS, EDC, TSTU. Many are described in International Patent Application Publication No. WO 98/42721. Conjugation may involve a carbonyl linker which may be formed by reaction of a free hydroxyl group of the saccharide with CDI (See Bethell *et al.*, 1979, J. Biol. Chem. 254:2572-4; Hearn *et al.*, 1981, J. Chromatogr. 218:509-18) followed by reaction of with a protein to form a carbamate linkage. This may involve reduction of the anomeric terminus to a primary hydroxyl group, optional protection/deprotection of the primary hydroxyl group, reaction of the primary hydroxyl group with CDI to form a CDI carbamate intermediate and coupling the CDI carbamate intermediate with an amino group on a protein.

After conjugation of the capsular polysaccharide to the carrier protein, the polysaccharide-protein conjugates are purified (enriched with respect to the amount of polysaccharide-protein conjugate) by one or more of a variety of techniques. Examples of these techniques are well known to the skilled artisan and include concentration/diafiltration operations, ultrafiltration, precipitation/elution, column chromatography, and depth filtration. See, e.g., U.S. Pat. No. 6,146,902.

After the individual glycoconjugates are purified, they are compounded to formulate the immunogenic composition of the present invention. These pneumococcal conjugates are prepared by separate processes and bulk formulated into a single dosage formulation.

An alternative method for characterizing the glycoconjugates of the invention is by the number of lysine residues in the carrier protein (e.g., CRM197) that become conjugated to the saccharide which can be characterized as a range of conjugated lysines (degree of conjugation). The evidence for lysine modification of the carrier protein, due to covalent linkages to the polysaccharides, can be obtained by amino acid analysis using routine methods known to those of skill in the art. Conjugation results in a reduction in the number of lysine

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residues recovered, compared to the carrier protein starting material used to generate the conjugate materials. In a preferred embodiment, the degree of conjugation of the glycoconjugate of the invention is between 2 and 15, between 2 and 13, between 2 and 10, between 2 and 8, between 2 and 6, between 2 and 5, between 2 and 4, between 3 and 15, between 3 and 13, between 3 and 10, between 3 and 8, between 3 and 6, between 3 and 5, between 3 and 4, between 5 and 15, between 5 and 10, between 8 and 15, between 8 and 12, between 10 and 15 or between 10 and 12. In an embodiment, the degree of conjugation of the glycoconjugate of the invention is about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11 , about 12, about 13, about 14 or about 15. In a preferred embodiment, the degree of conjugation of the glycoconjugate of the invention is between 4 and 7. In some such embodiments, the carrier protein is CRM197.

The glycoconjugates of the compositions of the invention may also be characterized by the ratio (weight/weight) of saccharide to carrier protein (Ps:Pr). In some embodiments, the ratio of polysaccharide to carrier protein of the glycoconjugates (w/w) in the composition is between 0.5 and 3.0 (e.g., about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1 , about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1 , about 2.2, about 2.3, about 2.4, about 2.5, about 2.6, about 2.7, about 2.8, about 2.9, or about 3.0). In other embodiments, the saccharide to carrier protein ratio (w/w) is between 0.5 and 2.5, between 0.5 and 1.5, between 0.8 and 2.5, between 0.5 and 1.0, between 1.0 and 1.5, between 1.0 and 2.0, between 0.8 and 2.4, between 0.8 and 2.3, between 0.8 and 2.2, between 0.8 and 2.1, between 0.8 and 2.0, between 0.8 and 1.9, between 0.8 and 1.8, between 0.8 and 1.7, between 0.8 and 1.6, between 0.8 and 1.5, between 0.8 and 1.4, between 0.8 and 1.3, between 0.9 and 2.4, between 0.9 and 2.3, between 0.9 and 2.2, between 0.9 and 2.1, between 0.9 and 2.0, between 0.9 and 1.9, between 0.9 and 1.8, between 0.9 and 1.7, between 0.9 and 1.6, between 0.9 and 1.5, between 0.9 and 1.4, between 0.9 and 1.3, between 0.9 and 1.2, between 1.0 and 2.4, between 1.0 and 2.3, between 1.0 and 2.2, between 1.0 and 2.1, between 1.0 and 2.0, between 1.0 and 1.9, between 1.0 and 1.8, between 1.0 and 1.7, between 1.0 and 1.6, between 1.0 and 1.5, between 1.0 and 1.4, between 1.0 and 1.3 or between 1.0 and 1.2. In further embodiments, the saccharide to carrier protein ratio (w/w) is between 0.8 and 1.2. In some such embodiments, the carrier protein is CRM197. The glycoconjugates and immunogenic compositions of the invention may contain free saccharide that is not covalently conjugated to the carrier protein, but is nevertheless present in the glycoconjugate composition. The free saccharide may be non-covalently associated with (i.e., non-covalently bound to, adsorbed to, or entrapped in or with) the glycoconjugate.

In specific embodiments, the saccharide to carrier protein ratio (w/w) for the serotype 15A conjugate is from about 1.0 to about 2.0, from about 1.25 to about 1.75, or from

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about 1.3 to about 1.7. In other embodiments, the saccharide to carrier protein ratio (w/w) for serotype 15A is about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, or 1.8.

In specific embodiments, the saccharide to carrier protein ratio (w/w) for the serotype 15C conjugate is from about 1.0 to about 2.0, from about 1.25 to about 1.75, or from about 1.3 to about 1.7. In other embodiments, the saccharide to carrier protein ratio (w/w) for serotype 15C is about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, or 1.8.

In specific embodiments, the saccharide to carrier protein ratio (w/w) for the serotype 33F conjugate is from about 1.0 to about 2.0, from about 1.25 to about 1.75, or from about 1.3 to about 1.7. In other embodiments, the saccharide to carrier protein ratio (w/w) for serotype 33F is about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, or 1.8.

In specific embodiments, the saccharide to carrier protein ratio (w/w) for the serotype 35B conjugate is from about 1.25 to about 2.25, from about 1.25 to about 2.0, or from about 1.3 to about 1.8. In other embodiments, the saccharide to carrier protein ratio (w/w) for serotype 33B is about 1.2, 1.3, 1.3, 1.5, 1.6, 1.7, 1.8, 1.9, or 2.0.

In specific embodiments, the saccharide to carrier protein ratio (w/w) for the serotype 24F conjugate is from about 0.5 to about 1.5, from about 0.75 to about 1.25, or from about 0.8 to about 1.0. In other embodiments, the saccharide to carrier protein ratio (w/w) for serotype 24F is about 0.5, 0.6, 0.7, 0.8, 0.9, or 1.0.

In a preferred embodiment, the glycoconjugate comprises less than about 50%, 45%, 40%, 35%, 30%, 25%, 20% or 15% of free polysaccharide compared to the total amount of polysaccharide. In a preferred embodiment the glycoconjugate comprises less than about 25% of free polysaccharide compared to the total amount of polysaccharide. In a preferred embodiment the glycoconjugate comprises less than about 20% of free polysaccharide compared to the total amount of polysaccharide. In a preferred embodiment the glycoconjugate comprises less than about 15% of free polysaccharide compared to the total amount of polysaccharide.

IV. Methods of Use

Embodiments of the invention also include one or more of the multivalent immunogenic compositions described herein (i) for use in, (ii) for use as a medicament or composition for, or (iii) for use in the preparation of a medicament for: (a) therapy (e.g., of the human body); (b) medicine; (c) inhibition of infection with *Streptococcus pneumoniae*; (d) induction of an immune response or a protective immune response against *S. pneumoniae*; (e) prophylaxis of infection by *S. pneumoniae*; (f) prevention of recurrence of *S. pneumoniae* infection; (g) reduction of the progression, onset or severity of pathological symptoms associated with *S. pneumoniae* infection including the prevention of associated complications such as brain damage, hearing loss, and seizures, (h) reduction of the likelihood of a *S. pneumoniae* infection or, (i) treatment, prophylaxis of, or delay in the onset, severity, or progression of pneumococcal

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disease(s), including, but not limited to: pneumococcal pneumonia, pneumococcal bacteremia, pneumococcal meningitis, otits media and sinusitis. In these uses, the multivalent pneumococcal polysaccharide-conjugate compositions of the invention can optionally be employed in combination with one or more adjuvants, or without an adjuvant.

Accordingly, the invention provides methods for the prophylactic treatment of (i.e. protection against) *S. pneumoniae* infection or pneumococcal disease comprising administering one or more of the multivalent immunogenic pneumococcal polysaccharide-protein conjugate compositions of the invention to a patient in need of treatment.

The compositions and formulations of the present invention can be used to protect or treat a human susceptible to infection, e.g., a pneumococcal infection, by means of administering a composition of the invention via a systemic or mucosal route.

In one embodiment, the invention provides a method of inducing an immune response to *S. pneumoniae*, comprising administering to a patient an immunologically effective amount of a multivalent immunogenic composition of the invention. In another embodiment, the invention provides a method of vaccinating a human against a pneumococcal infection, comprising the step of administering to the human an immunogically effective amount of a multivalent immunogenic composition of the invention.

Thus, in one aspect, the invention provides a method for (1) inducing an immune response in a human patient, (2) inducing a protective immune response in a human patient, (3) vaccinating a human patient against an infection with *S. pneumoniae*, or (4) reducing the likelihood of a *S. pneumoniae* infection in a human patient, the method comprising administering a multivalent immunogenic composition of the invention to the patient (i.e. any multivalent immunogenic composition described herein, such as the multivalent immunogenic compositions described in Section II, entitled "Multivalent Immunogenic Compositions," *supra*).

In one embodiment, the invention provides a method for the prevention of pneumococcal pneumoniae and invasive disease in adults 18 years of age and older. In another embodiment, the invention provides a method for the prevention of pneumococcal pneumoniae and invasive disease caused by the 24 Streptococcus pneumoniae strains (3, 6A, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 19A, 20A, 20B, 22F, 23A, 23B, 24F, 31, 33F, and 35B).

In one embodiment of the methods above, the composition comprises multiple *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise the set of serotypes: 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A. In one embodiment of the methods above, the composition comprises multiple *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae*

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serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise the set of serotypes: 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20. In one embodiment of the methods above, the composition comprises multiple *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* comprise the set of serotypes: 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B. In another embodiment of the methods above, the composition further comprises a *S. pneumoniae* polysaccharide protein conjugate of serotype 6A or 6C.

It has been shown that a pneumococcal conjugate vaccine comprising serotype 6A may provide some cross-protection against serotype 6C (Cooper *et al.*, *Vaccine* 29 (2011) 7207–7211). Therefore, in some embodiments of the methods above, the invention also provides use of multivalent immunogenic compositions that do not comprise serotype 6C, but instead comprise serotype 6A or serotypes 6A and 6B. In other embodiments, the immunogenic composition comprises pneumococcal conjugates of serotypes 6A, 6B, and 6C. In particular embodiments of the methods above, the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

I-a) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

I-b) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

I-c) 3, 7F, 19A, 22F, 33F, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; and

I-d) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A.

In particular embodiments of the set of serotypes above (I-a to I-d), serotype 20 or 20B may be substituted for serotype 20A.

In further embodiments of the methods above, the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

II-a) 15A, 16F, 23A, 23B, 24F, 31 and 35B;

II-b) 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B;

II-c) 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B;

II-d) 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B;

II-e) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

II-f) 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

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II-g) 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; and

II-h) 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A.

In particular embodiments of the set of serotypes above (II-e to IIh), serotype 20 or 20B may be substituted for serotype 20A.

In further embodiments of the methods above, the serotypes of *S. pneumoniae* comprise a set of serotypes as set forth in any of embodiments I-a), II-a) or II-e) and further comprise serotypes 6A, 6B, and 6C.

It has also been shown that a pneumococcal conjugate vaccine comprising serotype 10A may provide some cross-protection against serotype 39 (see WO 2017/ 085586). Therefore, in some embodiments of the methods above, the invention also provides use of multivalent immunogenic compositions that do not comprise serotype 10A, but instead comprise serotype 39. In other embodiments, the immunogenic composition comprises pneumococcal conjugates of serotypes 10A and 39. In particular embodiments of the methods above, the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

III-a) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 39, 11A, 12F, 15C, 17F, and 20A;

III-b) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 39, 11A, 12F, 15C, 17F, and 20A;

III-c) 3, 7F, 19A, 22F, 33F, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 39, 11A, 12F, 15C, 17F, and 20A;

III-d) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 39, 11A, 12F, 15C, 17F, and 20A;

III-e) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 39, 11A, 12F, 15C, 17F, and 20A;

III-f) 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 39, 11A, 12F, 15C, 17F, and 20A;

III-g) 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 39, 11A, 12F, 15C, 17F, and 20A; and

III-h) 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 39, 11A, 12F, 15C, 17F, and 20A.

In particular embodiments of the set of serotypes above (II-a to III-h), serotype 20 or 20B may be substituted for serotype 20A.

In further embodiments of the methods above, the serotypes of *S. pneumoniae* comprise a set of serotypes as set forth in any of embodiments III-a) to III-h) and further comprise serotype 10A.

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It has also been shown that immunogenic conjugates comprising *S. pneumoniae* serotype 15B capsular polysaccharide covalently linked to a carrier protein may provide some cross-protection against serotype 15C and/or serotype 15A (see WO 2015/110942). Therefore, in some embodiments of the methods above, the invention also provides use of multivalent immunogenic compositions that do not comprise serotype 15C (or de-O-acetylated 15B), but instead comprise serotype 15B (i.e. the serotype 15B polysaccharide is not de-O-acetylated). In other embodiments, the immunogenic composition comprises pneumococcal conjugates of serotypes 15B and 15C (or de-O-acetylated 15B). In particular embodiments of the methods above, the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

IV-a) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A;

IV-b) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A;

IV-c) 3, 7F, 19A, 22F, 33F, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A;

IV-d) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A;

IV-e) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A;

IV-f) 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A;

IV-g) 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A; and

IV-h) 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A.

In particular embodiments of the set of serotypes above (IVa to IVh), serotype 20 or 20B may be substituted for serotype 20A.

In further embodiments of the methods above, the serotypes of *S. pneumoniae* comprise a set of serotypes as set forth in any of embodiments IV-a) to IV-h) and further comprise serotype 15C (and/or de-O-acetylated 15B).

The compositions of the invention are useful in methods for providing complementary protection against *S. pneumoniae* in patients who had previously received a multivalent pneumococcal vaccine. In this use, the compositions of the invention can provide protection against particular *S. pneumoniae* serotypes that a patient had not been previously vaccinated against, can provide additional protection against *S. pneumoniae* serotypes that a patient had been previously vaccinated against, or can provide protection against both *S.*

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pneumoniae serotypes that a patient had not been previously vaccinated against and *S. pneumoniae* serotypes that a patient had been previously vaccinated against.

Thus, the invention provides a method of inducing an immune response, vaccinating, or inducing a protective immune response against *S. pneumoniae* in a patient, comprising administering a multivalent immunogenic composition to the patient, the composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates, wherein the polysaccharide protein conjugates comprise capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the patient had previously been vaccinated against *S. pneumoniae*. In embodiments of this aspect of the invention, the multivalent immunogenic composition can be any multivalent immunogenic composition described herein. In particular embodiments of the methods of the invention, the multivalent immunogenic composition is administered to a patient who was previously treated with a multivalent pneumococcal vaccine. The multivalent immunogenic vaccine may be any vaccine that is indicated for the prevention of pneumococcal disease caused by more than one serotype of *S. pneumoniae*.

In specific embodiments of the method above, the patient was previously treated with a multivalent pneumococcal vaccine that is indicated for the prevention of pneumococcal disease caused by *S. pneumoniae* serotypes selected from the group consisting of:

a) 4, 6B, 9V, 14, 18C, 19F and 23F;
b) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, and 19A;
c) 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F;
d) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, 19A, 22F, and 33F;
e) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 7F, 19A, 22F, 33F, 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20; and
f) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, 19A, 22F, 33F, 8, 10A, 11A, 12F and 15B.

In specific embodiments of the method above, the multivalent pneumococcal vaccine comprises capsular polysaccharides of *S. pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 7F, 19A, 22F, 33F, 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A. In specific embodiments of the method above, the multivalent pneumococcal vaccine comprises capsular polysaccharides of *S. pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 7F, 19A, 22F, 33F, 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20. In specific embodiments of the method above, the multivalent pneumococcal vaccine comprises capsular polysaccharides of *S. pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 7F, 19A, 22F, 33F, 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B.

In specific embodiments of the method above, the multivalent pneumococcal vaccine comprises multiple polysaccharide protein conjugates, wherein the polysaccharide

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protein conjugates comprise polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein. In other embodiments, the multivalent pneumococcal vaccine comprises multiple *S. pneumoniae* capsular polysaccharides that are not conjugated to a carrier protein.

In additional embodiments of the method above, the patient was previously treated with PREVNAR® 13 (Pneumococcal 13-valent Conjugate Vaccine [Diphtheria CRM197 Protein], Pfizer, Inc., Philadelphia, PA, USA).

In further embodiments of the method above, the patient was previously treated with PNEUMOVAX® 23 (Pneomoccoal Vaccine Polyvalent, Merck & Co., Inc., Kenilworth, NJ, USA).

In still further embodiments of the method above, the patient was previously treated with SYNFLORIX™ (Pneumococcal polysaccharide conjugate vaccine (adsorbed), GlaxoSmithKline Biologicals s.a., Rixensart, Belgium).

In embodiments of the method above, the multivalent immunogenic composition of the invention is administered to a patient at any time after the patient has received a multivalent pneumococcal vaccine, according to the treatment regimen provided by the medical professional, e.g. a physician. In particular embodiments, the multivalent immunogenic composition of the invention is administered to a patient from 1 month to 5 years after the patient has received the multivalent pneumococcal vaccine, alternatively, from 1 month to 1 year, from 1 month to 2 years, from 1 month to 3 years, from 1 month to 4 years, from 1 month to 6 months, from 2 months to 6 months, from 2 months to 1 year, from 1 year to 5 years, from 6 months to 5 years, from 6 months to 4 years, from 6 months to 3 years, from 6 months to 2 years, from 6 months to 1 year, from 1 year to 4 years, from 1 year to 3 years, or from 1 year to 2 years, after the patient has received the multivalent pneumococcal vaccine. In further embodiments, the multivalent immunogenic composition is administered to the patient about 1 month, about 2 months, about 3 months, about 4 months, about 5 months, about 6 months, about 7 months, about 8 months, about 9 months, about 10 months, about 11 months, about 1 year, about 1.25 years, about 1.5 years, about 1.75 years, about 2 years, about 2.25 years, about 2.5 years, about 2.75 years, about 3 years, about 3.25 years, about 3.5 years, about 3.75 years, about 4 years, about 4.25 years, about 4.5 years, about 4.75 years, or about 5 years after the patient has received the multivalent pneumococcal vaccine.

In further embodiments, the invention provides a method for (1) inducing an immune response in a human patient, (2) inducing a protective immune response in a human patient, (3) vaccinating a human patient against an infection with *S. pneumoniae*, or (4) reducing the likelihood of a *S. pneumoniae* infection in a human patient, the method comprising administering a multivalent immunogenic composition of the invention and administering a multivalent pneumococcal vaccine to the patient, in any order. The multivalent pneomococcal

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vaccine may be any vaccine indicated for the prevention of pneumococcal disease caused by more than one serotype of *S. pneumoniae*.

In specific embodiments of the method above, the patient is treated with a multivalent immunogenic composition of the invention and a multivalent pneumococcal vaccine that is indicated for the prevention of pneumococcal disease caused by *S. pneumoniae* serotypes selected from the group consisting of:

a) 4, 6B, 9V, 14, 18C, 19F and 23F;
b) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, and 19A;
c) 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F;
d) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, 19A, 22F, and 33F;
e) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 7F, 19A, 22F, 33F, 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20; and
f) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, 19A, 22F, 33F, 8, 10A, 11A, 12F and 15B.

In specific embodiments of the method above, the multivalent pneumococcal vaccine comprises capsular polysaccharides of *S. pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 7F, 19A, 22F, 33F, 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20A.

In specific embodiments of the method above, the multivalent pneumococcal vaccine comprises multiple polysaccharide protein conjugates, wherein the polysaccharide protein conjugates comprise polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein. In other embodiments, the multivalent pneumococcal vaccine comprises multiple *S. pneumoniae* capsular polysaccharides that are not conjugated to a carrier protein.

In additional embodiments of the method above, the patient is treated with a multivalent immunogenic composition of the invention and is treated with PREVNAR® 13 (Pneumococcal 13-valent Conjugate Vaccine [Diphtheria CRM197 Protein], Pfizer, Inc., Philadelphia, PA, USA), in any order. In one embodiment, the patient is administered PREVNAR® 13 first and the patient is administered a multivalent immunogenic composition of the invention second. In alternative embodiments, the patient is administered a multivalent immunogenic composition of the invention first and is administered PREVNAR® 13 second.

In further embodiments of the method above, the patient is treated with a multivalent immunogenic composition of the invention and is treated with PNEUMOVAX® 23 (pneomoccoal vaccine polyvalent, Merck & Co., Inc., Kenilworth, NJ, USA), in any order. In one embodiment, the patient is administered PNEUMOVAX ® 23 first and the patient is administered a multivalent immunogenic composition of the invention second. In alternative embodiments, the patient is administered a multivalent immunogenic composition of the invention first and is administered PNEUMOVAX ® 23 second.

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In still further embodiments of the method above, the patient is treated with a multivalent immunogenic composition of the invention and is treated with SYNFLORIX™ (Pneumococcal polysaccharide conjugate vaccine (adsorbed), GlaxoSmithKline Biologicals s.a., Rixensart, Belgium), in any order. In one embodiment, the patient is administered SYNFLORIX™ first and the patient is administered a multivalent immunogenic composition of the invention second. In alternative embodiment, the patient is administered a multivalent immunogenic composition of the invention first and is administered SYNFLORIX™ second.

In some embodiments of the method above, the multivalent immunogenic composition and the multivalent pneumococcal vaccine are administered concurrently. As used herein, "concurrent administration" is not limited to dosing of two compositions at the same time, but includes administration one right after the other in any order. In some embodiments, the multivalent immunogenic composition and the multivalent pneumococcal vaccine are administered via intramuscular or subcutaneous administration into separate anatomical sites, e.g. two different arms.

In some embodiments of the method above, the amount of time between administration of the multivalent immunogenic composition of the invention and the multivalent pneumococcal vaccine is from about 4 weeks to about 1 year. In alternative embodiments, the amount of time is from about 1 month to about 5 years.

In one embodiment, the patient is administered the multivalent pneumococcal vaccine first and the multivalent immunogenic composition of the invention second. In alternative embodiments, the patient is administered a multivalent immunogenic composition of the invention first and is administered the multivalent pneumococcal vaccine second.

Also provided is a method of inducing an immune response, vaccinating or inducing a protective immune response against *S. pneumoniae* in a patient, comprising:

(1) administering a multivalent immunogenic composition to the patient, the composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates, wherein each of the polysaccharide protein conjugates comprise capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein,

(2) waiting for a pre-determined amount of time to pass, and

(3) administering a multivalent pneumococcal vaccine to the patient.

In this method, the multivalent immunogenic composition can comprise any combination of *S. pneumoniae* polysaccharide protein conjugates set forth herein and the multivalent pneumococcal vaccine can be any vaccine indicated for the prevention of disease caused by more than one serotype of *S. pneumoniae*.

Also provided by the invention is a method of inducing an immune response, vaccinating or inducing a protective immune response against *S. pneumoniae* in a patient, comprising:

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(1) administering a multivalent pneumococcal vaccine to the patient,

(2) waiting for a pre-determined amount of time to pass, and

(3) administering a multivalent immunogenic composition to the patient, the composition comprising multiple *S. pneumoniae* polysaccharide protein conjugates, wherein each of the polysaccharide protein conjugates comprise capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein.

In this method, the multivalent immunogenic composition can comprise any combination of *S. pneumoniae* polysaccharide protein conjugates set forth herein and the multivalent pneumococcal vaccine can be any vaccine indicated for the prevention of disease caused by more than one serotype of *S. pneumoniae*.

In some embodiments of the methods above, the multivalent pneumococcal vaccine comprises multiple *S. pneumoniae* polysaccharide protein conjugates, wherein the polysaccharide protein conjugates comprise capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein. In alternative embodiments, the multivalent pneumococcal vaccine comprises *S. pneumoniae* capsular polysaccharides that are not conjugated to a carrier protein.

In any embodiments of the methods of the invention (i.e. any of the methods described herein), the method may further comprise administering one or more additional doses of a multivalent immunogenic composition of the invention to the pateint. In such methods, the patient may have already received a multivalent pneumococcal vaccine prior to receiving a first dose of a multivalent immunogenic composition of the invention, *supra*, or may not have been vaccinated against *S. pneumoniae* prior to receiving a multivalent immunogenic composition of the invention. Thus, in one embodiment, a patient who had received a multivalent pneumococcal vaccine indicated for the prevention of pneumococcal disease caused by *S. pneumoniae* is administered two or more doses of a multivalent immunogenic composition of the invention. In alternative embodiments, a patient who had not been previously treated with any vaccine indicated for the prevention of pneumococcal disease, is administered two or more doses of a multivalent immunogenic composition of the invention.

In embodiments of the method above, the two or more doses are of the same multivalent immunogenic composition of the invention. In alternative embodiments, the two or more doses are of different multivalent immunogenic compositions of the invention.

In specific embodiments of any of these methods, the patient is administered two, three, or four doses of a multivalent immunogenic composition of the invention. In particular embodiments, the patient is immunocompromised (e.g., on an immunosuppressive regimen following a stem cell transplant) and the number of doses is three.

In some embodiments, the amount of time between administration of each dose of multivalent immunogenic composition of the invention is from about 4 weeks to about 1 year. In

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alternative embodiment, the amount of time between administration of each dose of multivalent immunogenic composition of the invention is from about 1 month to about 5 years.

In embodiments of any of the methods of the invention, the patient to be treated with the composition(s) of the invention is a human. In certain embodiments, the human patient is a toddler (approximately 12 to 24 months), or young child (approximately 2 to 5 years). The compositions of this invention are also suitable for use with older children, adolescents and adults (e.g., aged 18 to 45 years, aged 18 to 50 years, aged 18 to 55 years, aged 18 to 60 years or 18 to 65 years). In other embodiments of any of the methods of the invention, the patient is from about 2 to about 18 years of age. In further embodiments of any of the methods of the invention, the patient is 18 years of age or older.

In further embodiments of the methods of the invention, the human patient is elderly. In some embodiments of any of the methods of the invention, the patient is 50 years of age or older. In some embodiments of any of the methods of the invention, the patient is 55 years of age or older. In some embodiments of any of the methods of the invention, the patient is 60 years of age or older. In still further embodiments of any of the methods of the invention, the patient is 65 years of age or older. In additional embodiments of any of the methods of the invention, the patient is 70 years of age or older.

In some embodiments of any of the methods of the invention, the patient to be treated with an immunogenic composition of the invention is immunocompromised.

In some embodiments of any of the methods of the invention, the multivalent immunogenic composition is administered concomitantly with a vaccine against influenza. In certain embodiments, the influenza vaccine is a "senior flu vaccine," a high dose flu vaccine indicated for the elderly, e.g. persons aged 65 and older.

The invention provides a method for inducing a protective immune response in a patient against a pneomococcal infection comprising the step of administering to the patient an immunologically effective amount of any of the multivalent immunogenic pneumococcal polysaccharide-protein conjugate compositions described herein. Optimal amounts of components for a particular vaccine (i.e. multivalent immunogenic composition) can be ascertained by standard studies involving observation of appropriate immune responses in subjects. For example, in another embodiment, the dosage for human vaccination is determined by extrapolation from animal studies to human data. In another embodiment, the dosage is determined empirically.

The methods of the invention can be used for the prevention and/or reduction of primary clinical syndromes caused by microbes, e.g., *S. pneumonia*, including both invasive infections (meningitis, pneumonia, and bacteremia), and noninvasive infections (acute otitis media, and sinusitis).

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Administration of the compositions of the invention can include one or more of: injection via the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or via mucosal administration to the oral/alimentary, respiratory or genitourinary tracts. In one embodiment, intranasal administration is used for the treatment of pneumonia or otitis media (as nasopharyngeal carriage of pneumococci can be more effectively prevented, thus attenuating infection at its earliest stage). In specific embodiments, the compositions of the invention are administered to the patient via intramuscular or subcutaneous administration.

All publications mentioned herein are incorporated by reference for the purpose of describing and disclosing methodologies and materials that might be used in connection with the present invention.

Having described different embodiments of the invention herein with reference to the accompanying drawings, it is to be understood that the invention is not limited to those precise embodiments, and that various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims.

The following examples illustrate, but do not limit the invention.

EXAMPLE 1

Preparation of *S. Pneumoniae* Capsular Polysaccharides

Methods of culturing pneumococci are well known in the art. *See, e.g.*, Chase, 1967, Methods of Immunology and Immunochemistry 1:52. Methods of preparing pneumococcal capsular polysaccharides are also well known in the art. See, e.g., European Patent No. EP 0 497 524 B1. The process described below generally follows the method described in European Patent No. EP 0 497 524 B1 and is generally applicable to all pneumococcal serotypes.

Isolates of pneumococcal strains for serotypes 6C, 23B, and 31 were obtained from Centers for Disease Control and Prevention (Atlanta, GA). Strains for serotypes 3, 8, 10A, 11A, 12F, 15B, 22F, and 33F were obtained from the University of Pennsylvania (Dr. Robert Austrian). Strains for serotypes 17F and 19A were obtained from the FDA Office of Biologics (Dr. John Robbins). Serotype 7F was obtained from the State University of New York, Downstate Medical Center (Dr. Gerald Schiffman). Isolates of pneumococcal serotypes not listed above were obtained from the American Type Culture Collection (Manassas, VA). Where needed, subtypes were differentiated on the basis of Quellung reaction using specific antisera. *See, e.g.*, U.S. Pat. No. 5,847,112. The obtained isolates were further clonally isolated by plating serially in two stages on agar plates consisting of an animal-component free medium containing soy peptone, yeast extract, and glucose without hemin. For serotype 7F, the agar plates used also

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contained hemin. Clonal isolates for each serotype were further expanded in liquid culture using animal-component free media containing soy peptone, yeast extract, HEPES, sodium chloride, sodium bicarbonate, potassium phosphate, glucose, and glycerol to prepare the pre-master cell banks.

The production of each serotype of pneumococcal polysaccharide consisted of a cell expansion and batch production fermentation followed by chemical inactivation prior to downstream purification. A thawed cell bank vial from each serotype was expanded using a shake flask or culture bottle containing a pre-sterilized animal-component free growth media containing soy peptone or soy peptone ultrafiltrate, yeast extract or yeast extract ultrafiltrate, HEPES, sodium chloride, sodium bicarbonate, potassium phosphate, and glucose. The cell expansion culture was grown in a sealed shake flask or bottle to minimize gas exchange with temperature and agitation control. For serotypes 3, 7F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 19A, 20, 22F, and 33F, a thawed cell bank vial was expanded using a fermentor containing the same media. During the cell expansion of these serotypes, temperature, pH, pressure, and agitation were controlled. Airflow overlay was also controlled as sparging was not used. After achieving a specified culture density, as measured by optical density at 600 nm, a portion of the cell expansion culture was transferred to a production fermentor containing pre-sterilized animal-component free growth media containing soy peptone or soy peptone ultrafiltrate, yeast extract or yeast extract ultrafiltrate, sodium chloride, potassium phosphate, and glucose. Temperature, pH, pressure, and agitation were controlled. Airflow overlay was also controlled as sparging was not used.

The batch fermentation was terminated via the addition of a chemical inactivating agent, phenol, when glucose was nearly exhausted. Pure phenol was added to a final concentration of 0.8 - 1.2% to inactivate the cells and liberate the capsular polysaccharide from the cell wall. Primary inactivation occurs for a specified time within the fermentor where temperature and agitation continue to be controlled. After primary inactivation, the batch was transferred to another vessel where it was held for an additional specified time at controlled temperature and agitation for complete inactivation. This was confirmed by either microbial plating techniques or by verification of the phenol concentration and specified time. The inactivated broth was then purified.

EXAMPLE 2

Purification of Pneumococcal Polysaccharides

The purification process for the pneumococcal polysaccharides consisted of several centrifugation, depth filtration, concentration/diafiltration operations, and precipitation steps. All procedures were performed at room temperature unless otherwise specified.

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Inactivated broth from the fermentor cultures of *S. pneumoniae* were flocculated with a cationic polymer (such as BPA-1000, TRETOLITE® (Baker Hughes Inc., Houston, TX), Spectrum 8160, poly(ethyleneimine), and Millipore pDADMAC). The cationic polymers binded to the impurity proteins, nucleic acids and cell debris. Following the flocculation step and an aging period, flocculated solids were removed via centrifugation and multiple depth filtration steps. Clarified broth was concentrated and diafiltered using a 100 kDa to 500 kDa MWCO (molecular weight cutoff) filter. Diafiltration was accomplished using Tris, $MgCl_2$ buffer and sodium phosphate buffer. Diafiltration removed residual nucleic acid and protein.

Removal of further impurities was accomplished by reprecipitation of the polysaccharide in sodium acetate and phenol with denatured alcohol and/or isopropanol. During the phenol precipitation step, sodium acetate in sodium phosphate saline buffer and phenol (liquefied phenols or solid phenols) were charged to the diafiltered retentate. Alcohol fractionation of the polysaccharide was then conducted in two stages. In the first stage a low percent alcohol was added to the preparation to precipitate cellular debris and other unwanted impurities, while the crude polysaccharide remained in solution. The impurities were removed via centrifugation followed by a depth filtration step. The polysaccharide was then recovered from the solution by adding additional isopropanol or denatured alcohol to the batch. The precipitated polysaccharide pellet was recovered by centrifugation, triturated and dried as a powder and stored frozen at -70°C.

EXAMPLE 3

Structure Identity Analysis of Certain Pneumococcal Serotypes by NMR Testing

Samples for NMR analysis were prepared by dissolving polysaccharide powder at 5mg powder/mL solution in deuterium oxide (D_2O) containing 0.01% dimethyl sulfoxide (DMSO) and 0.01% 2,2-Dimethyl-2-silapentane-5-sulfonate-d_6 sodium salt (DSS-d_6). DMSO is an internal standard that was used for quantitative analysis and DSS-d_6 was used to set the chemical shift scale to 0 ppm. A one-dimensional proton NMR data set was acquired at 50 °C and a portion of spectrum containing the anomeric resonances as then selectively written as x, y coordinates to an ASCII file for analysis using a Microsoft Excel workbook. The Y coordinates (i.e., spectral profile) were then compared to spectral profiles of capsular bacterial polysaccharides in a reference database. The reference profiles were generated in a similar manner on selected preparations of each serotype thereafter designated as the reference lot. A pairwise comparison was made of the y-values from the sample and the reference spectra to produce a correlation coefficient ($\rho_{x,y}$) as a measure of similarity between the spectra. A ρ-value of ≥ 0.95 with any of the reference spectra was taken as positive identification of the polysaccharide structure.

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FIGURES 1-4 provide the 600 MHz one-dimensional ^{1}H NMR spectra of capsular polysaccharides from *S. pneumonia* serotypes 6C, 15A, de-O-acetylated 15B and 35B, respectively. The ^{1}H NMR identity regions used for serotype identification of *S. pneumonia* serotypes 6C, 15A, de-O-acetylated 15B and 35B are provided in FIGURES 5-8, respectively.

Structure of capsular polysaccharides from S. pneumonia serotypes De-O-acetylated 15B and 15C

Immunogenicity studies were conducted using PCV21 in New Zealand White rabbits (see EXAMPLE 43, *infra*). In these studies, de-O-acetylated polysaccharide serotype 15B was used in the polyvalent composition in place of serotype 15C. NMR studies were conducted to confirm that de-O-acetylated 15B polysaccharide was equivalent to serotype 15C polysaccharide.

Structural differences between capsular polysaccharide serotypes show up as chemical shift differences in the NMR spectrum. The anomeric region (approximately 4.4 ppm to 6.0 ppm) is sensitive to every structural feature in the repeat unit of the polysaccharide. Differences in stereochemistry, monosaccharide composition, O-acetylation and glyosidic linkage impacts the chemical shifts of the anomeric signals leaving this region of the spectrum unique to each serotype. For de-O-acetylated serotype 15B polysaccharide, the complete ^{1}H NMR spectrum is identical to the ^{1}H NMR spectrum of serotype 15C polysaccharide, indicating the repeat unit of both polysaccharides consists of the same monosaccharide substituents and glyosidic linkage sites (see FIGURES 9B-9C and 10B-10C). It is also clearly shown that de-O-Acetylation step removed the O-Acetate group present in the 15B polysaccharide, and that there are essentially no observed O-Acetate groups left (see FIGURES 10A-10F).

EXAMPLE 4

Preparation of Serotype 3 Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in dimethylsulfoxide (DMSO). Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the

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Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 380 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.25 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 12 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 2 mg Ps/mL with sucrose concentration of 10% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 1.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.3. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 1 hour at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1.6 hours at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

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Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 1. Attributes of serotype 3 conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
186 / 253 kD	862/1468 kD	1.16	8.2	<1%	<1%

EXAMPLE 5

Preparation of Serotype 6C for Conjugate Monovalent Study Using DMSO Conjugation

Polysaccharide was dissolved, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Filtered dissolved polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.10 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

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The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 2.2 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.4. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 15 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 3 hours at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 2. Attributes of serotype 6C conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn /	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption	Free Ps / Total Ps	Free Protein / Total Protein

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Mw			(mol/ mol CRM197)		
144 / 229 kD	2038 / 4182 kD	1.20	9.2	3.1%	5.8%

EXAMPLE 6

Preparation of Serotype 6C Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Filtered dissolved polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.10 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

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Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 1.9 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.4. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 15 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 3 hours at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 3. Attributes of serotype 6C conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
144 / 229 kD	2104 / 5006 kD	1.11	8.4	4.9%	2.2%

Preparation of Serotype 6A Conjugate for Monovalent Studies Using DMSO Conjugation

Polysaccharide was dissolved, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

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Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 200 bar/5 passes. Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.10 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in Pseudomonas fluorescens as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 1.5 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.4. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 15 hour at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 3 hours at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at

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approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 3a. Attributes of serotype 6A conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
238 / 278 kD	2565 / 6310 kD	1.021	9.2	3%	5%

EXAMPLE 7

Preparation of Serotype 7F Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 150 bar/7 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide

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activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.24 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 4 hours at 4°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 2.6 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 4 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 3 hours at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

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The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 4. Attributes of serotype 7F conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
76 / 118 kD	1817 / 4026 kD	1.55	6.7	<1%	3.2%

EXAMPLE 8

Preparation of Serotype 8 Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 600 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.18 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 4 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

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Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 4.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. After the blend, the conjugation reaction proceeded for 2 hours at 22°C. Sodium cyanoborohydride was not added to the conjugation reaction because it was observed that sodium cyanoborohydride addition could result in irreversible precipitation during the conjugation reaction for serotype 8. Omitting sodium cyanoborohydride from the reaction avoided precipitation without significantly impacting conjugate attributes.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 5. Attributes of serotype 8 conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn/Mw	Conjugate Mn/Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein

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110 / 128 kD	1479 / 2196 kD	1.17	10.3	3.7%	<1%

EXAMPLE 9

Preparation of Serotype 9N Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 250 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.16 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 4 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

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Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.25 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 1 hour at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 6. Attributes of serotype 9N conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn/Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
195 / 226 kD	1407 / 3134 kD	1.33	10.1	1.9%	<1%

EXAMPLE 10

Preparation of Serotype 10A Conjugate for Monovalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide

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and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 200 bar/5 passes followed by 600 bar/5 passes to achieve a target molecular mass.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.15 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 5.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 2.0. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 4 hours at 22°C.

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Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 7. Attributes of serotype 10A conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
76 / 111 kD	5137 / 7061 kD	1.13	5.1	<1%	15%

EXAMPLE 11

Preparation of Serotype 10A Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the

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Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 600 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.16 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 4.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.75. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 4 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

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Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 8. Attributes of serotype 10A conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
94 / 130 kD	1540 / 3000 kD	1.54	7.7	8.5%	<1%

EXAMPLE 12

Preparation of Serotype 11A Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was size-reduced by acid hydrolysis by adding acetic acid to 200 mM, incubating at 92°C for 75 minutes, then neutralizing by adding cold potassium phosphate pH 7 buffer to 400 mM.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.13 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles

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aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO containing 25 mM sodium chloride. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.5 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 4 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ -60°C.

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Table 9. Attributes of serotype 11A conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn/Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
63 / 96 kD	1584 / 2804 kD	0.89	7.4	2.2%	2.8%

EXAMPLE 13

Preparation of Serotype 12F Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was size-reduced by acid hydrolysis by adding acetic acid to 200 mM, incubating at 90°C for 45 minutes, then neutralizing by adding cold potassium phosphate pH 7 buffer to 400 mM.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.26 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

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Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO containing 25 mM sodium chloride. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 4 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 3 hours at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 10. Attributes of serotype 12F conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
54 / 73 kD	1766 / 3119 Kd	1.20	9.6	1.3%	1.5%

EXAMPLE 14

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Preparation of Serotype 15A Conjugate for Monovalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 200 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 1.75 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 20 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO containing 25 mM sodium chloride. The polysaccharide and CRM197 solutions were blended to achieve a

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polysaccharide concentration of 6.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 2.0. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 2.5 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 11. Attributes of serotype 15A conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
158 / 200 kD	6949 / 9235 kD	1.05	9.7	10%	5.0%

EXAMPLE 15

Preparation of Serotype 15A Conjugate for 15A/B/C Cross Protection Study Using Aqueous Conjugation

Polysaccharide was dissolved, size reduced, chemically activated and buffer-exchanged by ultrafiltration. Purified CRM197 was then conjugated to the activated polysaccharide utilizing nickel chloride in the aqueous reaction mixture, and the resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

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Polysaccharide size reduction and oxidation

Purified pneumococcal capsular polysaccharide powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass. Homogenization pressure and number of passes through the homogenizer were controlled to 200 bar/5 passes to achieve a target molecular mass. Size-reduced polysaccharide was then concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.45 moles of sodium metaperiodate per mole of polysaccharide repeating unit. The oxidation reaction proceeded for 20 hours at 22°C. The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C. Further activation was achieved by adjusting the ultrafiltered product to 22°C and pH 5. Activation was conducted with the addition of 100 mM sodium metaperiodate solution with a charge of 2.0 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 20 hours at 22°C. The second activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Oxidized polysaccharide solution was mixed with water and 1.5 M potassium phosphate pH 6.0. The buffer pH selected was to improve the stability of activated polysaccharide during the conjugation reaction. Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was 0.2-micron filtered and combined with the buffered polysaccharide solution at a polysaccharide to CRM197 mass ratio of 0.6. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. The polysaccharide and phosphate concentrations were 9.75 g/L and 100 mM respectively. The polysaccharide concentration was selected to control the size of the resulting conjugate. Nickel chloride was added to approximately 2 mM using a 100 mM nickel chloride solution. Sodium cyanoborohydride (2 moles per mole of polysaccharide repeating unit) was added. Conjugation proceeded for 148 hours at 10°C to maximize consumption of polysaccharide and protein.

Reduction with sodium borohydride

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Following the conjugation reaction, the batch was diluted to a polysaccharide concentration of approximately 3.0 g/L, cooled to 2-8°C, and 1.2-micron filtered. The batch was diafiltered against 100 mM potassium phosphate, pH 7.0 at 2-8°C using a 100 kDa NMWCO tangential flow ultrafiltration membrane. The batch, recovered in the retentate, was then diluted to approximately 2.0 g polysaccharide/L and pH-adjusted with the addition of 1.2 M sodium bicarbonate, pH 9.4. Sodium borohydride (1 mole per mole of polysaccharide repeating unit) was added. 1.5 M potassium phosphate, pH 6.0 was later added.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM L-histidine in 150 mM sodium chloride, pH 7.0 at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane. Polysorbate 20 was added to the retentate batch to a concentration of 0.05% (w/v) then the batch was 0.2 micron filtered (with 0.5 micron prefilter).

The batch was adjusted to a polysaccharide concentration of 1.0 g/L with additional 10 mM L-histidine in 150 mM sodium chloride, pH 7.0 buffer with 0.03% (w/v) polysorbate 20. The batch was dispensed into aliquots and frozen at $\leq -60°C$.

Table 12. Attributes of serotype 15A conjugate for 15A/B/C cross protection study from aqueous conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
118 / 154 kDa	465 / 695 kDa	0.99	4.5	9.6%	<1%

EXAMPLE 16

Preparation of Serotype 15A Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the

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Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 200 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 1.75 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 20 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO which was pre-heated to 34°C. The polysaccharide solution was spiked with sodium chloride to a concentration of 50 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 5.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 2.0. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 2 hours at 34°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch

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was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 13. Attributes of serotype 15A conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
214 / 231 kD	1628 / 3518 kD	1.57	7.3	17%	7.8%

EXAMPLE 17

Preparation of Serotype 15B Conjugate for 15A/B/C Cross Protection Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 300 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide

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activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.20 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 4 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 2.0. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 5 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

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The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 14. Attributes of serotype 15B conjugate for 15A/B/C cross protection study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
203 / 252 kD	2329 / 3881 kD	1.67	7.9	3.6%	4.4%

EXAMPLE 18

Preparation of Serotype 15C Conjugate for Monovalent Study and 15A/B/C Cross Protection Study Using DMSO Conjugation

Polysaccharide derived from *Streptococcus pneumoniae* serotype 15B was dissolved, sized to a target molecular mass, subjected to mild base hydrolysis to release O-acetyl groups, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction, base hydrolysis and oxidation

Purified serotype 15B pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 300 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was heated to 60°C and sodium bicarbonate pH 9 buffer was added to a final concentration 50 mM. The batch was incubated with mixing for 13 hours at 60°C to release O-acetyl groups. Potassium phosphate pH 6 buffer was added to a final concentration of 136 mM to neutralize pH and the solution was cooled to ambient temperature. The solution was then concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

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The polysaccharide solution was adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.20 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 2.0. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 4 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

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The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 15. Attributes of serotype 15C conjugate for monovalent study and 15A/B/C cross protection study from DMSO conjugation

O-acetyl / Ps	Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
None detected	182 / 246 kD	1962 / 4019 kD	1.31	4.8	3.8%	13%

EXAMPLE 19

Preparation of Serotype 15C Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide derived from *Streptococcus pneumoniae* serotype 15B was dissolved, sized to a target molecular mass, subjected to mild base hydrolysis to release O-acetyl groups, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction, base hydrolysis and oxidation

Purified serotype 15B pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 300 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was heated to 60°C and sodium bicarbonate pH 9 buffer was added to a final concentration 50 mM. The batch was incubated with mixing for 13 hours at 60°C to release O-acetyl groups. Potassium phosphate pH 6 buffer was added to a final concentration of 136 mM to neutralize pH and the solution was cooled to ambient temperature.

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The solution was then concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.20 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.75. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 8 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

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The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 16. Attributes of serotype 15C conjugate ffor polyvalent study rom DMSO conjugation

O-acetyl / Ps	Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
None detected	182 / 246 kD	1540 / 3230 kD	1.34	6.6	9.2%	5.7%

EXAMPLE 20

Preparation of Serotype 16F Conjugate for Monovalent Study Using Aqueous Conjugation

Polysaccharide was dissolved, size reduced, chemically activated and buffer-exchanged by ultrafiltration. Purified CRM197 was then conjugated to the activated polysaccharide utilizing nickel chloride in the aqueous reaction mixture, and the resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular polysaccharide powder was dissolved in water, and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass. Homogenization pressure and number of passes through the homogenizer were controlled to 200 bar/5 passes followed by 500 bar/5 passes to achieve a target molecular mass. Size-reduced polysaccharide was then concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.15 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

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The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Oxidized polysaccharide solution was mixed with water and 1.5 M potassium phosphate pH 7.0. The buffer pH selected was to improve the stability of activated polysaccharide during the conjugation reaction. Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was 0.2-micron filtered and combined with the buffered polysaccharide solution at a polysaccharide to CRM197 mass ratio of 0.7. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. The polysaccharide and phosphate concentrations were 7.5 g/L and 100 mM respectively. The polysaccharide concentration was selected to control the size of the resulting conjugate. The solution was then 0.2-micron filtered. Nickel chloride was added to approximately 2 mM using a 100 mM nickel chloride solution. Sodium cyanoborohydride (2 moles per mole of polysaccharide repeating unit) was added. Conjugation proceeded for 122 hours at 22°C to maximize consumption of polysaccharide and protein.

Reduction with sodium borohydride

Following the conjugation reaction, the batch was diluted to a polysaccharide concentration of approximately 3.0 g/L, cooled to 2-8°C, and 1.2-micron filtered. The batch was diafiltered against 100 mM potassium phosphate, pH 7.0 at 2-8°C using a 100 kDa NMWCO tangential flow ultrafiltration membrane. The batch, recovered in the retentate, was then diluted to approximately 2.0 g polysaccharide/L and pH-adjusted with the addition of 1.2 M sodium bicarbonate, pH 9.4. Sodium borohydride (1 mole per mole of polysaccharide repeating unit) was added. 1.5 M potassium phosphate, pH 6.0 was later added.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM L-histidine in 150 mM sodium chloride, pH 7.0 at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane. Polysorbate 20 was added to the retentate batch to a concentration of 0.05% (w/v) then the batch was 0.2 micron filtered (with 0.5 micron prefilter).

The batch was adjusted to a polysaccharide concentration of 1.0 g/L with additional 10 mM L-histidine in 150 mM sodium chloride, pH 7.0 buffer with 0.03% (w/v) polysorbate 20. The batch was dispensed into aliquots and frozen at ≤ -60°C.

Table 17. Attributes of serotype 16F conjugate for monovalent study from aqueous conjugation

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Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
90 / 139 kD	1860 / 5539 kD	1.10	3.3	7.0%	<1%

EXAMPLE 21

Preparation of Serotype 16F Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 1000 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.15 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

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Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide solution was spiked with sodium chloride to a concentration of 50 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 2.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 2 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 18. Attributes of serotype 16F conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
91 / 177 kD	2075 / 3966 kD	1.29	11.0	<1%	<1%

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EXAMPLE 22

Preparation of Serotype 17F Conjugate for Monovalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 200 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.11 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The

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polysaccharide solution was spiked with sodium chloride to a concentration of 50 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 2.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 2 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 19. Attributes of serotype 17F conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
179/ 216 kD	2630 / 4632 kD	1.20	8.0	1.9%	4.5%

EXAMPLE 23

Preparation of Serotype 17F Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process

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parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 200 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.11 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide solution was spiked with sodium chloride to a concentration of 20 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 2.1 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 2 hours at 22°C.

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Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 20. Attributes of serotype 17F conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
150 / 212 kD	2650 / 4110 kD	1.10	7.7	2.8%	1.5%

EXAMPLE 24

Preparation of Serotype 19A Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. The polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

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The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The amount of sodium metaperiodate added was 0.26 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 20 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.8 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.33. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 1.5 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 3 hours at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kDa NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

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The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 21. Attributes of serotype 19A conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
96 / 186 kD	1714 / 3585 kD	1.22	9.1	7.0%	1.4%

EXAMPLE 25

Preparation of Serotype 20A Conjugate for Monovalent Study Using DMSO Conjugation

Polysaccharide previously determined to be serotype 20A (Calix *et al.*, Biochemical, Genetic, and Serological Characterization of Two Capsule Subtypes among *Streptococcus pneumoniae* Serotype 20 Strains, *J. Biol. Chem.* 287(33): 27885–27894, (2012)) was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 200 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. Sodium metaperiodate added at 0.11 moles of sodium metaperiodate per mole of polysaccharide

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repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 2.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 6 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

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Table 22. Attributes of serotype 20A conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
165 / 215 kD	5159 / 7778 kD	1.17	6.1	<1%	6.5%

EXAMPLE 26

Preparation of Serotype 20A Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 220 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. Sodium metaperiodate added at 0.16 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

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Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide solution was spiked with sodium chloride to a concentration of 20 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 1.4 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 4 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 23. Attributes of serotype 20A conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
178 / 215 kD	2478 / 3863 kD	1.10	8.3	1.0%	1.0%

EXAMPLE 27

Preparation of Serotype 22F Conjugate for Polyvalent Study Using DMSO Conjugation

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Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 400 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. Sodium metaperiodate added at 0.12 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 1.8 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium

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cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 1 hour at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 24. Attributes of serotype 22F conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
170 / 196 kD	1800 / 3970 kD	1.13	7.0	<1%	1.1%

EXAMPLE 28

Preparation of Serotype 23A Conjugate for Monovalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

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Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was size-reduced by acid hydrolysis by adding acetic acid to 200 mM, incubating at 90°C for 1.5 hours, then neutralizing by adding cold potassium phosphate pH 7 buffer to 400 mM.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The sodium metaperiodate added was 0.20 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide solution was spiked with sodium chloride to a concentration of 50 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added and conjugation proceeded for 2 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 3 hours at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at

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approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 25. Attributes of serotype 23A conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
70 / 97 kD	2183 / 3837 kD	1.20	11.8	<1%	<1%

EXAMPLE 29

Preparation of Serotype 23A Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was size-reduced by acid hydrolysis by adding acetic acid to 200 mM, incubating at 90°C for 1.5 hours, then neutralizing by adding cold potassium phosphate pH 7 buffer to 400 mM.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide

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activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The sodium metaperiodate added was 0.20 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.5 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added and conjugation proceeded for 2 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

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The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 26. Attributes of serotype 23A conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
116 / 175 kD	2156 / 4933 kD	1.12	6.3	3.3%	2.1%

EXAMPLE 30

Preparation of Serotype 23B Conjugate for Monovalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 400 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. Sodium metaperiodate added at 0.10 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

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Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide solution was spiked with sodium chloride to a final concentration of 50 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 5.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 2 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 27. Attributes of serotype 23B conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
155 / 179 kD	1322 / 3299 kD	1.28	6.2	13%	5.1%

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EXAMPLE 31

Preparation of Serotype 23B Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 400 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. Sodium metaperiodate added at 0.13 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The

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polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 5.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 4 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 28. Attributes of serotype 23B conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
172 / 197 kD	1076 / 2514 kD	1.26	7.4	12%	3.2%

EXAMPLE 32

Preparation of Serotype 24F Conjugate for Monovalent and Polyvalent Studies Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process

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parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was size-reduced by acid hydrolysis by adding acetic acid to 200 mM, incubating at 92°C for 50 minutes, then neutralizing by adding cold potassium phosphate pH 7 buffer to 400 mM.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The sodium metaperiodate added was 0.18 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 2 mg Ps/mL with sucrose concentration of 10% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide solution was spiked with sodium chloride to a final concentration of 50 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 1.5 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 2 hours at 22°C.

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Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 29. Attributes of serotype 24F conjugate for monovalent and polyvalent studies from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
56 / 100 kD	2233 / 4875 kD	0.94	7.3	4.4%	1.4%

EXAMPLE 33

Preparation of Serotype 31 Conjugate for Monovalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was size-reduced by acid hydrolysis by adding acetic

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acid to 200 mM, incubating at 90°C for 30 minutes, then neutralizing by adding cold potassium phosphate pH 7 buffer to 400 mM.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The sodium metaperiodate added was 0.16 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 4.0 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added and conjugation proceeded for 1 hour at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

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Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 30. Attributes of serotype 31 conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
96 / 119 kD	1818 / 2999 kD	1.15	9.5	<1%	<1%

EXAMPLE 34

Preparation of Serotype 31 Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 400 bar/5 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. Sodium metaperiodate added at 0.12 moles of sodium metaperiodate per mole of polysaccharide

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repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide solution was spiked with sodium chloride to a final concentration of 50 mM. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 3.5 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 1 hour at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

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Table 31. Attributes of serotype 31 conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
149 / 186 kD	2354 / 5971 kD	1.26	8.9	<1%	1.8%

EXAMPLE 35

Preparation of Serotype 33F Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, sized to a target molecular mass, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide size reduction and oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was homogenized to reduce the molecular mass of the Ps. Homogenization pressure and number of passes through the homogenizer were controlled to 350 bar/4 passes.

Size-reduced polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. Sodium metaperiodate was added at 0.12 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

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Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 materials were redissolved individually in equal volumes of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 1.8 g Ps/L and a polysaccharide to CRM197 mass ratio of 1.75. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Sodium cyanoborohydride (1 mole per mole of polysaccharide repeating unit) was added, and conjugation proceeded for 4 hours at 22°C.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 32. Attributes of serotype 33F conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
186 / 220 kD	1630 / 2470 kD	1.28	8.4	1.3%	5.4%

EXAMPLE 36

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Preparation of Serotype 35B Conjugate for Monovalent Study Using DMSO Conjugation

Polysaccharide was dissolved, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide oxidation

Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The sodium metaperiodate added was 0.05 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps and CRM197 were redissolved individually in equal volumes of DMSO containing 25 mM sodium chloride. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 10 g Ps/L and a polysaccharide to CRM197 mass ratio of 4.0. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Conjugation proceeded for 2.5 hours at 22°C. Sodium borohydride (0.025 moles per

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mole of polysaccharide repeating unit) was added over two spikes during the conjugation incubation.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1.5 hours at 22°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at $\leq -60°C$.

Table 33. Attributes of serotype 35B conjugate for monovalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
48 / 82 kD	5494 / 7385 kD	1.26	4.5	1.8%	11%

EXAMPLE 37

Preparation of Serotype 35B Conjugate for Polyvalent Study Using DMSO Conjugation

Polysaccharide was dissolved, chemically activated and buffer-exchanged by ultrafiltration. Activated polysaccharide and purified CRM197 were individually lyophilized and redissolved in DMSO. Redissolved polysaccharide and CRM197 solutions were then combined and conjugated as described below. The resulting conjugate was purified by ultrafiltration prior to a final 0.2-micron filtration. Several process parameters within each step, such as pH, temperature, concentration, and time were controlled to yield conjugates with desired attributes.

Polysaccharide oxidation

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Purified pneumococcal capsular Ps powder was dissolved in water and 0.45-micron filtered. Dissolved polysaccharide was concentrated and diafiltered against water using a 10 kDa NMWCO tangential flow ultrafiltration membrane.

The polysaccharide solution was then adjusted to 22°C and pH 5 with a sodium acetate buffer to minimize polysaccharide size reduction due to activation. Polysaccharide activation was initiated with the addition of a 100 mM sodium metaperiodate solution. The sodium metaperiodate added was 0.05 moles of sodium metaperiodate per mole of polysaccharide repeating unit to achieve a target level of polysaccharide activation (moles aldehyde per mole of polysaccharide repeating unit). The oxidation reaction proceeded for 2 hours at 22°C.

The activated product was diafiltered against 10 mM potassium phosphate, pH 6.4 followed by diafiltration against water using a 5 kDa NMWCO tangential flow ultrafiltration membrane. Ultrafiltration was conducted at 2-8°C.

Polysaccharide conjugation to CRM197

Purified CRM197, obtained through expression in *Pseudomonas fluorescens* as previously described (WO 2012/173876 A1), was diafiltered against 2 mM phosphate, pH 7.2 buffer using a 5 kDa NMWCO tangential flow ultrafiltration membrane and 0.2-micron filtered.

Activated polysaccharides were formulated for lyophilization at 6 mg Ps/mL with sucrose concentration of 5% w/v. CRM197 was formulated for lyophilization at 6 mg Pr/mL with sucrose concentration of 1% w/v.

Formulated Ps and CRM197 solutions were individually lyophilized. Lyophilized Ps was redissolved in DMSO containing 50 mM sodium chloride. Lyophilized CRM197 was redissolved in an equal volume of DMSO. The polysaccharide and CRM197 solutions were blended to achieve a polysaccharide concentration of 5.25 g Ps/L and a polysaccharide to CRM197 mass ratio of 3.5. The mass ratio was selected to control the polysaccharide to CRM197 ratio in the resulting conjugate. Conjugation proceeded for 3 hours at 34°C. Sodium borohydride (0.0375 moles per mole of polysaccharide repeating unit) was added over three spikes during the conjugation incubation.

Reduction with sodium borohydride

Sodium borohydride (2 moles per mole of polysaccharide repeating unit) was added following the conjugation reaction and incubated for 1 hour at 34°C. The batch was diluted into 150 mM sodium chloride, with approximately 0.025% (w/v) polysorbate 20, at approximately 4°C. Potassium phosphate buffer was then added to neutralize the pH. The batch was concentrated and diafiltered at approximately 4°C against 150 mM sodium chloride, 25 mM potassium phosphate pH 7, using a 30 kD NMWCO tangential flow ultrafiltration membrane.

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Final filtration and product storage

The batch was then concentrated and diafiltered against 10 mM histidine in 150 mM sodium chloride, pH 7.0, with 0.015% (w/v) polysorbate 20, at 4°C using a 300 kDa NMWCO tangential flow ultrafiltration membrane.

The retentate batch was 0.2 micron filtered (with 0.5 micron prefilter) then diluted with additional 10 mM histidine in 150 mM sodium chloride, pH 7.0 with 0.015% (w/v) polysorbate 20, dispensed into aliquots and frozen at ≤ −60°C.

Table 34. Attributes of serotype 35B conjugate for polyvalent study from DMSO conjugation

Oxidized Ps Mn / Mw	Conjugate Mn / Mw	Ps:Pr	Lysine Consumption (mol/ mol CRM197)	Free Ps / Total Ps	Free Protein / Total Protein
48 / 82 kD	1287 / 2585 kD	2.1	6.9	7.8%	4.6%

Some aqueous conjugation methods were not described for all serotypes in Examples 38-51. For those aqueous conjugation methods not described herein, similar methods can be found in WO2018/156491.

EXAMPLE 38

Formulation of Pneumococcal Conjugate Vaccines

Individual pneumococcal polysaccharide-protein conjugates prepared utilizing different chemistries as described in the Examples, *supra*, were used for the formulation of 1-, 7-, 8-, 14-, 15-, 16-, 21- and 31-valent pneumococcal conjugate vaccines referred to as PCV1, PCV7, PCV8, PCV14, PCV15, PCV16, PCV21, and PCV31, respectively.

The PCV1 vaccine drug product formulation contained serotype 3 conjugated either using reductive amination in a protic (aqueous) solvent or an aprotic (DMSO) solvent and formulated in 20 mM L-Histidine pH 5.8, 150 mM NaCl and 0.1% (w/v) PS-20 for a target final concentration of 84 µg/mL (w/v) Pneumococcal Polysaccharide (PnPs) in the vaccine.

The PCV7 vaccine drug product formulation contained serotypes 3, 8, 9N, 10A, 11A, 15A and 19A, conjugated either using reductive amination in a protic (aqueous) solvent or an aprotic (e.g. DMSO) and formulated in 20 mM L-Histidine pH 5.8, 150 mM NaCl and 0.1% (w/v) PS-20. Each polysaccharide-protein conjugate in PCV7 was formlulated at 12 µg/mL (w/v) Pneumococcal Polysaccharide (PnPs) for a target final concentration of 84 µg/mL PnPs in the vaccine.

The PCV8 vaccine drug product contained serotypes 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B conjugated either using reductive amination in an aprotic solvent (e.g DMSO) and formulated in 20 mM L-Histidine pH 5.8, 150 mM NaCl and 0.2% (w/v) PS-20. Each

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polysaccharide-protein conjugate in PCV8 was formlulated at 4 μg/mL (w/v) Pneumococcal Polysaccharide (PnPs) for a target final concentration of 32 μg/mL PnPs in the vaccine.

The PCV14 vaccine drug product contained serotypes 3, 7F, 8, 9N, 10A, 11A, 12F, 15A, 16F, 17F, 19A, 20A, 22F and 33F conjugated either using reductive amination in a protic (aqueous) solvent or an aprotic (e.g. DMSO)and formulated in 20 mM L-Histidine pH 5.8, 150 mM NaCl and 0.1% (w/v) PS-20. Each polysaccharide-protein conjugate in PCV14 was formulated at 6 μg/mL (w/v) Pneumococcal Polysaccharide (PnPs) for a target final concentration of 84 μg/mL PnPs in the vaccine.

The PCV15 vaccine drug product contained serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F conjugated in a protic (aqueous) solvent and formulated in 20 mM L-Histidine pH 5.8, 150 mM NaCl and 0.2% (w/v) PS-20. Each polysaccharide-protein conjugate was formulated at 4 μg/mL (w/v) Pneumococcal Polysaccharide (PnPs), except for 6B, which was formulated at 8 μg/mL, for a target final concentration of 64 μg/mL PnPs in the vaccine.

The PCV16 vaccine drug product contained serotypes 6C, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 20A, 23A, 23B, 24F, 31, and 35B, conjugated using reductive amination in an aprotic solvent (e.g. DMSO) and formulated in 20 mM L-Histidine pH 5.8 150 mM NaCl and 0.2% (w/v) PS-20. Each polysaccharide-protein conjugate was formulated at 4 μg/mL (w/v) or 8 μg/mL (w/v) Pneumococcal Polysaccharide (PnPs) for a target final concentration of 64 μg/mL or 128 μg/mL PnPs in the vaccine.

The PCV21 vaccine drug product contained serotypes 3, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 19A, 20A, 22F, 23A, 23B, 24F, 31, 33F and 35B, conjugated using reductive amination in an aprotic solvent (e.g. DMSO) and formulated in 20 mM L-Histidine pH 5.8 150 mM NaCl and various concentration of PS-20 as defined in each example. Each polysaccharide-protein conjugate was formulated at 4 μg/mL (w/v) or 8 μg/mL (w/v) Pneumococcal Polysaccharide (PnPs) for a target final concentration of 84 μg/mL or 168 μg/mL PnPs in the vaccine. In some examples, PCV21 was formulated with 250 μg [Al]/mL in the form of Aluminum Phosphate.

The PCV31 vaccine drug product contained serotypes 1, 3, 4, 5, 6A, 6B, 6C, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15A, 15C, 16F, 17F, 18C, 19A, 19F, 20A, 22F, 23A, 23B, 23F, 24F, 31, 33F and 35B conjugated using reductive amination in a protic solvent (aqueous) or an aprotic solvent (e.g. DMSO) and formulated in 20 mM L-Histidine pH 5.8 150 mM NaCl and 0.2% PS-20. Each polysaccharide-protein conjugate was formulated at 4 μg/mL (w/v) Pneumococcal Polysaccharide (PnPs), except for 6B, which was formulated at 8 μg/mL PnPs, for a target final concentration of 128 μg/mL PnPs in the vaccine. In some examples, PCV31 was formulated with 250 μg [Al]/mL in the form of Aluminum Phosphate.

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Additional monovalent PCV drug product formulations were prepared utilizing pnemococcal polysaccharide-protein conjugate serotypes 6A, 6B, 6C, 10A, 15A, 15B, 15C, 16F, 17F, 20A, 23A, 23B, 24F, 31 or 35B. Additional details are described in the specific examples for these formulations.

To prepare the PCV drug product formulation, the required volumes of bulk conjugates needed to obtain the indicated final concentration of (w/v) Pneumococcal Polysaccharide (also referred to as PnPs) were calculated based on the batch volume and the bulk polysaccharide concentration.

The formulation process consisted of a conjugate bulk blend preparation at 1X to 4X of final concentration of PnPs blends in 10-80 mM Histidine, 0.0-0.8% (w/v) PS-20, and150 mM sodium chloride, pH 5.8.

Histidine pH 5.8, PS-20 (if utilized) and 150 mM sodium chloride solutions were prepared and added to the formulation vessel. The individual pneumococcal polysaccharide-protein conjugates, stored frozen, were thawed at 2-8°C and then added to the formulation vessel. During the addition of polysaccharide-protein conjugate to the formulation buffer (conjugate blend), the vessel was mixed to ensure homogeneity using a magnetic sir bar or magnetic impeller. After all additions were made and the solution was stirred, the conjugate blend was passed through sterilizing filters and collected in a vessel with or without Aluminum phosphate adjuvant. In some cases, the sterilizing filters were chased with 150 mM sodium chloride to adjust the batch to target concentration.

The formulations were filled into plastic syringes, glass syringes, or vials.

EXAMPLE 39

Stability Assessment of Pneumococcal Conjugate Vaccines

The PCV16 (128 µg/mL PnPs) or two PCV21 pneumococcal conjugate vaccine drug products (84 µg/mL or 168 µg/mL PnPs) were filled in syringes or vials. The polysaccharide protein conjugates were made via reductive amination in an aprotic solvent (e.g. DMSO) and were formulated in 20 mM L-Histidine, pH 5.8, 150 mM NaCl, and 0.2% (w/v) PS-20. These drug products were placed at 25°C or 37°C for up to four weeks and at 4°C for up to twelve weeks. In some cases, the syringes were placed on a horizontal rotation platform and shaken for up to 18 hours after storage for 1 week at 4°C, 25°C or 37°C. This was done to simulate the shipping and handling stresses that may accompany drug product manufacture and distribution. To assess stability of the PCV16 or PCV21 drug products, HPSEC UV/MALS/RI was used. High Performance Size Exclusion Chromatography (HPSEC), using two Shodex columns (803 and 806), coupled together in series and Ultraviolet (UV) multi-angle light scattering (MALS) and refractive index (RI) detection to measure concentration and molar mass of PCV drug product formulations during storage. The concentration is calculated for each time

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interval across the peak and is then integrated at all intervals to achieve the final concentration value. Light scattering is proportional to the product of the molecular mass and the concentration of the analyte. Molar masses or Molecular weight at each interval is calculated from the detector signals. The weighted average (Mw) or number average (Mn) molecular mass is calculated across all intervals for the peak and reported as average molecular weight.

Storage may result in damage to protein and/or carbohydrate of a conjugate vaccine. Carbohydrate antigens that possess phosphodiester main-chain bonds or other instabilities in the repeat unit of the polysaccharide may be susceptible to depolymerization through hydrolysis upon storage for extended time and temperature. Furthermore, the carrier protein or carbohydrate that is used in the conjugate vaccine may be susceptible to aggregation upon storage and physical stresses.

As shown in FIGURE 11, the PCV16 and PCV21 drug products were stable through 4 weeks at25°C and 37°C and up to 12 wks at 4°C regardless of polysaccharide concentration. Moreover, upon horizontal agitation after thermal stress, the PCV16 and PCV21 drug products were shown to be stable. No loss of antigen quantity due to non-specific adsorption to the side of the containers orvessels was observed under these thermal and physical stresses. Additionally, no aggregation of the drug product was observed which would impact its performance on the column (e.g. irreversible binding to the column or unable to enter/exit the resolving columns) in estimating total dose of the vaccine (FIGURE 12).

HPSEC UV/MALs/RI was used to measure the molecular weight of a polysaccharide-protein conjugate. If the polysaccharide-protein conjugates in the drug product were degrading or depolymerizing, a decrease in molecular weight would be evident and if aggregation of the drug product was occurring, an increase in molecular weight would occur. This measurement provides additional characterization on the quality of the vaccine drug product or intermediate. As shown in FIGURE 13, the PCV16 and PCV21 drug products comprising drug substances that were each prepared using reductive amination in an aprotic solvent (e.g. DMSO) were stable against depolymerization or chemical degradation of the carbohydrate and stable against aggregation of the protein in the drug product formulation. This indicates that the PCV16 and PCV21 drug products using polysaccharide protein conjugates prepared using reductive amination in an aprotic solvent , such as DMSO, are stable through elevated temperature and physical stresses. The formulations demonstrate excellent stability in terms of both quantity and quality. The formulation does not lose total dose due to non-specific adsorption to the side of the containers or other surfaces and the formulation does not degrade or aggregate as indicated by the stable molecular weight of the vaccine drug product (weight averaged molecular weight (Mw) and number averaged molecular weight (Mn)).

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Impact of Conjugation Chemistry on Stability of a Pneumococcal Conjugate Vaccine

Individual pneumococcal polysaccharide-protein conjugates prepared utilizing different chemistries as described in the Examples, *supra*, were used for the formulation of 15- and a 16- valent pneumococcal conjugate vaccines referred to as PCV15 and PCV16 at 64 μg/mL. The PCV15 vaccine drug product contained serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F, conjugated using reductive amination in a protic solvent (all aqueous) and formulated in 20 mM L-Histidine pH 5.8, 150 mM NaCl and 0.2% w/v PS-20 (see EXAMPLE 38). The PCV16 drug product contained serotypes 6C, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 20A, 23A, 23B, 24F, 31, and 35B, conjugated using reductive amination in an aprotic solvent (e.g. all DMSO) and formulated in 20 mM L-Histidine, pH 5.8, 150 mM NaCl, and 0.2% (w/v) PS-20 (see EXAMPLE 38). Each polysaccharide-protein conjugate was formulated at 4 μg/mL (w/v) Pneumococcal Polysaccharide (PnPs) except for 6B in PCV15, which was formulated at 8 μg/mL, for a final concentration of 64 μg/mL PnPs in each vaccine.

The vaccine-filled containers were placed at 4°C or 37°C for up to 1 week. Intrinsic Protein Fluorescence Spectroscopy (IPFS) was used to assess the stability of pneumococcal polysaccharide-conjugated carrier protein, CRM197, in the PCV15 or PCV16 compositions upon storage for up to 1 week at 4 °C and 37 °C. Fluorescence emission spectra (Em 290-400 nm) of undiluted samples were collected using a Jasco FP-6500 Spectrofluorometer at ambient room temperature in a 1 cm path length quartz cuvette. An Excitation wavelength of 280 nm was used with a scan speed of 100 nm/min and excitation and emission bandpass of 3nm.

As shown in FIGURE 14, the drug product formulation containing drug substances prepared using reductive amination in an aprotic solvent (e.g. all DMSO) resulted in superior physical and chemical stability as compared to a vaccine utilizing drug substances prepared using a protic solvent during reductive amination in the conjugation process (all aqueous conjugation). In as little as 16 hours at 37°C, the fluorescence intensity of the PCV15 drug product, at 64 μg/mL PnPs, prepared using reductive amination in a protic solvent (all aqueous) for the conjugation process was reduced and the emission maximum of the vaccine shifted from 332 nm to 338 nm. The PCV16 vaccine drug product, at 64 μg/mL PnPs, utilizing drug substances prepared with reductive amination chemistry in an aprotic solvent (e.g. all DMSO) did not show a change in fluorescence intensity or emission maximum 338 nm. It should be understood that the resulting signal intensity and emission maximum acquired from the IPFS measurement is due to the collective stability of the individual drug substances in a multivalent complex drug product. We have shown that a vaccine drug product utilizing all drug substances prepared in an aprotic solvent is stable while a vaccine drug product utilizing all drug substances prepared in an a protic solvent is not stable.

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Without being bound to any particular theory, this stability could be due to conjugation in DMSO resulting in unfolding and denaturing the carrier protein. Conjugation to the denatured carrier protein would lock the conformation in denatured state post conjugation. If so, this study would also suggest the final conformation is stable under the stress study evaluated here. It is therefore preferred to use a vaccine with conjugate mostly prepared with aprotic solvents to provide a consistent and stable vaccine drug product.

If a vaccine drug product composition included a mixture of drug substances, glycoconjugates or polysaccharide protein conjugates utilizing different reductive amination solvents (aqueous or DMSO) in the conjugation processes, the average emission maximum or intensity measured from the drug product formulation would be weighted due to the contribution of each aqueous polysaccharide protein conjugate emission at 332 nm and each non-aqueous polysaccharide protein conjugate emission at 338 nm. It would be expected that a change would occur, in either intensity or emission maxima, upon elevated temperature for such a mixture that does not use all polysaccharide protein conjugates, prepared using reductive amination in an aprotic solvent, in the multivalent drug product formulation. It is therefore preferred to use a vaccine with conjugate mostly prepared with aprotic solvents to provide a consistent and stable vaccine drug product.

EXAMPLE 41

Compiled Monovalent Rabbit Immunogenicity Studies for Selected Serotypes

Adult New Zealand White rabbits (NZWR, n=3/group) were intramuscularly (IM) immunized with 0.25 ml or 0.5 mL (for 15C only) of respective monovalent conjugate vaccine on day 0 and day 14 (alternating sides). Monovalent pneumococcal vaccines, formulated in 20 mM L-Histidine, pH 5.8, 150 mM NaCl, and 0.2% (w/v) PS-20 and formulation process described in Example 38, were dosed as follows: (1) 1 μg PnPs (6C, 10A, 15A, 16F, 17F, 20A, 23A, 23B, 24F, 31 or 35B, each conjugated to CRM197) with 62.5 μg aluminum phosphate adjuvant (APA) per immunization, or (2) 2 μg PnPs (15C- CRM197 with 125 μg APA per immunization. Sera were collected prior to study start (pre-immune) and on days 14 (post-dose 1, PD1) and 28 (post-dose 2, PD2). NZWRs were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in NZWRs were deemed to be safe and well tolerated, as no vaccine-related adverse events were noted. All animal experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The NZWR experimental protocol was approved by the Institutional Animal Care and Use Committees at both Merck & Co., Inc. (Kenilworth, NJ) and Covance (Denver, PA).

NZWR sera were tested in ELISA assays to evaluate IgG immunogenicity using a 1-2 mg/mL respective PnPs coating concentration. Functional antibody was determined through

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opsonophagocytosis assays (OPA) based on previously described protocols available online at the Bacterial Respiratory Pathogen Reference Laboratory at the University of Alabama at Birmingham using Opsotiter® 3 software (UAB Research Foundation, Caro-Aguilar *et al.* Immunogenicity differences of a 15-valent pneumococcal polysaccharide conjugate vaccine (PCV15) based on vaccine dose, route of immunization and mouse strain. *Vaccine* 35(6):865-72 (2017); Burton *et al.* Development and validation of a fourfold multiplexed opsonization assay (MOPA4) for pneumococcal antibodies. *Clin Vaccine Immunol* 13(9):1004-9 (2006)).

All monovalent pneumococcal conjugate vaccines were found to be immunogenic in rabbits (FIGURE 15) and generate functional antibody which killed the respective bacterial strain (FIGURE 16).

EXAMPLE 42

Evaluation of Cross Protection of Serotypes 15A, 15B, 15C

Rabbits were immunized with 15A- CRM197/APA, 15B- CRM197/APA or 15C-CRM197/APA to evaluate cross-reactivity between each serotype.

Adult New Zealand White rabbits (NZWR, n=3/group) were intramuscularly (IM) immunized with 0.5 ml of respective monovalent conjugate vaccine on day 0 and day 14 (alternating sides). Monovalent pneumococcal conjugate vaccine, formulated in 20 mM L-Histidine, pH 5.8, 150 mM NaCl, and 0.2% (w/v) PS-20 and formulation process described in Example 38, was dosed at 2 µg PnPs (15A, 15B or 15C each conjugated to CRM197) with 125 µg APA per immunization. Sera were collected prior to study start (pre-immune) and on days 14 (post-dose 1, PD1) and 28 (post-dose 2, PD2). NZWRs were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in NZWRs were deemed to be safe and well tolerated, as no vaccine-related adverse events were noted. All animal experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The NZWR experimental protocol was approved by the Institutional Animal Care and Use Committees at both Merck & Co., Inc and Covance (Denver, PA).

NZWR sera were tested in ELISA assays to evaluate IgG immunogenicity using a 1-2 mg/mL respective PnPs coating concentration. Functional antibody was determined through OPA based on previously described protocols available online at the Bacterial Respiratory Pathogen Reference Laboratory at the University of Alabama at Birmingham using Opsotiter® 3 software (UAB Research Foundation (Caro-Aguilar *et al.*, 2017; Burton *et al.*, 2006).

All three monovalent pneumococcal conjugate vaccines of serogroup 15 were found to be immunogenic in rabbits (FIGURE 17) and generate functional antibody which killed

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the respective bacterial strain (FIGURE 18). In addition, rabbits immunized with serogroup 15 monovalent pneumococcal conjugate vaccines had equivalent PD2 IgG and OPA titers to the homologous and heterologous polysaccharide and bacterial strain, respectively. Rabbits immunized with 15A-CRM197/APA, 15B-CRM197/APA or 15C-CRM197/APA all had cross reactivity to each pneumococcal polysaccharide (15A, 15B, 15C) (FIGURE 17). Using post dose 2 (PD2) log transformed IgG data analyzed by One-way ANOVA with Tukey's multiple comparison test (P-value=0.354), there was no significant difference in the IgG titers across serogroup 15. In addition, rabbits immunized with 15A-CRM197/APA, 15B-CRM197/APA or 15C-CRM197/APA all had cross reactivity to each *S. pneumoniae* bacterial strain, (15A, 15B, 15C), as all rabbit hyper immune sera had functional antibody to each strain evaluated and killed the bacteria (FIGURE 18). Similarly, using post dose 2 (PD2) log transformed OPA data analyzed by One-way ANOVA with Tukey's multiple comparison test (P-Value=0.054), there was no significant difference in OPA titers across the serogroup 15.

EXAMPLE 43

Immunogenicity of PCV21 in New Zealand White Rabbits

Adult New Zealand White rabbits (NZWR, n=5/group) were intramuscularly (IM) immunized with 0.1-0.5 mL of pneumococcal conjugate vaccine on day 0 and day 14 (alternating sides). A PCV21 pneumococcal vaccine, formulated in 20 mM L-Histidine, pH 5.8, 150 mM NaCl, and 0.2% (w/v) PS-20 and formulation process described in Example 38, was dosed at 4, 2, 1, 0.4, 0.08 or 0.016 µg PnPs (3, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 19A, 20A, 22F, 23A, 23B, 24F, 31, 33F or 35B, each conjugated to CRM197) per immunization. Sera were collected prior to study start (pre-immune) and on days 14 (post-dose 1, PD1) and 28 (post-dose 2, PD2). NZWRs were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in NZWRs were deemed to be safe and well tolerated, as no vaccine-related adverse events were noted. All animal experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The NZWR experimental protocol was approved by the Institutional Animal Care and Use Committees at both Merck & Co., Inc and Covance (Denver, PA).

NZWR sera were evaluated for IgG immunogenicity using a multiplexed electrochemiluminescence (ECL) assay. This assay was developed for use with rabbit serum based on the human assay described by Marchese *et al.* (Optimization and validation of a multiplex, electrochemiluminescence-based detection assay for the quantitation of immunoglobulin G serotype-specific antipneumococcal antibodies in human serum. *Clin Vaccine Immunol.* 16(3): 387-96 (2009)) using technology developed by MesoScale Discovery (a division of MesoScale Diagnostics, LLC, Gaithersburg, MD) which utilizes a SULFO-TAG™

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label that emits light upon electrochemical stimulation. SULFO-TAG™-labeled anti-rabbit IgG was used as the secondary antibody for testing NZWR serum samples. Functional antibody was determined through multiplexed opsonophagocytic assays (MOPA) based on previously described protocols available online at the Bacterial Respiratory Pathogen Reference Laboratory at the University of Alabama at Birmingham using Opsotiter® 3 software (UAB Research Foundation, Caro-Aguilar *et al*, 2017, *supra*, Burton *et al.*, 2006, *supra*).

PCV21 pneumococcal conjugate vaccines were found to be immunogenic in rabbits (FIGURES 19A, 19B, 20A, and 20B) and generate functional antibody which killed vaccine-type bacterial strains (FIGURES 21A and 21B) at all doses tested.

Comparable PD1 immunogenicity was observed for PCV21 dosed at 4, 2, 1 and 0.4 µg per PnPs, with the exception of 24F which had higher immunogenicity in the 2 µg dose compared to the 4 or 1 µg doses and 15A, 15B and 15C which had higher immunogenicity in the 0.4 µg dose compared to the 2 µg dose (FIGURES 19A and 19B). Therefore, there was not much of a vaccine dose response at the higher PnPs doses (0.4-4 µg/PnPs). Lower immunogenicity was observed at the 0.08 and 0.016 µg PnPs vaccine doses, as many serotypes were significantly lower when compared to the 2 µg vaccine dose.

Comparable PD2 immunogenicity was observed for PCV21 dosed at 4, 2, 1 and 0.4 µg per PnPs (FIGURES 20A and 20B).

In general comparable PD2 MOPA titers were observed for PCV21 dosed at 4, 2, 1 and 0.4 µg per PnPs with MOPA titers trending lower for the 0.08 and 0.016 µg PnPs vaccine doses, although many not reaching statistical significance (data not shown). PCV21 dosed at 2 µg per PnPs was selected as a representative data set for MOPA. Specifically, rabbits immunized with PCV21 at the 2 µg dose had significantly higher PD1 MOPA titers for all serotypes compared to pre-immune rabbit sera with the exception of serotype 3 (FIGURE 21A). It should be noted that rabbit pre-immune sera from this study had higher background titers to serotypes 16F, 31 and 35B. Rabbits immunized with PCV21 at the 2 µg dose had significantly higher PD2 MOPA titers for all serotypes compared to pre-immune rabbit sera (FIGURE 21B). Log Transformed data were analyzed by One-way ANOVA with Dunnett's test to determine significance.

EXAMPLE 44

Materials and Methods

Free polysaccharide testing

Free polysaccharide (polysaccharide that is not conjugated with CRM197) in conjugate sample is measured by first precipitating free protein and conjugates with deoxycholate (DOC) and hydrochloric acid. Precipitates are then filtered out and the filtrates are analyzed for free polysaccharide concentration by HPSEC/UV/MALS/RI. Free polysaccharide is calculated as a percentage of total polysaccharide measured by HPSEC/UV/MALS/RI.

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Free protein testing

Free polysaccharide, polysaccharide-CRM197 conjugate, and free CRM197 in conjugate samples are separated by capillary electrophoresis in micellar electrokinetic chromatography (MEKC) mode. Briefly, samples are mixed with MEKC running buffer containing 25mM borate, 100mM SDS, pH 9.3, and are separated in a preconditioned bare-fused silica capillary. Separation is monitored at 200 nm and free CRM197 is quantified with a CRM197 standard curve. Free protein results are reported as a percentage of total protein content determined by the HPSEC/UV/MALS/RI procedure.

Molecular weight and concentration analysis of conjugates using HPSEC/UV/MALS/RI assay

Conjugate samples were injected and separated by high performance size-exclusion chromatography (HPSEC). Detection was accomplished with ultraviolet (UV), multi-angle light scattering (MALS) and refractive index (RI) detectors in series. Protein concentration was calculated from UV280 using an extinction coefficient. Polysaccharide concentration was deconvoluted from the RI signal (contributed by both protein and polysaccharide) using the dn/dc factors which are the change in a solution's refractive index with a change in the solute concentration reported in mL/g. Average molecular weight of the samples were calculated by Astra software (Wyatt Technology Corporation, Santa Barbara, CA) using the measured concentration and light scattering information across the entire sample peak. There are multiple form of average values of molecular weight for polydispersed molecules. For example number-average molecular weight Mn, weight-average molecular weight Mw, and z-average molecular weight Mz (Molecules, 2015, 20, 10313-10341). Unless specified, the molecular weights are weight-average molecular weight.

Determination of lysine consumption in conjugated protein as a measure of the number of covalent attachments between polysaccharide and carrier protein

The Waters AccQ-Tag amino acid analysis (AAA) was used to measure the extent of conjugation in conjugate samples. Samples were hydrolyzed using vapor phase acid hydrolysis in the Eldex workstation, to break the carrier proteins down into their component amino acids. The free amino acids were derivatized using 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC). The derivatized samples were then analyzed using UPLC with UV detection on a C18 column. The average protein concentration was obtained using representative amino acids other than lysine. Lysine consumption during conjugation (i.e., lysine loss) was determined by the difference between the average measured amount of lysine in the conjugate and the expected amount of lysine in the starting protein.

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EXAMPLE 45

Impact of Conjugation Process on Stability of a Pneumococcal Conjugate Vaccine

Individual pneumococcal polysaccharide-protein conjugates prepared utilizing different reductive amination solvents (aqueous or DMSO) as described in the Examples, supra, were used for the formulation of a 1-, 7-, 14-, and 21-valent pneumococcal conjugate vaccine drug product referred to as PCV1, PCV7, PCV14 and PCV21 at 84 μg/mL.

The drug product vaccine-filled containers were placed at 4°C or 37°C for up to 7 days. Intrinsic Protein Fluorescence Spectroscopy (IPFS) was used to assess the stability of pneumococcal polysaccharide-conjugated carrier protein, CRM197, in the PCV1, PCV7, PCV14 or PCV21 compositions upon storage for up to at 4°C and 37°C. Fluorescence emission spectra (Em 290-400 nm) of undiluted samples were collected using a Jasco FP-6500 Spectrofluorometer at ambient room temperature in a 1 cm path length quartz cuvette. An Excitation wavelength of 280 nm was used with a scan speed of 100 nm/min and excitation and emission bandpass of 3nm.

As shown in FIGURES 22 A-C, the drug product formulations containing all polysaccharide-protein conjugates prepared using reductive amination in an aprotic solvent (e.g. DMSO) resulted in superior physical and chemical stability as compared to a vaccine utilizing all polysaccharide-protein conjugates prepared using aqueous solvent during reductive amination in the conjugation process (protic). In as little as 16 hours at 37°C, the fluorescence intensity of the PCV1, PCV7 and PCV14 drug products prepared using all polysaccarhide-protein conjugates which were prepared using reductive amination in an aqueous solvent at 84 μg/mL PnPs was reduced and the emission maximum of the vaccine shifted from 332 nm to 338 nm. The PCV1, PCV7 and PCV14 vaccine drug products, at 84 μg/mL PnPs, utilizing drug substances prepared with reductive amination chemistry in an aprotic solvent (e.g. DMSO) did not show a change in fluorescence intensity or emission maximum at 338 nm. Moreover, a PCV21 drug product was also studied. It was formulated in 20 mM L-Histidine, pH 5.8, 150 mM NaCl, and 0.1% (w/v) PS-20 asdescribed in Example 38, containing polysaccharide-protein conjugates prepared using reductive amination in an aprotic solvent (e.g. DMSO). The study showed superior physical and chemical stability after storage at 37°C. PCV21 did not show a change in fluorescence intensity or emission maximum 338 nm (FIGURE 22D).

It should be understood that the resulting signal intensity and emission maximum acquired from the IPFS (EXAMPLE 40) measurement is due to the collective stability of the individual polysaccharide-protein conjugates in a multivalent complex drug product. We have shown that a vaccine drug product utilizing all polysaccharide-protein conjugates prepared in an aprotic solvent is stable while a vaccine drug product utilizing all drug substances prepared in a protic solvent is not stable under stressed conditions. If a vaccine drug product composition included a mixture of polysaccharide-protein conjugates using both protic and an aprotic

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conjugation process, the average emission maximum or intensity measured from the drug product would be weighted due to the contribution of each protic polysaccharide-protein conjugates emission at 332 nm and each aprotic of polysaccharide-protein conjugate emission at 338 nm. It would be expected that a weighted change would occur, in either intensity or emission maxima, upon elevated temperature for such a mixture due to the presence of the polysaccharide protein conjugates prepared using a protic conjugation process.

As shown in FIGURE 23, to assess stability of the PCV21 drug product prepared at 0.084 mg/mL PnPs, Nanoparticle Tracking Analysis (NTA) was utilized. The technique collects videos of directly tracked nanoparticle populations as they move by Brownian motion to extrapolate particle size and concentration. A class 1, 635 nm laser focuses an 80 micron red laser beam through the liquid sample, illuminating particles as rapidly diffusing points of light. A CCD camera records a 30 frame per second video to track the movement of each individual illimunated particle over time. The system software identifies the center of each individual particle from the video and tracks the distance independently traversed to determine the mean square displacement. This tracking is peformed simultaneously for every particle within the sample population in each frame until the raw data collected from the entire video is analyzed. By simultaneously measuring the mean square displacement of every individual particle tracked, its diffusion coefficient (Dt) and the spherical equivalent hydrodynamic radius (rh) are determined by applying the Stokes-Einstein equation. The software then represents this accumulated data as a particle size and concentration distribution. Raw data information on not only particle size and concentration, but also intensity, or brightness of the individual particle are gathered. Taken together the data are fitted and plotted indivdiually as particle intensity relative to particle size, and particle concentration relative to particle size, and then on three dimensional contour plots comparing particle size, concentration, and intensity of all particle populations.

A conjugate vaccine comprised of carrier protein and polysaccharide antigen may together or separately be susceptible to aggregation within the 10-1000 nm particle size range, thereby making NTA a suitable stability indicating technique for assessing and quantitating aggregation phenomena. Upon exposure of drug product to 4°C and 37°C for 1 week, no significant aggregation of drug product was observed by NTA for formulations containing PS-20 at concentrations of 0.025% w/v, 0.05% w/v, 0.1% w/v, 0.15% w/v, and 0.2% w/v PS20 (as shown in FIGURE 23). Howevever, after subjecting formulations to agitation for 6 hr. at 4°C, the formulation lacking polysorbate 20 was ineffective at mitigating agitation induced aggregation of the drug product as evidenced by increased D90 particle size, broadened particle size distribution, and increased particle intensity as provided by NTA. Therefore it is preferred to keep polysorbate 20 at 0.025 to 0.2% or higher levels (w/v) to stabilize the drug product during routine manufacturing, storage, shipping and handling.

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As described in Example 39, HPSEC UV/MALS/RI was used to measure the molecular weight of a 21-valent (PCV21) polysaccharide-protein conjugate drug product, formulated in 20 mM L-Histidine, pH 5.8, 150 mM NaCl, and 0 to 0.2% (w/v) PS-20 and formulation process described in Example 38. The formulations were placed at 4°C or 37°C for up to one week. In some cases, the syringes were placed on a horizontal rotation platform and shaken for up to 6 hours after storage for 1 week at 4°C or 37°C. This was done to simulate the shipping and handling stresses that may accompany drug product manufacture and distribution. If the polysaccharide-protein conjugates in the drug products were degrading or depolymerizing, a decrease in molecular weight would be evident and if aggregation of the drug product formulation was occurring, an increase in molecular weight would occur. This measurement provides additional characterization on the quality of the vaccine drug product or polysaccharide-protein conjugates as a function of PS-20 concentration. As shown in FIGURE 24, the PCV21 drug product formulations comprising all drug substances that were each prepared using reductive amination in an aprotic solvent (e.g. DMSO) were stable against depolymerization or chemical degradation of the carbohydrate and stable against aggregation of the protein in the drug product formulation due to heat treatment between 0.05% and 0.15% PS-20 . Collectively, this indicates that a PCV21 drug product is stable through elevated temperature and physical stresses when formulated using PS-20 from 0.025% and 0.2% or higher levels.

EXAMPLE 46

PCV21 Protection from Challenge in Mice

Young female Swiss Webster mice (6-8 weeks old, n=10/group) were intraperitoneally (IP) immunized with 0.1-0.5 mL of a 21-valent pneumococcal conjugate vaccine (PCV21) on days 0, day 14, and day 28. PCV21 pneumococcal vaccine was dosed at 4, 2, 0.4, 0.08 or 0.016 μg PnPs (3, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 19A, 20A, 22F, 23A, 23B, 24F, 31, 33F and 35B each conjugated to CRM197) per immunization. Mice were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in mice were deemed to be safe and well tolerated, as no vaccine-related adverse events were noted. On day 52 the mice were intratracheally challenged with *Streptococcus pneumoniae* serotype 24F. Exponential phase cultures of S. pneumoniae were centrifuged, washed, and suspended in sterile PBS. Mice were anesthetized with isoflurane prior to challenge. 105 cfu of S. pneumoniae in 0.1 mL of PBS was placed in the throat of mice hung upright by their incisors. Aspiration of the bacteria was induced by gently pulling the tongue outward and covering the nostrils. Mice were weighed daily and euthanized if weight loss exceeded 20% of starting weight. Blood was collected at 24 hours, 48 hours, and 72 hours to assess for bacteremia. Mice were observed at least twice daily by trained animal care staff for

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any signs of illness or distress. All animal experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The mouse experimental protocol was approved by the Institutional Animal Care and Use Committee at Merck & Co., Inc.

Mouse sera were evaluated for IgG immunogenicity using a multiplexed electrochemiluminescence (ECL) assay. This assay was developed for use with mouse serum based on the human assay described by Marchese et al., Clin Vaccine Immunol. (2009) 16(3):387-96, using technology developed by MesoScale Discovery (a division of MesoScale Diagnostics, LLC, Gaithersburg, MD) which utilizes a SULFO-TAG™ label that emits light upon electrochemical stimulation. SULFO-TAG™-labeled anti-mouse IgG was used as the secondary antibody for testing mouse serum samples. Functional antibody was determined through multiplexed opsonophagocytic assays (MOPA) based on previously described protocols at www.vaccine.uab.edu and Opsotiter® 3 software owned by and licensed from University of Alabama (UAB) Research Foundation (See, Caro-Aguilar I. et al., Vaccine (2017) 35(6):865-72 and Burton R.L. and Nahm M.H. Clin. Vaccine Immunol. (2006) 13(9):1004-9).

PCV21 immunization generated antibody titers in Swiss Webster mice for all serotypes in the vaccine (data not shown). PCV21 was also immunogenic in Balb/c and CD1 mice (data not shown). PCV21 immunized Swiss Webster mice were also protected from challenge with S. pneumoniae serotype 24F (FIGURE 25). Mantel Cox log-rank test indicates that all PCV21 immunized groups were significantly protected from challenge when compared to the naïve group ($P<0.05$). Likewise, PCV21 immunized mouse groups had little to no bacteremia, which was significantly less when compared to the naïve group (data not shown).

EXAMPLE 47

PCV Immunogenicity and Functional Antibody in Rabbits

Adult New Zealand white rabbits (NZWR, n=5/group) were intramuscularly (IM) immunized with 0.1 mL of PCV on day 0 and day 14 (alternating sides). PCV was dosed at 0.4 µg PnPs per serotype per immunization with with 25 µg [Al] in the form of Aluminum Phosphate Adjuvant (APA). In the case of PCV31, the dosing concentration of serotype 6B was doubled to 0.8 µg PnPs per immunization. PCV21 (as described in EXAMPLE 38) included purified polysaccharides from serotypes 3, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 19A, 20A, 22F, 23A, 23B, 24F, 31, 33F and 35B each conjugated to CRM197. Each polysaccharide-protein conjugate was prepared unadjuvanted or formulated with 250 µg [Al]/mL in the form of Aluminum Phosphate or APA to evaluate the effect of adjuvant (PCV21/APA). Lower valent PCVs were also formulated (as described in EXAMPLE 38) to include 8 novel STs (PCV8: 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B each conjugated to CRM197, unadjuvanted), the 16 STs not included in any licensed PCV (PCV16 : 6C, 8, 9N, 10A, 11A, 12F, 15A, 15C,

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16F, 17F, 20, 23A, 23B, 24F, 31 and 35B each conjugated to CRM197, unadjuvanted) or PCV31 (1, 3, 4, 5, 6A, 6B, 6C, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15A, 15C, 16F, 17F, 18C, 19A, 19F, 20A, 22F, 23A, 23B, 23F, 24F, 33F and 35B each conjugated to CRM197, formulated with 250 µg [Al]/mL in the form of Aluminum Phosphate) to evaluate carrier suppression as vaccine valency increased (PCV31/APA). Each vaccine used polysaccharide protein conjugates formulated at 4 µg/mL (w/v) Pneumococcal Polysaccharide (PnPs), except for 6B which was formulated at 8 µg/mL (w/v) Pneumococcal Polysaccharide (PnPs) in PCV31or PCV31/APA. The final concentration of Pneumococcal Polysaccharide (PnPs) in each vaccine was 128 µg/mL PnPs in PCV31, 84 µg/mL PnPs in PCV21, 64 µg/mL in PCV16 and 32 µg/mL in PCV8.

Sera were collected prior to study start (pre-immune) and on days 14 (PD1) and 28 (PD2). NZWRs were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in NZWRs were deemed to be safe and well tolerated, as no vaccine-related adverse events were noted. All animal experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The NZWR experimental protocol was approved by the Institutional Animal Care and Use Committees at both Merck & Co., Inc and Covance (Denver, PA).

Rabbit sera were evaluated for IgG immunogenicity using a multiplexed electrochemiluminescence (ECL) assay. This assay was developed for use with rabbit serum based on the human assay described by Marchese et al., Clin Vaccine Immunol. (2009) 16(3):387-96, using technology developed by MesoScale Discovery (a division of MesoScale Diagnostics, LLC, Gaithersburg, MD) which utilizes a SULFO-TAG™ label that emits light upon electrochemical stimulation. SULFO-TAG™-labeled anti-rabbit IgG was used as the secondary antibody for testing NZWR serum samples. Functional antibody was determined through multiplexed opsonophagocytic assays (MOPA) based on previously described protocols at www.vaccine.uab.edu and Opsotiter® 3 software owned by and licensed from University of Alabama (UAB) Research Foundation (See, Caro-Aguilar I. et al., Vaccine (2017) 35(6):865-72 and Burton R.L. and Nahm M.H. Clin. Vaccine Immunol. (2006) 13(9):1004-9).

Rabbit sera were tested as a pool for pre-immune and PD1, and individually for PD2 in multiplexed electrochemiluminescent assays to determine antibody titers. PCVs generated antibody titers in rabbits for all serotypes following immunizations with the vaccine (data not shown). Serotype 15B polysaccharide was not included in PCV16, PCV21 or PCV31, however antibody titers to serotype 15B were observed following immunization with both PCV16, PCV21 and PCV31.

PCV21 without adjuvant had comparable to or significantly higher immunogenicity (serotypess 3, 7F, 10A, 19A, 23A and 23B) when compared to PCV21 with

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APA (FIGURE 26A-B and FIGURE 28), suggesting no additional immunogenicity benefit in rabbits with APA included in the vaccine.

Carrier induced epitopic suppression refers to interference with the antibody response to an antigen (such as capsular polysaccharide) coupled to the same carrier protein (such as CRM197). Interference is thought to arise from competition for a limited number of carrier specific primed T helper cells. As a result, there may be a decrease in response to the capsular polysaccharide. Pfizer has observed a decrease in vaccine immunogenicity of shared serotypes as the vaccine valency increased from a 7-valent to 13-valent [Comparison of IgG antibody GMC of Prevnar (7 valent) vs Prevnar13. (table 9, page 29 of PCV13 monograph)]. Therefore, we sought to investigate the immunogenicity of lower valent PCVs compared to PCV21 (FIGURE 27A-C). PCV8 (8 novel serotypess) had comparable immunogenicity relative to PCV21 for the 8 shared serotypess (FIGURE 29). PCV16 (PCV21 minus 5 overlapping serotypess from PCV15) had comparable immunogenicity relative to PCV21 for 15 of the 16 shared serotypes (FIGURE 29). Serotype 31 had higher immunogenicity in PCV16 (FIGURE 29). Overall, there were no major trends of carrier suppression with PCV21 in NZWR. PCV31/APA had comparable immunogenicity relative to PCV21/APA for the 12 of the 21 shared serotypess. 10 serotypes (6C, 7F, 9N, 12F, 15A, 15B (not in PCV21), 15C, 17F, 19A, and 23A) had higher immunogenicity in PCV31, ranged between 2.9-fold and 11-fold (FIGURE 30). Overall, there were no major trends of carrier suppression with PCV31 in NZWR.

PCVs were found to be immunogenic in rabbits and generated functional antibodies which killed vaccine-type bacterial strains. Rabbit sera were tested in multiplexed opsonophagocytic assays (MOPA) to determine functional antibody titers. PCV21 had comparable to or higher PD2 OPA titers when compared to PCV21/APA, PCV8 and PCV16 (FIGURE 31). Log Transformed data were analyzed by One-way ANOVA with Dunnett's test to determine significance. PCV21 had significantly higher OPA titers for serotype 16F compared to PCV8 and for serotypes 12F, 23A and 19A compared to PCV21/APA. PCV31 had significantly higher OPA titers for serotype 23A compared to PCV21.

EXAMPLE 48

PCV21 Immunogenicity in Adult Rhesus Macaques

PCV21 was also assessed in adult Rhesus macaque immunogenicity models. Rhesus macaques were intramuscularly immunized with PCV21 on days 0, 28 and 56. PCV21 was dosed at 1 μg PnPs in a 0.25 mL volume (3, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 19A, 20A, 22F, 23A, 23B, 24F, 31, 33F and 35B each conjugated to CRM197) per immunization. Sera were collected prior to study start (pre-immune, day 0) and on days 14 (PD1), 28, 42 (PD2), 56, 70 (PD3) and 84.

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Rhesus sera were evaluated for IgG immunogenicity using a multiplexed electrochemiluminescence (ECL) assay. This assay was developed for use with Rhesus serum based on the human assay described by Marchese et al. and Skinner et al. (Marchese R.D. et al., Clin. Vaccine Immunol. (2009) 16(3):387-96 and Skinner, J.M. et al.,Vaccine (2011) 29(48):8870-8876) using technology developed by MesoScale Discovery (a division of MesoScale Diagnostics, LLC, Gaithersburg, MD) which utilizes a SULFO-TAG™ label that emits light upon electrochemical stimulation. SULFO-TAG™-labeled anti-human IgG was used as the secondary antibody for testing Rhesus serum samples. Functional antibody was determined through multiplexed opsonophagocytic assays (MOPA) based on previously described protocols at www.vaccine.uab.edu and Opsotiter® 3 software owned by and licensed from University of Alabama (UAB) Research Foundation (See, Caro-Aguilar I. et al., Vaccine (2017) 35(6):865-72 and Burton R.L. and Nahm M.H. Clin. Vaccine Immunol. (2006) 13(9):1004-9).

PCV21 was found to be immunogenic in adult monkeys and generated functional antibodies which killed vaccine-type bacterial strains at all time points tested (FIGURE 32 and 33). It is also of note that PCV21, which contains polysaccharide conjugates 15A-CRM197 and 15C-CRM197, also provides cross-reactivity to 15B, as evidenced in ECL. PCV21 was immunogenic with one dose of vaccine in the monkeys (FIGURE 32). Most serotypes reached their maximum titer at PD1, with the exception of STs 6C, 12F and 24F, which benefited from additional immunizations. The adult Rhesus monkeys had higher pre-existing antibody titers as compared to former PCV studies in infant Rhesus monkeys. The pre-existing antibody titers were most apparent when testing the sera samples in MOPA, which made determining the opsonophagocytic titer difficult. All study time points were tested in MOPA as pooled or individual samples, but for easier viewing, only pre-immune, PD1 and PD3 are shown (FIGURE 33), as there was not much difference in the OPA titers between PD1 and PD3.

PCV21 immunized adult Rhesus macaque sera were evaluated for cross protection to other S. pneumoniae bacteria (FIGURE 34). PCV21 immunized macaque sera had cross protection with serotypes 6A, 6B and 23F but not 19F. The cross protection to 6A and 6B is likely due to immunization with polysaccharide conjugate 6C-CRM197 as part of a multivalent PCV21. Similarly, immunization with polysaccharide conjugates 23A-CRM197 and 23B-CRM197 as part of a multivalent PCV resulted in cross protection to serotype 23F. Immunization with PCV21 including polysaccharide conjugate 19A-CRM197 did not provide cross protection to 19F.

EXAMPLE 49

PCV21 Immunogenicity in Adult Rhesus Macaques - Evaluation of Adjuvant and Carrier Suppression

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Another monkey study included evaluation of PCV21 with and without APA and comparison of shared serotype immunogenicity to evaluate carrier suppression. Adult Rhesus macaques (n=5/group) were intramuscularly immunized with vaccines at a half human dose on day 0 of the study. PCV21 was dosed at 1 μg PnPs in a 0.25 mL volume (3, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 19A, 20, 22F, 23A, 23B, 24F, 31, 33F and 35B each conjugated to CRM197) per immunization. One group included PCV21, adjuvanted with 62.5 μg [Al] in the form of Aluminum Phosphate . Another group included PCV15 dosed at 1 μg PnPs in a 0.25 mL volume (1, 3, 4, 5, 6A, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F and 33F each conjugated to CRM197 with 6B dosed at 2 μg) per immunization and adjuvanted with 62.5 μg [Al] in the form of Aluminum Phosphate. Another group included Prevnar13® (PCV13) dosed at 1.1 μg PnPs in a 0.25 mL volume (1, 3, 4, 5, 6A, 7F, 9V, 14, 18C, 19A, 19F and 23F each conjugated to CRM197 with 6B dosed at 2.2 μg) per immunization. Sera were collected prior to study start (pre-immune, day 0) and on day 28.

PCV21 was found to be immunogenic in adult monkeys and generated functional antibodies which killed vaccine-type bacterial strains. PCV21 without adjuvant had comparable to or significantly higher immunogenicity (serotypes 15A and 15B) when compared to PCV21 with APA (FIGURE 35). PCV21 did not include serotype 15B.

PCV21 immunized monkeys were compared to those immunized with PCV15 and Prevnar13. PCV21 and PCV15 have 5 shared serotypes (3, 7F, 19A, 22F, 33F); there was no difference in immunogenicity of those 5 serotypes between monkeys immunized with PCV21 or PCV15 (FIGURE 36). PCV21 and Prevnar13 have 3 shared serotypes (3, 7F, 19A); there was no difference in immunogenicity for serotypes 7F and 19A. Prevnar13 immunized monkeys had lower antibody titers for serotype 3 compared to PCV21 (FIGURE 36). This finding is consistent with the PCV15 human clinical data, showing serotype 3 to be more immunogenic in Merck's PCV15 vaccine. Taken together, these results suggest that carrier suppression was not observed in adult Rhesus macaques immunized with PCV21 when compared to Prevnar13 or PCV15.

EXAMPLE 50

Serotype 6A, 6B and 6C Cross Reactivity – Monovalent Study

The monovalent drug product was prepared using Pneumococcal polysaccharide 6A-CRM197 conjugate or Pneumococcal polysaccharide 6B-CRM197 conjugate and was formulated in 20 mM histidine pH 5.8 and 150 mM sodium chloride and 0.1% w/v polysorbate-20 (PS-20) at a target total polysaccharide concentration of 4.0 μg/mL of either serotype. The conjugates were prepared by individually conjugating the CRM197 protein to Pneumococcal polysaccharide (PnPs) types (either -6A or -6B). The required volume of bulk conjugates needed to obtain the target concentration of individual serotypes were calculated based on batch

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volume and concentration of individual bulk polysaccharide concentrations. The individual conjugates were added to a solution of histidine, sodium chloride and PS-20 to create a 2X conjugate blend. The formulation vessel containing the 2X conjugate blend were mixed using a magnetic stir bar, and sterile filtered into another vessel. The sterile filtered 2X blend was then diluted with saline to achieve the desired target total polysaccharide and excipient concentrations. The formulations were then filled into vials and stored at 2-8°C.

Rabbits were immunized with 6A-CRM197 or 6B-CRM197 to evaluate cross-reactivity within serogroup 6. Adult New Zealand White rabbits (NZWR, n=3/group) were intramuscularly (IM) immunized with 0.25 mL of respective monovalent conjugate vaccine on day 0 and day 14 (alternating sides). Monovalent pneumococcal conjugate vaccine, formulated in 20 mM L-Histidine pH 5.8 150 mM NaCl and 0.1% (w/v) PS-20 with a formulation process described in Example 38, was dosed at 1 μg PnPs (6A or 6B each conjugated to CRM197). Sera were collected prior to study start (pre-immune) and on days 14 (post-dose 1, PD1) and 28 (post-dose 2, PD2). NZWRs were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in NZWRs were deemed to be safe and well tolerated, as no vaccine-related adverse events were noted. All animal experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The NZWR experimental protocol was approved by the Institutional Animal Care and Use Committees at both Merck & Co., Inc and Covance (Denver, PA).

NZWR sera were tested in ELISA assays to evaluate IgG immunogenicity using a 2 μg/mL respective PnPs coating concentration. Functional antibody was determined through opsonophagocytosis assays (OPA) based on previously described protocols at www.vaccine.uab.edu and Opsotiter® 3 software owned by and licensed from UAB Research Foundation (See, Caro-Aguilar I. et al., Vaccine (2017) 35(6):865-72 and Burton R.L. and Nahm M.H. Clin. Vaccine Immunol. (2006) 13(9):1004-9).

Both 6A-CRM197 and 6B-CRM197 were found to be immunogenic in rabbits (FIGURE 37) and generate functional antibody which killed the respective bacterial strain (FIGURE 38). In addition, rabbits immunized with serogroup 6 monovalent pneumococcal conjugate vaccines had equivalent PD2 IgG and OPA titers to the homologous and heterologous polysaccharide and bacterial strain, respectively. Rabbits immunized with 6A-CRM197 or 6B-CRM197 all had cross reactivity to each pneumococcal polysaccharide (PnPs 6A, PnPs 6B and PnPs 6C) (FIGURE 37). Using post-dose 2 (PD2) log transformed IgG data analyzed by One-way ANOVA, there was no significant difference in the IgG titers across serogroup 6. In addition, rabbits immunized with 6A-CRM197 or 6B-CRM197 all had cross reactivity to each S. pneumoniae bacterial strain (6A, 6B and 6C), as all rabbit hyper immune sera had functional antibody to each strain evaluated and killed the bacteria (FIGURE 38).

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Similarly, using post-dose 2 (PD2) log transformed OPA data analyzed by One-way ANOVA, there was no significant difference in OPA titers across the serogroup 6.

EXAMPLE 51

Serotype 20A and 20B Cross Reactivity – Monvalent Study

The monovalent drug product was prepared using Pneumococcal polysaccharide 20A-CRM197 conjugate and was formulated in 20 mM histidine pH 5.8 and 150 mM sodium chloride and 0.2% w/v Polysorbate-20 (PS-20) at 4.0 µg/mL. The formulation was prepared with 250.0 µg [Al]/mL in the form of Aluminum Phosphate as the adjuvant (20A-CRM197/APA). The conjugate was prepared by individually conjugating the CRM197 protein to Pneumococcal polysaccharide (PnPs) type 20. The required volume of bulk conjugate needed to obtain the target concentration of individual serotype was calculated based on batch volume and concentration of individual bulk polysaccharide concentration. The single conjugate was added to a solution of histidine, sodium chloride and PS-20 to produce a 4X conjugate blend at 16.0 µg/mL. The formulation vessel containing the conjugate blend was mixed using a magnetic stir bar and the sterile was filtered into another vessel. The sterile filtered 4X blend was then added to another vessel containing Aluminum Phosphate Adjuvant to achieve the desired target total polysaccharide, excipient and adjuvant concentrations. The formulations were then filled into glass vials and stored at 2-8°C.

Rabbits were immunized with 20A-CRM197/APA or PCV21 to evaluate cross-reactivity within serogroup 20. Adult New Zealand White rabbits (NZWR, n=3/group) were intramuscularly (IM) immunized with pneumococcal conjugate vaccine on day 0 and day 14 (alternating sides). Monovalent pneumococcal conjugate vaccine, formulated as described above, was dosed at 1 µg PnPs in 0.25 ml (ST20A conjugated to CRM197 and 62.5 µg [Al] in the form of Aluminum Phosphate was used as the adjuvant), a PCV21 multivalent pneumococcal conjugate vaccine (84 µg/mL PnPs) , formulated in 20 mM L-Histidine pH 5.8 150 mM NaCl and 0.2% (w/v) PS-20 with a formulation process described in Example 38, was dosed at 0.4 µg PnPs in 0.1 ml (serotypes 3, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15C, 16F, 17F, 19A, 20, 22F, 23A, 23B, 24F, 31, 33F and 35B each conjugated to CRM197). Sera were collected prior to study start (pre-immune) and on days 14 (post-dose 1, PD1) and 28 (post-dose 2, PD2). NZWRs were observed at least daily by trained animal care staff for any signs of illness or distress. The vaccine formulations in NZWRs were deemed to be safe and well tolerated, as no vaccine-related adverse events were noted. All animal experiments were performed in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The NZWR experimental protocol was approved by the Institutional Animal Care and Use Committees at both Merck & Co., Inc and Covance (Denver, PA).

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NZWR sera were tested in opsonophagocytosis assays (OPA) to evaluate functional antibody based on previously described protocols at www.vaccine.uab.edu and Opsotiter® 3 software owned by and licensed from UAB Research Foundation (See, Caro-Aguilar I. et al., Vaccine (2017) 35(6):865-72 and Burton R.L. and Nahm M.H. Clin. Vaccine Immunol. (2006) 13(9):1004-9).

Rabbits immunized with both monovalent 20A-CRM197/APA and multivalent PCV21 vaccines generated functional antibody which killed both S. pneumoniae serotypes 20A and 20B (FIGURE 39). This suggests that serotype 20A and 20B share structural similarity, resulting in cross-reactivity within the serogroup.

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WHAT IS CLAIMED IS:

(1) A multivalent immunogenic composition comprising *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein, wherein the serotypes of *S. pneumoniae* in the polysaccharide protein conjugates comprise a set of serotypes selected from the group consisting of:

a) 15A, 16F, 23A, 23B, 24F, 31 and 35B;

b) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20; and

c) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20.

(2) The multivalent immunogenic composition of claim 1, wherein the set of serotypes of *S. pneumoniae* listed in a), b) or c) further comprises:

(i) serotype 6C,

(ii) serotype 6A, or

(iii) serotypes 6A and 6B.

(3) The multivalent immunogenic composition of claim 1, wherein the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

a) 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20;

b) 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20; and

c) 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20

wherein serotype 20 in sets a) to c) may be optionally substituted with either serotype 20A or serotype 20B.

(4) The multivalent immunogenic composition of claim 1, wherein the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

a) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

b) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; and

c) 3, 7F, 19A, 22F, 33F, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A.

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(5) The multivalent immunogenic composition of claim 1 wherein the serotypes of *S. pneumoniae* comprise serotypes selected from the group consisting of 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A.

(6) The multivalent immunogenic composition of claim 1 wherein the serotypes of *S. pneumoniae* comprise serotypes selected from the group consisting of 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A.

(7) The multivalent immunogenic composition of any of claims 1 to 6, wherein the immunogenic composition does not comprise any further *S. pneumoniae* polysaccharide protein conjugates.

(8) The multivalent immunogenic composition of any of claims 1 to 7, wherein at least one of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent.

(9) The multivalent immunogenic composition of any of claims 1 to 8, wherein each of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent.

(10) The multivalent immunogenic composition of claims 1 to 9, wherein the total polysaccharide concentration in the composition is from about 0.02 to about 0.175 mg/mL

(11) The multivalent immunogenic composition of any of claims 1 to 10, wherein the weight average molecular weight (Mw) of the *S. pneumoniae* polysaccharide protein conjugates in the composition is from about 1,000 to about 6,000 kDa.

(12) The multivalent immunogenic composition of any of claims 1 to 11, wherein the number average molecular weight (Mn) of the *S. pneumoniae* polysaccharide protein conjugates in the composition is from about 500 to about 4,000 kDa.

(13) The multivalent immunogenic composition of any of claims 1 to 12, wherein the composition is stable for up to 4 weeks or more at 37°C .

(14) The multivalent immunogenic composition of any of claims 1 to12, wherein the composition is stable for up to 12 weeks or more at 4°C.

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(15) The multivalent immunogenic composition of claim 13 or 14, wherein stability is determined using HPSEC UV/MALS/RI.

(16) The multivalent immunogenic composition of any of claims 1 to 15, wherein the emission maximum of the composition measured using intrinsic protein fluorescence spectroscopy with an excitation wavelength at 280 nanometers (nm) is from about 335 nm to about 342 nm.

(17) The multivalent immunogenic composition of claim 16, wherein the emission maximum remains from about 335 nm to about 342 nm and the fluorescence intensity is stable for at least 1 week at 37°C

(18) The multivalent immunogenic composition of any of claims 8-17, wherein the aprotic solvent is dimethylsulfoxide (DMSO).

(19) The multivalent immunogenic composition of claim 8 or 9, wherein the conjugation reaction is reductive amination.

(20) The multivalent immunogenic composition of any one of claims 1 to 19, wherein the carrier protein is selected from the group consisting of Outer Membrane Protein Complex (OMPC), tetanus toxoid, diphtheria toxoid, protein D and CRM197.

(21) The multivalent immunogenic composition of any one of claims 1 to 20, wherein the carrier protein is CRM197.

(22) The multivalent immunogenic composition of any one of claims 1 to 21, wherein the composition further comprises an adjuvant.

(23) The multivalent immunogenic composition of any one of claims 1 to 21, wherein the composition does not comprise an adjuvant.

(24) The multivalent immunogenic composition of any one of claims 1 to 23, further comprising 10-80 mM histidine pH 5.8 and 150 mM NaCl.

(25) The multivalent immunogenic composition of claim 24, further comprising from 0.025% to 0.8% w/v polysorbate 20.

(26) A multivalent immunogenic composition comprising *S. pneumoniae* polysaccharide protein conjugates, wherein each of the conjugates comprises a polysaccharide

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from an *S. pneumoniae* serotype conjugated to CRM197, wherein the serotypes of *S. pneumoniae* in the polysaccharide protein conjugates consist of a set of serotypes selected from the group consisting of:

a) 15A, 16F, 23A, 23B, 24F, 31 and 35B;
b) 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B;
c) 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B;
d) 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B;
e) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
f) 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
g) 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
h) 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
i) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
j) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
k) 3, 7F, 19A, 22F, 33F, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; and
l) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

wherein serotype 20A in sets e) to l) may be optionally substituted with either serotype 20 or serotype 20B; wherein each of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent and wherein the composition does not comprise an adjuvant.

(27) A multivalent immunogenic composition of claim 26 comprising *S. pneumoniae* polysaccharide protein conjugates, wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to CRM197, wherein the serotypes of *S. pneumoniae* in the polysaccharide protein conjugates consist of serotypes selected from 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; wherein serotype 20A may be optionally substituted with either serotype 20 or serotype 20B; wherein each of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent and wherein the composition does not comprise an adjuvant.

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(28) A multivalent immunogenic composition of claim 26 comprising *S. pneumoniae* polysaccharide protein conjugates, wherein each of the conjugates comprises a polysaccharide from an *S. pneumoniae* serotype conjugated to CRM197, wherein the serotypes of *S. pneumoniae* in the polysaccharide protein conjugates consist of serotypes selected from 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; wherein serotype 20A may be optionally substituted with either serotype 20 or serotype 20B; wherein each of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent and wherein the composition does not comprise an adjuvant.

(29) A method for inducing a protective immune response in a human patient comprising administering the multivalent immunogenic composition of any one of claims 1 to 28 to the patient.

(30) The method of claim 29, wherein the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

a) 3, 7F, 19A, 22F, 33F, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
b) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
c) 3, 7F, 19A, 22F, 33F, 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; and
d) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

wherein serotype 20A in sets a) to d) may be optionally substituted with either serotype 20 or serotype 20B.

(31) The method of claim 29, wherein the serotypes of *S. pneumoniae* comprise a set of serotypes selected from the group consisting of:

a) 15A, 16F, 23A, 23B, 24F, 31 and 35B;
b) 6A, 15A, 16F, 23A, 23B, 24F, 31 and 35B 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31 and 35B;
c) 6C, 15A, 16F, 23A, 23B, 24F, 31 and 35B;
d) 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
e) 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;
f) 6A, 6B, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; and

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g) 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

wherein serotype 20A in sets d) to g) may be optionally substituted with either serotype 20 or serotype 20B.

(32) The method of claim 30, wherein the serotypes of *S. pneumoniae* comprise serotypes selected from 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A.

(33) The method of claim 30, wherein the serotypes of *S. pneumoniae* comprise serotypes selected from 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A.

(34) The method of any of claims 29 to 33, wherein the patient was previously treated with a multivalent pneumococcal vaccine.

(35) The method of claim 34, wherein the multivalent pneumococcal vaccine is indicated for the prevention of pneumococcal disease caused by serotypes selected from the group consisting of:

a) 4, 6B, 9V, 14, 18C, 19F and 23F;
b) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, and 19A;
c) 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F;
d) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, 19A, 22F, and 33F;
e) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 7F, 19A, 22F, 33F, 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20;
f) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, 19A, 22F, 33F, 8, 10A, 11A, 12F and 15B; and
g) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 6C, 7F, 19A, 22F, 33F, 8, 10A, 11A, 12F and 15B

(36) The method of claim 34 or 35, wherein the multivalent pneumococcal vaccine comprises multiple polysaccharide protein conjugates, wherein the polysaccharide protein conjugates comprise polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein.

(37) The method of any of claims 31 to 36, further comprising administering one or more additional doses of the multivalent immunogenic composition to the patient.

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(38) The method of claim 37, wherein the amount of time between doses is from about 4 weeks to about 1 year.

(39) The method of claim 37 or claim 38, wherein two additional doses are administered to the patient and the patient is immunocompromised.

(40) The method of any of claims 31 to 33, further comprising administering a multivalent pneumococcal vaccine to the patient in any order, wherein the multivalent immunogenic composition and the multivalent pneumococcal vaccine are not the same.

(41) The method of claim 40, wherein the multivalent pneumococcal vaccine is indicated for the prevention of pneumococcal disease caused by *S. pneumoniae* serotypes selected from the group consisting of:

a) 4, 6B, 9V, 14, 18C, 19F and 23F;
b) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, and 19A;
c) 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F;
d) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, 19A, 22F, and 33F;
e) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 7F, 19A, 22F, 33F, 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20;
f) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F, 19A, 22F, 33F, 8, 10A, 11A, 12F and 15B; and
g) 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 6C, 7F, 19A, 22F, 33F, 8, 10A, 11A, 12F and 15B.

(42) The method of claim 41, wherein the multivalent pneumococcal vaccine is comprised of multiple *S. pneumoniae* polysaccharide protein conjugates wherein each of the conjugates comprises polysaccharide from an *S. pneumoniae* serotype conjugated to a carrier protein.

(43) The method of any of claims 40-42, wherein the multivalent immunogenic composition and the multivalent pneumococcal vaccine are administered concurrently.

(44) The method of any of claims 40-42, wherein the the amount of time between administration of the multivalent immunogenic composition and the multivalent pneumococcal vaccine is from about 4 weeks to about 1 year.

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(45) The method of any of claims 40-42 or 44, wherein the multivalent immunogenic composition is administered before the multivalent pneumococcal vaccine.

(46) The method of any of claims 40-42 or 44, wherein the multivalent pneumococcal vaccine is administered before the multivalent immunogenic composition

(47) The method of any of claims 31 to 46, wherein the patient is 50 years of age or older.

(48) The method of any of claims 31 to 46, wherein the patient is between 2 and 18 years of age.

(49) The method of any of claims 31 to 46, wherein the patient is 18 years of age or older.

(50) The method of any of claims 31 to 46, wherein the patient is 65 years of age or older.

(51) The method of any of claims 31 to 50, wherein the patient is immunocompromised.

(52) The method of any of claims 31 to 51, wherein the immunogenic composition is administered by subcutaneous or intramuscular injection.

(53) The method of any of claims 31 to 52, wherein the immunogenic composition is administered concomitantly with a vaccine against influenza.

(54) A method for preparing a serotype 8 *Streptococcus pneumoniae* polysaccharide-protein conjugate utilizing a conjugation reaction in an aprotic solvent, wherein the conjugation reaction does not use cyanoborohydride.

(55) The method of claim 54, wherein the conjugation reaction is a Schiff base reduction or reductive amination.

(56) The method of claims 54 and 55, wherein the protein is tetanus toxoid, diphtheria toxoid, or CRM197.

(57) The method of claim 56, wherein the protein is CRM197.

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(58) The method of any one of claims 54 to 57, wherein the conjugation reaction is reductive amination.

(59) The method of any of claims 54 to 48, wherein the reductive amination is performed in dimethylsulfoxide (DMSO).

(60) A method for inducing a protective immune response in a human patient comprising administering the multivalent immunogenic composition of claims 1 to 28 to the human patient, wherein the polysaccharide dose of each serotype is from about 0.4 to about 4 μg.

(61) A multivalent immunogenic composition comprising *S. pneumoniae* polysaccharide protein conjugates, wherein each of the conjugates comprises a polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, and wherein the polysaccharide protein conjugates include polysaccharides of *S. pneumoniae* serotypes selected from the group consisting of:

I) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20;

II) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A;

III) 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B;

IV) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20;

V) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A; and

VI) 3, 7F, 19A, 22F, 33F, 6C, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20B.

(62) The multivalent immunogenic composition of claim 61, wherein the immunogenic composition does not comprise polysaccharide protein conjugates having polysaccharides from any further *S. pneumoniae* serotypes.

(63) The multivalent immunogenic composition of any of claims 61 or 62, wherein at least one of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent.

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(64) The multivalent immunogenic composition of any of claims 61 to 63, wherein each of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent.

(65) The multivalent immunogenic composition of any of claims 63 to 64, wherein the aprotic solvent is dimethylsulfoxide (DMSO).

(66) The multivalent immunogenic composition of any of claims 61 to 65, wherein the carrier protein is selected from the group consisting of Outer Membrane Protein Complex (OMPC), tetanus toxoid, diphtheria toxoid, protein D and CRM197.

(67) The multivalent immunogenic composition of any of claims 61 to 66, wherein the carrier protein is CRM197.

(68) The multivalent immunogenic composition of any of claims 61 to 67, wherein the composition further comprises an adjuvant.

(69) The multivalent immunogenic composition of any of claims 61 to 67, wherein the composition does not comprise an adjuvant.

(70) A method for inducing a protective immune response in a human patient comprising administering the multivalent immunogenic composition of any of claims 61 to 69 to a patient.

(71) The method of claim 70, wherein the patient was previously treated with a multivalent pneumococcal vaccine.

(72) The method of any of claims 70 to 71, further comprising administering one or more additional doses of the multivalent immunogenic composition to the patient.

(73) The method of any of claims 70 to 72, wherein the amount of time between doses is from about 4 weeks to about 1 year.

(74) The method of any of claims 70 to 73, wherein two additional doses are administered to the patient and the patient is immunocompromised.

(75) The method of any of claims 70 to 74, further comprising administering a multivalent pneumococcal vaccine to the patient in any order, wherein the multivalent immunogenic composition and the multivalent pneumococcal vaccine are not the same.

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(76) The method of claim 75, wherein the multivalent pneumococcal vaccine is comprised of multiple *S. pneumonia* polysaccharide protein conjugates wherein each of the conjugates comprises polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein.

(77) The method of any of claims 75 to 76, wherein the multivalent immunogenic composition and the multivalent pneumococcal vaccine are administered concurrently.

(78) The method of any of claims 75 to 77, wherein the amount of time between administration of the multivalent immunogenic composition and the multivalent pneumococcal vaccine is from about 4 weeks to about 1 year.

(79) The method of any of claims 75 to 78, wherein the multivalent immunogenic composition is administered before the multivalent pneumococcal vaccine.

(80) The method of any of claims 75 to 78, wherein the multivalent pneumococcal vaccine is administered before the multivalent immunogenic composition.

(81) The method of any of claims 70 to 80 , wherein the patient is 50 years of age or older.

(82) The method of any of claims 70 to 81, wherein the patient is between 2 and 18 years of age.

(83) The method of any of claims 70 to 82, wherein the patient is 18 years of age or older.

(84) The method of any of claims 70 to 83, wherein the patient is 65 years of age or older.

(85) The method of any of claims 70 to 84, wherein the patient is immunocompromised.

(86) The method of any of claims 70 to 85, wherein the immunogenic composition is administered by subcutaneous or intramuscular injection.

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(87) The method of any of claims 70 to 86, wherein the immunogenic composition is administered concomitantly with a vaccine against influenza.

(88) A method for the prevention of pneumococcal pneumoniae and invasive disease in adults 18 years of age and older comprising administering the multivalent immunogenic composition of claim 62 to a patient.

(89) A method for the prevention of pneumococcal pneumoniae and invasive disease caused by the 24 *Streptococcus pneumoniae* strains (3, 6A, 6C, 7F, 8, 9N, 10A, 11A, 12F, 15A, 15B, 15C, 16F, 17F, 19A, 20A, 20B, 22F, 23A, 23B, 24F, 31, 33F, and 35B) comprising administering the multivalent immunogenic composition of claim 62 to a patient.

(90) A multivalent immunogenic composition comprising 21 distinct polysaccharide protein conjugates, wherein each of the conjugates comprises a capsular polysaccharide from a *S. pneumoniae* serotype conjugated to a carrier protein, wherein the polysaccharide are prepared from *S. pneumoniae* serotypes 3, 7F, 19A, 22F, 33F, 6A, 15A, 16F, 23A, 23B, 24F, 31, 35B, 8, 9N, 10A, 11A, 12F, 15C, 17F, and 20A and wherein the carrier protein is CRM197.

(91) The multivalent immunogenic composition of claim 90, wherein the immunogenic composition does not comprise polysaccharide protein conjugates prepared from any other *S. pneumoniae* serotypes.

(92) The multivalent immunogenic composition of claim 90, wherein each of the polysaccharide protein conjugates is formed by a conjugation reaction comprising an aprotic solvent, wherein the aprotic solvent is dimethylsulfoxide (DMSO).

(93) The multivalent immunogenic composition of claim 90, wherein the composition does not comprise an adjuvant.

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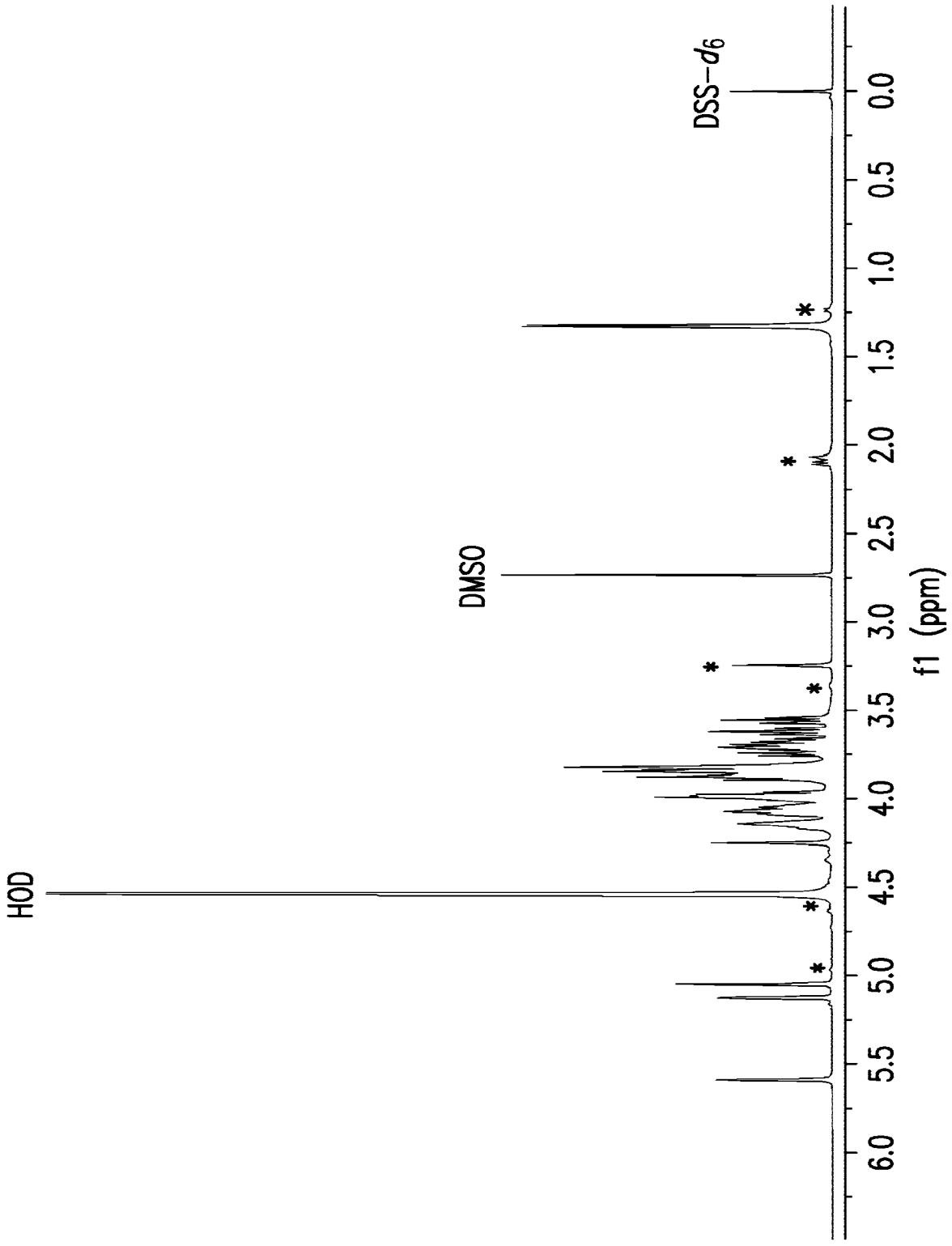


FIG.1

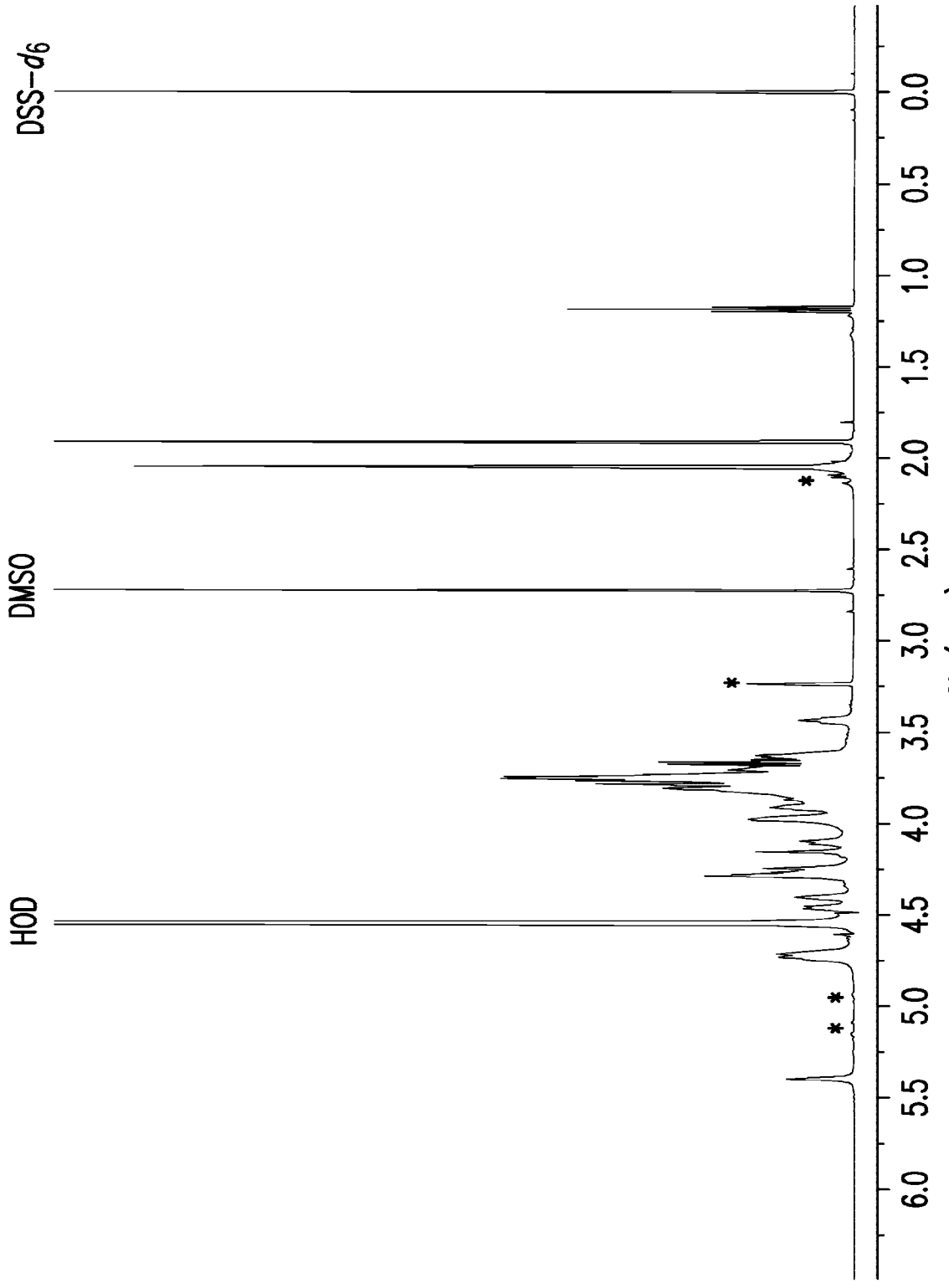


FIG.2

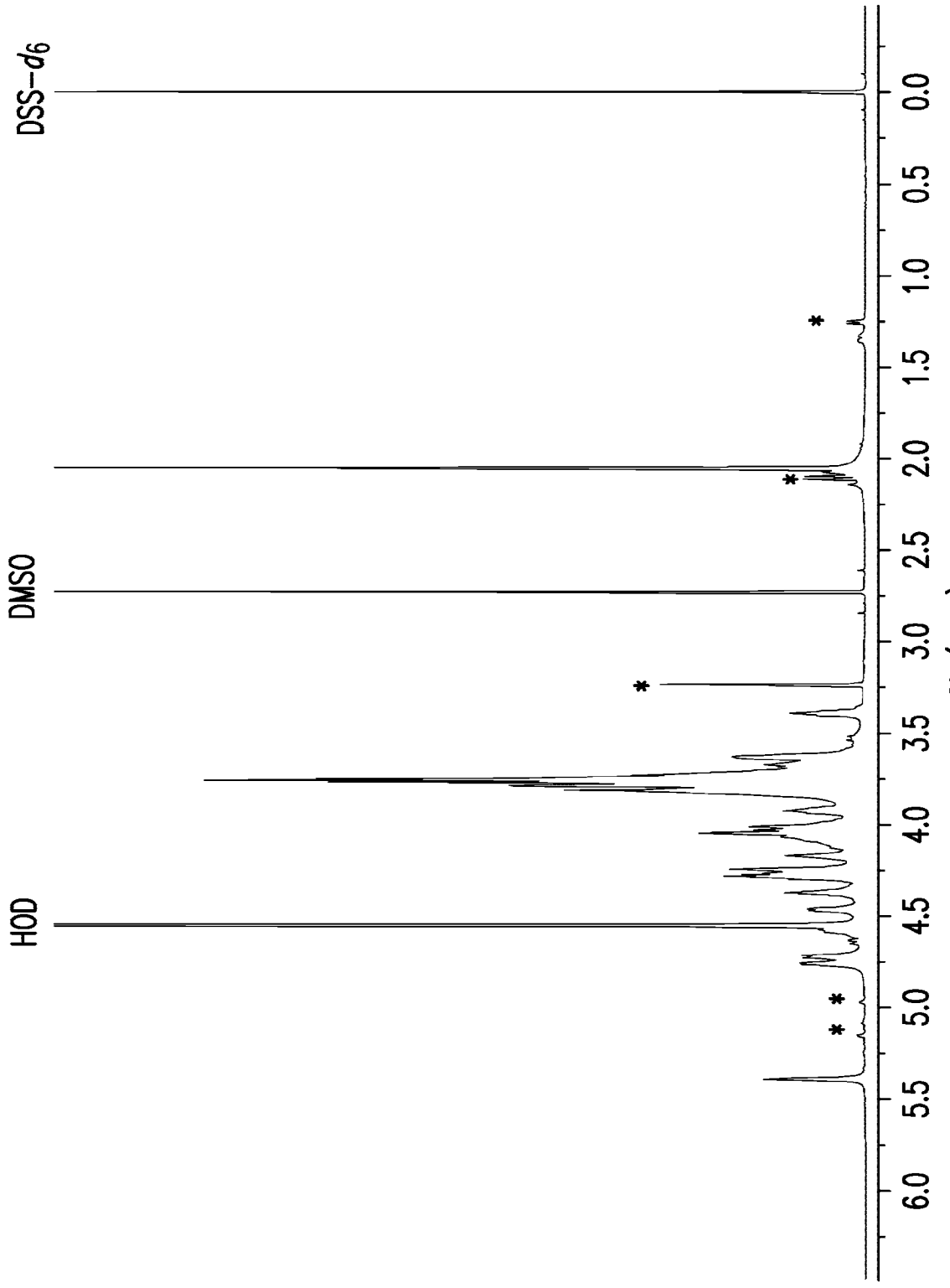


FIG.3

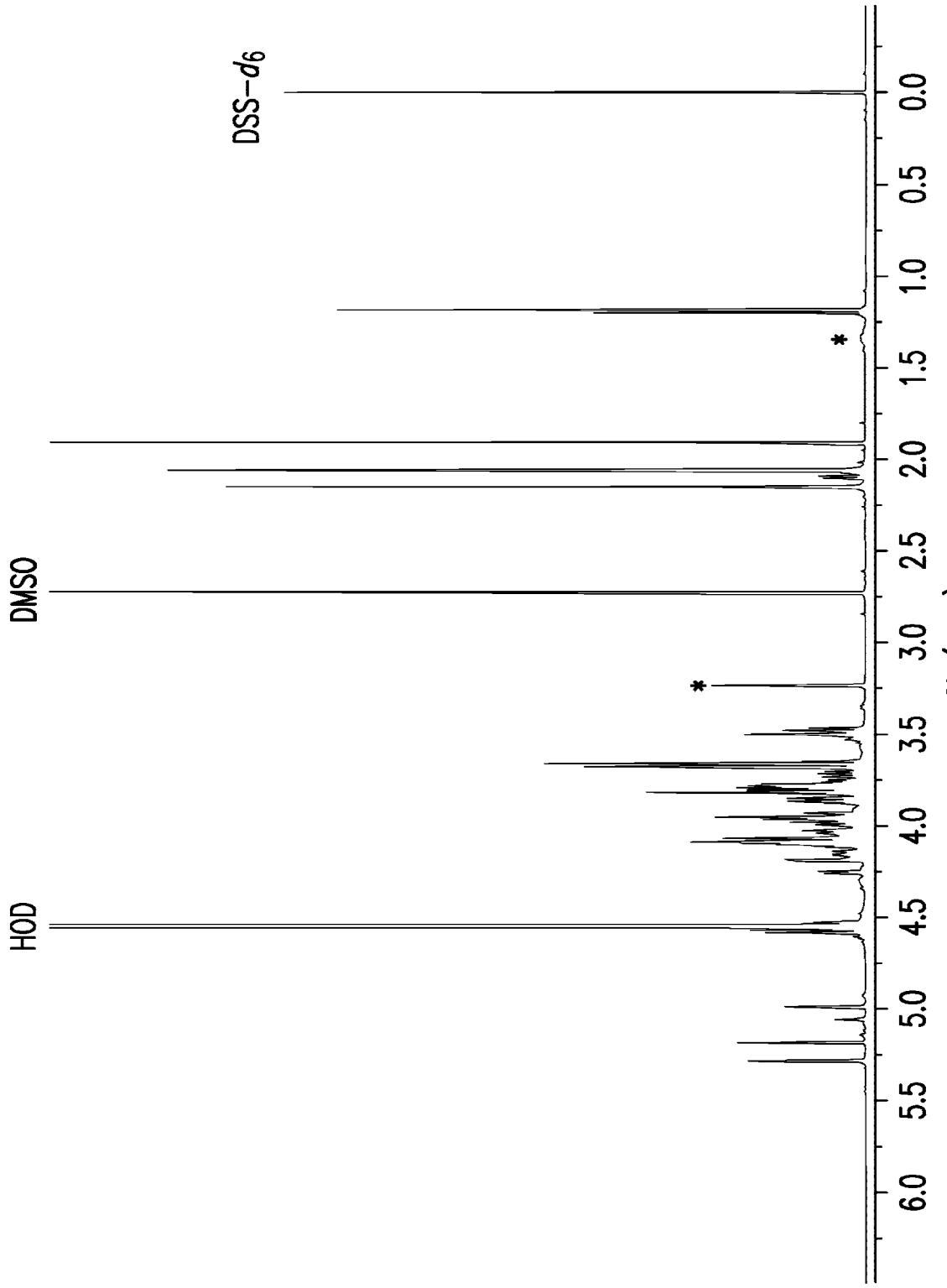


FIG.4

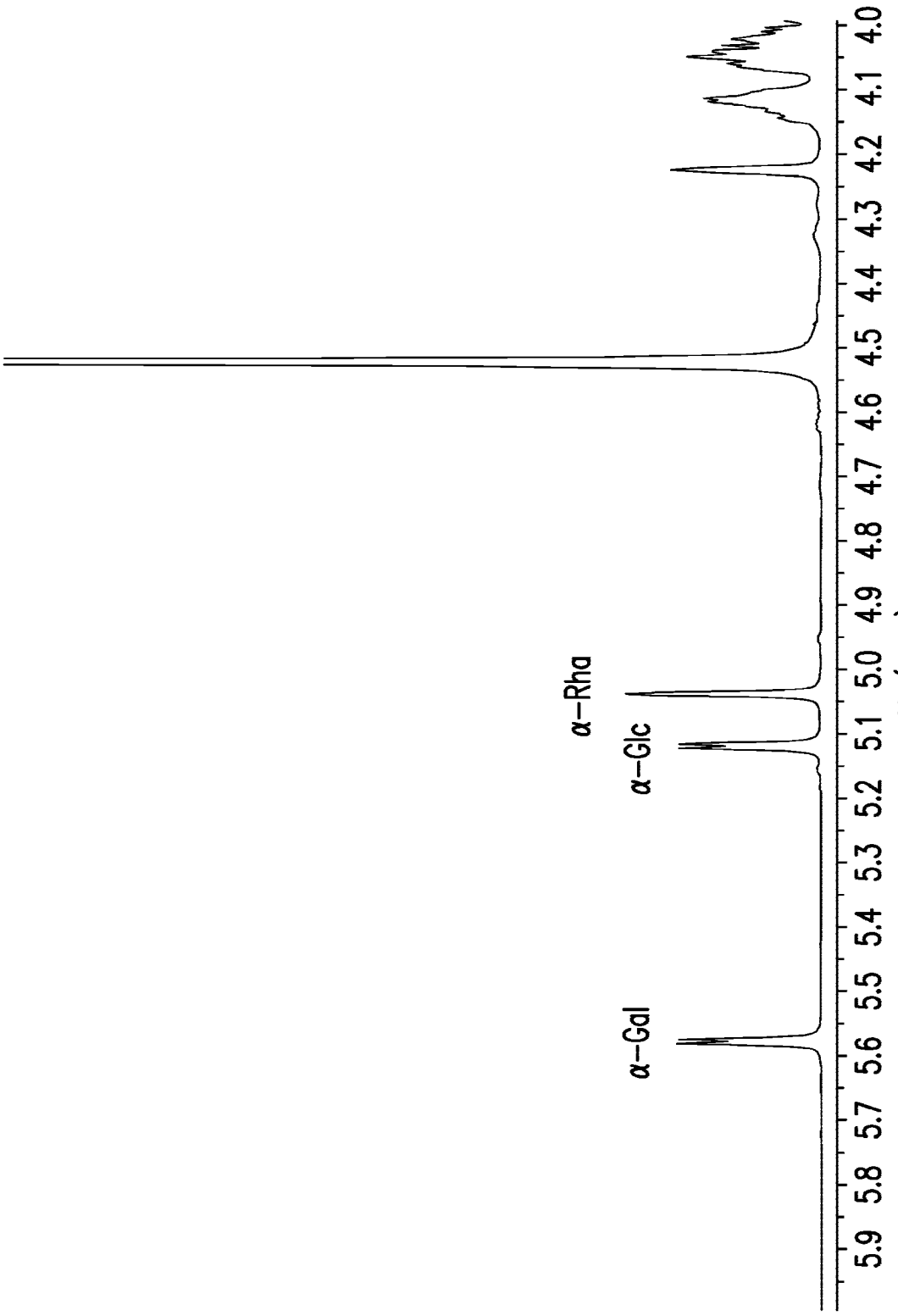

α–Rha
α–Glc
α–Gal
5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0
f1 (ppm)

FIG.5

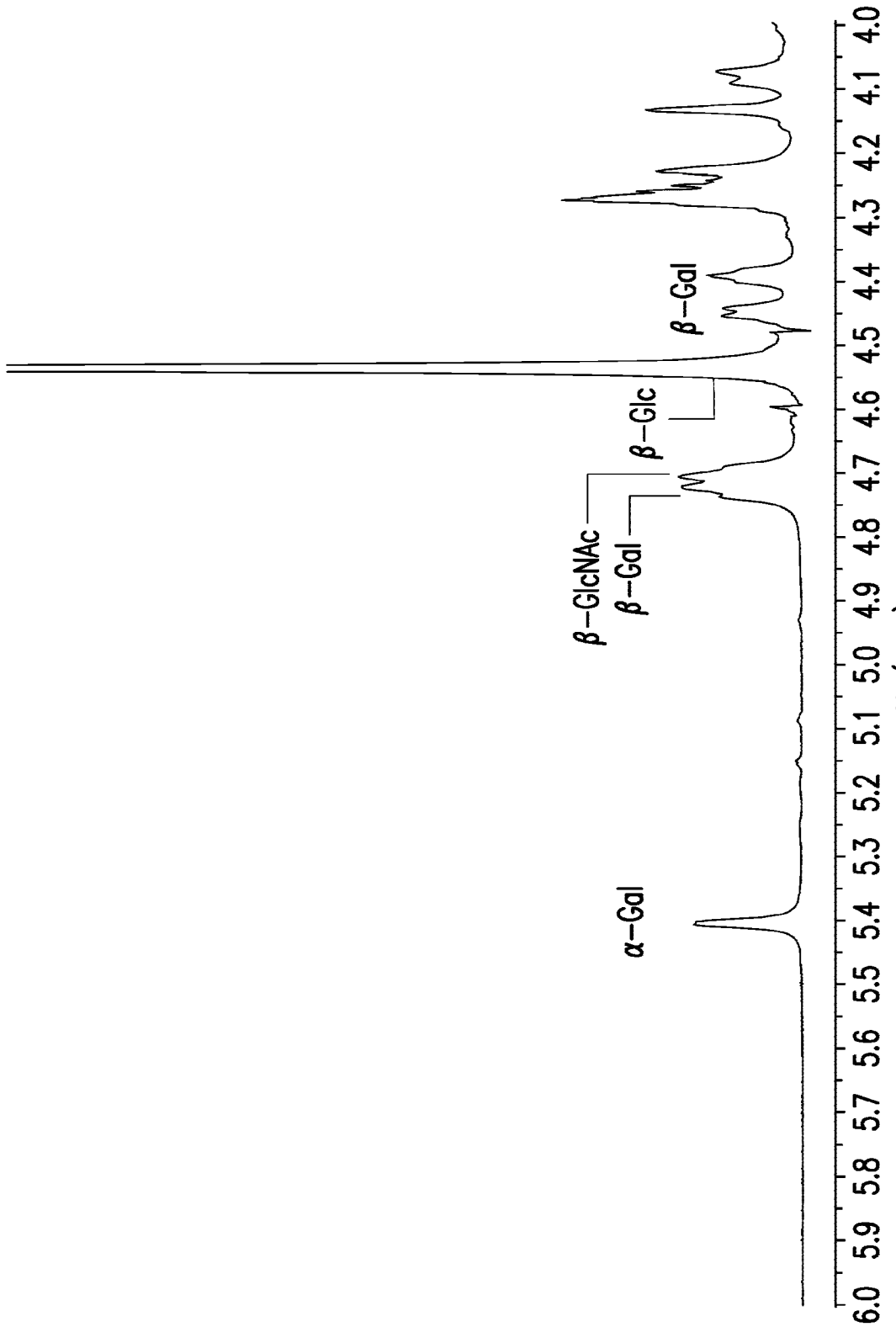

β–Gal
β–Glc
β–GlcNAc
β–Gal
α–Gal
6.0 5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0
f1 (ppm)

FIG.6

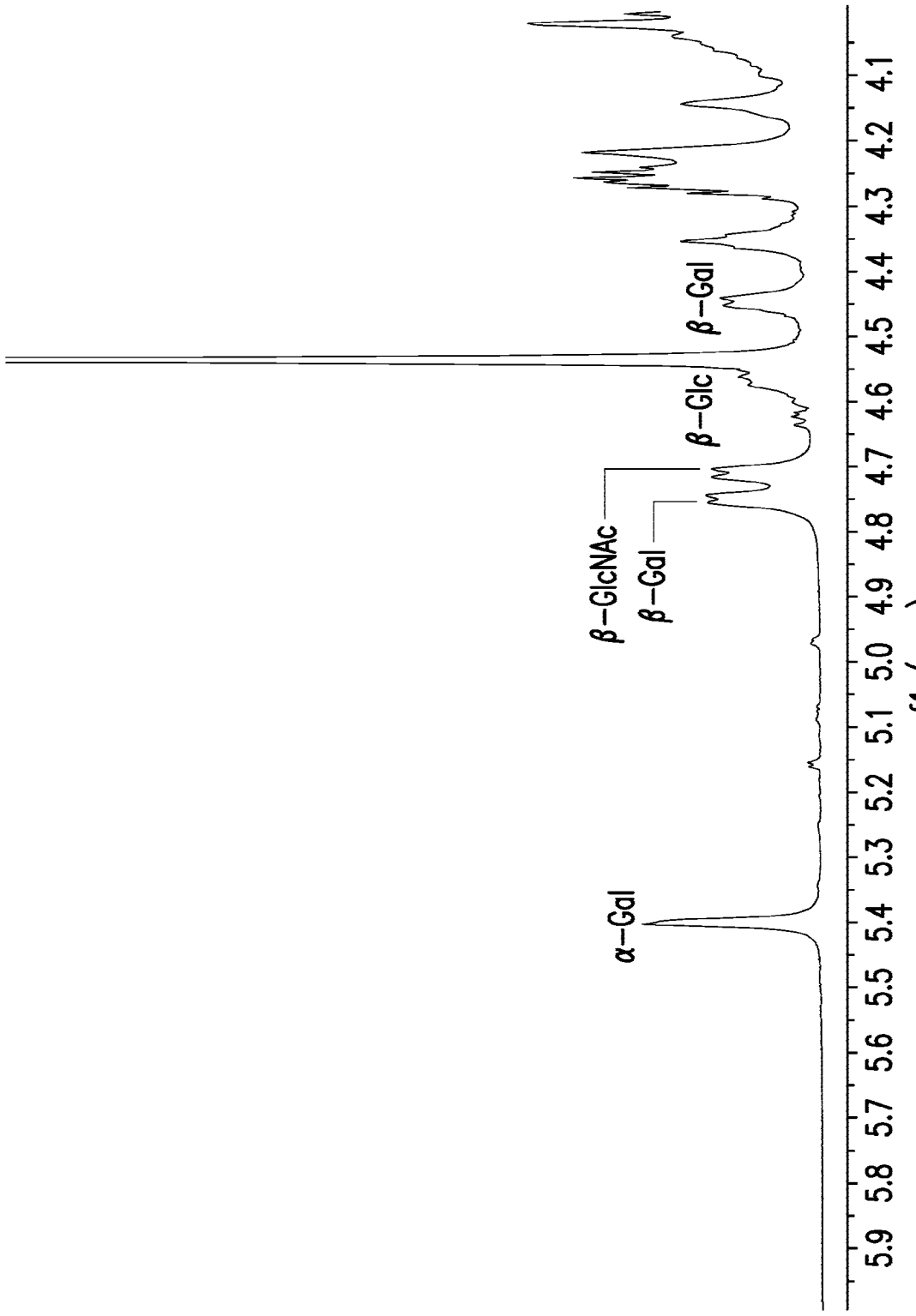

α–Gal
β–GlcNAc
β–Gal
β–Glc
β–Gal
5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1
f1 (ppm)

FIG. 7

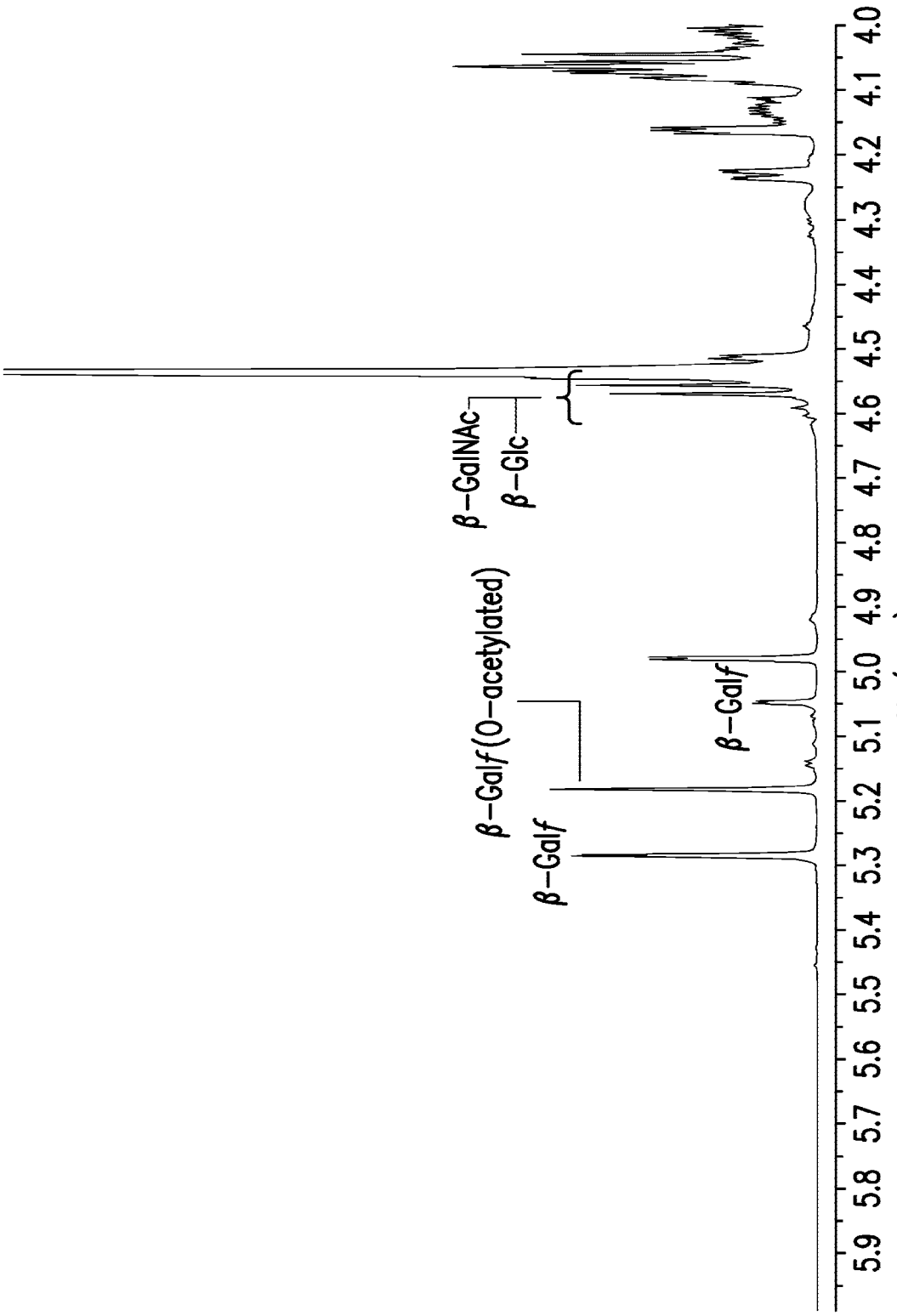

β-GalNAc
β-Glc
β-Galf(O-acetylated)
β-Galf
β-Galf
f1 (ppm)
4.0
4.1
4.2
4.3
4.4
4.5
4.6
4.7
4.8
4.9
5.0
5.1
5.2
5.3
5.4
5.5
5.6
5.7
5.8
5.9

FIG.8

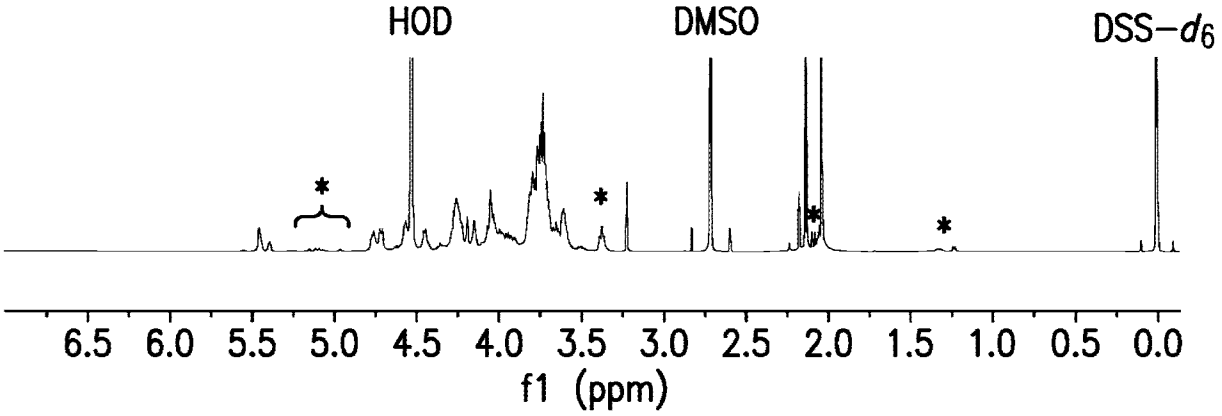


FIG.9A

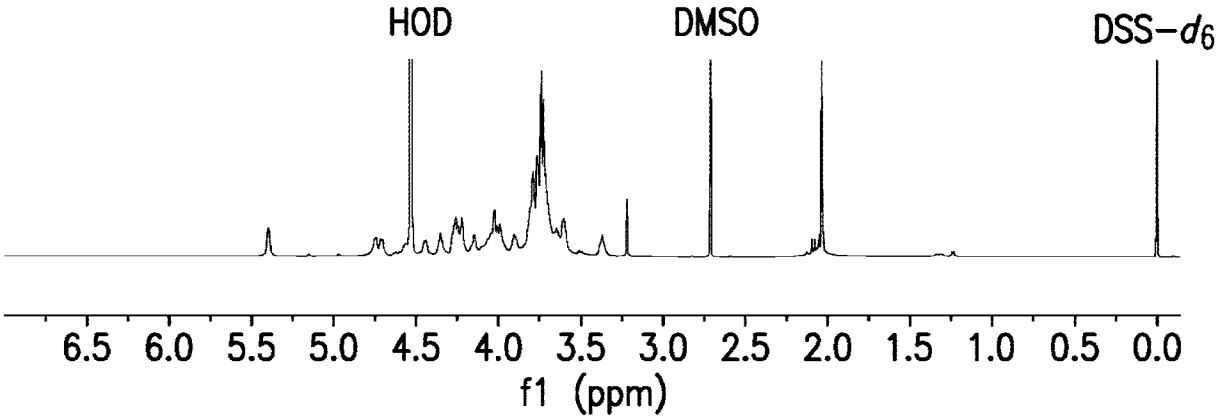


FIG.9B

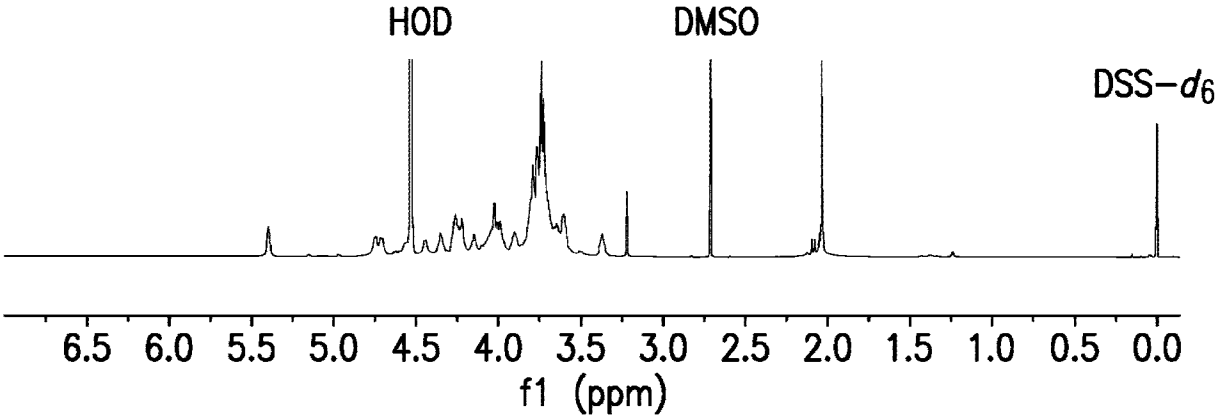


FIG.9C

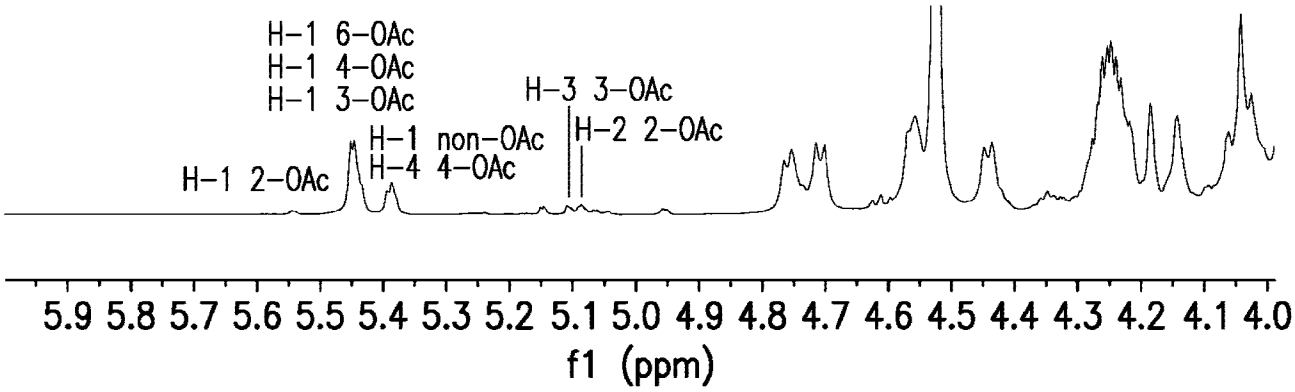


FIG.10A

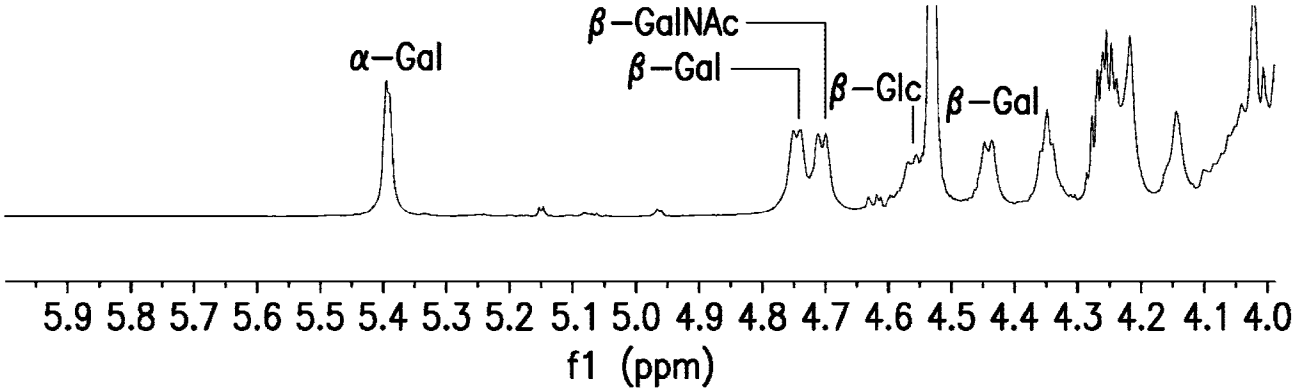


FIG.10B

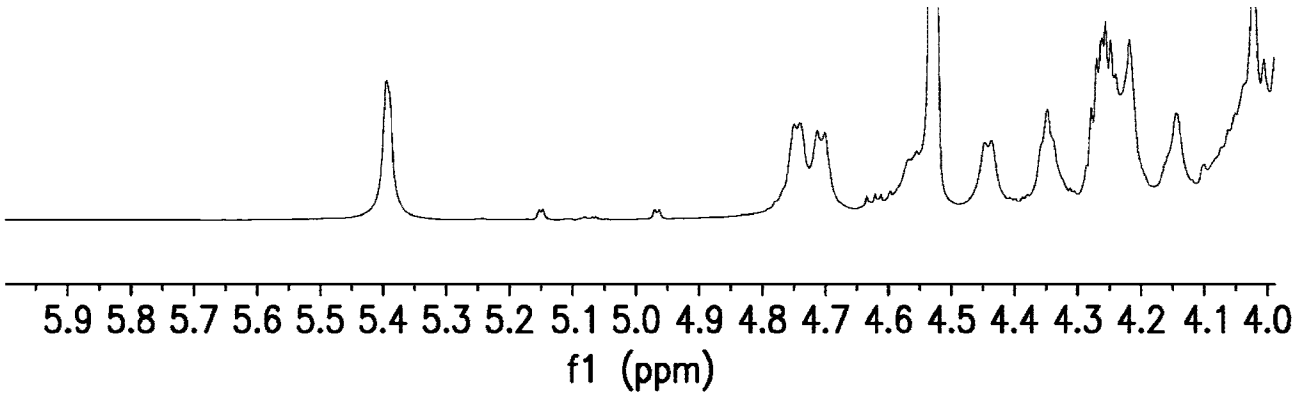


FIG.10C

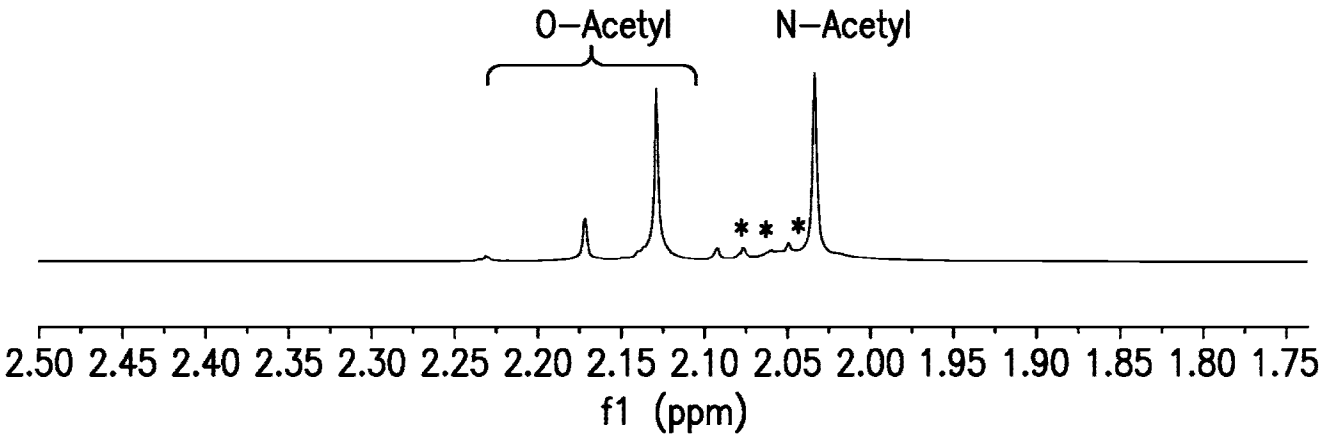


FIG.10D

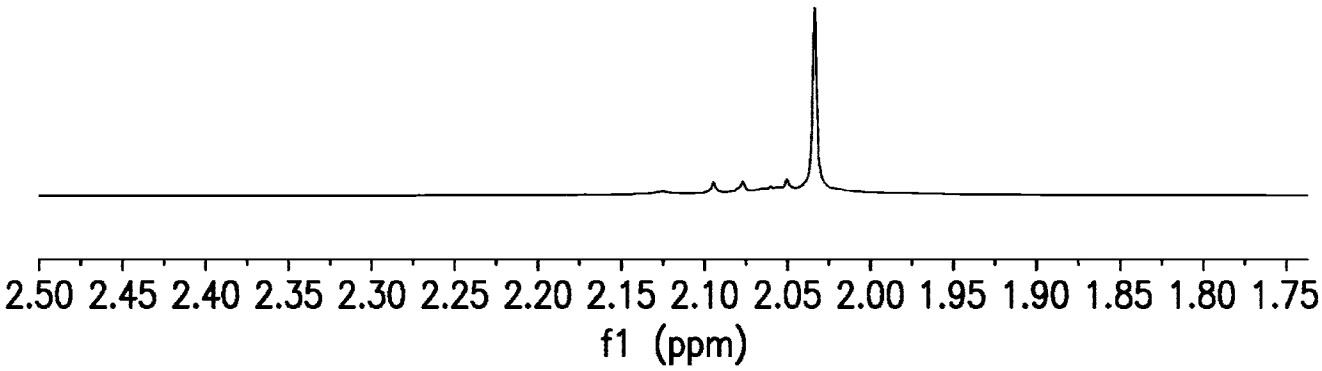


FIG.10E

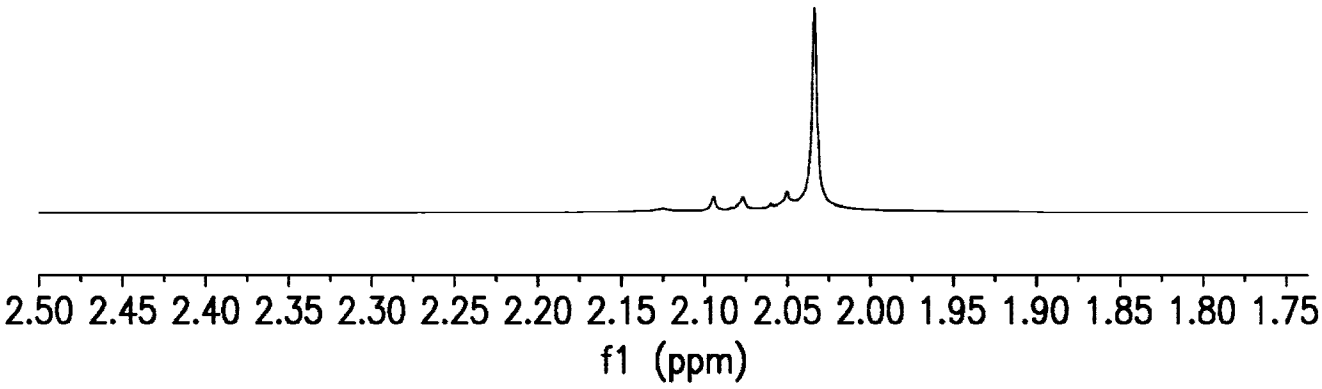


FIG.10F

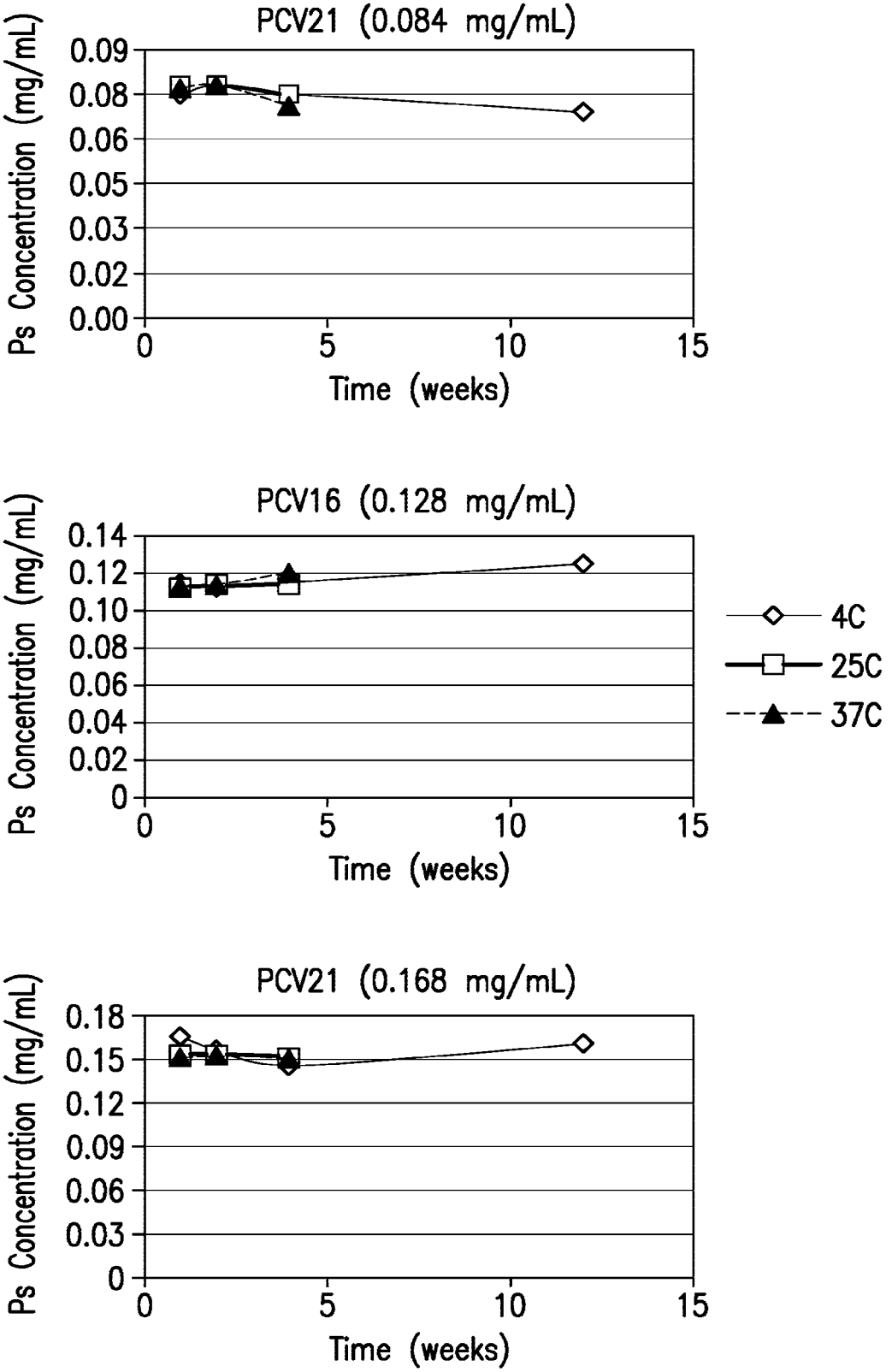

PCV21 (0.084 mg/mL)
Ps Concentration (mg/mL)
0.09
0.08
0.06
0.05
0.03
0.02
0.00
0
5
10
15
Time (weeks)
PCV16 (0.128 mg/mL)
Ps Concentration (mg/mL)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
Time (weeks)
4C
25C
37C
PCV21 (0.168 mg/mL)
Ps Concentration (mg/mL)
0.18
0.15
0.12
0.09
0.06
0.03
0
Time (weeks)

FIG.11

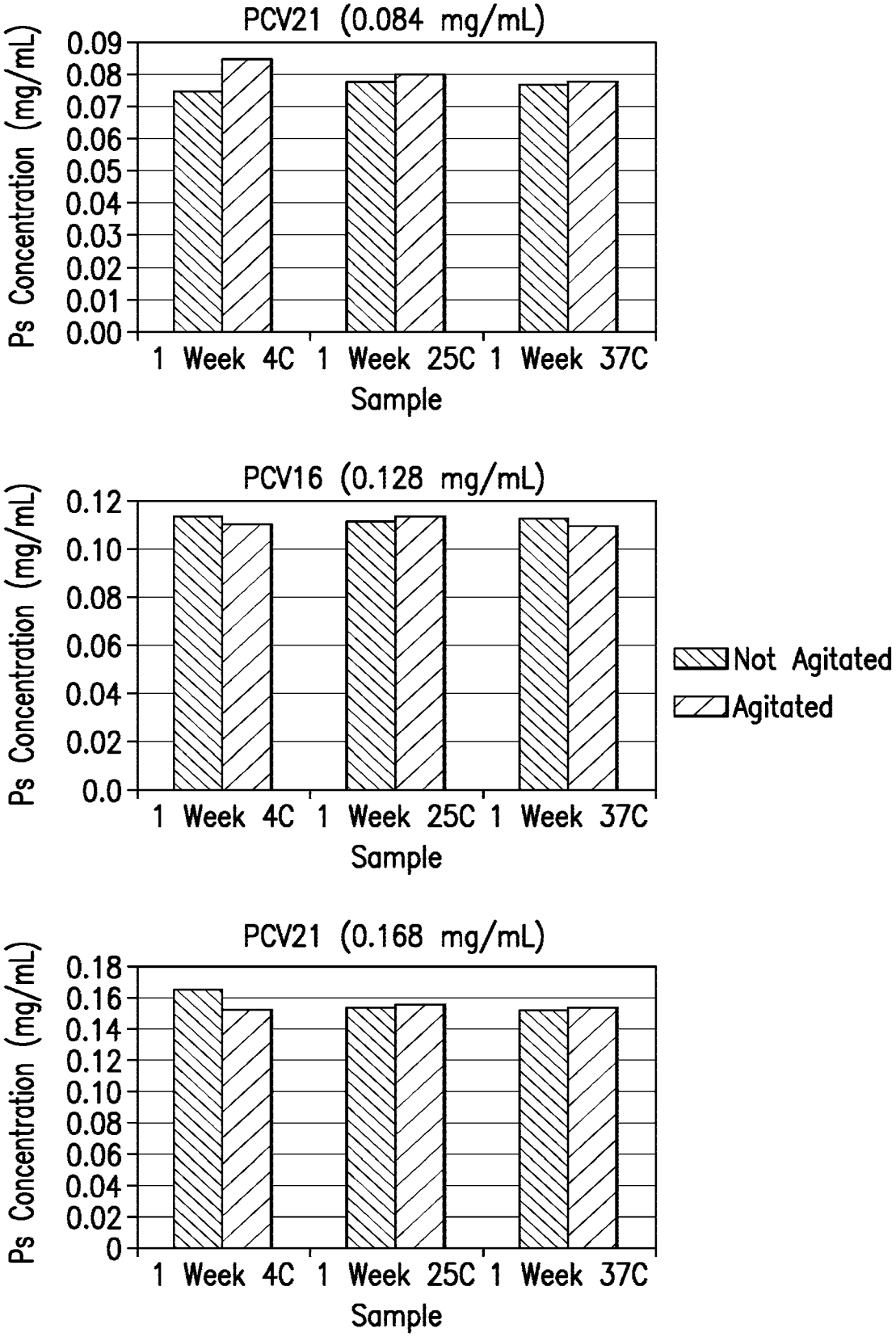

PCV21 (0.084 mg/mL)
Ps Concentration (mg/mL)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
1 Week 4C
1 Week 25C
1 Week 37C
Sample
PCV16 (0.128 mg/mL)
Ps Concentration (mg/mL)
0.12
0.10
0.08
0.06
0.04
0.02
0.0
1 Week 4C
1 Week 25C
1 Week 37C
Sample
Not Agitated
Agitated
PCV21 (0.168 mg/mL)
Ps Concentration (mg/mL)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
1 Week 4C
1 Week 25C
1 Week 37C
Sample

FIG.12

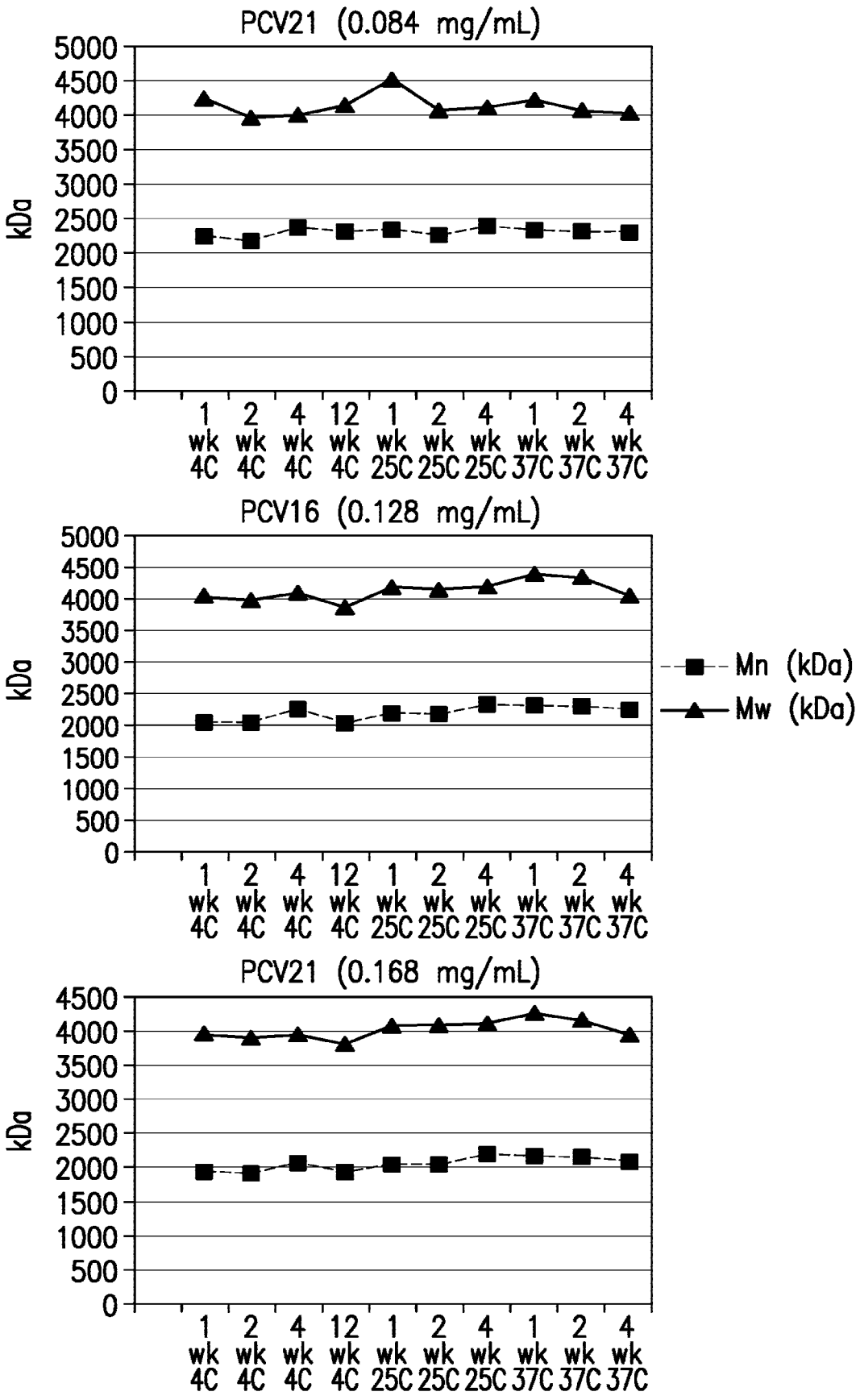

PCV21 (0.084 mg/mL)
PCV16 (0.128 mg/mL)
PCV21 (0.168 mg/mL)
kDa
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1 wk 4C
2 wk 4C
4 wk 4C
12 wk 4C
1 wk 25C
2 wk 25C
4 wk 25C
1 wk 37C
2 wk 37C
4 wk 37C
Mn (kDa)
Mw (kDa)

FIG. 13

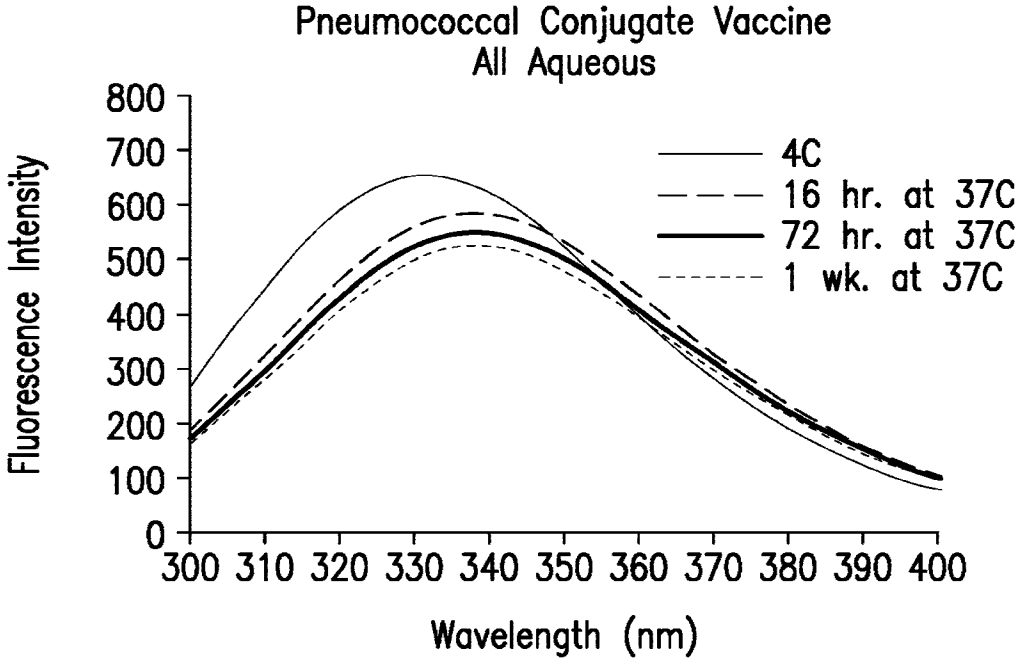


FIG.14A

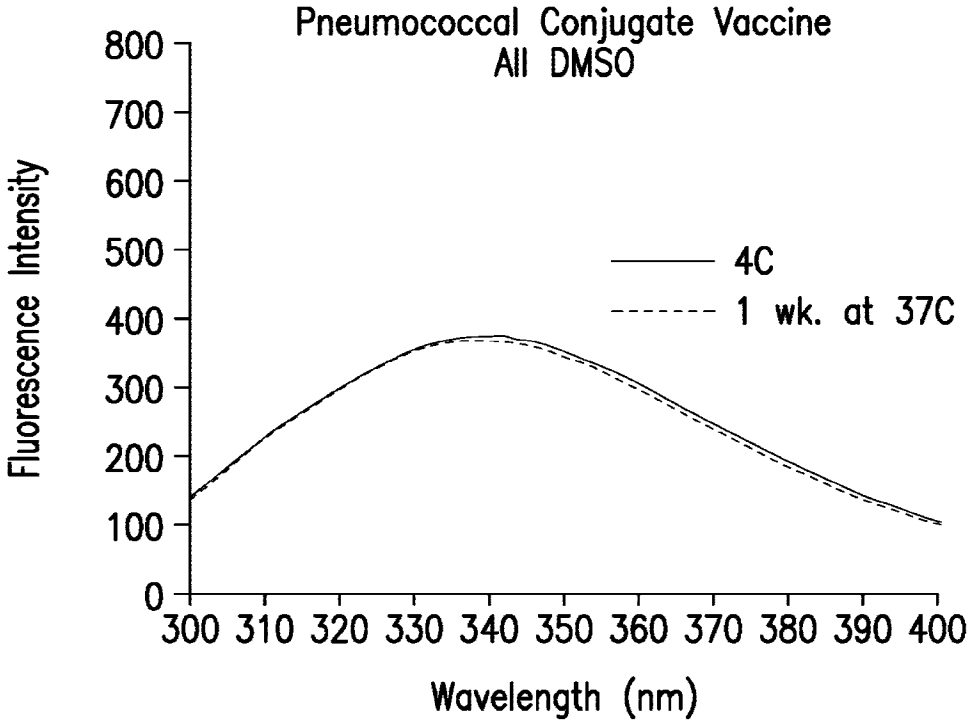


FIG.14B

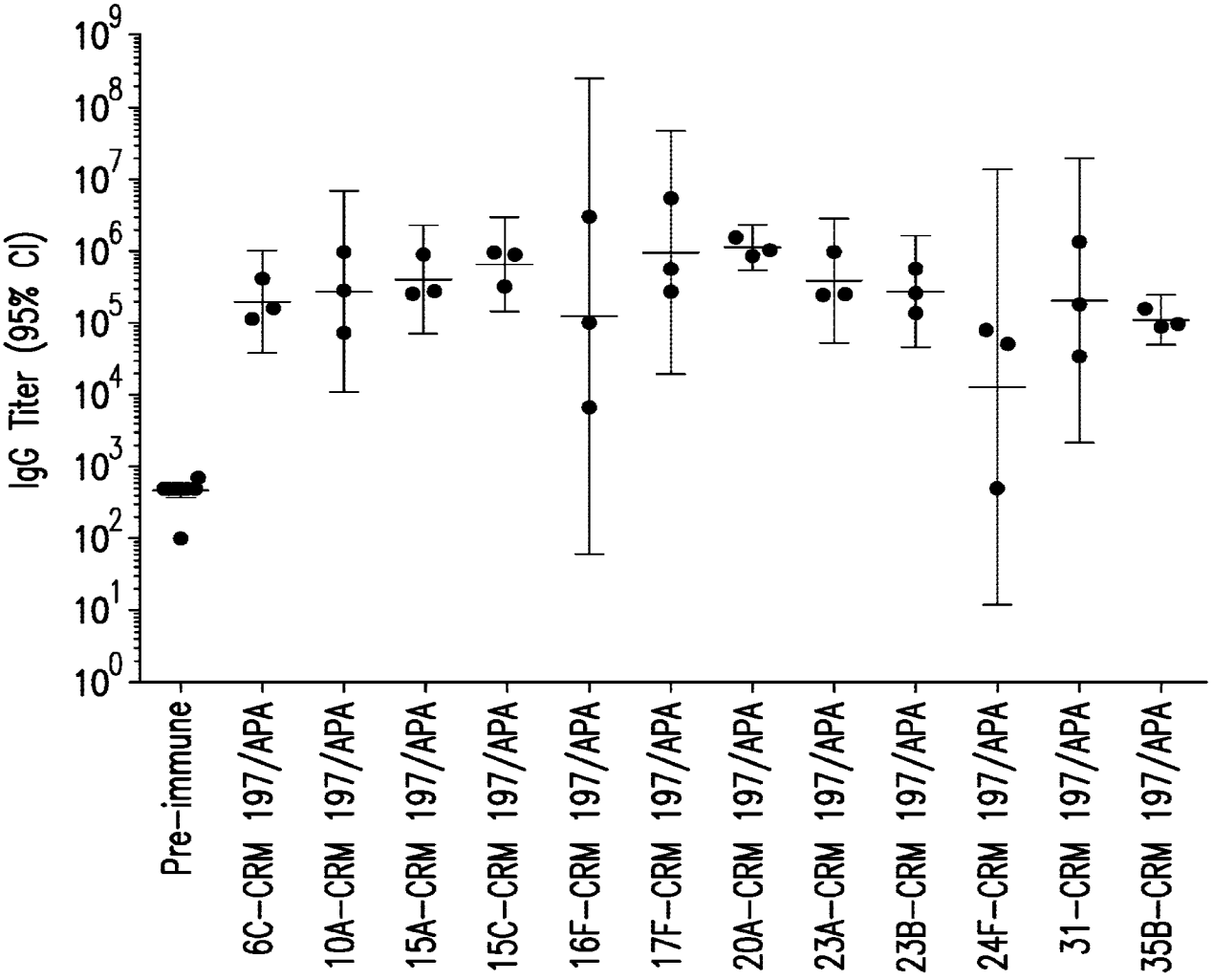

IgG Titer (95% CI)
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Pre-immune
6C-CRM 197/APA
10A-CRM 197/APA
15A-CRM 197/APA
15C-CRM 197/APA
16F-CRM 197/APA
17F-CRM 197/APA
20A-CRM 197/APA
23A-CRM 197/APA
23B-CRM 197/APA
24F-CRM 197/APA
31-CRM 197/APA
35B-CRM 197/APA

FIG.15

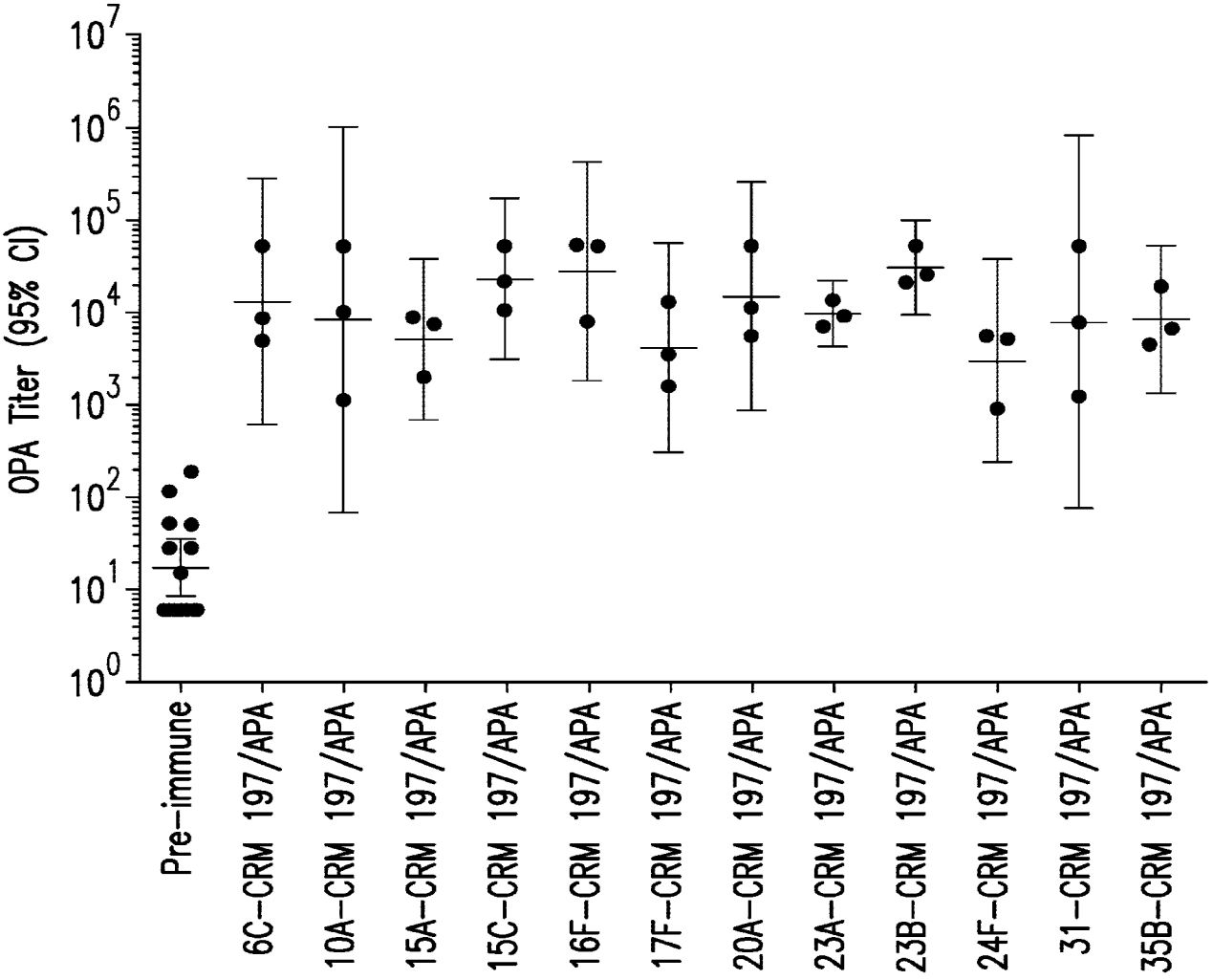

OPA Titer (95% CI)
10^0
10^1
10^2
10^3
10^4
10^5
10^6
10^7
Pre-immune
6C-CRM 197/APA
10A-CRM 197/APA
15A-CRM 197/APA
15C-CRM 197/APA
16F-CRM 197/APA
17F-CRM 197/APA
20A-CRM 197/APA
23A-CRM 197/APA
23B-CRM 197/APA
24F-CRM 197/APA
31-CRM 197/APA
35B-CRM 197/APA

FIG.16

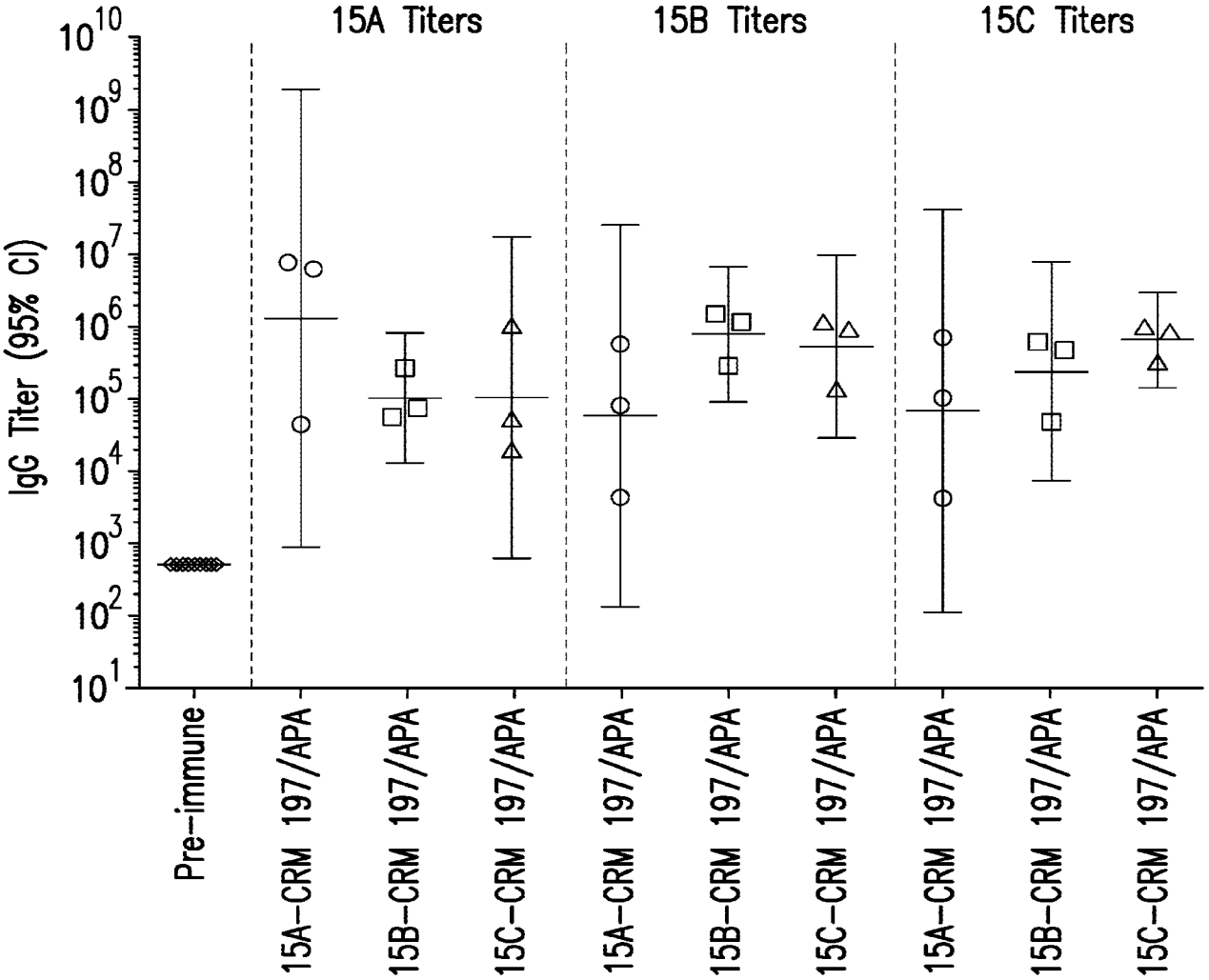

15A Titers
15B Titers
15C Titers
IgG Titer (95% CI)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
Pre-immune
15A-CRM 197/APA
15B-CRM 197/APA
15C-CRM 197/APA
15A-CRM 197/APA
15B-CRM 197/APA
15C-CRM 197/APA
15A-CRM 197/APA
15B-CRM 197/APA
15C-CRM 197/APA

FIG.17

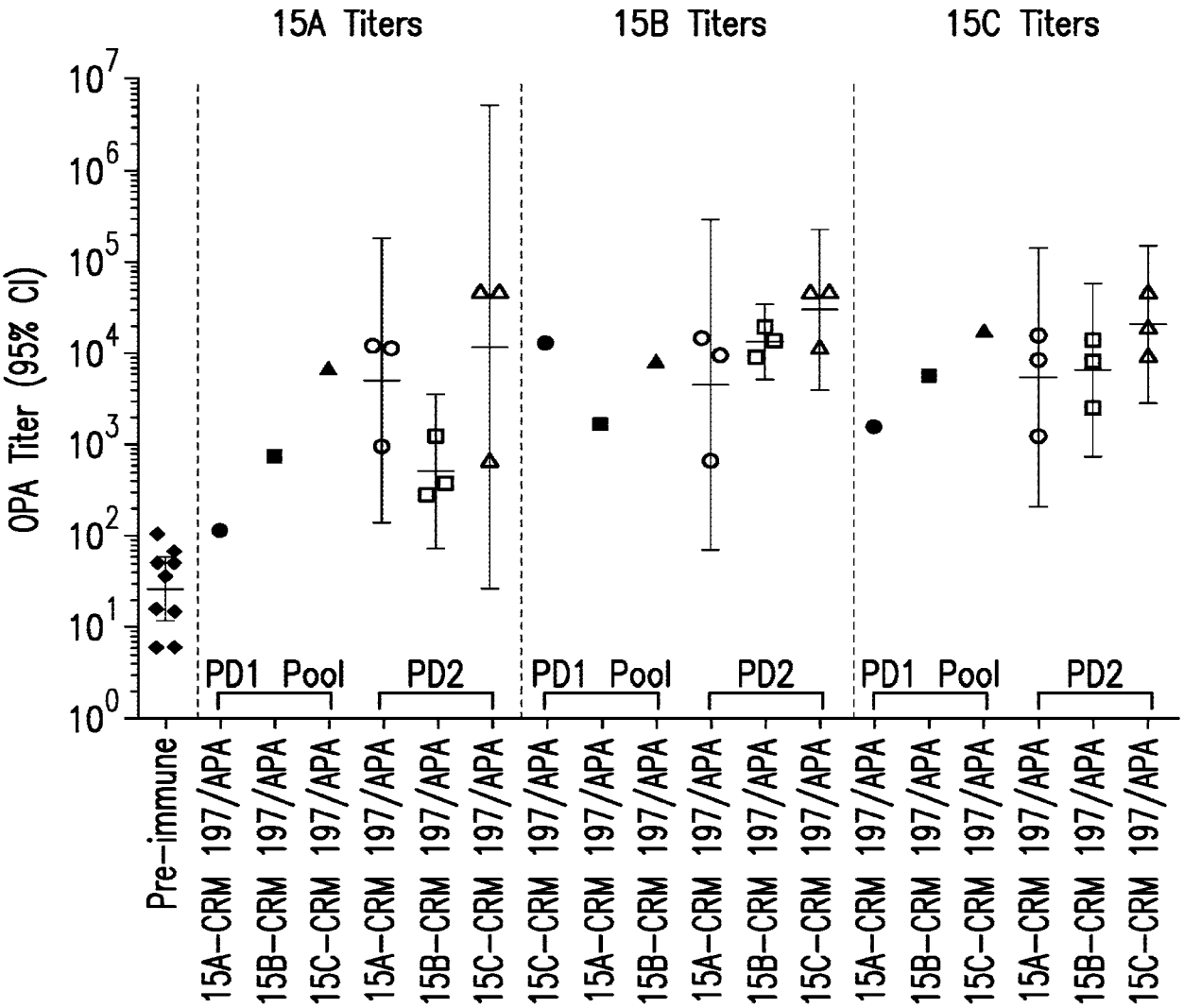

15A Titers
15B Titers
15C Titers
OPA Titer (95% CI)
10^0
10^1
10^2
10^3
10^4
10^5
10^6
10^7
PD1 Pool
PD2
Pre-immune
15A-CRM 197/APA
15B-CRM 197/APA
15C-CRM 197/APA

FIG.18

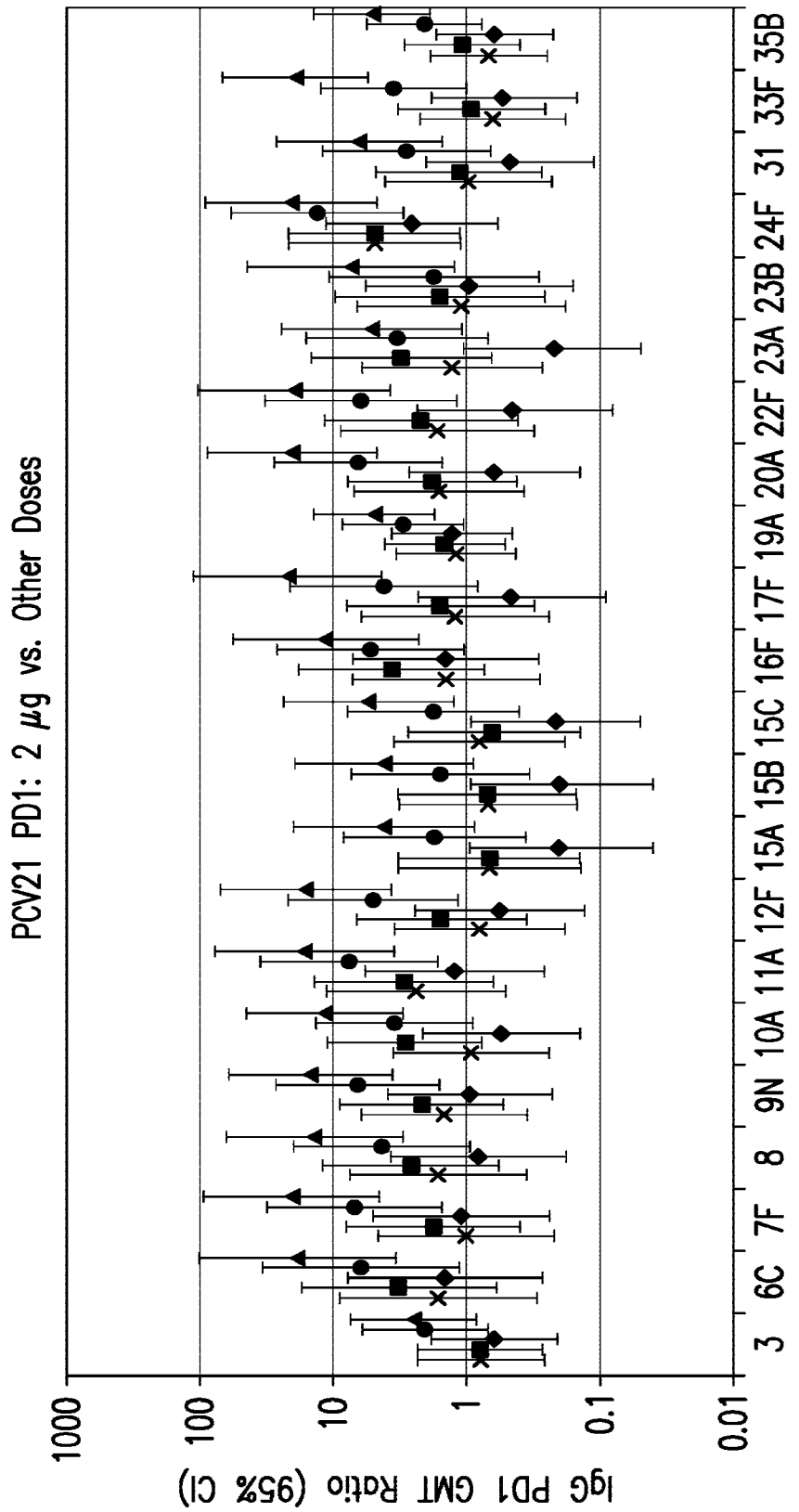

PCV21 PD1: 2 μg vs. Other Doses
lgG PD1 GMT Ratio (95% CI)
1000
100
10
1
0.1
0.01
3 6C 7F 8 9N 10A 11A 12F 15A 15B 15C 16F 17F 19A 20A 22F 23A 23B 24F 31 33F 35B
Pn Type
× 2 μg vs. 4 μg
■ 2 μg vs. 1 μg
◆ 2 μg vs. 0.4 μg
● 2 μg vs. 0.08 μg
▲ 2 μg vs. 0.016 μg

FIG.19A

Serotype	2 μg v. 4 μg	2 μg v. 1 μg	2 μg v. 0.4 μg	2 μg v. 0.08 μg	2 μg v. 0.016 μg
3	0.78 (0.26, 2.3)	0.79 (0.27, 2.3)	0.62 (0.21, 1.84)	2.1 (0.7, 6.1)	2.5 (0.85, 7.5)
6C	1.64 (0.3, 8.9)	3.2 (0.6, 18)	1.44 (0.27, 7.8)	6.3 (1.15, 34)	19 (3.5, 102)
7F	1.01 (0.22, 4.6)	1.76 (0.39, 8)	1.11 (0.24, 5.1)	6.9 (1.52, 32)	21 (4.5, 94)
8	1.64 (0.36, 7.5)	2.6 (0.57, 12)	0.81 (0.18, 3.7)	4.3 (0.94, 20)	14 (3, 63)
9N	1.46 (0.35, 6)	2.1 (0.52, 8.9)	0.94 (0.23, 3.9)	6.5 (1.58, 27)	15 (3.5, 60)
10A	0.92 (0.24, 3.5)	2.9 (0.75, 11)	0.55 (0.14, 2.1)	3.5 (0.9, 13)	12 (3, 44)
11A	2.4 (0.51, 11)	2.9 (0.62, 14)	1.21 (0.26, 5.7)	7.5 (1.61, 35)	16 (3.5, 77)
12F	0.79 (0.18, 3.5)	1.54 (0.35, 6.7)	0.55 (0.13, 2.4)	5 (1.14, 22)	16 (3.7, 70)
15A	0.68 (0.14, 3.3)	0.67 (0.14, 3.2)	0.2 (0.041, 0.95)	1.73 (0.36, 8.3)	4.1 (0.86, 20)
15B	0.68 (0.15, 3.1)	0.7 (0.15, 3.3)	0.2 (0.043, 0.93)	1.57 (0.34, 7.3)	4.2 (0.89, 19)
15C	0.81 (0.18, 3.5)	0.63 (0.14, 2.8)	0.21 (0.49, 0.93)	1.77 (0.4, 7.7)	5.4 (1.24, 24)
16F	1.42 (0.28, 7.1)	3.7 (0.73, 18)	1.43 (0.29, 7.2)	5.3 (1.05, 29)	11 (2.3, 57)
17F	1.22 (0.24, 6.2)	1.58 (0.31, 8.1)	0.46 (0.09, 2.3)	4.2 (0.82, 21)	22 (4.3, 112)
19A	1.19 (0.42, 3.4)	1.45 (0.51, 4.1)	1.29 (0.45, 3.7)	3 (1.05, 8.5)	5 (1.74, 14)
20A	1.59 (0.37, 6.9)	1.82 (0.42, 7.9)	0.62 (0.14, 2.7)	6.5 (1.52, 28)	20 (4.7, 88)
22F	1.65 (0.31, 8.8)	2.2 (0.41, 12)	0.45 (0.084, 2.4)	6.2 (1.17, 33)	20 (3.7, 105)
23A	1.27 (0.27, 6)	3.1 (0.65, 15)	0.22 (0.046, 1.04)	3.3 (0.7, 16)	5.1 (1.08, 25)
23B	1.09 (0.18, 6.7)	1.59 (0.26, 9.7)	0.95 (0.16, 5.8)	1.8 (0.29, 11)	7.4 (1.21, 45)
24F	4.9 (1.1, 21)	4.9 (1.11, 22)	2.6 (0.58, 11)	13 (3, 59)	21 (4.7, 92)
31	0.96 (0.23, 4.1)	1.13 (0.27, 4.8)	0.47 (0.11, 2)	2.1 (0.66, 12)	6.4 (1.51, 27)
33F	0.64 (0.18, 2.2)	0.92 (0.26, 3.3)	0.53 (0.15, 1.85)	3.6 (1.03, 13)	19 (5.5, 68)
35B	0.69 (0.25, 1.87)	1.08 (0.4, 2.9)	0.63 (0.23, 1.69)	2.1 (0.77, 5.6)	5.1 (1.9, 14)

FIG.19B

Petitioner Merck Ex-1015, 0838

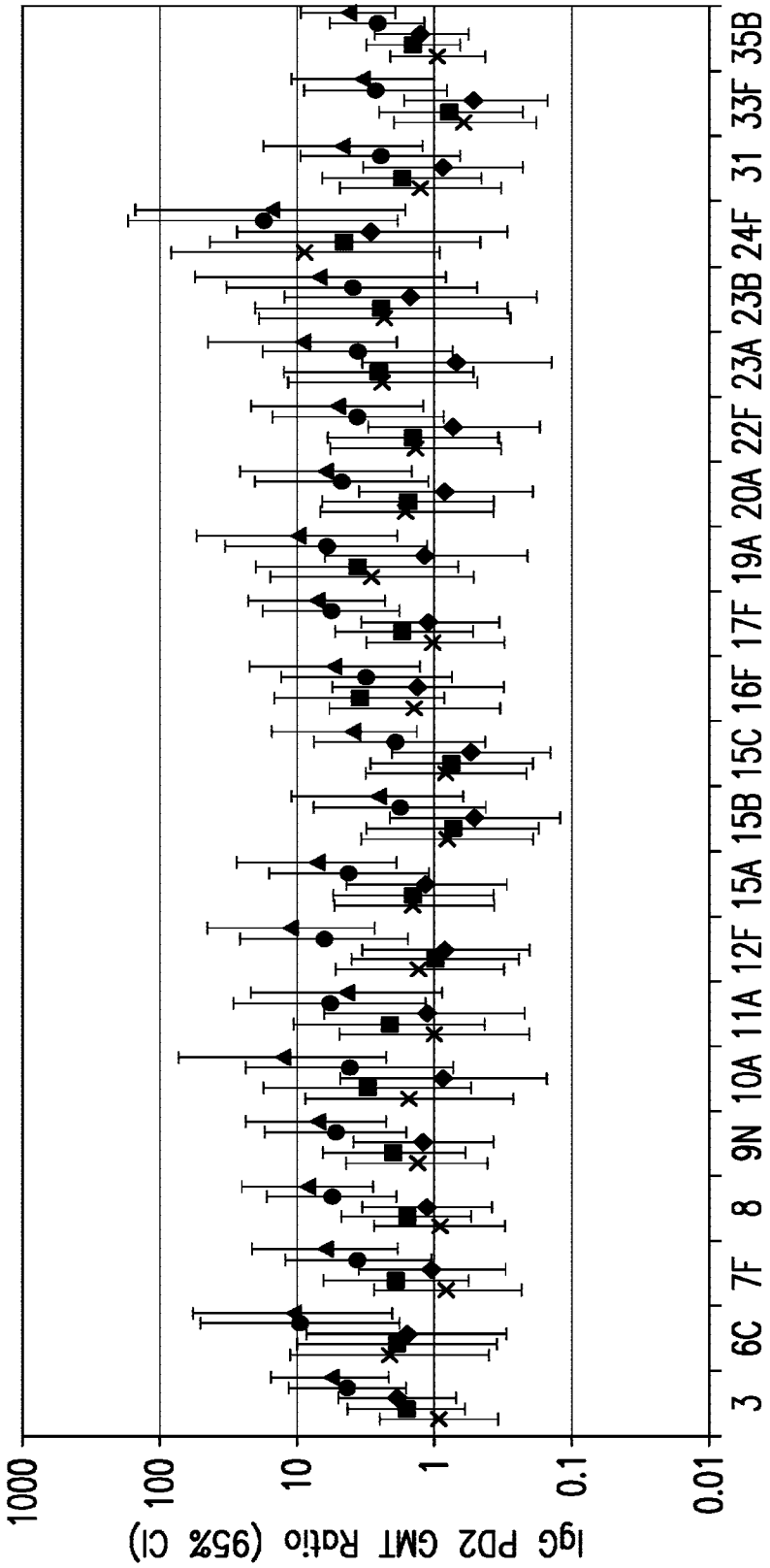

1000
100
10
1
0.1
0.01
IgG PD2 GMT Ratio (95% CI)
3 6C 7F 8 9N 10A 11A 12F 15A 15B 15C 16F 17F 19A 20A 22F 23A 23B 24F 31 33F 35B
Pn Type
× 2 μg vs. 4 μg
■ 2 μg vs. 1 μg
◆ 2 μg vs. 0.4 μg
● 2 μg vs. 0.08 μg
▲ 2 μg vs. 0.016 μg

FIG.20A

Serotype	2 μg v. 4 μg	2 μg v. 1 μg	2 μg v. 0.4 μg	2 μg v. 0.08 μg	2 μg v. 0.016 μg
3	0.91 (0.34, 2.5)	1.58 (0.59, 4.3)	1.86 (0.69, 5.0)	4.3 (1.59, 12.0)	5.7 (2.1, 15.0)
6C	2.1 (0.39, 11.0)	1.87(0.35, 10)	1.6 (0.3, 8.6)	9.6 (1.79, 51)	11 (1.98, 56)
7F	0.8 (0.23, 2.7)	1.88 (0.55, 6.4)	1.03 (0.3, 3.5)	3.6 (1.05, 12)	6.2 (1.83, 21)
8	0.9 (0.3, 2.7)	1.57 (0.53, 4.7)	1.1 (0.37, 3.3)	5.5 (1.84, 17)	8.3 (2.8, 25)
9N	1.31 (0.4, 4.3)	1.92 (0.58, 6.3)	1.17 (0.36, 3.8)	5.1 (1.56, 17)	7.1 (2.2, 23)
10A	1.49 (0.26, 8.6)	3 (0.53, 17)	0.84 (0.15, 4.8)	4.1 (0.71, 23)	13 (2.2, 72)
11A	0.98 (0.2, 4.9)	2.1 (0.42, 10)	1.09 (0.22, 5.4)	5.7 (1.15, 29)	4.3 (0.86, 22)
12F	1.28 (0.31, 5.2)	0.96 (0.24, 3.9)	0.8 (0.2, 3.3)	6.3 (1.53, 26)	11 (2.7, 45)
15A	1.39 (0.36, 5.3)	1.39 (0.36, 5.3)	1.12 (0.29, 4.3)	4.1 (1.06, 16)	7.1 (1.85, 27)
15B	0.79 (0.19, 3.3)	0.72 (0.17, 3)	0.49 (0.12, 2.1)	1.75 (0.41, 7.4)	2.5 (0.6, 11)
15C	0.81 (0.21, 3.1)	0.74 (0.19, 2.9)	0.53 (0.14, 2)	1.92 (0.5, 7.4)	3.9 (1.03, 15)
16F	1.37 (0.33, 5.8)	3.5 (0.83, 15)	1.31 (0.31, 5.5)	3.1 (0.73, 13)	5.2 (1.24, 22)
17F	0.99 (0.31, 3.1)	1.65 (0.52, 5.2)	1.06 (0.33, 3.4)	5.6 (1.76, 18)	7.1 (2.3, 23)
19A	2.8 (0.51, 16)	3.6 (0.65, 20)	1.13 (0.21, 6.2)	6.1 (1.0, 33)	9.8 (1.78, 54)
20A	1.59 (0.37, 6.8)	1.53 (0.36, 6.5)	0.83 (0.19, 3.5)	4.7 (1.1, 20)	6.2 (1.45, 26)
22F	1.34 (0.32, 5.7)	1.42 (0.34, 6)	0.72 (0.17, 3)	3.6 (0.84, 15)	5.1 (1.2, 21)
23A	2.4 (0.48, 12)	2.5 (0.51, 12)	0.67 (0.14, 3.3)	3.6 (0.74, 18)	9.1 (1.84, 45)
23B	2.3 (0.28, 19)	2.4 (0.29, 20)	1.49 (0.18, 12)	4 (0.48, 33)	6.8 (0.82, 56)
24F	8.6 (0.9, 83)	4.5 (0.46, 43)	2.9 (0.3, 28)	18 (1.85, 172)	16 (1.61, 150)
31	1.26 (0.33, 4.8)	1.17 (0.45, 6.5)	0.86 (0.23, 3.3)	2.5 (0.64, 9.4)	4.7 (1.22, 18)
33F	0.6 (0.18, 1.97)	0.77 (0.23, 2.5)	0.5 (0.15, 1.65)	2.7 (0.81, 8.9)	3.4 (1.02, 11)
35B	0.94 (0.43, 2.1)	1.41 (0.64, 3.1)	1.24 (0.56, 2.8)	2.6 (1.18, 5.8)	4.3 (1.94, 9.6)

FIG.2OB

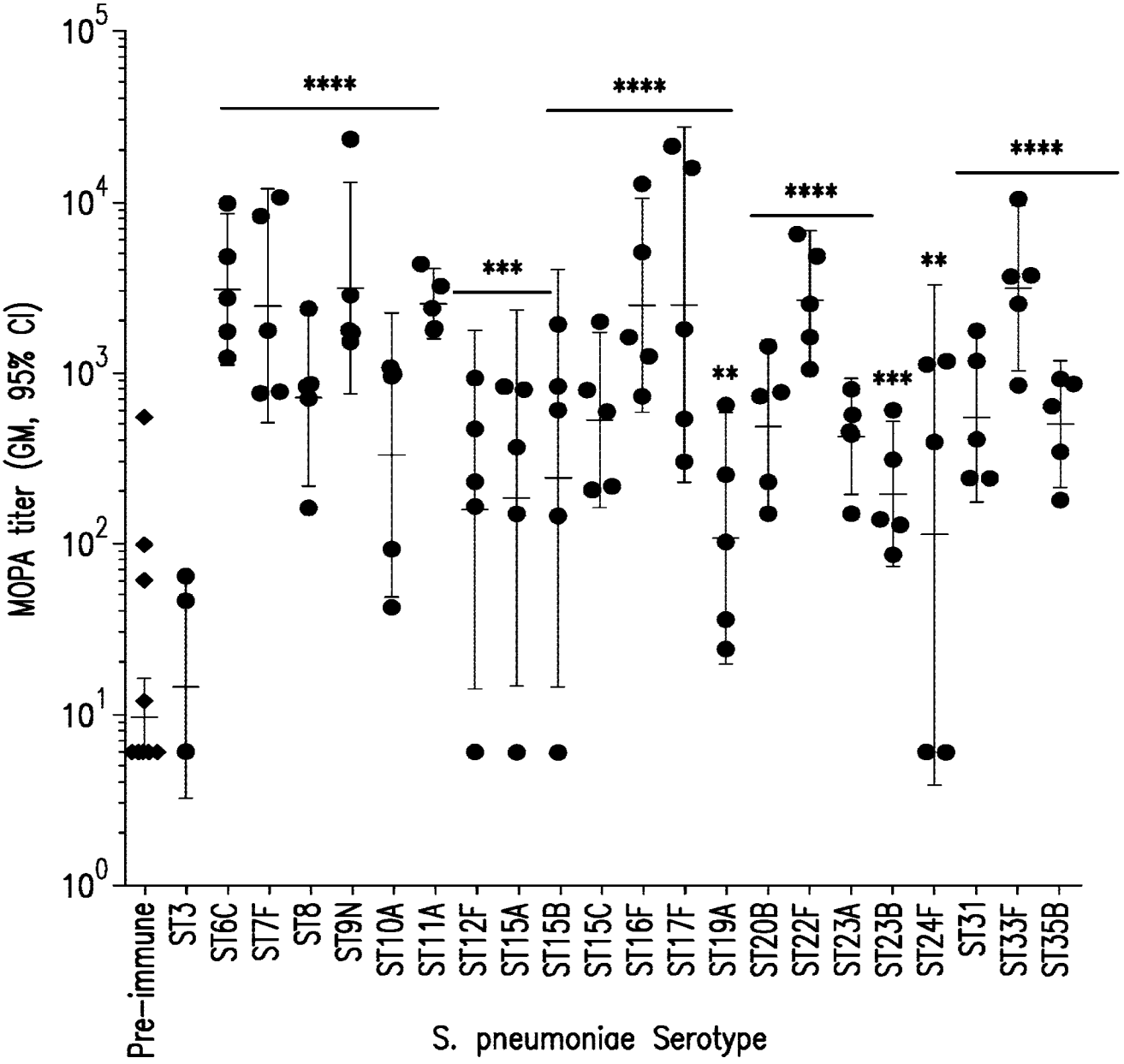

MOPA titer (GM, 95% CI)
10^0
10^1
10^2
10^3
10^4
10^5
****
****
***
****
**
***
**
****
Pre-immune
ST3
ST6C
ST7F
ST8
ST9N
ST10A
ST11A
ST12F
ST15A
ST15B
ST15C
ST16F
ST17F
ST19A
ST20B
ST22F
ST23A
ST23B
ST24F
ST31
ST33F
ST35B
S. pneumoniae Serotype

FIG.21A

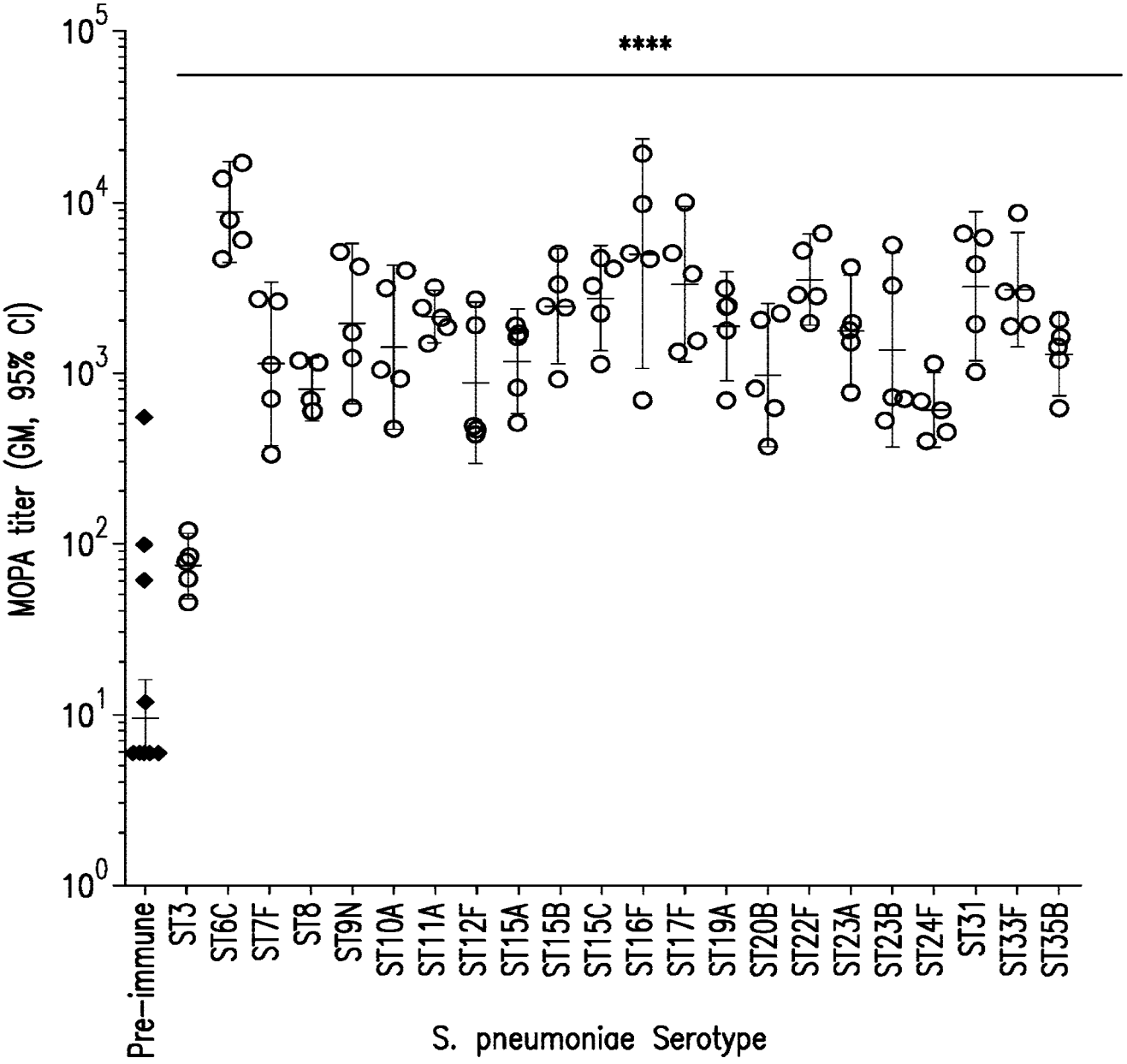

****
MOPA titer (GM, 95% CI)
10^0
10^1
10^2
10^3
10^4
10^5
Pre-immune
ST3
ST6C
ST7F
ST8
ST9N
ST10A
ST11A
ST12F
ST15A
ST15B
ST15C
ST16F
ST17F
ST19A
ST20B
ST22F
ST23A
ST23B
ST24F
ST31
ST33F
ST35B
S. pneumoniae Serotype

FIG.21B

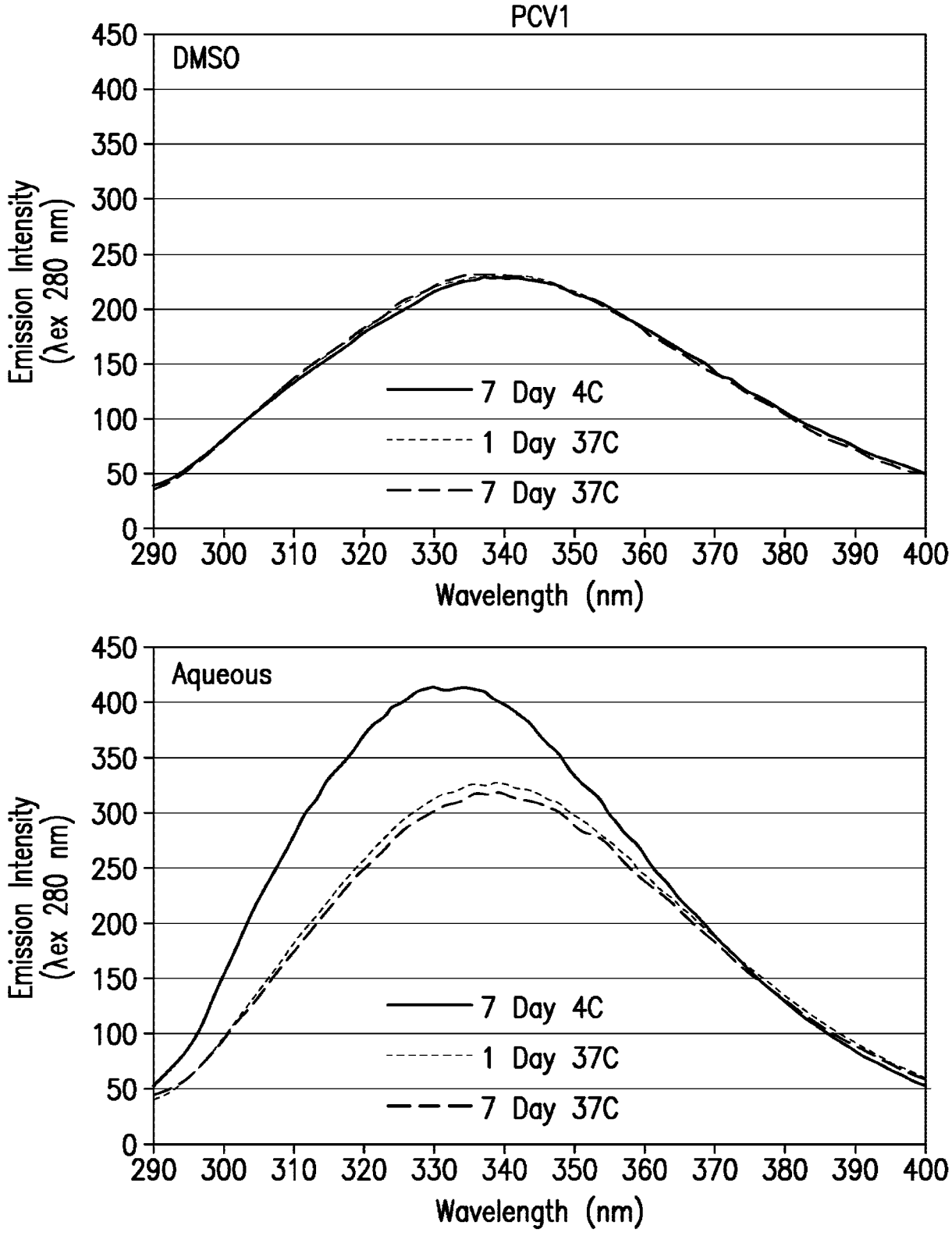

PCV1
DMSO
Emission Intensity (λex 280 nm)
450
400
350
300
250
200
150
100
50
0
290 300 310 320 330 340 350 360 370 380 390 400
Wavelength (nm)
7 Day 4C
1 Day 37C
7 Day 37C
Aqueous
Emission Intensity (λex 280 nm)
Wavelength (nm)
7 Day 4C
1 Day 37C
7 Day 37C

FIG.22A

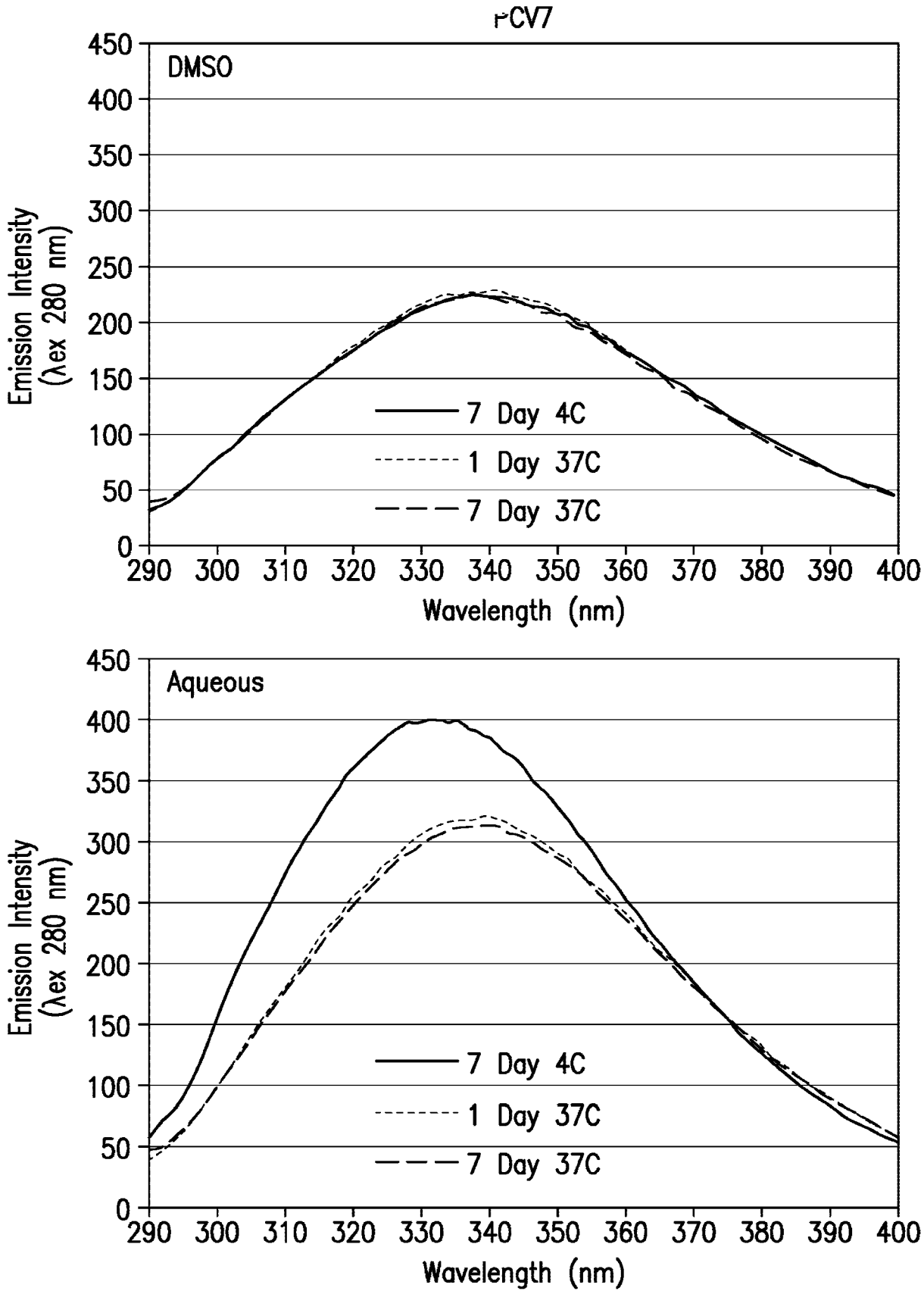

PCV7
DMSO
Emission Intensity (λex 280 nm)
450
400
350
300
250
200
150
100
50
0
7 Day 4C
1 Day 37C
7 Day 37C
290 300 310 320 330 340 350 360 370 380 390 400
Wavelength (nm)
Aqueous
Emission Intensity (λex 280 nm)
450
400
350
300
250
200
150
100
50
0
7 Day 4C
1 Day 37C
7 Day 37C
290 300 310 320 330 340 350 360 370 380 390 400
Wavelength (nm)

FIG.22B

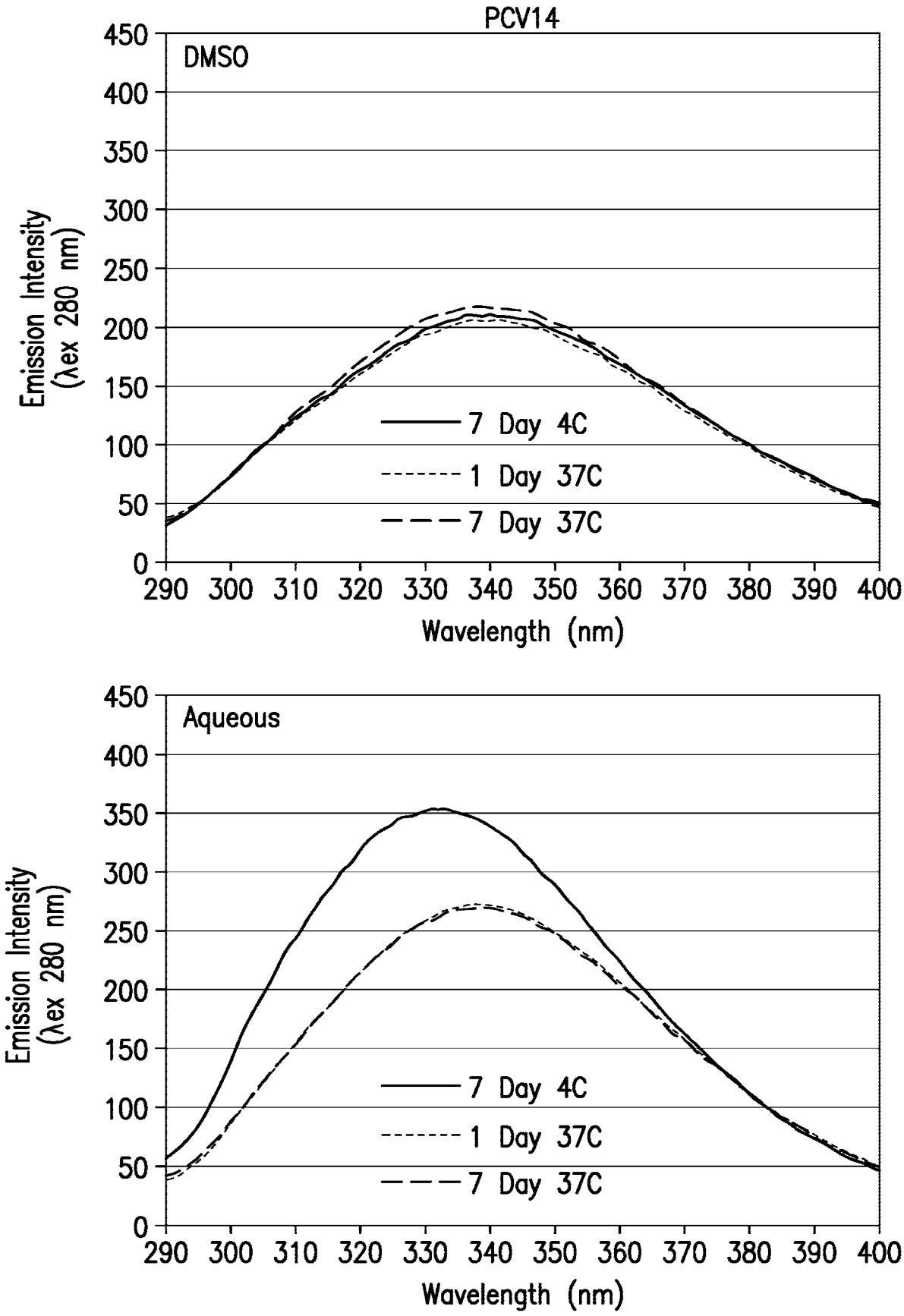

PCV14
DMSO
Emission Intensity (λex 280 nm)
450
400
350
300
250
200
150
100
50
0
7 Day 4C
1 Day 37C
7 Day 37C
290 300 310 320 330 340 350 360 370 380 390 400
Wavelength (nm)
Aqueous
Emission Intensity (λex 280 nm)
450
400
350
300
250
200
150
100
50
0
7 Day 4C
1 Day 37C
7 Day 37C
290 300 310 320 330 340 350 360 370 380 390 400
Wavelength (nm)

FIG.22C

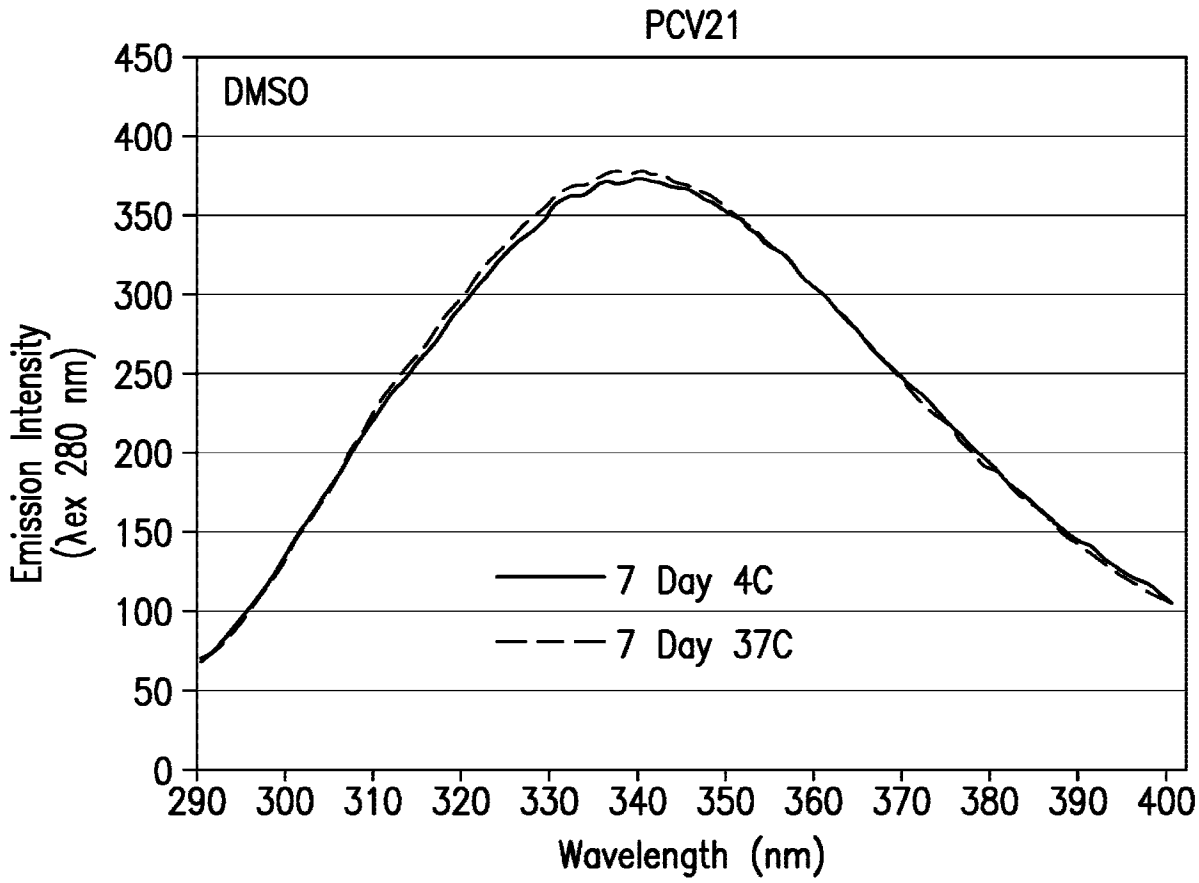

PCV21
DMSO
Emission Intensity
(λex 280 nm)
450
400
350
300
250
200
150
100
50
0
7 Day 4C
7 Day 37C
290 300 310 320 330 340 350 360 370 380 390 400
Wavelength (nm)

FIG.22D

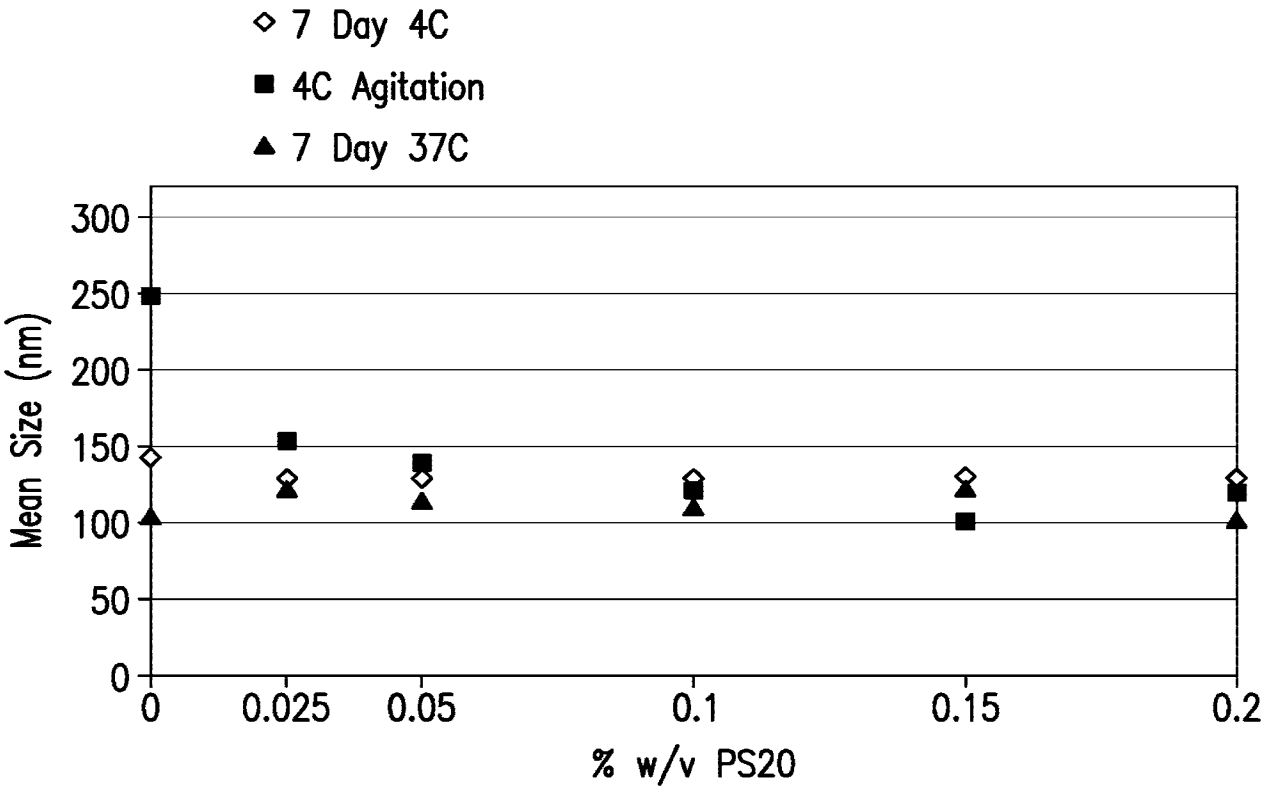

◇ 7 Day 4C
■ 4C Agitation
▲ 7 Day 37C
Mean Size (nm)
300
250
200
150
100
50
0
0
0.025
0.05
0.1
0.15
0.2
% w/v PS20

FIG.23

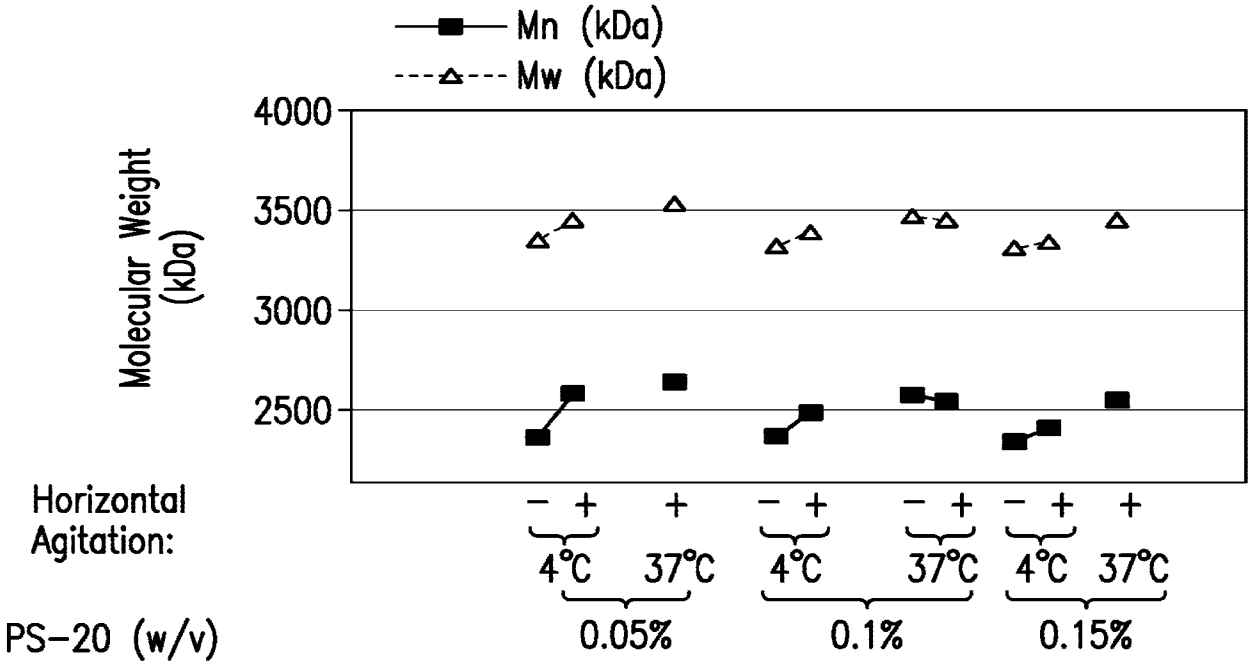

Mn (kDa)
Mw (kDa)
Molecular Weight (kDa)
4000
3500
3000
2500
Horizontal Agitation:
PS-20 (w/v)
4°C
37°C
0.05%
0.1%
0.15%

FIG.24

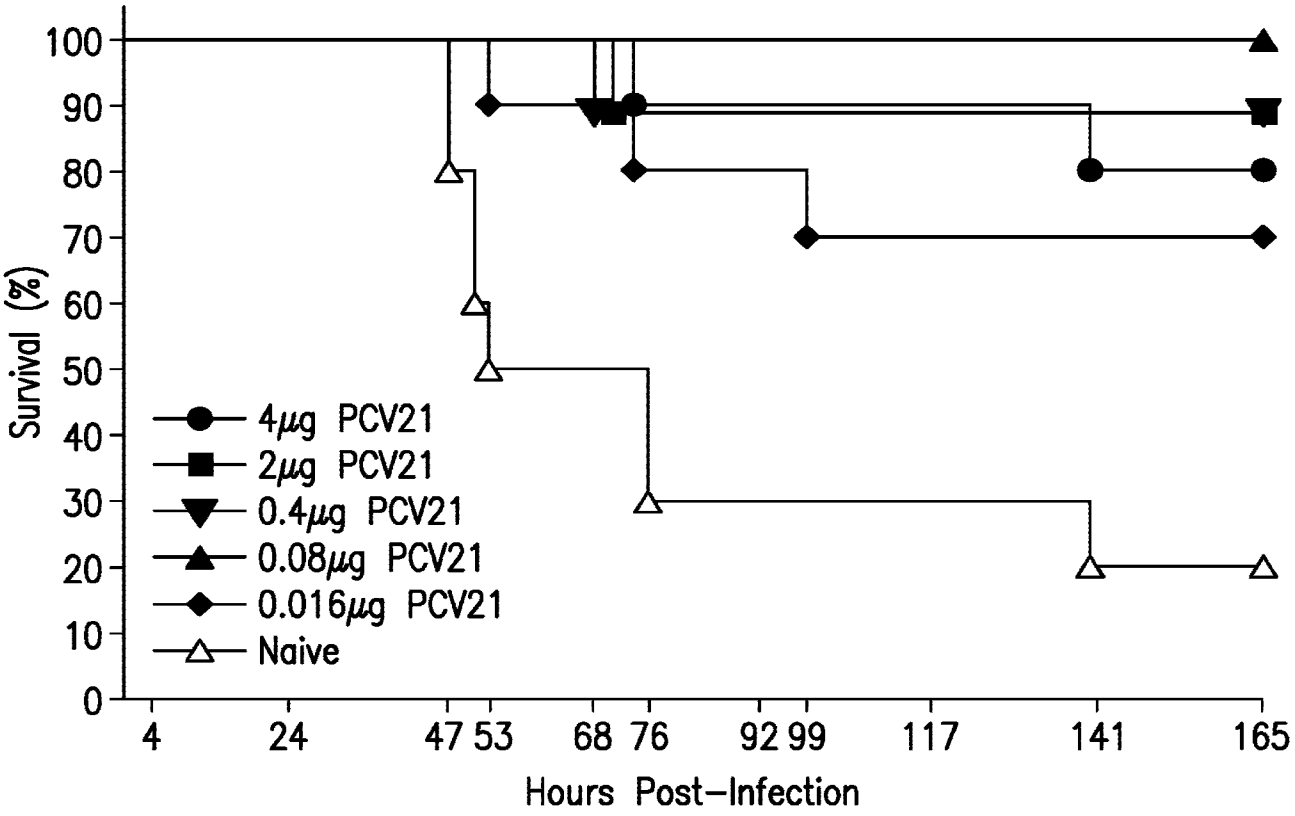

100
90
80
70
60
50
40
30
20
10
0
Survival (%)
4μg PCV21
2μg PCV21
0.4μg PCV21
0.08μg PCV21
0.016μg PCV21
Naive
4
24
47 53
68 76
92 99
117
141
165
Hours Post-Infection

FIG.25

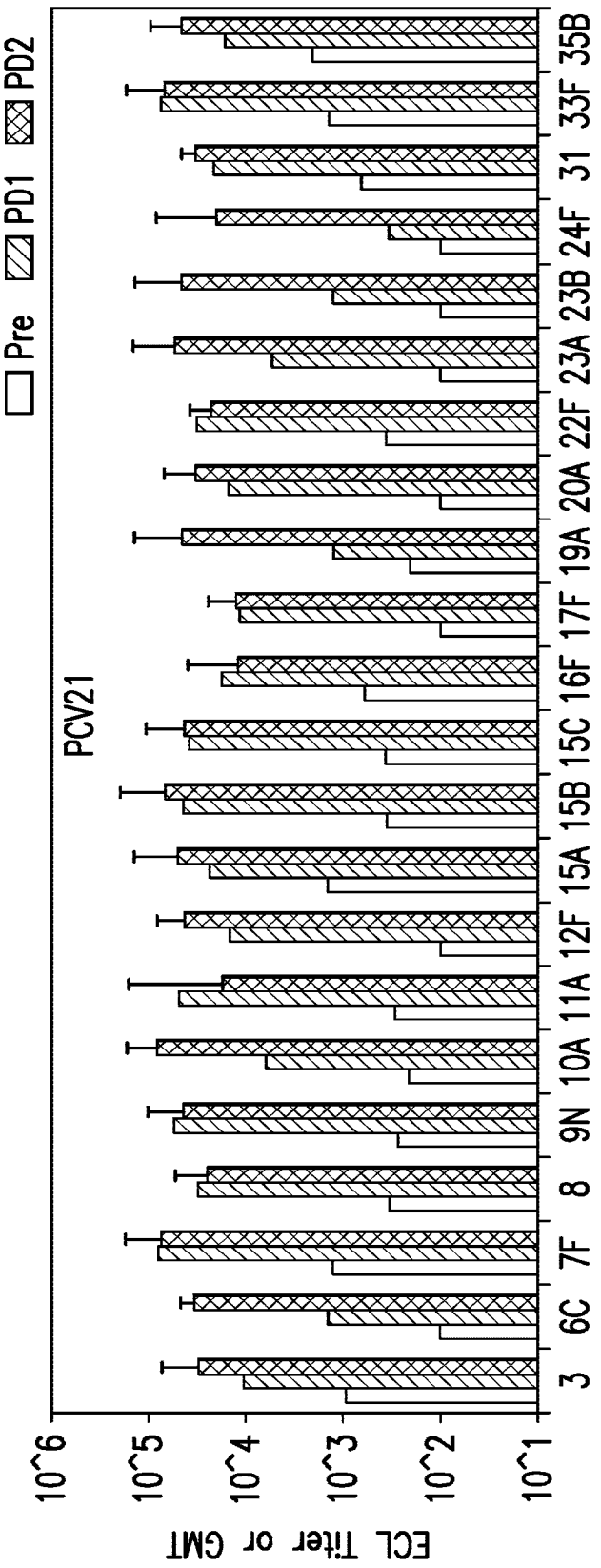

Pre
PD1
PD2
PCV21
ECL Titer or GMT
10^6
10^5
10^4
10^3
10^2
10^1
3
6C
7F
8
9N
10A
11A
12F
15A
15B
15C
16F
17F
19A
20A
22F
23A
23B
24F
31
33F
35B
Serotype

FIG.26A

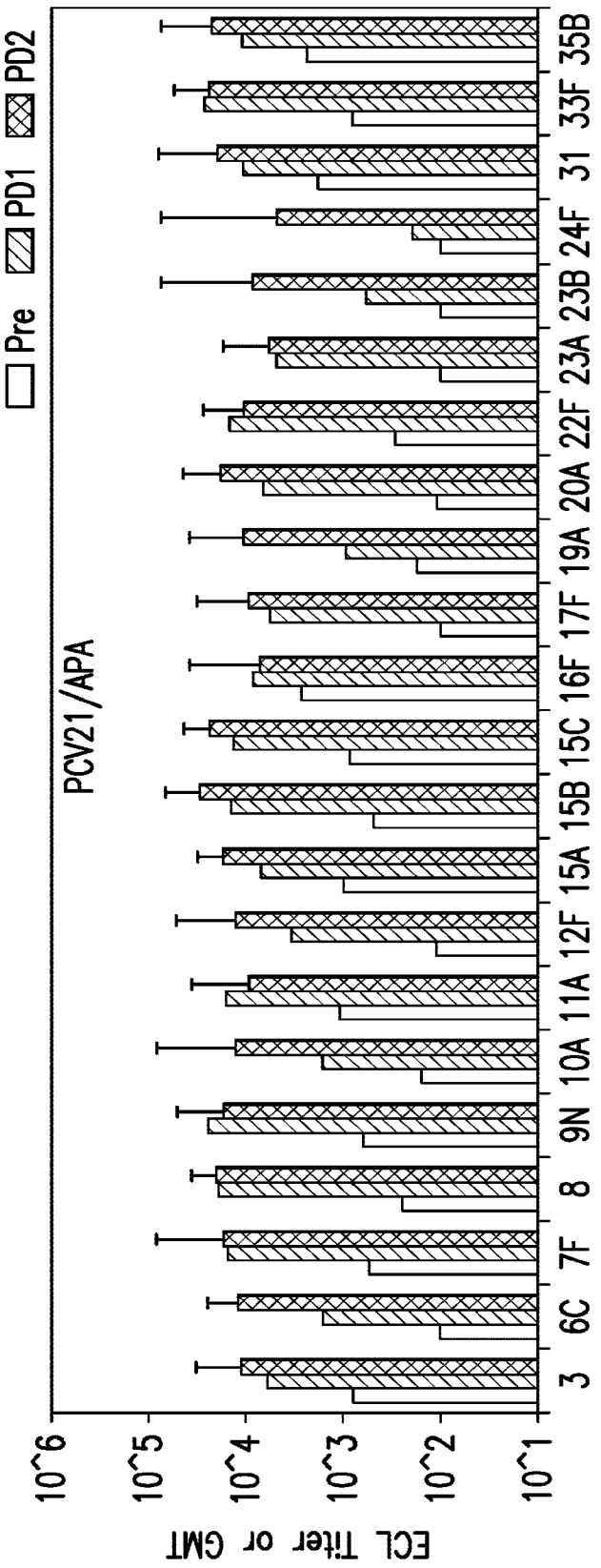

Pre
PD1
PD2
PCV21/APA
ECL Titer or GMT
10^6
10^5
10^4
10^3
10^2
10^1
3
6C
7F
8
9N
10A
11A
12F
15A
15B
15C
16F
17F
19A
20A
22F
23A
23B
24F
31
33F
35B
Serotype

FIG.26B

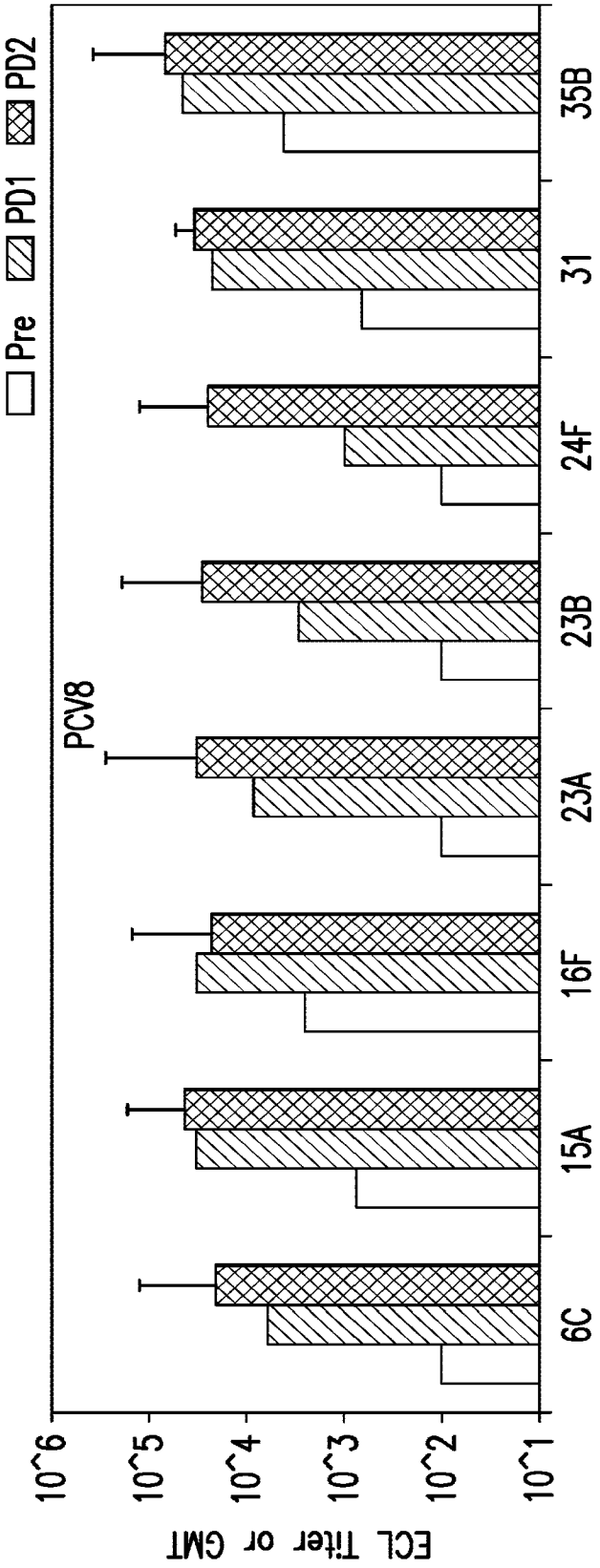

Pre
PD1
PD2
PCV8
6C
15A
16F
23A
23B
24F
31
35B
Serotype
ECL Titer or GMT
10^1
10^2
10^3
10^4
10^5
10^6

FIG.27A

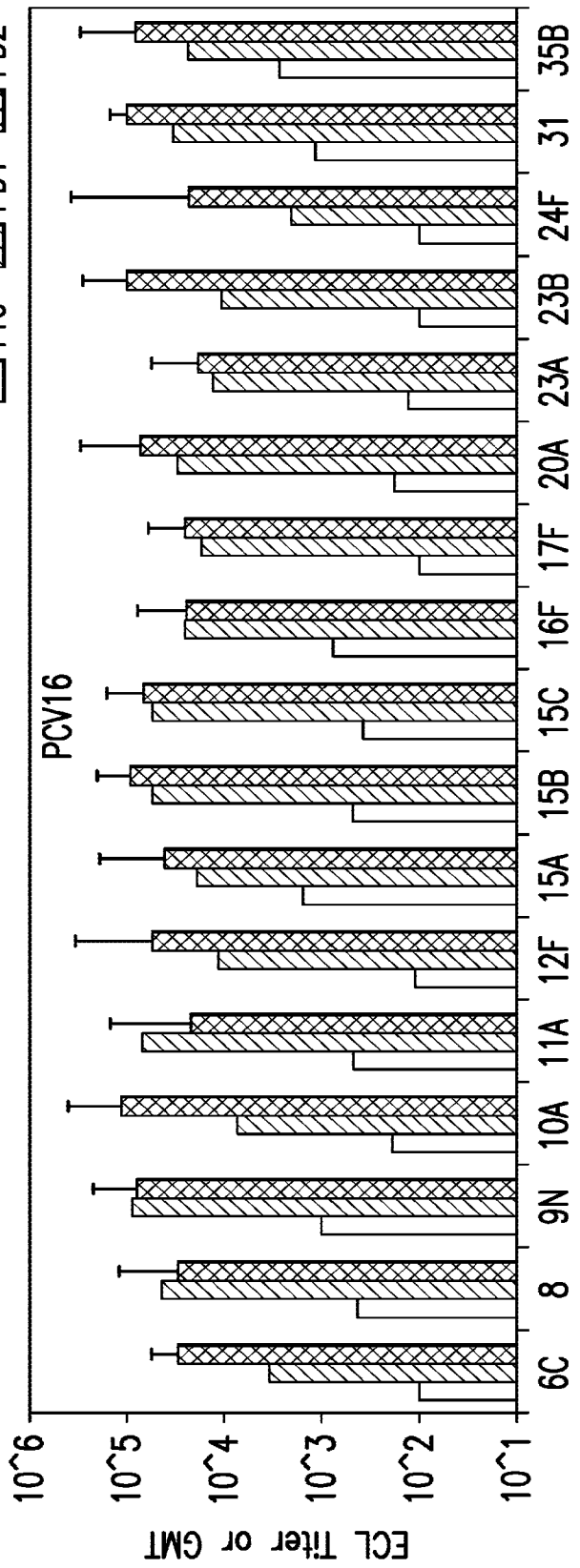

Pre
PD1
PD2
PCV16
ECL Titer or GMT
10^6
10^5
10^4
10^3
10^2
10^1
6C
8
9N
10A
11A
12F
15A
15B
15C
16F
17F
20A
23A
23B
24F
31
35B
Serotype

FIG.27B

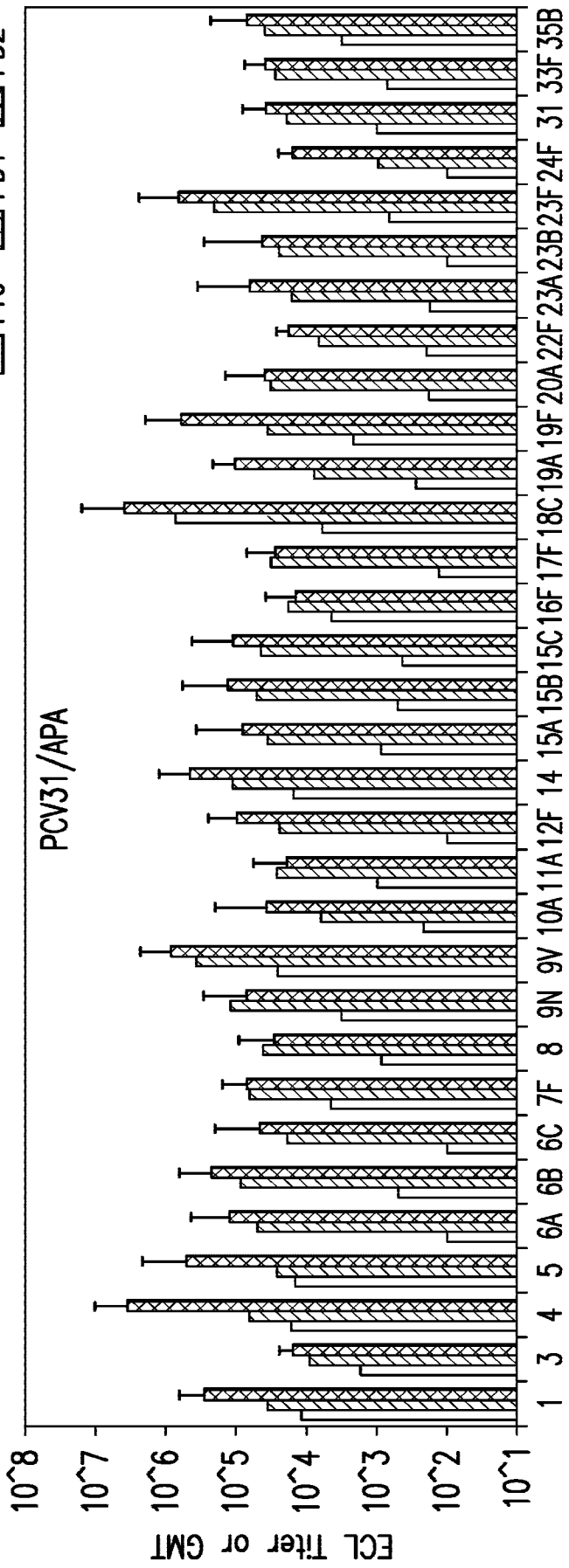

Pre
PD1
PD2
PCV31/APA
ECL Titer or GMT
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
1 3 4 5 6A 6B 6C 7F 8 9N 9V 10A 11A 12F 14 15A 15B 15C 16F 17F 18C 19A 19F 20A 22F 23A 23B 23F 24F 31 33F 35B
Serotype

FIG.27C

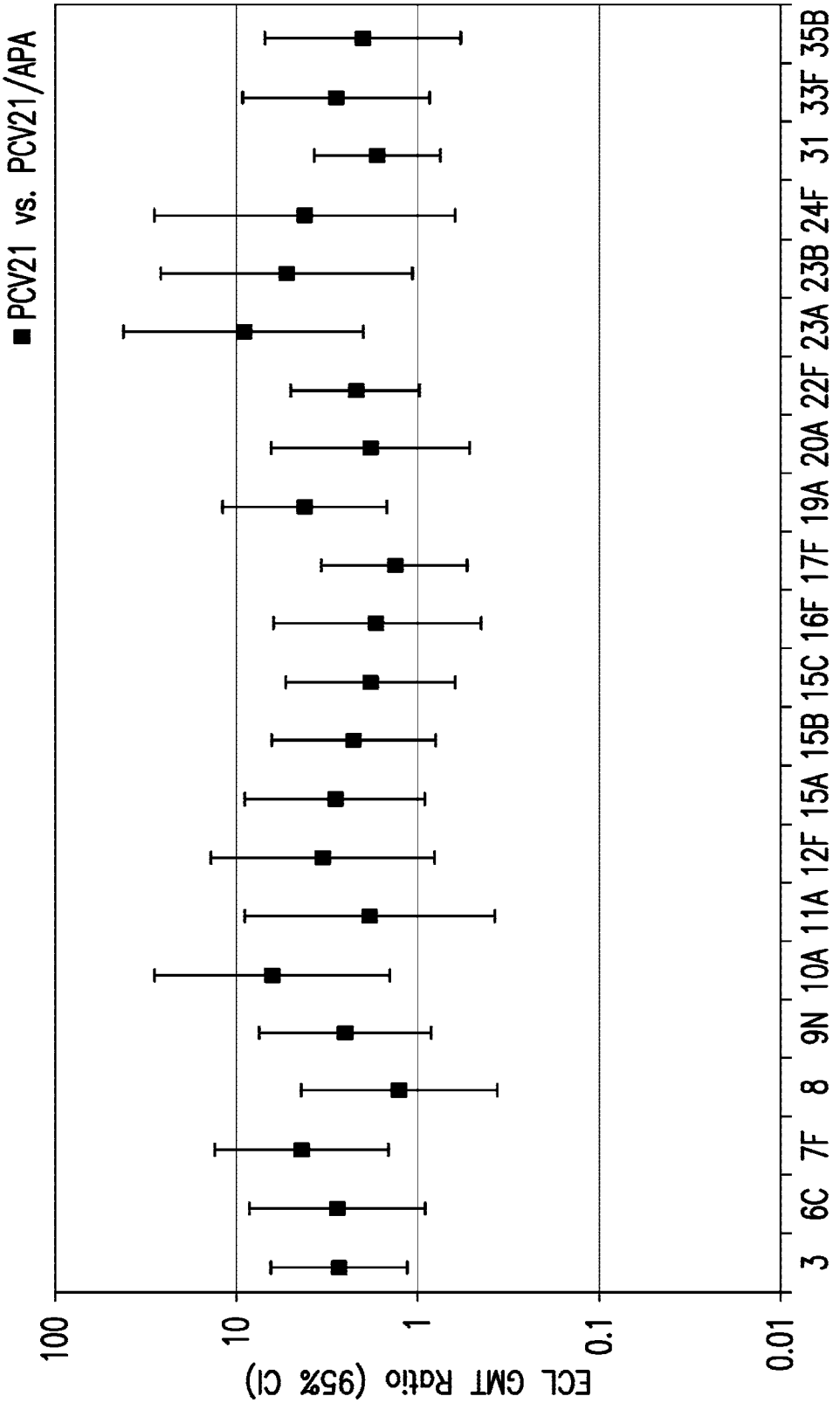

PCV21 vs. PCV21/APA
ECL GMT Ratio (95% CI)
100
10
1
0.1
0.01
3 6C 7F 8 9N 10A 11A 12F 15A 15B 15C 16F 17F 19A 20A 22F 23A 23B 24F 31 33F 35B
Serotype

FIG.28

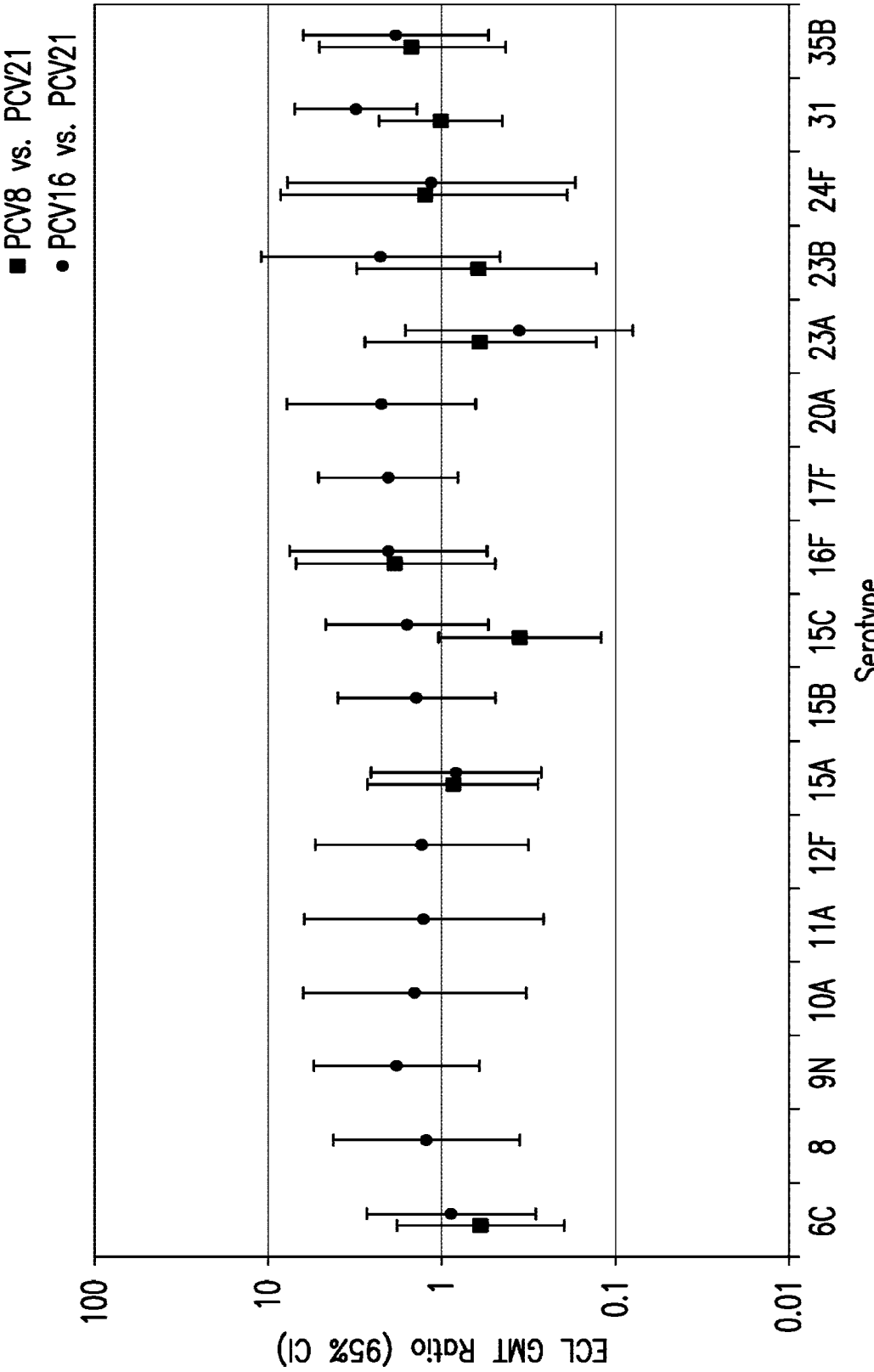

■PCV8 vs. PCV21
●PCV16 vs. PCV21
ECL GMT Ratio (95% CI)
100
10
1
0.1
0.01
6C
8
9N
10A
11A
12F
15A
15B
15C
16F
17F
20A
23A
23B
24F
31
35B
Serotype

FIG.29

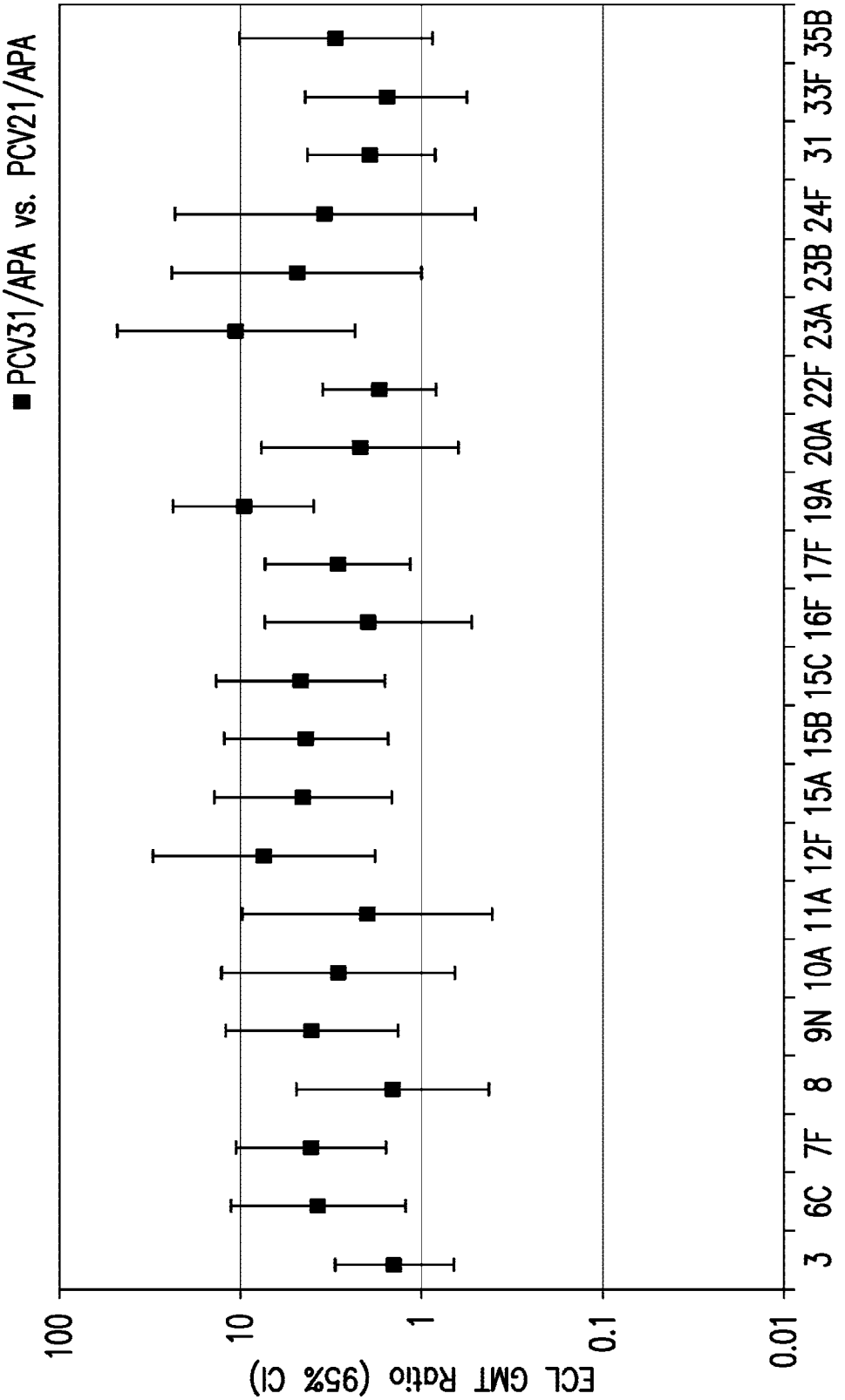

PCV31/APA vs. PCV21/APA
ECL GMT Ratio (95% CI)
100
10
1
0.1
0.01
3 6C 7F 8 9N 10A 11A 12F 15A 15B 15C 16F 17F 19A 20A 22F 23A 23B 24F 31 33F 35B
Serotype

FIG.3O

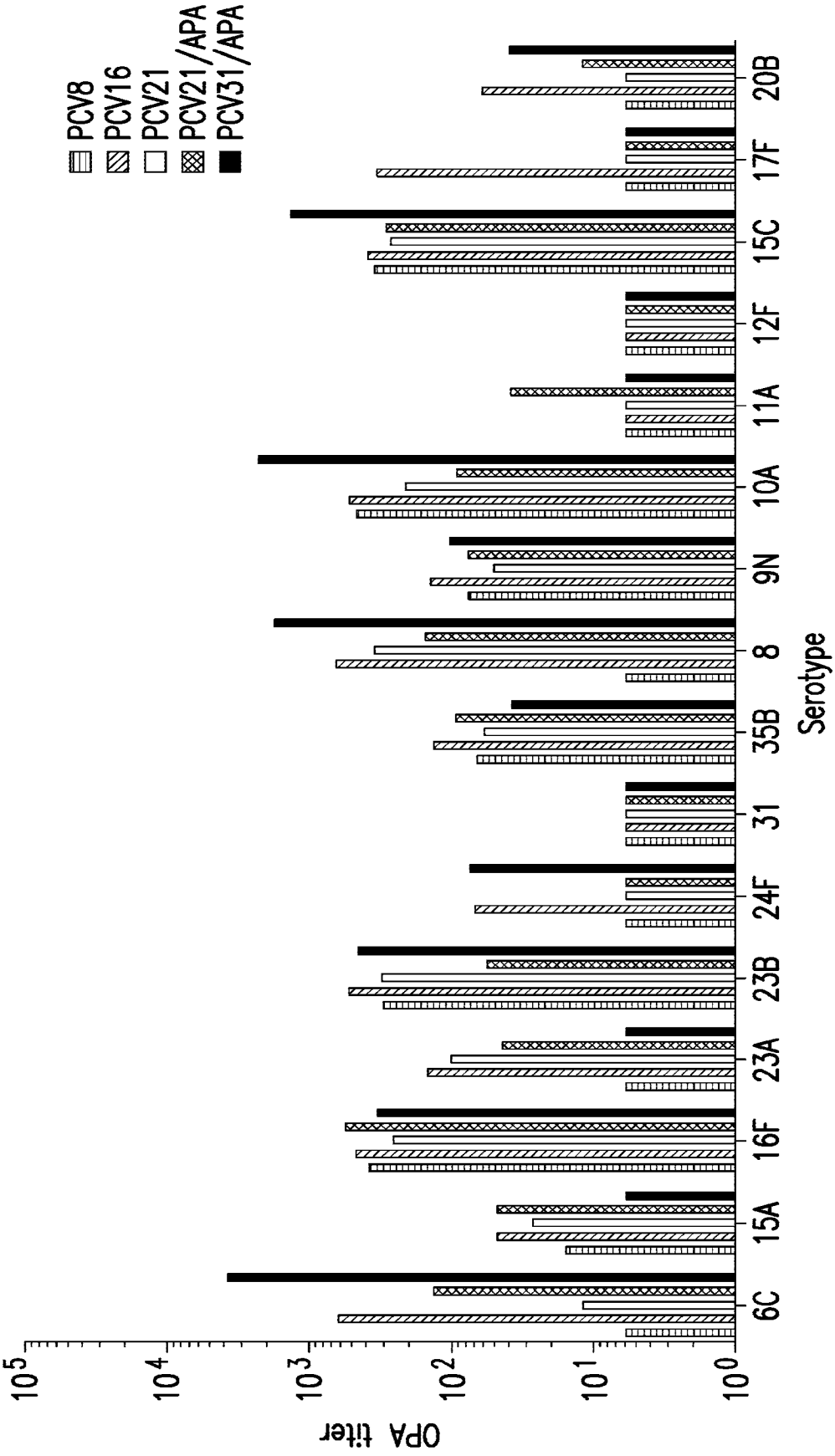

PCV8
PCV16
PCV21
PCV21/APA
PCV31/APA
OPA titer
10^0
10^1
10^2
10^3
10^4
10^5
6C
15A
16F
23A
23B
24F
31
35B
8
9N
10A
11A
12F
15C
17F
20B
Serotype

FIG.31A

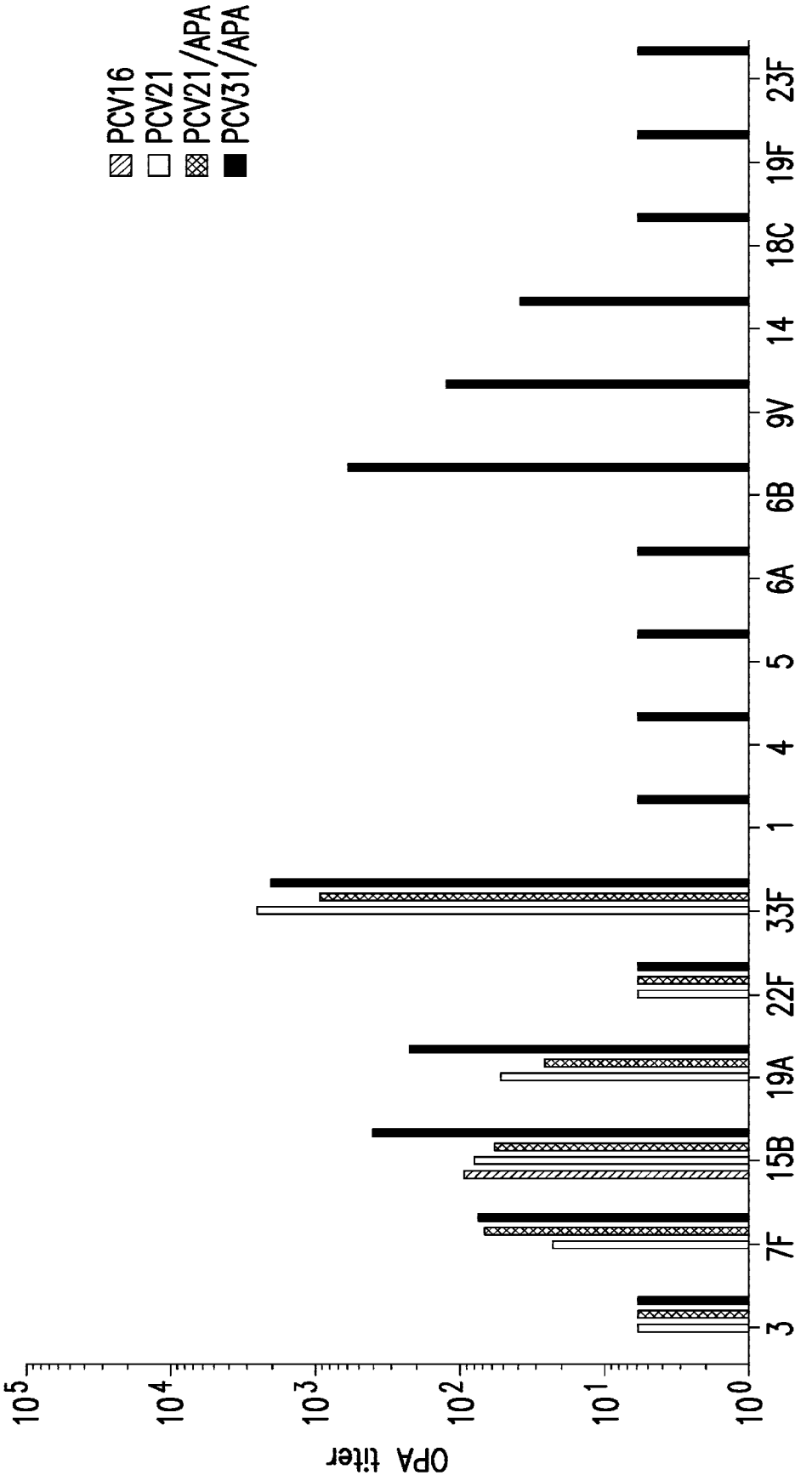

PCV16
PCV21
PCV21/APA
PCV31/APA
OPA titer
10^0
10^1
10^2
10^3
10^4
10^5
3
7F
15B
19A
22F
33F
1
4
5
6A
6B
9V
14
18C
19F
23F
Serotype

FIG.31B

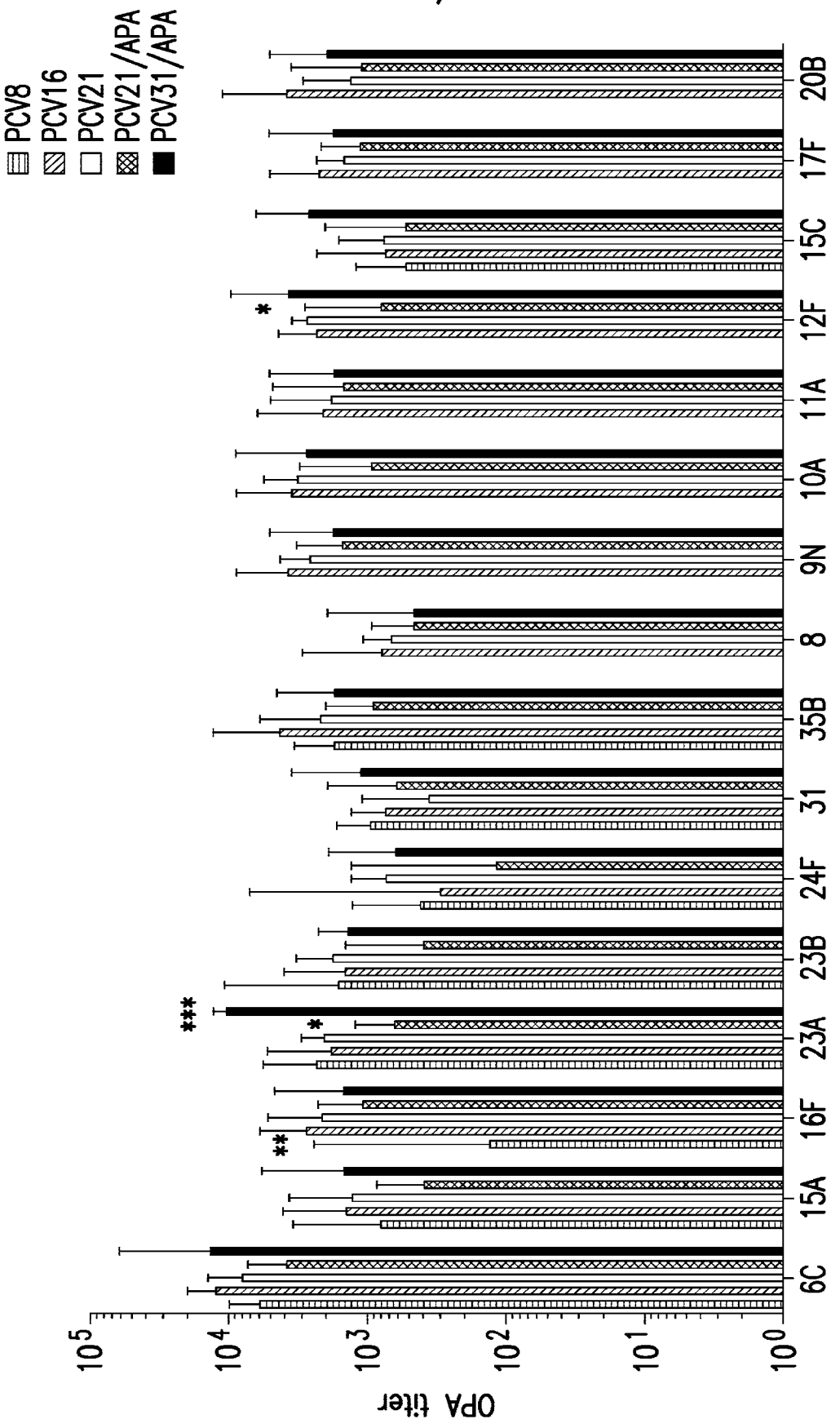

PCV8
PCV16
PCV21
PCV21/APA
PCV31/APA
OPA titer
10^5
10^4
10^3
10^2
10^1
10^0
6C
15A
16F
23A
23B
24F
31
35B
8
9N
10A
11A
12F
15C
17F
20B
Serotype
*
**
***

FIG.31C

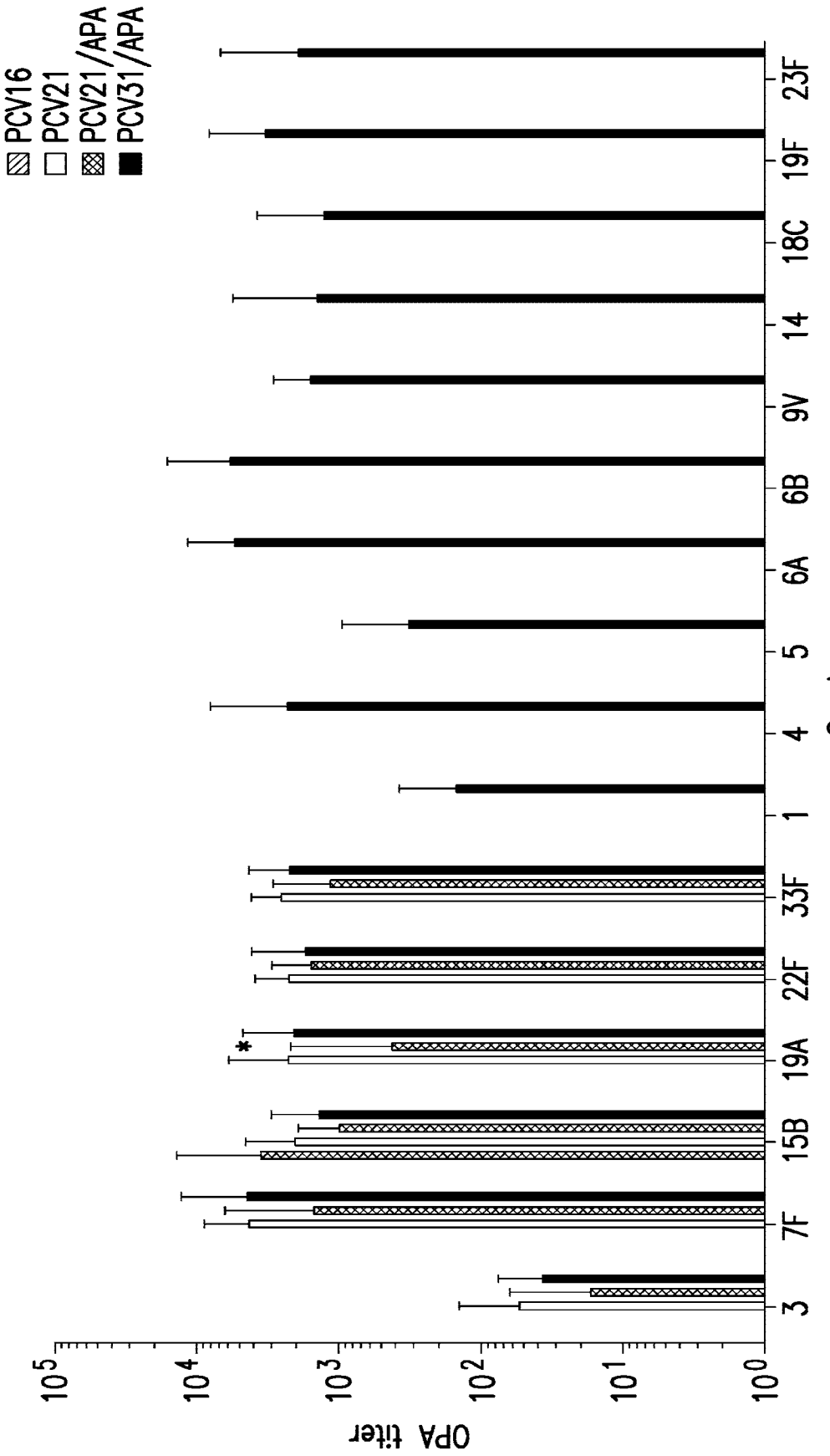

PCV16
PCV21
PCV21/APA
PCV31/APA
OPA titer
10^0
10^1
10^2
10^3
10^4
10^5
3
7F
15B
19A
22F
33F
1
4
5
6A
6B
9V
14
18C
19F
23F
Serotype
*

FIG.31D

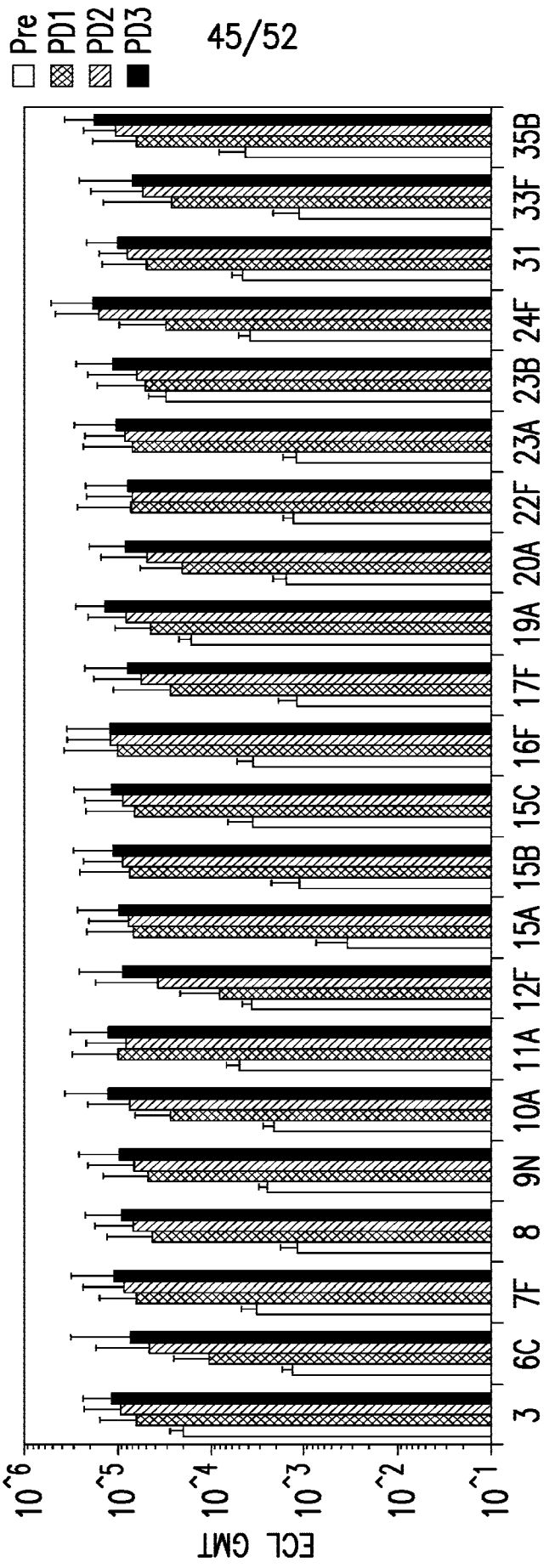

Pre
PD1
PD2
PD3
ECL GMT
10^6
10^5
10^4
10^3
10^2
10^1
3
6C
7F
8
9N
10A
11A
12F
15A
15B
15C
16F
17F
19A
20A
22F
23A
23B
24F
31
33F
35B
Serotype

FIG.32

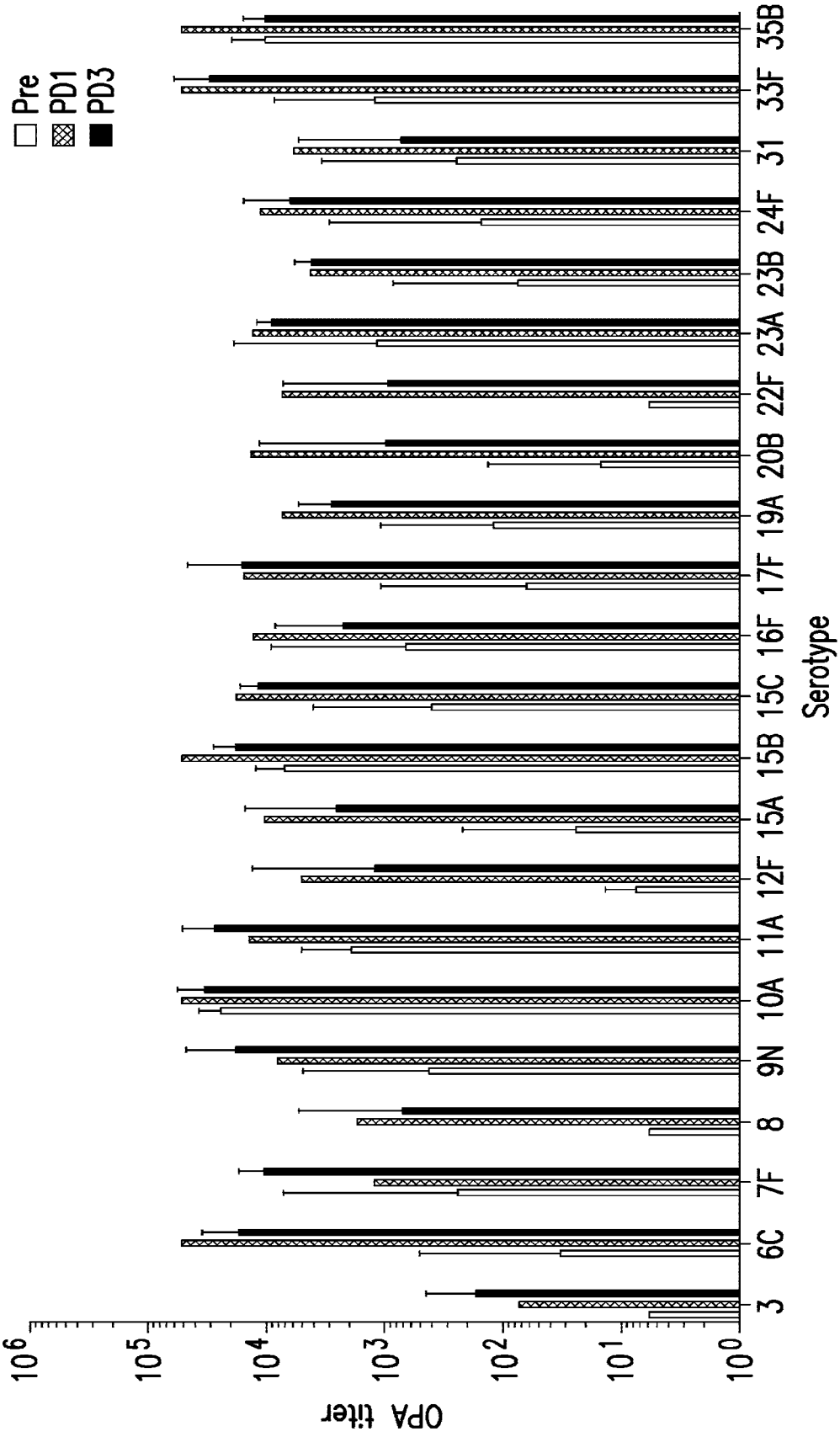

Pre
PD1
PD3
OPA titer
10^0
10^1
10^2
10^3
10^4
10^5
10^6
3
6C
7F
8
9N
10A
11A
12F
15A
15B
15C
16F
17F
19A
20B
22F
23A
23B
24F
31
33F
35B
Serotype

FIG.33

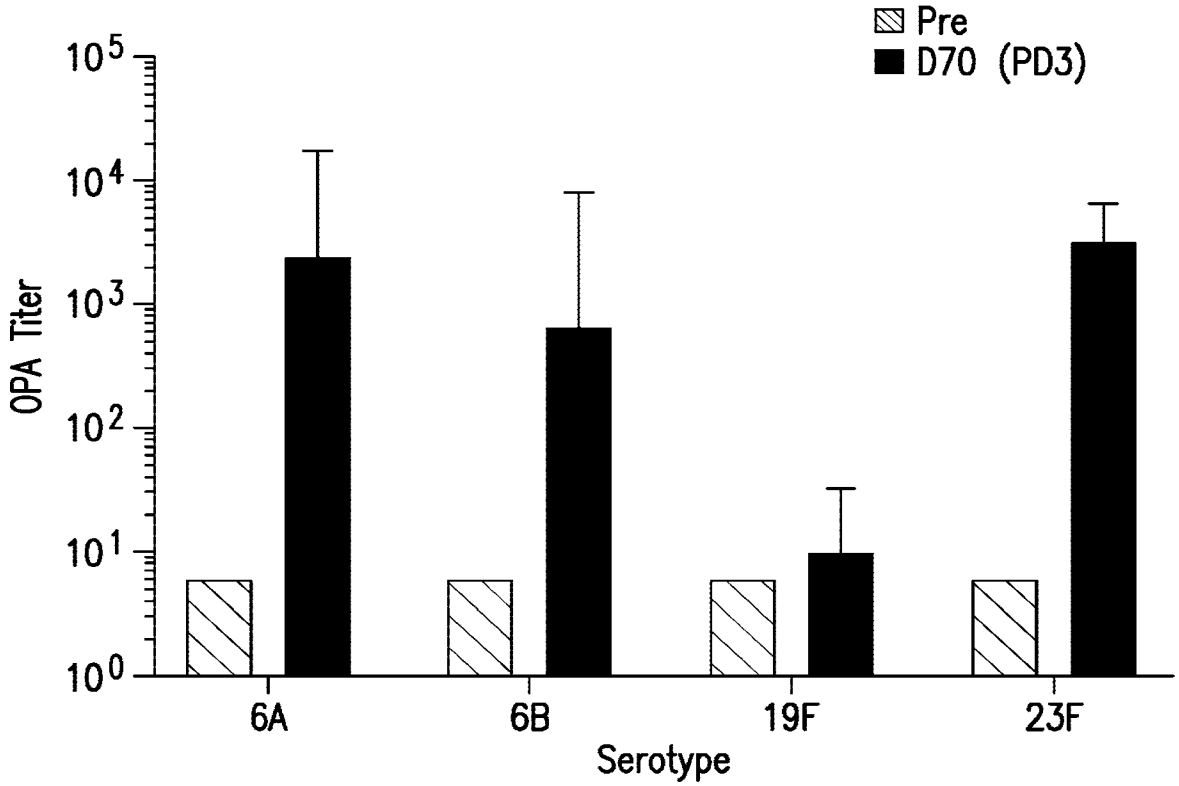

Pre
D70 (PD3)
OPA Titer
10^5
10^4
10^3
10^2
10^1
10^0
6A
6B
19F
23F
Serotype

FIG.34

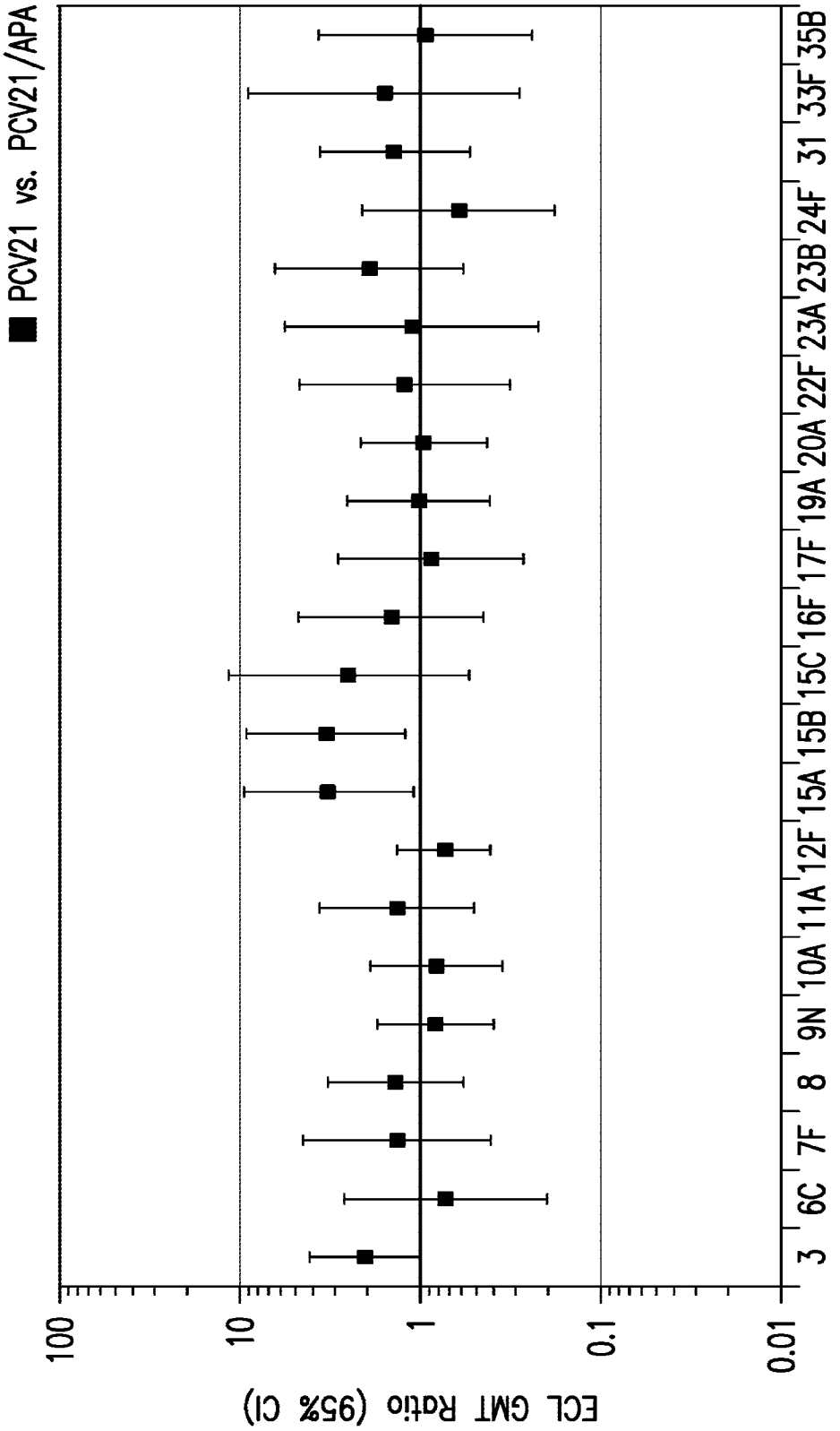

PCV21 vs. PCV21/APA
ECL GMT Ratio (95% CI)
100
10
1
0.1
0.01
3
6C
7F
8
9N
10A
11A
12F
15A
15B
15C
16F
17F
19A
20A
22F
23A
23B
24F
31
33F
35B
Serotype

FIG.35

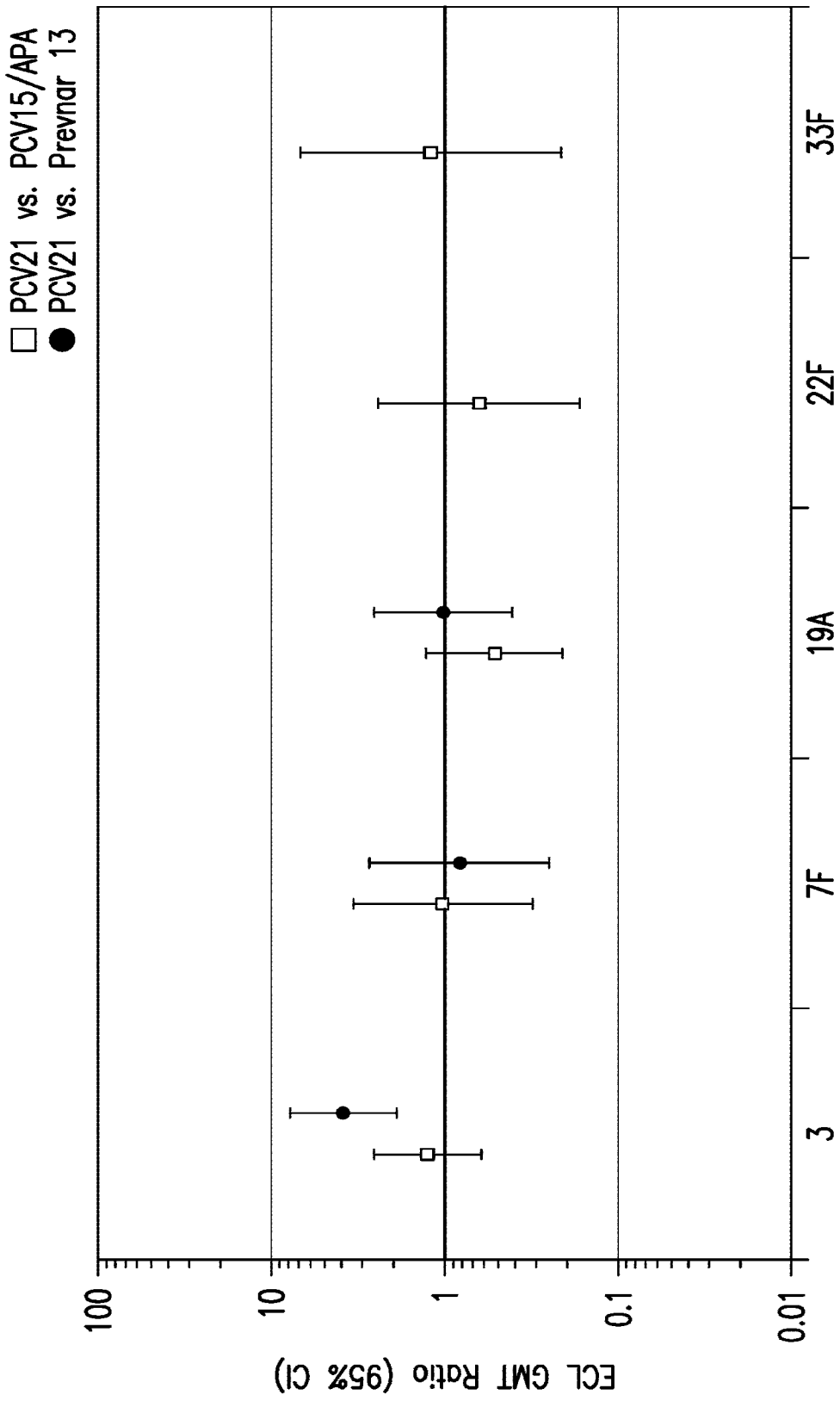

PCV21 vs. PCV15/APA
PCV21 vs. Prevnar 13
ECL GMT Ratio (95% CI)
100
10
1
0.1
0.01
3
7F
19A
22F
33F
Serotype

FIG.36

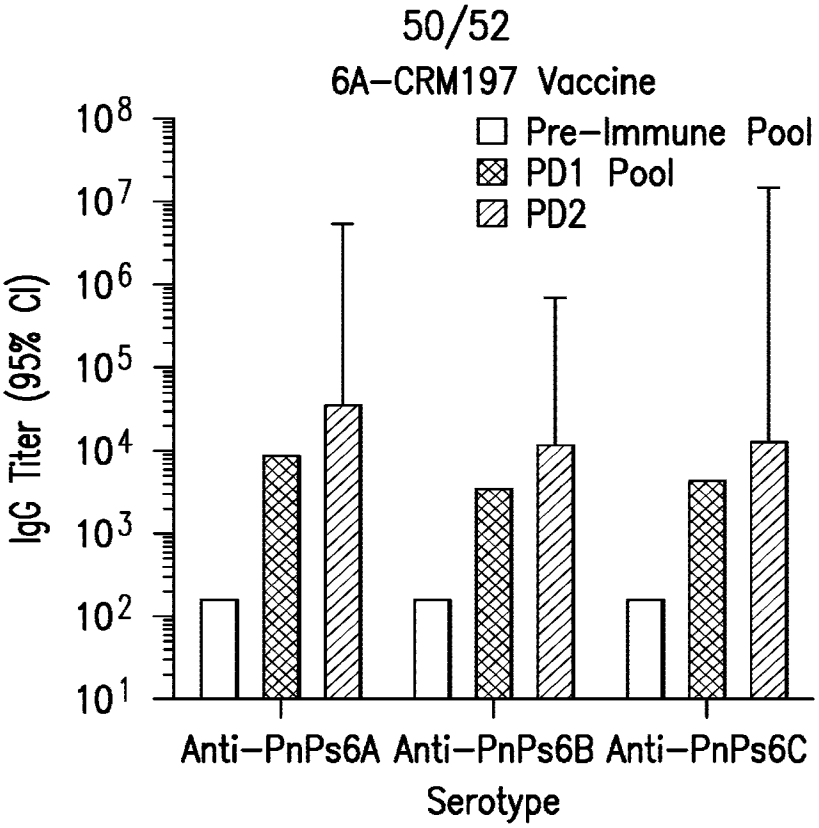


FIG.37A

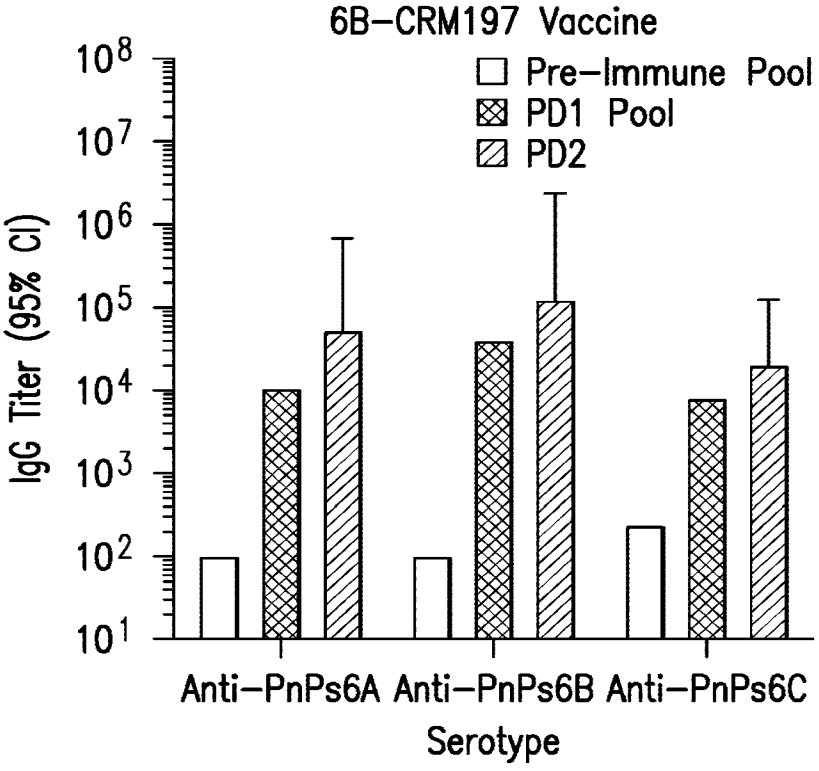


FIG.37B

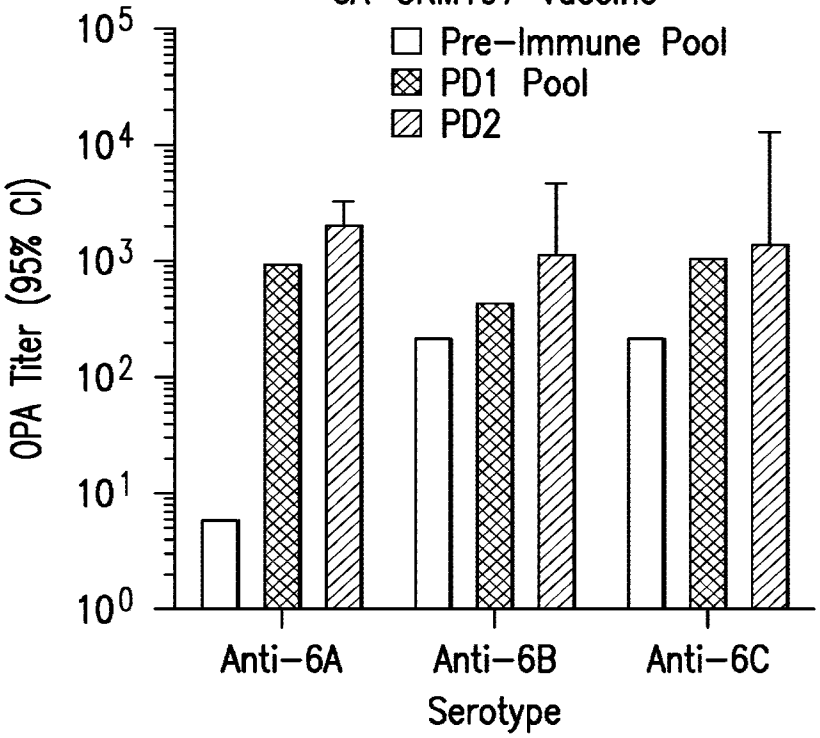


FIG.38A

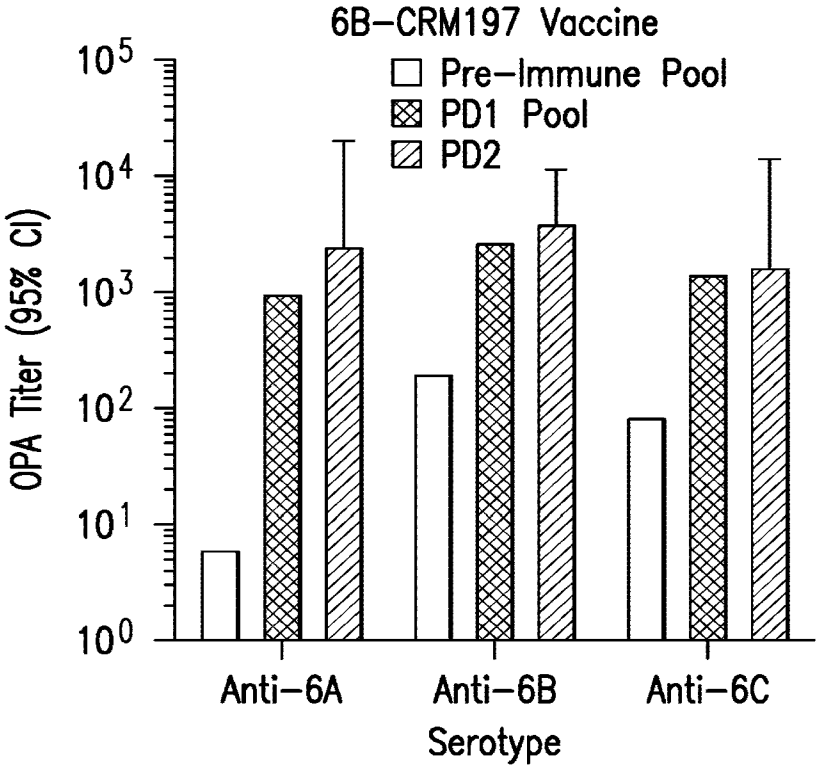


FIG.38B

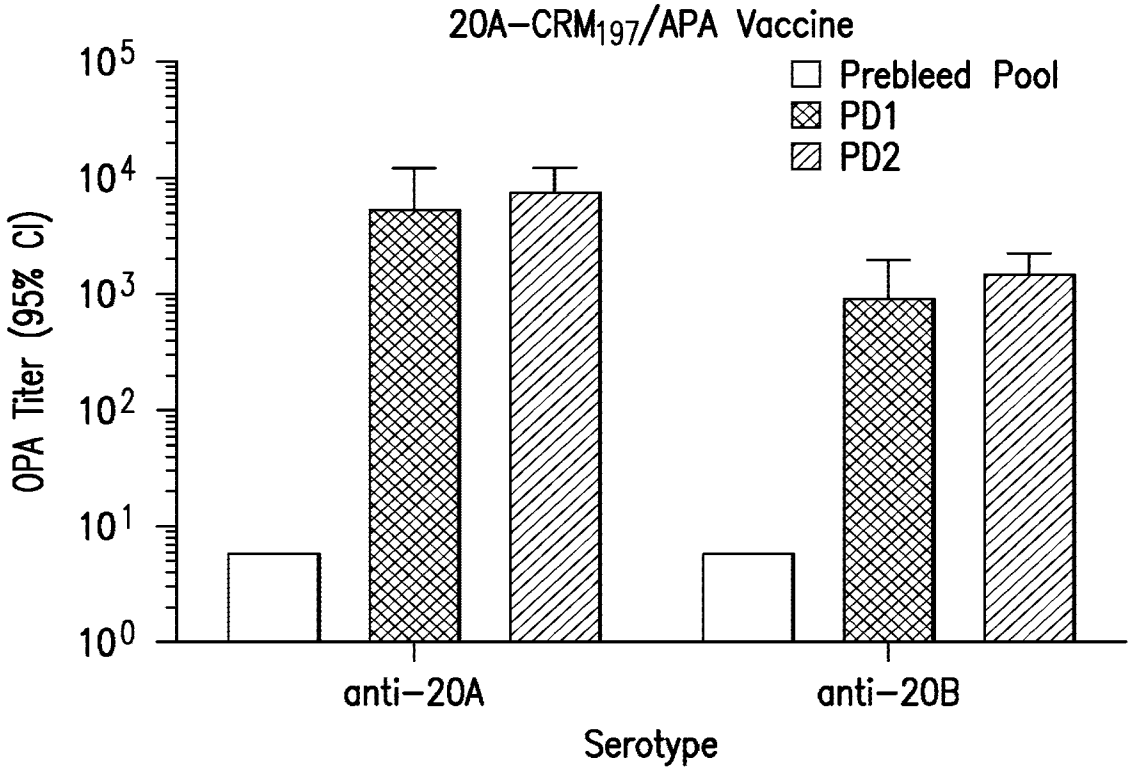


FIG.39A

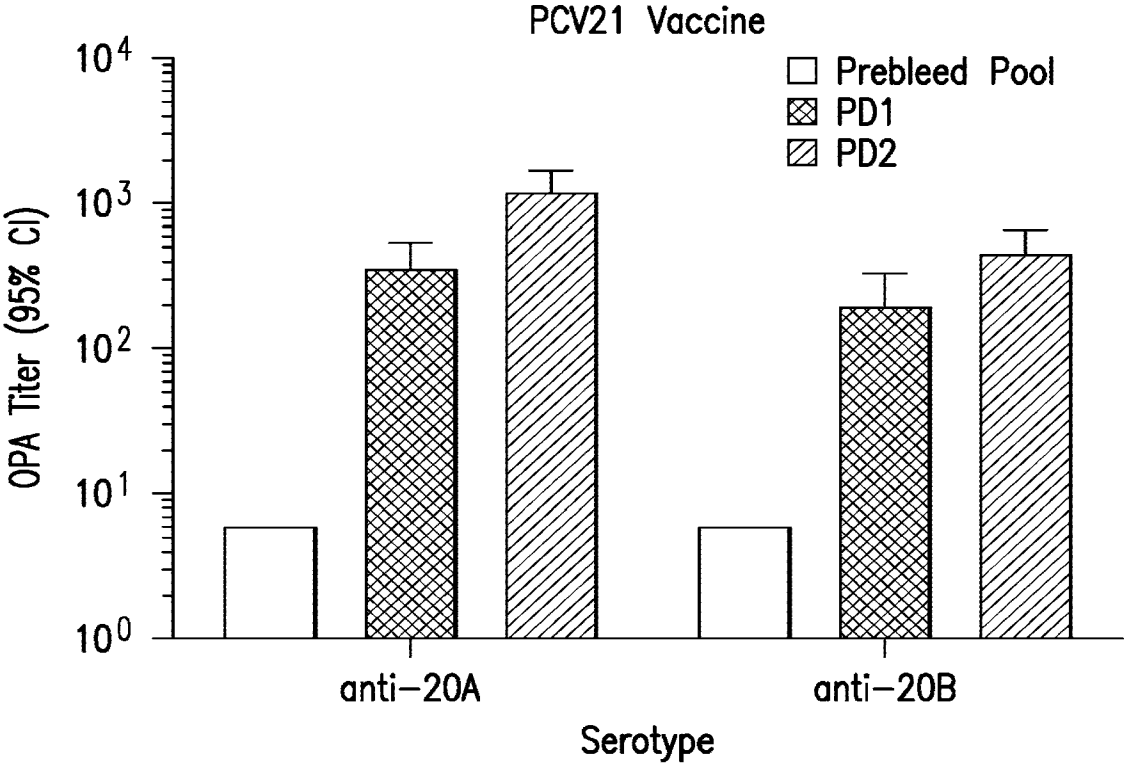


FIG.39B

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(54) Title: MULTIVALENT PNEUMOCOCCAL VACCINES

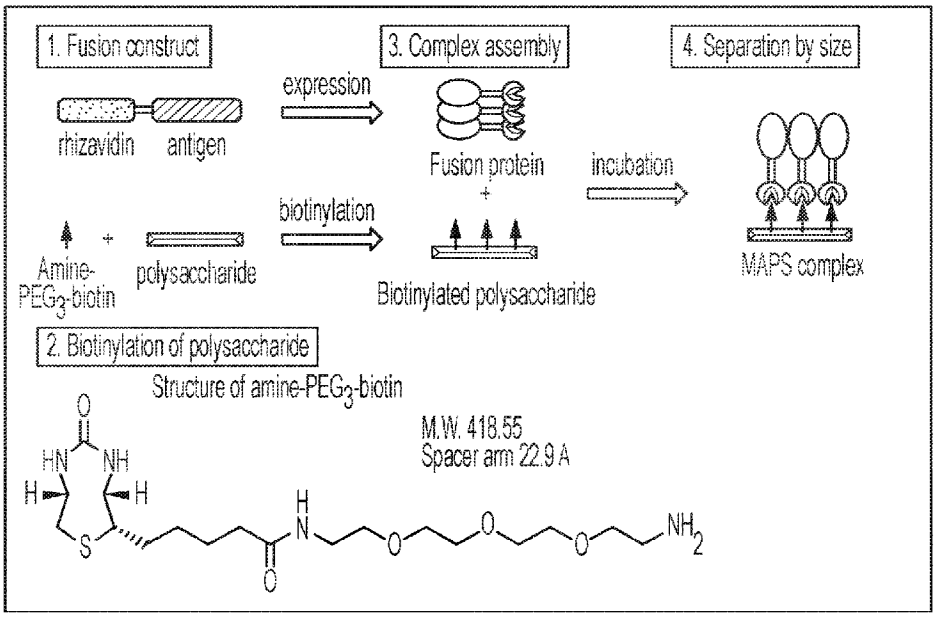


FIG. 1

(57) Abstract: Technologies for the prevention and/or treatment of pneumococcal infections.

[Continued on next page]

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TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

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MULTIVALENT PNEUMOCOCCAL VACCINES

Cross-Reference to Related Applications

[0001] This application claims the benefit of U.S. Provisional Application No. 62/730,471 filed September 12, 2018, the contents of which are hereby incorporated herein in their entirety.

Background

[0002] *Streptococcus pneumoniae* remains a leading cause of serious illness, including bacteremia, sepsis, meningitis and pneumonia, among children and adults worldwide. Morbidity and mortality among infants, young children, the elderly and subjects who have certain underlying medical conditions is high.

[0003] *S. pneumoniae* is a Gram-positive encapsulated coccus that colonizes the nasopharynx in about 5-10% of healthy adults and 20-40% of healthy children. Normal colonization becomes infectious when *S. pneumoniae* is carried into the Eustachian tubes, nasal sinuses, lungs, bloodstream, meninges, joint spaces, bones and peritoneal cavity. *S. pneumoniae* infection is the most frequent cause of bacteremia, pneumonia, meningitis, sinusitis and acute otitis media [CDC, 2010].

[0004] Pneumococcal disease can be invasive or non-invasive. The most common form of non-invasive disease, non-bacteremic pneumococcal pneumonia, remains one of the most frequent causes for pneumonia hospitalizations. Invasive pneumococcal disease (IPD) is defined as *S. pneumoniae* isolated from a normally sterile site (*e.g.*, cerebrospinal fluid, blood, joint fluid, pleural fluid or peritoneal fluid). The highest incidence of IPD is found at the extremes of age – in elderly adults and in young children younger than 2 years of age. In the U.S., prior to advent of the first pneumococcal vaccine, *S. pneumoniae* caused approximately 17,000 cases of invasive disease each year among children younger than 5 years of age, including 700 cases of meningitis and 200 deaths [CDC, 2000]. The highest morbidity and mortality rates have been reported in developing countries, but the disease burden is also considerable in industrialized countries.

[0005] *S. pneumoniae* has several virulence factors that enable the organism to evade the immune system. Examples include a polysaccharide capsule that prevents phagocytosis by host immune cells, proteases that inhibit complement-mediated opsonization, and proteins that

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cause lysis of host cells. In the polysaccharide capsule, the presence of complex polysaccharides forms the basis for dividing pneumococci into different serotypes. To date, close to 100 serotypes of *S. pneumoniae* have been identified.

[0006] Two vaccines for *S. pneumoniae* are currently available in the U.S.; PCV13 and PPSV23. PCV13 cannot confer protection against most of the known serotypes of *S. pneumoniae*. While PPSV23 includes polysaccharide components of more serotypes of *S. pneumoniae* than PCV13, it induces an immune response that is neither long-lasting nor anamnestic upon subsequent challenge. PPSV23 protects adults and the elderly against invasive pneumococcal disease; however, no consistent effect has been observed in the prevention of pneumonia [Gruber et al, 2008].

[0007] Thus, there is a medical need for a vaccine that provides T-cell dependent immunity against a broad range of serotypes of *S. pneumoniae*.

Summary

[0008] The present disclosure addresses the lack of suitable technologies for the prevention and/or treatment of pneumococcal infection. Among other things, the present disclosure addresses challenges in providing vaccines with sufficient immunogenicity to protect against invasive pneumococcal disease and pneumonia, by inducing a T- and B-cell response providing immunity against a broad range of *S. pneumoniae* serotypes including those serotypes not included in the vaccine.

[0009] Among other things, the present disclosure provides compositions and methods for prevention and/or treatment of pneumococcal infections in patient populations in need thereof.

[0010] In some embodiments, a vaccine comprises an immunogenic complex, wherein the immunogenic complex comprises: (a) a biotinylated polysaccharide antigen; and (b) a fusion protein comprising: (i) a biotin-binding moiety; and (ii) at least one polypeptide antigen; wherein the biotinylated polysaccharide antigen is non-covalently associated with the biotin-binding moiety of the fusion protein to form an immunogenic complex.

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[0011] In some embodiments, the at least one polypeptide antigen comprises: an SP1500 polypeptide or antigenic fragment thereof; or an SP0785 polypeptide or antigenic fragment thereof. In some embodiments, the at least one polypeptide antigen comprises: (a) a first polypeptide antigen comprising an SP1500 polypeptide or antigenic fragment thereof; and (b) a second polypeptide antigen comprising an SP0785 polypeptide or antigenic fragment thereof.

[0012] In some embodiments, the biotinylated polysaccharide antigen comprises a polysaccharide of *Streptococcus pneumoniae*. In some embodiments, the biotinylated polysaccharide antigen comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10C, 10F, 11A, 11B, 11C, 11D, 11E, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20A, 20B, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33C, 33D, 33E, 33F, 34, 35A, 35B, 35C, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, and 48.

[0013] In some embodiments, the biotinylated polysaccharide antigen comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F.

[0014] In some embodiments, the biotinylated polysaccharide antigen comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F.

[0015] In some embodiments, the biotinylated polysaccharide antigen comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B.

[0016] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising:(a) a plurality of biotinylated polysaccharide antigens, wherein the plurality comprises polysaccharide antigens of one or more of *Streptococcus pneumoniae* serotypes 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10C, 10F, 11A, 11B, 11C, 11D, 11E, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F,

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16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20A, 20B, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33C, 33D, 33E, 33F, 34, 35A, 35B, 35C, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, and 48; and (b) a plurality of fusion proteins, each fusion protein comprising (i) a biotin-binding moiety; (ii) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (iii) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof; wherein each of the first plurality of biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of one or more of the plurality of fusion proteins to form an immunogenic complex.

[0017] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising: (a) a plurality of biotinylated polysaccharide antigens, wherein the plurality comprises polysaccharide antigens of one or more of *Streptococcus pneumoniae* serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F; and (b) a plurality of fusion proteins, each fusion protein comprising (i) a biotin-binding moiety; (ii) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (iii) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof; wherein each of the first plurality of biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of one or more of the plurality of fusion proteins to form an immunogenic complex.

[0018] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising: (a) a plurality of biotinylated polysaccharide antigens, wherein the plurality comprises polysaccharide antigens of each of *Streptococcus pneumoniae* serotypes 1, 2,

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3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F; and (b) a plurality of fusion proteins, each fusion protein comprising (i) a biotin-binding moiety; (ii) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (iii) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof; wherein each of the first plurality of biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of one or more of the plurality of fusion proteins to form an immunogenic complex.

[0019] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising: (a) a plurality of biotinylated polysaccharide antigens, wherein the plurality comprises polysaccharide antigens of one or more of *Streptococcus pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F; and (b) a plurality of fusion proteins, each fusion protein comprising (i) a biotin-binding moiety; (ii) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (iii) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof; wherein each of the first plurality of biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of one or more of the plurality of fusion proteins to form an immunogenic complex.

[0020] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising: (a) a plurality of biotinylated polysaccharide antigens, wherein the plurality comprises polysaccharide antigens of each of *Streptococcus pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F; and (b) a plurality of fusion proteins, each fusion protein comprising (i) a biotin-binding moiety; (ii) a first polypeptide

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antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (iii) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof; wherein each of the first plurality of biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of one or more of the plurality of fusion proteins to form an immunogenic complex.

[0021] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising: (a) a plurality of biotinylated polysaccharide antigens, wherein the plurality comprises polysaccharide antigens of one or more of *Streptococcus pneumoniae* serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B; and (b) a plurality of fusion proteins, each fusion protein comprising (i) a biotin-binding moiety; (ii) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (iii) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof; wherein each of the first plurality of biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of one or more of the plurality of fusion proteins to form an immunogenic complex.

[0022] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising: (a) a plurality of biotinylated polysaccharide antigens, wherein the plurality comprises polysaccharide antigens of each of *Streptococcus pneumoniae* serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B; and (b) a plurality of fusion proteins, each fusion protein comprising (i) a biotin-binding moiety; (ii) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5

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or an antigenic fragment thereof; and (iii) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof; wherein each of the first plurality of biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of one or more of the plurality of fusion proteins to form an immunogenic complex.

[0023] In some embodiments, a vaccine comprises a biotin-binding moiety, wherein the biotin-binding moiety is a polypeptide that has or comprises an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:1 or a biotin-binding fragment thereof; or a polypeptide that has or comprises an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:2 or a biotin-binding fragment thereof. In some embodiments, a vaccine comprises a fusion protein that is or comprises an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:6. In some embodiments, a vaccine comprises the fusion protein CP1.

[0024] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising one or more species of immunogenic complexes, wherein a species refers to complexes comprising a polysaccharide antigen of a single *Streptococcus pneumoniae* serotype. In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising two or more species of immunogenic complexes, wherein a species refers to complexes comprising a polysaccharide antigen of a single *Streptococcus pneumoniae* serotype. In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising nine or more species of immunogenic complexes, wherein a species refers to complexes comprising a polysaccharide antigen of a single *Streptococcus pneumoniae* serotype.

[0025] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising fifteen or more species of immunogenic complexes, wherein a species refers to complexes comprising a polysaccharide antigen of a single *Streptococcus pneumoniae* serotype.

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[0026] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising twenty-four or more species of immunogenic complexes, wherein a species refers to complexes comprising a polysaccharide antigen of a single *Streptococcus pneumoniae* serotype.

[0027] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising: a first species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 1 non-covalently complexed with a fusion protein; a second species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 2 non-covalently complexed with a fusion protein; a third species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 3 non-covalently complexed with a fusion protein; a fourth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 4 non-covalently complexed with a fusion protein; a fifth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 5 non-covalently complexed with a fusion protein; a sixth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 6A non-covalently complexed with a fusion protein; a seventh species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 6B non-covalently complexed with a fusion protein; an eighth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 7F non-covalently complexed with a fusion protein; a ninth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 8 non-covalently complexed with a fusion protein; a tenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 9N non-covalently complexed with a fusion protein; an eleventh species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 9V non-covalently complexed with a fusion protein; a twelfth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 10A non-covalently complexed with a fusion protein; a thirteenth species of immunogenic complex comprising a biotinylated

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polysaccharide antigen of *Streptococcus pneumoniae* serotype 11A non-covalently complexed with a fusion protein; a fourteenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 12F non-covalently complexed with a fusion protein; a fifteenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 14 non-covalently complexed with a fusion protein; a sixteenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 15B non-covalently complexed with a fusion protein; a seventeenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 17F non-covalently complexed with a fusion protein; an eighteenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 18C non-covalently complexed with a fusion protein; a nineteenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 19A non-covalently complexed with a fusion protein; a twentieth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 19F non-covalently complexed with a fusion protein; a twenty-first species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 20B non-covalently complexed with a fusion protein; a twenty-second species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 22F non-covalently complexed with a fusion protein; a twenty-third species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 23F non-covalently complexed with a fusion protein; a twenty-fourth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 33F non-covalently complexed with a fusion protein; wherein each fusion protein comprises (a) a biotin-binding moiety;(b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof;

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and wherein each of the first through twenty-fourth biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of at least one fusion protein to form immunogenic complexes.

[0028] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising: a first species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 1 non-covalently complexed with a fusion protein; a second species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 3 non-covalently complexed with a fusion protein; a third species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 4 non-covalently complexed with a fusion protein; a fourth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 5 non-covalently complexed with a fusion protein; a fifth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 6A non-covalently complexed with a fusion protein; a sixth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 6B non-covalently complexed with a fusion protein; a seventh species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 7F non-covalently complexed with a fusion protein; an eighth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 9V non-covalently complexed with a fusion protein; a ninth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 14 non-covalently complexed with a fusion protein; a tenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 18C non-covalently complexed with a fusion protein; an eleventh species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 19A non-covalently complexed with a fusion protein; a twelfth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 19F non-covalently complexed with a fusion protein; a thirteenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 22F non-covalently complexed

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with a fusion protein; a fourteenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 23F non-covalently complexed with a fusion protein; a fifteenth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 33F non-covalently complexed with a fusion protein; wherein each fusion protein comprises (a) a biotin-binding moiety;(b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof; and wherein each of the first through fifteenth biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of at least one fusion protein to form immunogenic complexes.

[0029] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising: a first species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 2 non-covalently complexed with a fusion protein; a second species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 8 non-covalently complexed with a fusion protein; a third species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 9N non-covalently complexed with a fusion protein; a fourth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 10A non-covalently complexed with a fusion protein; a fifth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 11A non-covalently complexed with a fusion protein; a sixth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 12F non-covalently complexed with a biotin-binding fusion protein; a seventh species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 15B non-covalently complexed with a fusion protein; an eighth species of immunogenic complex comprising a

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biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 17F non-covalently complexed with a fusion protein; and a ninth species of immunogenic complex comprising a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 20B non-covalently complexed with a fusion protein; wherein each fusion protein comprises (a) a biotin-binding moiety;(b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof; and wherein each of the first through ninth biotinylated polysaccharide antigens is non-covalently associated with the biotin-binding moiety of at least one fusion protein to form immunogenic complexes.

[0030] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising two or more species of immunogenic complexes, wherein each species of immunogenic complexes contributes a stoichiometrically equal ratio, by weight, of the polysaccharide antigen.

[0031] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising two or more species of immunogenic complexes, wherein at least one species of immunogenic complex contributes a stoichiometrically different ratio, by weight, of the polysaccharide antigen.

[0032] In some embodiments, a vaccine comprises a plurality of immunogenic complexes comprising two or more species of immunogenic complexes, wherein different species of immunogenic complexes contribute a stoichiometrically different ratio, by weight, of the polysaccharide antigen.

[0033] In some embodiments, a vaccine comprises a fusion protein comprising a biotin-binding moiety, wherein the biotin-binding moiety is a polypeptide comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:1

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or a biotin-binding fragment thereof; or a polypeptide comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:2 or a biotin-binding fragment thereof.

[0034] In some embodiments, an immunogenic complex comprises a fusion protein comprising a biotin-binding moiety, wherein the biotin-binding moiety is a polypeptide comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:1 or a biotin-binding fragment thereof; or a polypeptide comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:2 or a biotin-binding fragment thereof.

[0035] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* non-covalently associated with a fusion protein, wherein the fusion protein comprises a biotin-binding moiety and at least one polypeptide antigen.

[0036] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen, wherein the biotinylated polysaccharide antigen comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10C, 10F, 11A, 11B, 11C, 11D, 11E, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20A, 20B, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33C, 33D, 33E, 33F, 34, 35A, 35B, 35C, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, and 48.

[0037] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen, wherein the biotinylated polysaccharide antigen comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F.

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[0038] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen, wherein the biotinylated polysaccharide antigen comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F.

[0039] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen, wherein the biotinylated polysaccharide antigen comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B.

[0040] In some embodiments, an immunogenic complex comprises at least one peptide antigen, wherein the at least one polypeptide antigen comprises an SP1500 polypeptide or antigenic fragment thereof; an SP0785 polypeptide or antigenic fragment thereof; or a combination thereof.

[0041] In some embodiments, an immunogenic complex comprises at least one peptide antigen, wherein the at least one polypeptide antigen comprises: (a) a first polypeptide antigen comprising an SP1500 polypeptide or antigenic fragment thereof; and (b) a second polypeptide antigen comprising an SP0785 polypeptide or antigenic fragment thereof.

[0042] In some embodiments, an immunogenic complex comprises at least one peptide antigen, wherein the at least one polypeptide antigen comprises: (a) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (b) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0043] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 1 non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or

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at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0044] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 2 non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0045] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 3 non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0046] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 4 non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%,

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at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0047] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 5 non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0048] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 6A non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0049] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 6B non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

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[0050] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 7F non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0051] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 8 non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0052] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 9N non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0053] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 9V non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first

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polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0054] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 10A non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0055] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 11A non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0056] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 12F non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second

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polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0057] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 14 non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0058] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 15B non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0059] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 17F non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

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[0060] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 18C non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0061] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 19A non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0062] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 19F non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0063] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 20B non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first

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polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0064] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 22F non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0065] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 23F non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0066] In some embodiments, an immunogenic complex comprises a biotinylated polysaccharide antigen of *Streptococcus pneumoniae* serotype 33F non-covalently associated with a fusion protein, wherein the fusion protein comprises (a) a biotin-binding moiety; (b) a first polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:5 or an antigenic fragment thereof; and (c) a second

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polypeptide antigen comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:4 or an antigenic fragment thereof.

[0067] In some embodiments, an immunogenic complex comprises a biotin-binding moiety, wherein the biotin-binding moiety is a polypeptide comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:1 or a biotin-binding fragment thereof; or a polypeptide comprising an amino acid sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or at least 99.5% identical to SEQ ID NO:2 or a biotin-binding fragment thereof.

[0068] In some embodiments, an immunogenic complex comprises a fusion protein and a polysaccharide antigen, wherein the ratio of fusion protein to polysaccharide antigen in the complex is about 1:1, about 2:1, about 3:1, about 4:1, about 5:1, about 6:1, about 7:1, about 8:1, about 9:1, or about 10:1 by weight. In some embodiments, a vaccine comprises one or more immunogenic complexes disclosed herein.

[0069] In some embodiments, a pharmaceutical composition comprises a vaccine or vaccine composition disclosed herein, and a pharmaceutically acceptable carrier. In some embodiments, a pharmaceutical composition comprises an immunogenic complex disclosed herein, and a pharmaceutically acceptable carrier. In some embodiments, a pharmaceutical composition disclosed herein comprises one or more adjuvants. In some embodiments, the one or more adjuvants is or comprises a co-stimulation factor.

[0070] In some embodiments, the one or more adjuvants are selected from the group consisting of aluminum phosphate, aluminum hydroxide, and phosphated aluminum hydroxide. In some embodiments, wherein the one or more adjuvants is or comprises aluminum phosphate. In some embodiments, a pharmaceutical composition disclosed herein is formulated for injection.

[0071] In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein induces an immune response. In some embodiments, the immune

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response comprises an innate immune response. In some embodiments, the immune response comprises an antibody or B cell response. In some embodiments, the immune response comprises a CD4+ T cell response (*e.g.*, T_H1, T_H2, or T_H17 response), a CD8+ T cell response, a CD4+ and CD8+ T cell response, or a CD4-/CD8- T cell response. In some embodiments, the immune response comprises (i) an antibody or B cell response, and (ii) a T cell response. In some embodiments, the immune response comprises (i) an antibody or B cell response, (ii) a T cell response, and (iii) an innate immune response. In some embodiments, the immune response is to at least one polysaccharide antigen or at least one polypeptide antigen of a fusion protein. In some embodiments, the immune response comprises (i) an antibody or B cell response to at least one polysaccharide antigen, and (ii) a CD4+ T cell response (*e.g.*, T_H1, T_H2, or T_H17 response), a CD8+ T cell response, a CD4+ and CD8+ T cell response, or a CD4-/CD8- T cell response to at least one polypeptide of a fusion protein. In some embodiments, the immune response comprises (i) an antibody or B cell response to at least one polysaccharide antigen, and (ii) an antibody or B cell response to at least one polypeptide of a fusion protein. In some embodiments, the immune response comprises (i) an antibody or B cell response to at least one polysaccharide antigen, and (ii) an antibody or B cell response, and a CD4+ T cell response, including a T_H1, T_H2, or T_H17 response, a CD8+ T cell response, a CD4+ and CD8+ T cell response, or a CD4-/CD8- T cell response, to at least one polypeptide of a fusion protein.

[0072] In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein induces an opsonic/bactericidal response against one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such an opsonic/bactericidal response may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such an opsonic/bactericidal response may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such an opsonic/bactericidal response may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus*

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pneumoniae, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, an opsonic/bactericidal response can include production of antibodies that opsonize one or more serotypes of *Streptococcus pneumoniae*, which leads to killing of such serotype(s) by one or more immune pathways. In some embodiments, such an opsonic/bactericidal response can be determined by an opsonophagocytic assay (OPA) or a concentrated opsonophagocytic assay (COPA) known in the art and/or as described herein. In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein inhibits transmission of one or more serotypes of *Streptococcus pneumoniae* from the subject to another subject. In some embodiments, such inhibition of transmission may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of transmission may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of transmission may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0073] In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein inhibits or reduces the rate of occurrence of Invasive Pneumonoccal Disease (IPD) associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition of IPD or reduction in the rate of occurrence of IPD may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described

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herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of IPD or reduction in the rate of occurrence of IPD may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of IPD or reduction in the rate of occurrence of IPD may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein reduces the severity of Invasive Pneumonoccal Disease (IPD) associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of IPD may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of IPD may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of IPD may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0074] In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein inhibits or reduces the rate of occurrence of bacteremia, sepsis, and/or meningitis associated with or induced by one or more serotypes of *Streptococcus*

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pneumoniae. In some embodiments, such inhibition, or reduction in the rate of occurrence, of bacteremia, sepsis, and/or meningitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of bacteremia, sepsis, and/or meningitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of bacteremia, sepsis, and/or meningitis may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein reduces the severity of bacteremia, sepsis, and/or meningitis associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of bacteremia, sepsis, and/or meningitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of bacteremia, sepsis, and/or meningitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of bacteremia, sepsis, and/or meningitis may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include

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polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0075] In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein inhibits or reduces the rate of occurrence of organ damage associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in the rate of occurrence, of organ damage may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of organ damage may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of organ damage may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein reduces, the severity of organ damage associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of organ damage may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of organ damage may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of organ damage may be

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directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0076] In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein inhibits or reduces the rate of occurrence of pneumonia associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in the rate of occurrence, of pneumonia may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of pneumonia may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of pneumonia may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein reduces the severity of pneumonia associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of pneumonia may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of pneumonia may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a

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pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of pneumonia may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0077] In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein inhibits or reduces the rate of occurrence of otitis media associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in the rate of occurrence, of otitis media may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of otitis media may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of otitis media may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein reduces the severity of otitis media associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of otitis media may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s)

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present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of otitis media may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of otitis media may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0078] In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein inhibits or reduces the rate of occurrence of sinusitis associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in the rate of occurrence, of sinusitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of sinusitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of sinusitis may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein reduces the severity of sinusitis associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some

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embodiments, such reduction in the severity of sinusitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of sinusitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of sinusitis may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0079] In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein inhibits colonization of mucosal surface(s) by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition of colonization of mucosal surface(s) may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of colonization of mucosal surface(s) may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of colonization of mucosal surface(s) may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, upon administration to a subject, a pharmaceutical composition disclosed herein inhibits colonization

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of the nasopharynx by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition of colonization of nasopharynx may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of colonization of nasopharynx may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of colonization of nasopharynx may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0080] Some embodiments provide a method of making a vaccine, the method comprising non-covalently complexing a plurality of biotinylated polysaccharide antigens with a plurality of a fusion protein comprising at least one polypeptide antigen selected from: an SP1500 polypeptide or antigenic fragment thereof; an SP0785 polypeptide or antigenic fragment thereof; or a combination thereof. In some embodiments, the plurality of biotinylated polysaccharide antigens comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10C, 10F, 11A, 11B, 11C, 11D, 11E, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20A, 20B, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33C, 33D, 33E, 33F, 34, 35A, 35B, 35C, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, and 48. In some embodiments, the plurality of biotinylated polysaccharide antigens comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F. In some embodiments, the plurality of biotinylated

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polysaccharide antigens comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from each of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F. In some embodiments, the plurality of biotinylated polysaccharide antigens comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F. In some embodiments, the plurality of biotinylated polysaccharide antigens comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from each of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F. In some embodiments, the plurality of biotinylated polysaccharide antigens comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from one or more of 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B. In some embodiments, the plurality of biotinylated polysaccharide antigens comprises a polysaccharide of *Streptococcus pneumoniae* having a serotype selected from each of 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B.

[0081] Some embodiments provide for a method of immunizing a subject against *Streptococcus pneumoniae* infection and/or colonization, the method comprising administering to the subject an immunologically effective amount of a vaccine disclosed herein. In some embodiments, a method of immunizing a subject against *Streptococcus pneumoniae* infection and/or colonization, comprises administering to a subject an immunologically effective amount of an immunogenic complex disclosed herein. In some embodiments, a method of immunizing a subject against *Streptococcus pneumoniae* infection and/or colonization, comprises administering to a subject an immunologically effective amount of a pharmaceutical composition disclosed herein.

[0082] In some embodiments, administration of the vaccine, immunogenic complex, or pharmaceutical composition induces an immune response. In some embodiments, the immune response comprises an innate immune response. In some embodiments, the immune response comprises an antibody or B cell response. In some embodiments, the immune response comprises a CD4+ T cell response (*e.g.*, T_H1, T_H2, or T_H17 response), a CD8+ T cell response, a CD4+ and CD8+ T cell response, or a CD4-/CD8- T cell response. In some embodiments, the immune response comprises (i) an antibody or B cell response and (ii) a T cell response. In some embodiments, the immune response comprises (i) an antibody or B cell response, (ii) a T

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cell response, and (iii) an innate immune response. In some embodiments, the immune response is to at least one polysaccharide antigen or at least one polypeptide of a fusion protein. In some embodiments, the immune response comprises (i) an antibody or B cell response to at least one polysaccharide antigen, and (ii) a CD4+ T cell response (*e.g.*, T_H1, T_H2, or T_H17 response), a CD8+ T cell response, a CD4+ and CD8+ T cell response, or a CD4-/CD8- T cell response to at least one polypeptide of a fusion protein. In some embodiments, the immune response comprises (i) an antibody or B cell response to at least one polysaccharide antigen, and (ii) an antibody or B cell response to at least one polypeptide of a fusion protein. In some embodiments, the immune response comprises (i) an antibody or B cell response to at least one polysaccharide antigen, and (ii) an antibody or B cell response, and a CD4+ T cell response, including T_H1, T_H2, or T_H17 response, or a CD8+ T cell response, a CD4+ and CD8+ T cell response, or a CD4-/CD8- T cell response, to at least one polypeptide of a fusion protein.

[0083] In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) induces an opsonic/bactericidal response against one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such an opsonic/bactericidal response may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such an opsonic/bactericidal response may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such an opsonic/bactericidal response may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, administration of a vaccine, immunogenic complex,

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or pharmaceutical composition (*e.g.*, ones as described and/or utilized herein) inhibits transmission of one or more serotypes of *Streptococcus pneumoniae* from the subject to another subject. In some embodiments, such inhibition of transmission may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of transmission may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of transmission may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0084] In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) inhibits or reduces the rate of occurrence of Invasive Pneumococcal Disease (IPD) associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in the rate of occurrence, of IPD may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of IPD may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of IPD may be directed against two or more

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(*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) reduces the severity of Invasive Pneumococcal Disease (IPD) associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of IPD may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of IPD may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of IPD may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0085] In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) inhibits or reduces the rate of occurrence of bacteremia, sepsis, and/or meningitis associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in rate of occurrence, of bacteremia, sepsis, and/or meningitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or

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more) of such serotype(s). In some embodiments, such inhibition, or reduction in rate of occurrence, of bacteremia, sepsis, and/or meningitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in rate of occurrence, of bacteremia, sepsis, and/or meningitis may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) reduces the severity of bacteremia, sepsis, and/or meningitis associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of bacteremia, sepsis, and/or meningitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of bacteremia, sepsis, and/or meningitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of bacteremia, sepsis, and/or meningitis may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

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[0086] In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) inhibits or reduces the rate of occurrence of organ damage associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in the rate of occurrence, of organ damage may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of organ damage may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of organ damage may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) reduces the severity of organ damage associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of organ damage may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of organ damage may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some

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embodiments, such reduction in the severity of organ damage may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0087] In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) inhibits or reduces the rate of occurrence of pneumonia associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in the rate of occurrence, of pneumonia may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of pneumonia may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of pneumonia may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) reduces the severity of pneumonia associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of pneumonia may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition

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described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of pneumonia may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of pneumonia may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0088] In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) inhibits or reduces the rate of occurrence of otitis media associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in the rate of occurrence, of otitis media may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of otitis media may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of otitis media may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more)

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of such serotype(s). In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) reduces the severity of otitis media associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of otitis media may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of otitis media may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of otitis media may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0089] In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) inhibits or reduces the rate of occurrence of sinusitis associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition, or reduction in the rate of occurrence, of sinusitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of sinusitis be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more)

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of such serotype(s). In some embodiments, such inhibition, or reduction in the rate of occurrence, of sinusitis may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) reduces the severity of sinusitis associated with or induced by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such reduction in the severity of sinusitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of sinusitis may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such reduction in the severity of sinusitis may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

[0090] In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) inhibits colonization of mucosal surface(s) by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition of colonization of mucosal surface(s) may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein

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includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of colonization of mucosal surface(s) may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of colonization of mucosal surface(s) may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, administration of a vaccine, immunogenic complex, or pharmaceutical composition (*e.g.*, ones described and/or utilized herein) inhibits colonization of nasopharynx by one or more serotypes of *Streptococcus pneumoniae*. In some embodiments, such inhibition of colonization of nasopharynx may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of colonization of nasopharynx may be directed against one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s). In some embodiments, such inhibition of colonization of nasopharynx may be directed against two or more (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) serotypes of *Streptococcus pneumoniae*, wherein a vaccine, immunogenic complex, or pharmaceutical composition described herein (i) includes polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s); and (ii) does not include polysaccharide(s) present in at least one or more (*e.g.*, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) of such serotype(s).

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[0091] In some embodiments of the methods disclosed herein, the *Streptococcus pneumoniae* has a serotype selected from one or more of 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10C, 10F, 11A, 11B, 11C, 11D, 11E, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20A, 20B, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33C, 33D, 33E, 33F, 34, 35A, 35B, 35C, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, and 48. In some embodiments, the *Streptococcus pneumoniae* has a serotype selected from one or more of 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F. In some embodiments, the *Streptococcus pneumoniae* has a serotype selected from one or more of 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F. In some embodiments, the *Streptococcus pneumoniae* has a serotype selected from one or more of 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B.

[0092] In some embodiments, the subject is immunized against *Streptococcus pneumoniae* infection and/or colonization with one dose of a vaccine. In some embodiments, the subject is immunized against *Streptococcus pneumoniae* infection and/or colonization with two doses of a vaccine. In some embodiments, the subject is immunized against *Streptococcus pneumoniae* infection and/or colonization with three doses of a vaccine.

Brief Description of the Drawings

[0093] The present teachings described herein will be more fully understood from the following description of various illustrative embodiments, when read together with the accompanying drawings. It should be understood that the drawings described below are for illustration purposes only and are not intended to limit the scope of the present teachings in any way.

[0094] Figure 1 is a schematic representation of an exemplary Multiple Antigen Presenting System (MAPS). In the exemplary embodiment shown, MAPS immunogenic complexes comprise one or more polypeptide antigens fused to the biotin-binding protein rhizavidin, or a biotin-binding domain or biotin-binding fragment thereof, and a biotinylated

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antigenic polysaccharide. In this figure, each MAPS complex is formed between one or more fusion proteins and a biotinylated polysaccharide by non-covalent binding of a truncated rhizavidin to biotin.

[0095] Figure 2 is a schematic of an exemplary CP1 fusion protein. Such an exemplary CP1 fusion protein comprises a biotin-binding protein such as, *e.g.*, a truncated rhizavidin protein (*e.g.*, amino acids 45-179 of a wild-type rhizavidin protein), a first linker (*e.g.*, a GGGGSSS linker), a SP1500 polypeptide (*e.g.*, amino acids 27-278 of a full-length *S. pneumoniae* SP1500 polypeptide), a second linker (*e.g.*, the amino acid sequence AAA), and a SP0785 polypeptide (*e.g.*, amino acids 33-399 of a full length *S. pneumoniae* SP0785 polypeptide). In some embodiments, a CP1 fusion protein may further comprise a detectable or purification tag (*e.g.*, His tag). The amino acid sequence AAA can be from the Not I site on a PET21/24b plasmid, or synthesized. For a GGGGSSS linker, the SSS amino acid sequence can be from the Sac I site on a PET21/24b plasmid, with the GGGG amino acid sequence added to create a flexible linker with minimal steric hindrance. Alternatively, the GGGGSSS linker can be synthesized.

[0096] Figure 3 is a table showing exemplary structures of *S. pneumoniae* antigenic polysaccharides of serotypes 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F.

[0097] Figure 4 is a schematic of an exemplary pET24a(+) CP1 construct.

[0098] Figure 5 is a flow-chart depicting an exemplary upstream manufacturing process for fusion protein CP1. An exemplary CP1 polypeptide is a fusion protein comprising a truncated rhizavidin [aa 45-179 of a full-length rhizavidin protein], a first linker (*e.g.*, a GGGGSSS linker), a SP1500 polypeptide, a second linker (*e.g.*, the amino acid sequence AAA), and a SP0785 polypeptide. SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

[0099] Figure 6 is a flow-chart depicting an exemplary downstream manufacturing process for fusion protein CP1. An exemplary CP1 polypeptide is a fusion protein comprising a truncated rhizavidin [aa 45-179 of a full-length rhizavidin protein], a first linker (*e.g.*, a GGGGSSS linker), a SP1500 polypeptide, a second linker (*e.g.*, the amino acid sequence AAA),

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and a SP0785 polypeptide. DS: drug substance; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TFF: tangential flow filtration.

[0100] Figure 7 is a flow-chart depicting an exemplary manufacturing process for MAPS immunogenic complexes, comprising biotinylation of antigenic polysaccharides and assembly with fusion protein CP1. MAPS: Multiple Antigen Presenting System.

[0101] Figure 8 includes Panel A and Panel B, which are flow-charts depicting exemplary manufacturing processes for a MAPS vaccine. For example, such exemplary processes can be used to manufacture a MAPS24 vaccine, comprising MAPS immunogenic complexes that comprise capsular polysaccharides from 24 different *S. pneumoniae* serotypes. DMAP: 4-Dimethylaminopyridine; MAPS: Multiple Antigen Presenting System; PS: polysaccharide. † In-process tests

[0102] Figure 9 illustrates immunogenicity of exemplary formulations of the 24-valent MAPS24 vaccine as compared to the 13-valent Prevnar 13 vaccine after a single dose. The graph depicts serotype-specific IgG antibody titers to polysaccharides of the indicated *S. pneumoniae* serotypes, obtained from sera collected two weeks after a first immunization (P1) with MAPS24 vaccine or Prevnar 13. MAPS24 vaccine used for immunization was non-adjuvanted, or adjuvanted with passivated aluminum hydroxide or aluminum phosphate, as indicated. Results are expressed as ratios of the geometric mean titer of IgG antibodies from MAPS24 sera relative to Prevnar 13 sera. Each circle on the graph shows the geometric mean titer ratio for the indicated MAPS24 sera, against polysaccharide(s) of the indicated *S. pneumoniae* serotype (top of graph). Dotted line shows a geometric mean titer ratio of 1. Error bars show the ± 95% confidence interval. Abbreviations: 24v MAPS: 24-valent MAPS24 vaccine; $AlPO_4$: aluminum phosphate; pAlOH: passivated aluminum hydroxide; GMT Ratio: geometric mean titer ratio.

[0103] Figure 10 illustrates immunogenicity of exemplary formulations of the 24-valent MAPS24 vaccine as compared to the 13-valent Prevnar 13 vaccine after a second dose. The graph depicts serotype-specific IgG antibody titers to polysaccharides of the indicated *S. pneumoniae* serotypes, obtained from sera collected two weeks after a second immunization (P2) with MAPS24 vaccine or Prevnar 13. MAPS24 vaccine used for immunization was non-

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adjuvanted, or adjuvanted with passivated aluminum hydroxide or aluminum phosphate, as indicated. Results are expressed as ratios of the geometric mean titer of IgG antibodies from MAPS24 sera relative to Prevnar 13 sera. Each circle on the graph shows the geometric mean titer ratio for the indicated MAPS24 sera, against polysaccharide(s) of the indicated *S. pneumoniae* serotype (top of graph). Dotted line shows a geometric mean titer ratio of 1. Error bars show the ± 95% confidence interval. Abbreviations: 24v MAPS: 24-valent MAPS24 vaccine; $AlPO_4$: aluminum phosphate; pAlOH: passivated aluminum hydroxide; GMT Ratio: geometric mean titer ratio.

[0104] Figure 11 illustrates immunogenicity of exemplary formulations of the 15-valent MAPS15 vaccine as compared to the 13-valent Prevnar 13 vaccine after a single dose. The graph depicts serotype-specific IgG antibody titers to polysaccharides of the indicated *S. pneumoniae* serotypes, obtained from sera collected two weeks after a first immunization (P1) with MAPS15 vaccine or Prevnar 13. MAPS15 vaccine used for immunization was non-adjuvanted, or adjuvanted with passivated aluminum hydroxide or aluminum phosphate, as indicated. Results are expressed as ratios of the geometric mean titer of IgG antibodies from MAPS24 sera relative to Prevnar 13 sera. Each circle on the graph shows the geometric mean titer ratio for the indicated MAPS24 sera, against polysaccharide(s) of the indicated *S. pneumoniae* serotype (top of graph). Dotted line shows a geometric mean titer ratio of 1. Error bars show the ± 95% confidence interval. Abbreviations: 15v MAPS: 15-valent MAPS15 vaccine; $AlPO_4$: aluminum phosphate; pAlOH: passivated aluminum hydroxide; GMT Ratio: geometric mean titer ratio.

[0105] Figure 12 illustrates immunogenicity of exemplary formulations of the 15-valent MAPS15 vaccine as compared to the 13-valent Prevnar 13 vaccine after a second dose. The graph depicts serotype-specific IgG antibody titers to polysaccharides of the indicated *S. pneumoniae* serotypes, obtained from sera collected two weeks after a second immunization (P2) with MAPS15 vaccine or Prevnar 13. MAPS15 vaccine used for immunization was non-adjuvanted, or adjuvanted with passivated aluminum hydroxide or aluminum phosphate, as indicated. Results are expressed as ratios of the geometric mean titer of IgG antibodies from MAPS24 sera relative to Prevnar 13 sera. Each circle on the graph shows the geometric mean titer ratio for the indicated MAPS24 sera, against polysaccharide(s) of the indicated *S.*

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pneumoniae serotype (top of graph). Dotted line shows a geometric mean titer ratio of 1. Error bars show the ± 95% confidence interval. Abbreviations: 15v MAPS: 15-valent MAPS15 vaccine; $AlPO_4$: aluminum phosphate; pAlOH: passivated aluminum hydroxide; GMT Ratio: geometric mean titer ratio.

[0106] Figure 13 illustrates immunogenicity of exemplary formulations of the 24-valent MAPS24 and 15-valent MAPS15 vaccines, as compared to the 13-valent Prevnar 13 vaccine. The graph depicts serotype-specific IgG antibody titers against capsular polysaccharide of representative *S. pneumoniae* serotype 1, obtained from sera collected prior to immunization (P0) and at two timepoints following immunization with MAPS24, MAPS15, or Prevnar 13 vaccines: P1= two weeks after first immunization, and P2 = two weeks after second immunization. Vaccines used for immunization were non-adjuvanted, or adjuvanted with passivated aluminum hydroxide or aluminum phosphate, as indicated. Results are expressed in arbitrary units per ml. Each vertical bar on the graph shows geometric mean IgG antibody titer against capsular polysaccharide of *S. pneumoniae* serotype 1 for the indicated sera (top of graph) at the indicated timepoint (bottom of graph). Error bars show the 95% confidence interval. Abbreviations: CPS: capsular polysaccharide; Non-Adj: non-adjuvanted; 24V MAPS: 24-valent MAPS24 vaccine; 15V MAPS: 15-valent MAPS15 vaccine; $AlPO_4$: aluminum phosphate; p-AlOH: passivated aluminum hydroxide.

[0107] Figure 14 illustrates immunogenicity of an exemplary formulation of the 24-valent MAPS24 vaccine as compared to the 13-valent Prevnar 13 vaccine after one and two doses. The graph depicts the induction ratio (fold change from baseline) for serotype-specific IgG antibodies against polysaccharides of *S. pneumoniae* serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F. Sera were collected prior to immunization (P0) and at two timepoints following immunization with MAPS24 or Prevnar 13 vaccines: P1 (day 14) = two weeks after first immunization, and P2 (day 28) = two weeks after second immunization. Each vertical bar on the graph represents the IgG antibody geometric mean titer induction ratio (P1/P0 or P2/P0) for the indicated sera, against polysaccharide(s) of the indicated *S. pneumoniae* serotype (bottom of graph). Error bars show the 95% confidence interval. Abbreviations: PCV13: 13-valent Prevnar 13 vaccine; MAPS24: 24-valent MAPS24 vaccine.

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[0108] Figure 15 illustrates immunogenicity of an exemplary formulation of the 24-valent MAPS24 vaccine as compared to the 13-valent Prevnar 13 vaccine after one and two doses. The graph depicts the induction ratio (fold change from baseline) for serotype-specific IgG antibodies against polysaccharides of *S. pneumoniae* serotypes 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, 20B, 22F, and 33F. Sera were collected prior to immunization (P0) and at two timepoints following immunization with MAPS24 or Prevnar 13 vaccines: P1 (day 14) = two weeks after first immunization, and P2 (day 28) = two weeks after second immunization. Each vertical bar on the graph represents the IgG antibody geometric mean titer induction ratio (P1/P0 or P2/P0) for the indicated sera, against polysaccharide(s) of the indicated *S. pneumoniae* serotype (bottom of graph). Error bars show the 95% confidence interval. Abbreviations: PCV13: 13-valent Prevnar 13 vaccine; MAPS24: 24-valent MAPS24 vaccine.

[0109] Figure 16 illustrates T_H17 responses to exemplary vaccines comprising MAPS immunogenic complexes. Vaccines used for immunization were a 12-valent MAPS12 vaccine on CP1, a 6-valent MAPS6 vaccine on CP1, a 6-valent MAPS6 vaccine on an alternate fusion protein in combination with the preceding 6-valent MAPS6 vaccine on CP1, and an inactivated whole-cell *S. pneumoniae* vaccine (WCB). Peripheral blood samples of immunized mice were stimulated with whole-cell *S. pneumoniae* antigen (WCA; left panel), or a combination of SP1500 and SP0785 proteins (right panel). T_H17 responses of stimulated cells are shown by secretion of IL-17A in the media. Each point on the graph shows the concentration of secreted IL-17A for one mouse. Horizontal bars show the geometric mean concentration of secreted IL-17A for each group. Data were analyzed using Mann-Whitney U test (***: $p<0.001$). Abbreviations: BPL: betapropiolactone; CP1: rhizavidin [aa 45-179]-GGGGSSS-SP1500-AAA-SP0785; CP2a: alternate fusion protein; MAPS: Multiple Antigen Presenting System; 12V CP1: 12-valent MAPS12 vaccine on CP1; 6V CP1: 6-valent MAPS6 vaccine on CP1; 6V CP2a: 6-valent MAPS6 vaccine on CP2a; p-AlOH: passivated aluminum hydroxide.

[0110] Figure 17 illustrates protective effects of exemplary vaccines comprising immunogenic MAPS complexes. The graph depicts protection from colonization by *S. pneumoniae* following immunization with the indicated vaccines and intranasal challenge with *S. pneumoniae* serotype 6B (polysaccharide(s) of which is/are not included in the vaccines). Vaccines used for immunization were a 12-valent MAPS12 vaccine on CP1, a 6-valent MAPS6

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vaccine on CP1, a 6-valent MAPS6 vaccine on an alternate fusion protein in combination with the preceding 6-valent MAPS6 vaccine on CP1, and an inactivated whole-cell *S. pneumoniae* vaccine (WCB). Each point on the graph represents *S. pneumoniae* CFU per nasal wash for one mouse. Horizontal bars represent the geometric mean *S. pneumoniae* CFU per nasal wash for each group. Data were analyzed using Mann-Whitney U test. Abbreviations: BPL: betapropiolactone; CFU: colony forming unit; CP1: rhizavidin [aa 45-179]-GGGGSSS-SP1500-AAA-SP0785; CP2a: alternate fusion protein; MAPS: Multiple Antigen Presenting System; 12V CP1: 12-valent MAPS12 vaccine on CP1; 6V CP1: 6-valent MAPS6 vaccine on CP1; 6V CP2a: 6-valent MAPS6 vaccine on CP2a; p-AlOH: passivated aluminum hydroxide.

[0111] Figure 18 illustrates T_H17 responses to exemplary vaccines comprising MAPS immunogenic complexes. Vaccines used for immunization were (i) a 24-valent MAPS24 vaccine on CP1 (24V) in combination with aluminum phosphate ($AlPO_4$) adjuvant, and (ii) a 24-valent MAPS vaccine on CP1 (24V) in combination with passivated aluminum hydroxide (p-AlOH) adjuvant. Splenocytes of immunized mice were stimulated with a fusion of SP1500 and SP0785 proteins. T_H17 responses of stimulated cells are shown by secretion of IL-17A in the media. Each point on the graph shows the concentration of secreted IL-17A for one mouse. Horizontal bars show the geometric mean concentration of secreted IL-17A for each group. Statistical analyses were performed in comparison with the p-AlOH control group using Mann-Whitney U test (***: $p<0.001$).

[0112] Figure 19 illustrates protective effects of exemplary vaccines comprising MAPS immunogenic complexes. The graph depicts protection from colonization by *S. pneumoniae* following immunization with the indicated vaccines and intranasal challenge with *S. pneumoniae* serotype 6B (polysaccharide(s) of which is/are not included in the vaccines). Vaccines used for immunization were (i) a 23-valent MAPS23 vaccine (lacking serotype 6B) with CP1 (23V) in combination with aluminum phosphate ($AlPO_4$) adjuvant, and (ii) a 23-valent MAPS23 vaccine (lacking serotype 6B) with CP1 (23V) in combination with passivated aluminum hydroxide (p-AlOH) adjuvant. Each point on the graph represents *S. pneumoniae* colony forming unit (CFU) per nasal wash for one mouse. Horizontal bars represent the geometric mean *S. pneumoniae* CFU per nasal wash for each group. Statistical analyses were performed in comparison with the p-AlOH control group using Mann-Whitney U test (*: $p=0.2$, **: $p=0.02$).

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[0113] Figure 20 illustrates immunogenicity of an exemplary formulation of the 24-valent MAPS24 vaccine as compared to the 13-valent Prevnar 13 vaccine. The graph depicts IgG antibody titers against whole-cell *S. pneumoniae* of the indicated serotypes, polysaccharides of which are not included in MAPS24 vaccine or Prevnar 13 (non-vaccine types, NVTs). Sera were collected prior to immunization (P0) and two weeks following second immunization (P2) with MAPS24 vaccine or Prevnar 13. Results are expressed in arbitrary units per ml. Each vertical bar on the graph denotes the geometric mean titer of whole-cell binding IgG antibodies for the indicated sera, against whole-cell *S. pneumoniae* of the indicated serotype (bottom of graph). Error bars show the ± 95% confidence interval. Abbreviations: AU: arbitrary units; Prevnar: 13-valent Prevnar 13 vaccine; MAPS24: 24-valent MAPS24 vaccine;. *$p<0.05$ and **$p<0.01$ for Wilcoxon matched pairs test; ns: not statistically significant.

[0114] Figure 21 illustrates immunogenicity of an exemplary formulation of the 24-valent MAPS24 vaccine as compared to the 13-valent Prevnar 13 vaccine after two doses. The graph depicts the induction ratio (fold-change from baseline) of IgG antibodies against whole-cell *S. pneumoniae* of the indicated serotypes, polysaccharides of which are not included in MAPS24 vaccine or Prevnar 13 (non-vaccine types, NVTs). Sera were collected prior to immunization (P0) and two weeks following second immunization (P2) with MAPS24 vaccine or Prevnar 13. Each vertical bar on the graph represents the IgG antibody geometric mean titer induction ratio (P2/P0) for the indicated sera, against whole-cell *S. pneumoniae* of the indicated serotype (bottom of graph). Error bars show the ± 95% confidence interval. Abbreviations: Prevnar: 13-valent Prevnar 13 vaccine; MAPS24: 24-valent MAPS24 vaccine; P2/P0: post-2nd immunization (P2) IgG titer divided by pre-immune (P0) IgG titer; **$p<0.01$ and *** $p<0.001$ for Mann-Whitney test.

[0115] Figure 22 illustrates the presence of functional antibodies against representative non-vaccine serotypes of *S. pneumoniae* in MAPS24 immune sera, using an opsonophagocytic killing assay. *S. pneumoniae* serotypes 15A, 16F, 23A, 31, 35B, and 35F, polysaccharides of which are not included in MAPS24 vaccine or Prevnar 13, were separately incubated in a modified concentrated opsonophagocytic assay (COPA) with heat-inactivated sera from rabbits immunized with an exemplary formulation of the 24-valent MAPS24 vaccine. The presence of functional antibodies is shown by killing of incubated *S. pneumoniae*. Sera were collected prior

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to immunization (P0) and two weeks following second immunization (P2). Results are expressed as percent killing activity, *i.e.*, the percent reduction in *S. pneumoniae* colony forming units (CFU) following incubation with immune (P2) sera, relative to incubation with matched pre-immune (P0) sera. Each vertical bar of Panels A-F represents the percent killing activity observed with the indicated dilution of MAPS24 immune sera (bottom of each panel) against the indicated *S. pneumoniae* serotype (top of each panel).

[0116] Figure 23 illustrates the presence of functional antibodies against a representative non-vaccine serotype of *S. pneumoniae* (*e.g.*, serotype16F) in MAPS24 immune sera, compared to Prevnar 13 immune sera, using an opsonophagocytic killing assay. *S. pneumoniae* serotype 16F, polysaccharide(s) of which is/are not included in MAPS24 vaccine or Prevnar 13, was incubated in a modified concentrated opsonophagocytic assay (COPA) with heat-inactivated sera from rabbits immunized with an exemplary formulation of the 24-valent MAPS24 vaccine or with Prevnar 13. The presence of functional antibodies was shown by killing of incubated *S. pneumoniae*. Sera were collected prior to immunization (P0) and two weeks following second immunization (P2). Results are expressed as percent survival, *i.e.*, the percent reduction in *S. pneumoniae* colony forming units (CFU) following incubation with immune (P2) sera, relative to incubation with matched pre-immune (P0) sera. Each vertical bar of the graphs represents the percent survival of *S. pneumoniae* serotype 16F observed with MAPS24 immune sera (Panel A) or Prevnar 13 immune sera (Panel B) at the indicated dilution (bottom of each graph). Error bars show the ± 95% confidence interval. Abbreviations: Prevnar: 13-valent Prevnar 13 vaccine; MAPS24: 24-valent MAPS24 vaccine; *$p<0.05$ and **$p<0.01$ for Wilcoxon matched pairs test; ns: not statistically significant.

[0117] Figure 24 illustrates the presence of functional antibodies against a representative non-vaccine serotype of *S. pneumoniae* (*e.g.*, serotype 15A) in MAPS24 immune sera, compared to Prevnar 13 immune sera, using an opsonophagocytic killing assay. *S. pneumoniae* serotype 15A, polysaccharide(s) of which is/are not included in MAPS24 vaccine or Prevnar 13, was incubated in a modified concentrated opsonophagocytic assay (COPA) with heat-inactivated sera from rabbits immunized with an exemplary formulation of the 24-valent MAPS24 vaccine or with Prevnar 13. The presence of functional antibodies was shown by killing of incubated *S. pneumoniae*. Sera were collected prior to immunization (P0) and two weeks following second

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immunization (P2). Results are expressed as percent survival, *i.e.*, the percent reduction in *S. pneumoniae* colony forming units (CFU) following incubation with immune (P2) sera, relative to incubation with matched pre-immune (P0) sera. Each vertical bar of the graphs represents the percent survival of *S. pneumoniae* serotype 15A observed with MAPS24 immune sera (Panel A) or Prevnar 13 immune sera (Panel B) at the indicated dilution (bottom of each graph). Error bars show the ± 95% confidence interval. Abbreviations: Prevnar: 13-valent Prevnar 13 vaccine; MAPS24: 24-valent MAPS24 vaccine; *$p<0.05$ and **$p<0.01$ for Wilcoxon matched pairs test; ns: not statistically significant.

[0118] Figure 25 illustrates specificity of functional antibodies against a representative non-vaccine serotype of *S. pneumoniae* (*e.g.*, serotype 16F), polysaccharide(s) of which is/are not included in MAPS24 vaccine. The specificity of functional antibodies was assayed using an inhibition variant of the modified concentrated opsonophagocytic assay (iCOPA), in which immune sera are pre-incubated with a specific inhibitory reagent (*e.g.*, purified fusion protein CP1) that inhibits or competes with binding of functional antibodies to a target antigen. Pre-incubated sera are then incubated with *S. pneumoniae* to determine whether killing by functional antibodies is inhibited. Rabbits were immunized with an exemplary formulation of the 24-valent MAPS24 vaccine. Sera were collected prior to immunization (P0) and two weeks following second immunization (P2). Results are expressed as percent killing activity, *i.e.*, the percent reduction in *S. pneumoniae* colony forming units (CFU) following incubation with immune (P2) sera, relative to incubation with matched pre-immune (P0) sera. Each vertical bar on the graph represents the percent killing activity against *S. pneumoniae* serotype 16F observed with MAPS24 immune sera, pre-incubated as indicated with purified fusion protein CP1 (+ CP1) or no inhibitor, at the indicated dilution (bottom of graph).

[0119] Figure 26 illustrates the presence of functional antibodies against a representative non-vaccine serotype of *S. pneumoniae* (*e.g.*, serotype 16F) in SP1500 and SP0785 immune sera, using an opsonophagocytic killing assay. *S. pneumoniae* serotype 16F, polysaccharide(s) of which is/are not included in MAPS24 vaccine or Prevnar 13, was incubated in a modified concentrated opsonophagocytic assay (COPA) with heat-inactivated sera from rabbits immunized with SP1500 or SP0785 proteins. The presence of functional antibodies was shown by killing of incubated *S. pneumoniae*. Sera were collected prior to immunization (P0) and two

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weeks following third immunization (P3). Results are expressed as percent killing activity, *i.e.*, the percent reduction in *S. pneumoniae* colony forming units (CFU) following incubation with immune (P3) sera, relative to incubation with matched pre-immune (P0) sera. Each vertical bar of the graph represents the percent killing activity against *S. pneumoniae* serotype 16F observed with the indicated SP1500 or SP0785 immune sera at the indicated dilution.

Definitions

[0120] In this application, unless otherwise clear from context, (i) the term "a" may be understood to mean "at least one"; (ii) the term "or" may be understood to mean "and/or"; (iii) the terms "comprising" and "including" may be understood to encompass itemized components or steps whether presented by themselves or together with one or more additional components or steps; and (iv) the terms "about" and "approximately" may be understood to permit standard variation as would be understood by those of ordinary skill in the art; and (v) where ranges are provided, endpoints are included.

[0121] **About:** The term "about", when used herein in reference to a value, refers to a value that is similar, in context to the referenced value. In general, those skilled in the art, familiar with the context, will appreciate the relevant degree of variance encompassed by "about" in that context. For example, in some embodiments, the term "about" may encompass a range of values that within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less of the referred value.

[0122] **Administration**: As used herein, the term "administration" typically refers to the administration of a composition to a subject or system to achieve delivery of an agent that is, or is included in, the composition. Those of ordinary skill in the art will be aware of a variety of routes that may, in appropriate circumstances, be utilized for administration to a subject, for example a human. For example, in some embodiments, administration may be ocular, oral, parenteral, topical, etc. In some particular embodiments, administration may be bronchial (*e.g.*, by bronchial instillation), buccal, dermal (which may be or comprise, for example, one or more of topical to the dermis, intradermal, interdermal, transdermal, etc.), enteral, intra-arterial, intradermal, intragastrical, intramedullary, intramuscular, intranasal, intraperitoneal, intrathecal, intravenous, intraventricular, within a specific organ (*e.g.*, intrahepatic), mucosal, nasal, oral,

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rectal, subcutaneous, sublingual, topical, tracheal (*e.g.*, by intratracheal instillation), vaginal, vitreal, etc. In some embodiments, administration may involve only a single dose. In some embodiments, administration may involve application of a fixed number of doses. In some embodiments, administration may involve dosing that is intermittent (*e.g.*, a plurality of doses separated in time) and/or periodic (*e.g.*, individual doses separated by a common period of time) dosing. In some embodiments, administration may involve continuous dosing (*e.g.*, perfusion) for at least a selected period of time.

[0123] **Agent**: In general, the term "agent", as used herein, may be used to refer to a compound or entity of any chemical class including, for example, a polypeptide, nucleic acid, saccharide, lipid, small molecule, metal, or combination or complex thereof. In appropriate circumstances, as will be clear from context to those skilled in the art, the term may be utilized to refer to an entity that is or comprises a cell or organism, or a fraction, extract, or component thereof. Alternatively or additionally, as context will make clear, the term may be used to refer to a natural product in that it is found in and/or is obtained from nature. In some instances, again as will be clear from context, the term may be used to refer to one or more entities that is man-made in that it is designed, engineered, and/or produced through action of the hand of man and/or is not found in nature. In some embodiments, an agent may be utilized in isolated or pure form; in some embodiments, an agent may be utilized in crude form. In some embodiments, potential agents may be provided as collections or libraries, for example that may be screened to identify or characterize active agents within them. In some cases, the term "agent" may refer to a compound or entity that is or comprises a polymer; in some cases, the term may refer to a compound or entity that comprises one or more polymeric moieties. In some embodiments, the term "agent" may refer to a compound or entity that is not a polymer and/or is substantially free of any polymer and/or of one or more particular polymeric moieties. In some embodiments, the term may refer to a compound or entity that lacks or is substantially free of any polymeric moiety.

[0124] **Amino acid**: In its broadest sense, the term "amino acid", as used herein, refers to any compound and/or substance that can be incorporated into a polypeptide chain, *e.g.*, through formation of one or more peptide bonds. In some embodiments, an amino acid has the general structure H_2N–C(H)(R)–COOH. In some embodiments, an amino acid is a naturally-

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occurring amino acid. In some embodiments, an amino acid is a non-natural amino acid; in some embodiments, an amino acid is a D-amino acid; in some embodiments, an amino acid is an L-amino acid. "Standard amino acid" refers to any of the twenty standard L-amino acids commonly found in naturally occurring peptides. "Non-standard amino acid" refers to any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or obtained from a natural source. In some embodiments, an amino acid, including a carboxy- and/or amino-terminal amino acid in a polypeptide, can contain a structural modification as compared with the general structure above. For example, in some embodiments, an amino acid may be modified by methylation, amidation, acetylation, pegylation, glycosylation, phosphorylation, and/or substitution (*e.g.*, of the amino group, the carboxylic acid group, one or more protons, and/or the hydroxyl group) as compared with the general structure. In some embodiments, such modification may, for example, alter the circulating half-life of a polypeptide containing the modified amino acid as compared with one containing an otherwise identical unmodified amino acid. In some embodiments, such modification does not significantly alter a relevant activity of a polypeptide containing the modified amino acid, as compared with one containing an otherwise identical unmodified amino acid. As will be clear from context, in some embodiments, the term "amino acid" may be used to refer to a free amino acid; in some embodiments it may be used to refer to an amino acid residue of a polypeptide.

[0125] **Antibody**: As used herein, the term "antibody" refers to a polypeptide that includes canonical immunoglobulin sequence elements sufficient to confer specific binding to a particular target antigen. As is known in the art, intact antibodies as produced in nature are approximately 150 kDa tetrameric agents comprised of two identical heavy chain polypeptides (about 50 kDa each) and two identical light chain polypeptides (about 25 kDa each) that associate with each other into what is commonly referred to as a "Y-shaped" structure. Each heavy chain is comprised of at least four domains (each about 110 amino acids long)– an amino-terminal variable (VH) domain (located at the tips of the Y structure), followed by three constant domains: CH1, CH2, and the carboxy-terminal CH3 (located at the base of the Y's stem). A short region, known as the "switch", connects the heavy chain variable and constant regions. The "hinge" connects CH2 and CH3 domains to the rest of the antibody. Two disulfide bonds in this hinge region connect the two heavy chain polypeptides to one another in an intact antibody.

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Each light chain is comprised of two domains – an amino-terminal variable (VL) domain, followed by a carboxy-terminal constant (CL) domain, separated from one another by another "switch". Intact antibody tetramers are comprised of two heavy chain-light chain dimers in which the heavy and light chains are linked to one another by a single disulfide bond; two other disulfide bonds connect the heavy chain hinge regions to one another, so that the dimers are connected to one another and the tetramer is formed. Naturally-produced antibodies are also glycosylated, typically on the CH2 domain. Each domain in a natural antibody has a structure characterized by an "immunoglobulin fold" formed from two beta sheets (*e.g.*, 3-, 4-, or 5-stranded sheets) packed against each other in a compressed antiparallel beta barrel. Each variable domain contains three hypervariable loops known as "complement determining regions" (CDR1, CDR2, and CDR3) and four somewhat invariant "framework" regions (FR1, FR2, FR3, and FR4). When natural antibodies fold, the FR regions form the beta sheets that provide the structural framework for the domains, and the CDR loop regions from both the heavy and light chains are brought together in three-dimensional space so that they create a single hypervariable antigen binding site located at the tip of the Y structure. The Fc region of naturally-occurring antibodies binds to elements of the complement system, and also to receptors on effector cells, including for example effector cells that mediate cytotoxicity. As is known in the art, affinity and/or other binding attributes of Fc regions for Fc receptors can be modulated through glycosylation or other modification. In some embodiments, antibodies produced and/or utilized in accordance with the present invention include glycosylated Fc domains, including Fc domains with modified or engineered such glycosylation. For purposes of the present invention, in certain embodiments, any polypeptide or complex of polypeptides that includes sufficient immunoglobulin domain sequences as found in natural antibodies can be referred to and/or used as an "antibody", whether such polypeptide is naturally produced (*e.g.*, generated by an organism reacting to an antigen), or produced by recombinant engineering, chemical synthesis, or other artificial system or methodology. In some embodiments, an antibody is polyclonal; in some embodiments, an antibody is monoclonal. In some embodiments, an antibody has constant region sequences that are characteristic of mouse, rabbit, primate, or human antibodies. In some embodiments, antibody sequence elements are humanized, primatized, chimeric, etc., as is known in the art. Moreover, the term "antibody" as used herein, can refer in appropriate

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embodiments (unless otherwise stated or clear from context) to any of the art-known or developed constructs or formats for utilizing antibody structural and functional features in alternative presentation. For example, embodiments, an antibody utilized in accordance with the present invention is in a format selected from, but not limited to, intact IgA, IgG, IgE or IgM antibodies; bi- or multi- specific antibodies (*e.g.*, Zybodies®, etc.); antibody fragments such as Fab fragments, Fab' fragments, F(ab')2 fragments, Fd' fragments, Fd fragments, and isolated CDRs or sets thereof; single chain Fvs; polypeptide-Fc fusions; single domain antibodies (*e.g.*, shark single domain antibodies such as IgNAR or fragments thereof); cameloid antibodies; masked antibodies (*e.g.*, Probodies®); Small Modular ImmunoPharmaceuticals ("SMIPs™"); single chain or Tandem diabodies (TandAb®); VHHs; Anticalins®; Nanobodies® minibodies; BiTE®s; ankyrin repeat proteins or DARPINs®; Avimers®; DARTs; TCR-like antibodies;, Adnectins®; Affilins®; Trans-bodies®; Affibodies®; TrimerX®; MicroProteins; Fynomers®, Centyrins®; and KALBITOR®s. In some embodiments, an antibody may lack a covalent modification (*e.g.*, attachment of a glycan) that it would have if produced naturally. In some embodiments, an antibody may contain a covalent modification (*e.g.*, attachment of a glycan, a payload [*e.g.*, a detectable moiety, a therapeutic moiety, a catalytic moiety, etc.], or other pendant group [*e.g.*, poly-ethylene glycol, etc.]).

[0126] **Antigen**: The term "antigen", as used herein, refers to (i) an agent that induces an immune response; and/or (ii) an agent that binds to a T cell receptor (*e.g.*, when presented by an MHC molecule) or to an antibody. In some embodiments, an antigen induces a humoral response (*e.g.*, including production of antigen-specific antibodies); in some embodiments, an antigen induces a cellular response (*e.g.*, involving T cells whose receptors specifically interact with the antigen). In some embodiments, an antigen induces a humoral response and a cellular response. In some embodiments, an antigen binds to an antibody and may or may not induce a particular physiological response in an organism. In general, an antigen may be or include any chemical entity such as, for example, a small molecule, a nucleic acid, a polypeptide, a carbohydrate, a lipid, a polymer (in some embodiments other than a biologic polymer (*e.g.*, other than a nucleic acid or amino acid polymer)), etc. In some embodiments, an antigen is or comprises a polypeptide. In some embodiments, an antigen is or comprises a polysaccharide. Those of ordinary skill in the art will appreciate that, in general, an antigen may be provided in

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isolated or pure form, or alternatively may be provided in crude form (*e.g.*, together with other materials, for example in an extract such as a cellular extract or other relatively crude preparation of an antigen-containing source). In some embodiments, antigens utilized in accordance with the present invention are provided in a crude form. In some embodiments, an antigen is a recombinant antigen. In some embodiments, an antigen is a polypeptide or a polysaccharide that, upon administration to a subject, induces a specific and/or clinically relevant immune response to such polypeptide or polysaccharide. In some embodiments, an antigen is selected to induce a specific and/or clinically relevant immune response to such polypeptide or polysaccharide.

[0127] **Associated with**: Two entities are "associated" with one another, as that term is used herein, if the presence, level and/or form of one is correlated with that of the other. In some embodiments, two or more entities are physically "associated" with one another if they interact, directly or indirectly, so that they are and/or remain in physical proximity with one another. In some embodiments, two or more entities that are physically associated with one another are covalently linked to one another. In some embodiments, two or more entities that are physically associated with one another are not covalently linked to one another but are non-covalently associated, for example by means of affinity interactions, electrostatic interactions, hydrogen bonds, van der Waals interaction, hydrophobic interactions, magnetism, and combinations thereof.

[0128] **Binding**: It will be understood that the term "binding", as used herein, typically refers to a non-covalent association between or among two or more entities. "Direct" binding involves physical contact between entities or moieties; indirect binding involves physical interaction by way of physical contact with one or more intermediate entities. Binding between two or more entities can typically be assessed in any of a variety of contexts – including where interacting entities or moieties are studied in isolation or in the context of more complex systems (*e.g.*, while covalently or otherwise associated with a carrier entity and/or in a biological system or cell).

[0129] **Carrier protein**: As used herein, the term "carrier protein" refers to a protein or peptide that is coupled, or complexed, or otherwise associated with a hapten (*e.g.*, a small peptide or lipid) or less immunogenic antigen (*e.g.*, a polysaccharide) and that induces or

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improves an immune response to such a coupled, or complexed, or otherwise associated hapten (*e.g.*, a small peptide or lipid) or less immunogenic antigen (*e.g.*, a polysaccharide). In some embodiments, such an immune response is or comprises a response to a hapten or less immunogenic antigen that is coupled, or complexed, or otherwise associated with such a carrier protein. In some embodiments, such an immune response is or comprises a response to both a carrier protein and a hapten or less immunogenic antigen that is coupled, or complexed, or otherwise associated with such a carrier protein. In some embodiments, no significant immune response to a carrier protein itself occurs. In some embodiments, immune response to a carrier protein may be detected; in some embodiments, immune response to such a carrier protein is strong. In some embodiments, a carrier protein is coupled, or complexed, or otherwise associated with one or more other molecules.

[0130] **Colonization:** As used herein, the term "colonization" generally refers to the ability of a microbe to grow at a target site or surface. For example, the terms "colonization" refers to the ability of a microbe (*e.g.*, a bacterium) to grow at an anatomical site (*e.g.*, a mucosal membrane, gastrointestinal tract, injury site, organ, *etc.*) of a host.

[0131] **Combination therapy**: As used herein, the term "combination therapy" refers to those situations in which a subject is exposed to two or more therapeutic regimens (*e.g.*, two or more therapeutic agents). In some embodiments, the two or more regimens may be administered simultaneously; in some embodiments, such regimens may be administered sequentially (*e.g.*, all "doses" of a first regimen are administered prior to administration of any doses of a second regimen); in some embodiments, such agents are administered in overlapping dosing regimens. In some embodiments, "administration" of combination therapy may involve administration of one or more agent(s) or modality(ies) to a subject receiving the other agent(s) or modality(ies) in the combination. For clarity, combination therapy does not require that individual agents be administered together in a single composition (or even necessarily at the same time), although in some embodiments, two or more agents, or active moieties thereof, may be administered together in a combination composition, or even in a combination compound (*e.g.*, as part of a single chemical complex or covalent entity).

[0132] **Derivative**: As used herein, the term "derivative", or grammatical equivalents thereof, refers to a structural analogue of a reference substance. That is, a "derivative" is a

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substance that shows significant structural similarity with the reference substance, for example sharing a core or consensus structure, but also differs in certain discrete ways. Such a substance would be said to be "derived from" said reference substance. In some embodiments, a derivative is a substance that can be generated from the reference substance by chemical manipulation. In some embodiments, a derivative is a substance that can be generated through performance of a synthetic process substantially similar to (*e.g.*, sharing a plurality of steps with) one that generates the reference substance.

[0133] **Domain:** The term "domain" as used herein refers to a section or portion of an entity. In some embodiments, a "domain" is associated with a particular structural and/or functional feature of the entity so that, when the domain is physically separated from the rest of its parent entity, it substantially or entirely retains the particular structural and/or functional feature. Alternatively or additionally, a domain may be or include a portion of an entity that, when separated from that (parent) entity and linked with a different (recipient) entity, substantially retains and/or imparts on the recipient entity one or more structural and/or functional features that characterized it in the parent entity. In some embodiments, a domain is a section or portion of a molecule (*e.g.*, a small molecule, carbohydrate, lipid, nucleic acid, or polypeptide). In some embodiments, a domain is a section of a polypeptide; in some such embodiments, a domain is characterized by a particular structural element (*e.g.*, a particular amino acid sequence or sequence motif, α-helix character, β-sheet character, coiled-coil character, random coil character, etc.), and/or by a particular functional feature (*e.g.*, binding activity, enzymatic activity, folding activity, signaling activity, etc.).

[0134] **Dosage form or unit dosage form**: Those skilled in the art will appreciate that the term "dosage form" may be used to refer to a physically discrete unit of an active agent (*e.g.*, a therapeutic or diagnostic agent) for administration to a subject. Typically, each such unit contains a predetermined quantity of active agent. In some embodiments, such quantity is a unit dosage amount (or a whole fraction thereof) appropriate for administration in accordance with a dosing regimen that has been determined to correlate with a desired or beneficial outcome when administered to a relevant population (*i.e.*, with a therapeutic dosing regimen). Those of ordinary skill in the art appreciate that the total amount of a therapeutic composition or agent

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administered to a particular subject is determined by one or more attending physicians and may involve administration of multiple dosage forms.

[0135] **Dosing regimen:** Those skilled in the art will appreciate that the term "dosing regimen" may be used to refer to a set of unit doses (typically more than one) that are administered individually to a subject, typically separated by periods of time. In some embodiments, a given therapeutic agent has a recommended dosing regimen, which may involve one or more doses. In some embodiments, a dosing regimen comprises a plurality of doses each of which is separated in time from other doses. In some embodiments, individual doses are separated from one another by a time period of the same length; in some embodiments, a dosing regimen comprises a plurality of doses and at least two different time periods separating individual doses. In some embodiments, all doses within a dosing regimen are of the same unit dose amount. In some embodiments, different doses within a dosing regimen are of different amounts. In some embodiments, a dosing regimen comprises a first dose in a first dose amount, followed by one or more additional doses in a second dose amount different from the first dose amount. In some embodiments, a dosing regimen comprises a first dose in a first dose amount, followed by one or more additional doses in a second dose amount same as the first dose amount. In some embodiments, a dosing regimen is correlated with a desired or beneficial outcome when administered across a relevant population (*i.e.*, is a therapeutic dosing regimen).

[0136] **Fragment**: A "fragment" of a material or entity as described herein has a structure that includes a discrete portion of the whole, but lacks one or more moieties found in the whole. In some embodiments, a fragment consists of such a discrete portion. In some embodiments, a fragment includes a discrete portion of the whole which discrete portion shares one or more functional characteristics found in the whole. In some embodiments, a fragment consists of such a discrete portion. In some embodiments, a fragment consists of or comprises a characteristic structural element or moiety found in the whole. In some embodiments, a fragment of a polymer, *e.g.*, a polypeptide or a polysaccharide, comprises or consists of at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 275, 300, 325, 350, 375, 400, 425, 450, 475, 500 or more monomeric units (*e.g.*, residues) as found in the whole polymer. In some embodiments, a polymer fragment comprises or consists of at least

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about 5%, 10%, 15%, 20%, 25%, 30%, 25%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more of the monomeric units (*e.g.*, residues) found in the whole polymer. The whole material or entity may in some embodiments be referred to as the "parent" of the whole.

[0137] **Homology**: As used herein, the term "homology" refers to the overall relatedness between polymeric molecules, *e.g.*, between nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical. In some embodiments, polymeric molecules are considered to be "homologous" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% similar (*e.g.*, containing residues with related chemical properties at corresponding positions). For example, as is well known by those of ordinary skill in the art, certain amino acids are typically classified as similar to one another as "hydrophobic" or "hydrophilic" amino acids, and/or as having "polar" or "non-polar" side chains. Substitution of one amino acid for another of the same type may often be considered a "homologous" substitution.

[0138] **Identity**: As used herein, the term "identity" refers to the overall relatedness between polymeric molecules, *e.g.*, between nucleic acid molecules (*e.g.*, DNA molecules and/or RNA molecules) and/or between polypeptide molecules. In some embodiments, polymeric molecules are considered to be "substantially identical" to one another if their sequences are at least 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical. Calculation of the percent identity of two nucleic acid or polypeptide sequences, for example, can be performed by aligning the two sequences for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second sequence for optimal alignment and non-identical sequences can be disregarded for comparison purposes). In certain embodiments, the length of a sequence aligned for comparison purposes is at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or substantially 100% of the length of a reference sequence. The nucleotides at corresponding

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positions are then compared. When a position in the first sequence is occupied by the same residue (*e.g.*, nucleotide or amino acid) as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which needs to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two nucleotide sequences can be determined using the algorithm of Meyers and Miller, 1989, which has been incorporated into the ALIGN program (version 2.0). In some exemplary embodiments, nucleic acid sequence comparisons made with the ALIGN program use a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. The percent identity between two nucleotide sequences can, alternatively, be determined using the GAP program in the GCG software package using an NWSgapdna.CMP matrix.

[0139] **Improve, increase, inhibit** or **reduce:** As used herein, the terms "improve", "increase", "inhibit', "reduce", or grammatical equivalents thereof, indicate values that are relative to a baseline or other reference measurement. In some embodiments, an appropriate reference measurement may be or comprise a measurement in a particular system (*e.g.*, in a single subject) under otherwise comparable conditions absent presence of (*e.g.*, prior to and/or after) a particular agent or treatment, or in presence of an appropriate comparable reference agent. In some embodiments, an appropriate reference measurement may be or comprise a measurement in comparable system known or expected to respond in a particular way, in presence of the relevant agent or treatment.

[0140] **Immunologically effective amount** or **immunologically effective dose:** As used herein, "immunologically effective amount" or "immunologically effective dose" refers to an amount of an antigenic or immunogenic substance, *e.g.*, an antigen, immunogen, immunogenic complex, immunogenic composition, vaccine, or pharmaceutical composition, which when administered to a subject, either in a single dose or as part of a series of doses, that is sufficient to enhance a subject's own immune response against a subsequent exposure to a pathogen. In some embodiments, the pathogen is *S. pneumoniae*. In some embodiments, the immune response is against one or more different serotypes of *S. pneumoniae*. In some embodiments, the immune

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response is against two or more different serotypes of *S. pneumoniae*. In some embodiments, the immune response is against nine or more different serotypes of *S. pneumoniae*. In some embodiments, the immune response is against thirteen or more different serotypes of *S. pneumoniae*. In some embodiments, the immune response is against fifteen or more different serotypes of *S. pneumoniae*. In some embodiments, the immune response is against twenty-three or more different serotypes of *S. pneumoniae*. In some embodiments, the immune response is against twenty-four or more different serotypes of *S. pneumoniae*. An immunologically effective amount may vary based on the subject to be treated, the species of the subject, the degree of immune response desired to induce, etc. In some embodiments, an immunologically effective amount is sufficient for treatment or protection of a subject having or at risk of having disease. In some embodiments, an immunologically effective amount refers to a non-toxic but sufficient amount that can be an amount to treat, attenuate, or prevent infection and/or disease (*e.g.*, bacterial infection, pneumococcal infection, bacterial colonization, pneumococcal colonization, complications associated with bacterial infection, complications associated with pneumococcal infection, etc.) in any subject. In some embodiments, an immunologically effective amount is sufficient to induce an immunoprotective response upon administration to a subject.

[0141] **Immunoprotective response** or **protective response:** As used herein, "immunoprotective response" or "protective response" refers to an immune response that mediates antigen or immunogen-induced immunological memory. In some embodiments, an immunoprotective response is induced by the administration of a substance, *e.g.*, an antigen, immunogen, immunogenic complex, immunogenic composition, vaccine, or pharmaceutical composition to a subject. In some embodiments, immunoprotection involves one or more of active immune surveillance, a more rapid and effective response upon immune activation as compared to a response observed in a naïve subject, efficient clearance of the activating agent or pathogen, followed by rapid resolution of inflammation. In some embodiments, an immunoprotective response is an adaptive immune response. In some embodiments, an immunoprotective response is sufficient to protect an immunized subject from productive infection by a particular pathogen or pathogens to which a vaccine is directed (*e.g.*, *S. pneumoniae* infection).

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[0142] **Immunization:** As used herein, "immunization", or grammatical equivalents thereof, refers to a process of inducing an immune response to an infectious organism or agent in a subject ("active immunization"), or alternatively, providing immune system components against an infectious organism or agent to a subject ("passive immunization"). In some embodiments, immunization involves the administration of one or more antigens, immunogens, immunogenic complexes, vaccines, immune molecules such as antibodies, immune sera, immune cells such as T cells or B cells, or pharmaceutical compositions to a subject. In some embodiments, immunization is performed by administering an immunologically effective amount of a substance, *e.g.*, an antigen, immunogen, immunogenic complex, immunogenic composition, vaccine, immune molecule such as an antibody, immune serum, immune cell such as a T cell or B cell, or pharmaceutical composition to a subject. In some embodiments, immunization results in an immunoprotective response in the subject. In some embodiments, active immunization is performed by administering to a subject an antigenic or immunogenic substance, *e.g.*, an antigen, immunogen, immunogenic complex, vaccine, or pharmaceutical composition. In some embodiments, passive immunization is performed by administering to a subject an immune system component, *e.g.*, an immune molecule such as an antibody, immune serum, or immune cell such as a T cell or B cell.

[0143] **Isolated:** As used herein, the term "isolated", or grammatical equivalents thereof, refers to a substance and/or entity that has been (1) separated from at least some of the components with which it was associated when initially produced (whether in nature and/or in an experimental setting), and/or (2) designed, produced, prepared, and/or manufactured by the hand of man. Isolated substances and/or entities may be separated from about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% of the other components with which they were initially associated. In some embodiments, isolated agents are about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99%, or more than about 99% pure. As used herein, a substance is "pure" if it is substantially free of other components. In some embodiments, as will be understood by those skilled in the art, a substance may still be considered "isolated" or even "pure", after having been combined with

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certain other components such as, for example, one or more carriers or excipients (*e.g.*, buffer, solvent, water, etc.); in such embodiments, percent isolation or purity of the substance is calculated without including such carriers or excipients. To give but one example, in some embodiments, a biological polymer such as a polypeptide or polysaccharide that occurs in nature is considered to be "isolated" when, a) by virtue of its origin or source of derivation is not associated with some or all of the components that accompany it in its native state in nature; b) it is substantially free of other polypeptides or nucleic acids of the same species from the species that produces it in nature; c) is expressed by or is otherwise in association with components from a cell or other expression system that is not of the species that produces it in nature. Thus, for instance, in some embodiments, a polypeptide or polysaccharide that is chemically synthesized or is synthesized in a cellular system different from that which produces it in nature is considered to be an "isolated" polypeptide or polysaccharide. Alternatively or additionally, in some embodiments, a polypeptide or polysaccharide that has been subjected to one or more purification techniques may be considered to be an "isolated" polypeptide or polysaccharide to the extent that it has been separated from other components a) with which it is associated in nature; and/or b) with which it was associated when initially produced.

[0144] **Linker**: As used herein, the term "linker" is used to refer to an entity that connects two or more elements to form a multi-element agent. For example, those of ordinary skill in the art appreciate that a polypeptide whose structure includes two or more functional or organizational domains often includes a stretch of amino acids between such domains that links them to one another. In some embodiments, a polypeptide comprising a linker element has an overall structure of the general form S1-L-S2, wherein S1 and S2 may be the same or different and represent two domains associated with one another by the linker (L). In some embodiments, a polypeptide linker is at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more amino acids in length. In some embodiments, a linker is characterized in that it tends not to adopt a rigid three-dimensional structure, but rather provides flexibility to the polypeptide. A variety of different linker elements that can appropriately be used when engineering polypeptides (*e.g.*, fusion polypeptides) are known in the art (Holliger et al, 1993; Poljak, 1994).

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[0145] **Non-inferior**: As used herein, the term "non-inferior" in the context of evaluating a test pharmaceutical composition refers to a test pharmaceutical composition that is (*e.g.*, in terms of immunogenicity and/or functional antibody titer generated by the test composition) at least as effective as a reference composition. In some embodiments, non-inferiority is demonstrated when the lower bound of the 95% confidence interval (CI) for the geometric mean titer (GMT) ratio of a test pharmaceutical composition over a reference composition is at least greater than or equal to 0.4 or above, including, *e.g.*, at least 0.5, at least 0.6, at least 0.7, at least 0.8, at least 0.9, at least 0.95, at least 0.98, at least 1.0, or higher. For example, in some embodiments, an immunogenic composition or vaccine described herein is non-inferior to a reference vaccine (*e.g.*, PCV13 or PPSV23) when the lower bound of the 95% confidence interval (CI) for the geometric mean titer (GMT) ratio of the immunogenic composition or vaccine over the reference vaccine is at least greater than or equal to 0.4 or above, including, *e.g.*, at least 0.5, at least 0.6, at least 0.7, at least 0.8, at least 0.9, at least 0.95, at least 0.98, at least 1.0, or higher. In some embodiments, an immunogenic composition or vaccine described herein is non-inferior to a reference vaccine (*e.g.*, PCV13 or PPSV23) when the lower bound of the 95% confidence interval (CI) for the geometric mean titer (GMT) ratio of the immunogenic composition or vaccine over the reference vaccine is at least greater than or equal to 0.95 or above, including, *e.g.*, at least 0.96, at least 0.97, at least 0.98, at least 0.99, at least 1.0, at least 1.1, at least 1.3, at least 1.5, or higher. In some embodiments, an immunogenic composition or vaccine described herein is non-inferior to a reference vaccine (*e.g.*, PCV13 or PPSV23) when the seroconversion rates, or percentages of vaccine recipients with immune responses, are above a pre-defined threshold, *e.g.*, the lower bound of the 95% confidence interval for the difference between the percentage of subjects who seroconvert, following immunization with an immunogenic composition or vaccine described herein or immunization with the reference vaccine (*e.g.*, PCV13 or PPSV23), is greater than -0.10.

[0146] **Pharmaceutical composition**: As used herein, the term "pharmaceutical composition" refers to a composition in which an active agent is formulated together with one or more pharmaceutically acceptable carriers. In some embodiments, the active agent is present in unit dose amount appropriate for administration in a therapeutic regimen that shows a statistically significant probability of achieving a predetermined therapeutic effect when

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administered to a relevant population. In some embodiments, a pharmaceutical composition may be specially formulated for administration in solid or liquid form, including those adapted for the following: oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, *e.g.*, those targeted for buccal, sublingual, and systemic absorption, boluses, powders, granules, pastes for application to the tongue; parenteral administration, for example, by subcutaneous, intramuscular, intravenous or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; topical application, for example, as a cream, ointment, or a controlled-release patch or spray applied to the skin, lungs, or oral cavity; intravaginally or intrarectally, for example, as a pessary, cream, or foam; sublingually; ocularly; transdermally; or nasally, pulmonary, and to other mucosal surfaces.

[0147] **Pharmaceutically acceptable**: As used herein, the term "pharmaceutically acceptable" applied to the carrier, diluent, or excipient used to formulate a composition as disclosed herein means that the carrier, diluent, or excipient must be compatible with the other ingredients of the composition and not deleterious to the recipient thereof.

[0148] **Polysaccharide**: The term "polysaccharide" as used herein refers to a polymeric carbohydrate molecule composed of long chains of monosaccharide units bound together by glycosidic, phosphodiester, or other linkages and on hydrolysis give the constituent monosaccharides or oligosaccharides. Polysaccharides range in structure from linear to highly branched. Examples include storage polysaccharides such as starch and glycogen, structural polysaccharides such as cellulose and chitin and microbial polysaccharides, and antigenic polysaccharides found in microorganisms including, but not limited to, capsular polysaccharides (CPS), O polysaccharides (OPS), core O polysaccharides (COPS), and lipopolysaccharides (LPS).

[0149] **Polypeptide**: The term "polypeptide", as used herein, generally has its art-recognized meaning of a polymer of at least three amino acids, *e.g*, linked to each other by peptide bonds. Those of ordinary skill in the art will appreciate that the term "polypeptide" is intended to be sufficiently general as to encompass not only polypeptides having a complete sequence recited herein, but also to encompass polypeptides that represent functional fragments (*i.e.*, fragments retaining at least one activity) of such complete polypeptides. Moreover, those of ordinary skill in the art understand that protein sequences generally tolerate some substitution

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without destroying activity. Thus, any polypeptide that retains activity and shares at least about 30-40% overall sequence identity, often greater than about 50%, 60%, 70%, or 80%, and further usually including at least one region of much higher identity, often greater than 90% or even 95%, 96%, 97%, 98%, or 99% in one or more highly conserved regions, usually encompassing at least 3-4 and often up to 20 or more amino acids, with another polypeptide of the same class, is encompassed within the relevant term "polypeptide" as used herein. Polypeptides may contain L-amino acids, D-amino acids, or both and may contain any of a variety of amino acid modifications or analogs known in the art. Useful modifications include, *e.g.*, terminal acetylation, amidation, methylation, etc. In some embodiments, proteins may comprise natural amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof.

[0150] **Prevention**: The term "prevent" or "prevention", as used herein in connection with a disease, disorder, and/or medical condition, refers to reducing the risk of developing the disease, disorder and/or condition, and/or a delay of onset, and/or reduction in frequency and/or severity of one or more characteristics or symptoms of a particular disease, disorder or condition. In some embodiments, prevention is assessed on a population basis such that an agent is considered to "prevent" a particular disease, disorder or condition if a statistically significant decrease in the development, frequency, and/or intensity of one or more symptoms of the disease, disorder or condition is observed in a population susceptible to the disease, disorder, or condition. In some embodiments, prevention may be considered complete when onset of a disease, disorder or condition has been delayed for a pre-defined period of time.

[0151] **Protein**: As used herein, the term "protein" encompasses a polypeptide. Proteins may include moieties other than amino acids (*e.g.*, may be glycoproteins, proteoglycans, etc.) and/or may be otherwise processed or modified. Those of ordinary skill in the art will appreciate that a "protein" can be a complete polypeptide chain as produced by a cell (with or without a signal sequence), or can be a characteristic portion thereof. Those of ordinary skill will appreciate that a protein can sometimes include more than one polypeptide chain, for example linked by one or more disulfide bonds or associated by other means. Polypeptides may contain l-amino acids, d-amino acids, or both and may contain any of a variety of amino acid modifications or analogs known in the art. Useful modifications include, *e.g.*, terminal acetylation, amidation, methylation, etc. In some embodiments, proteins may comprise natural

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amino acids, non-natural amino acids, synthetic amino acids, and combinations thereof. The term "peptide" is generally used to refer to a polypeptide having a length of less than about 100 amino acids, less than about 50 amino acids, less than 20 amino acids, or less than 10 amino acids. In some embodiments, proteins are antibodies, antibody fragments, biologically active portions thereof, and/or characteristic portions thereof.

[0152] **Recombinant**: As used herein, the term "recombinant" is intended to refer to polypeptides that are designed, engineered, prepared, expressed, created, manufactured, and/or isolated by recombinant means, such as polypeptides expressed using a recombinant expression vector transfected into a host cell; polypeptides isolated from a recombinant, combinatorial human polypeptide library; polypeptides isolated from an animal (*e.g.*, a mouse, rabbit, sheep, fish, etc.) that is transgenic for or otherwise has been manipulated to express a gene or genes, or gene components that encode and/or direct expression of the polypeptide or one or more component(s), portion(s), element(s), or domain(s) thereof; and/or polypeptides prepared, expressed, created or isolated by any other means that involves splicing or ligating selected nucleic acid sequence elements to one another, chemically synthesizing selected sequence elements, and/or otherwise generating a nucleic acid that encodes and/or directs expression of the polypeptide or one or more component(s), portion(s), element(s), or domain(s) thereof. In some embodiments, one or more of such selected sequence elements is found in nature. In some embodiments, one or more of such selected sequence elements is designed in silico. In some embodiments, one or more such selected sequence elements results from mutagenesis (*e.g.*, *in vivo* or *in vitro*) of a known sequence element, *e.g.*, from a natural or synthetic source such as, for example, in the germline of a source organism of interest (*e.g.*, of a human, a mouse, etc.).

[0153] **Reference**: As used herein, the term "reference" describes a standard or control relative to which a comparison is performed. For example, in some embodiments, an agent, animal, subject, population, sample, sequence or value of interest is compared with a reference or control agent, animal, subject, population, sample, sequence or value. In some embodiments, a reference or control is tested and/or determined substantially simultaneously with the testing or determination of interest. In some embodiments, a reference or control is a historical reference or control, optionally embodied in a tangible medium. Typically, as would be understood by those skilled in the art, a reference or control is determined or characterized under comparable

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conditions or circumstances to those under assessment. Those skilled in the art will appreciate when sufficient similarities are present to justify reliance on and/or comparison to a particular possible reference or control.

[0154] **Response:** As used herein, a "response" to treatment may refer to any beneficial alteration in a subject's condition that occurs as a result of or correlates with treatment. Such alteration may include stabilization of the condition (*e.g.*, prevention of deterioration that would have taken place in the absence of the treatment), amelioration of symptoms of the condition, and/or improvement in the prospects for cure of the condition, etc. It may refer to a subject's response or to a tumor's response. Subject or tumor response may be measured according to a wide variety of criteria, including clinical criteria and objective criteria. Techniques for assessing response include, but are not limited to, clinical examination, positron emission tomography, chest X-ray CT scan, MRI, ultrasound, endoscopy, laparoscopy, presence or level of biomarkers in a sample obtained from a subject, cytology, and/or histology. The exact response criteria can be selected in any appropriate manner, provided that when comparing groups of subjects and/or tumors, the groups to be compared are assessed based on the same or comparable criteria for determining response rate. One of ordinary skill in the art will be able to select appropriate criteria.

[0155] **Risk**: As will be understood from context, "risk" of a disease, disorder, and/or condition refers to a likelihood that a particular subject will develop the disease, disorder, and/or condition. In some embodiments, risk is expressed as a percentage. In some embodiments, risk is from 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90 up to 100%. In some embodiments risk is expressed as a risk relative to a risk associated with a reference sample or group of reference samples. In some embodiments, a reference sample or group of reference samples have a known risk of a disease, disorder, condition and/or event. In some embodiments a reference sample or group of reference samples are from subjects comparable to a particular subject. In some embodiments, relative risk is 0,1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more.

[0156] **Serotype**: As used herein, the term "serotype", also referred to as a serovar, refers to a distinct variation within a species of bacteria or virus or among immune cells of different subjects. These microorganisms, viruses, or cells are classified together based on their cell surface antigens, allowing the epidemiologic classification of organisms to the sub-species

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level. A group of serovars with common antigens may be referred to as a serogroup or sometimes serocomplex.

[0157] **Subject:** As used herein, the term "subject" refers an organism, typically a mammal (*e.g.*, a human, in some embodiments including prenatal human forms). In some embodiments, a subject is suffering from a relevant disease, disorder or condition. In some embodiments, a subject is susceptible to a disease, disorder, or condition. In some embodiments, a subject displays one or more symptoms or characteristics of a disease, disorder or condition. In some embodiments, a subject does not display any symptom or characteristic of a disease, disorder, or condition. In some embodiments, a subject is someone with one or more features characteristic of susceptibility to or risk of a disease, disorder, or condition. In some embodiments, a subject is a patient. In some embodiments, a subject is an subject to whom diagnosis and/or therapy is and/or has been administered.

[0158] **Superior:** As used herein, the term "superior" in the context of evaluating a test pharmaceutical composition refers to a test pharmaceutical composition that performs (*e.g.*, in terms of immunogenicity and/or functional antibody titer generated by the test composition) better than a reference composition. In some embodiments, superiority is demonstrated when the upper bound of the 95% confidence interval (CI) for the geometric mean titer (GMT) ratio of a test pharmaceutical composition over a reference composition is at least 1.3 or above, including, *e.g.*, at least 1.4, at least 1.5, at least 2, at least 2.5, at least 3, at least 4, or higher. For example, in some embodiments, an immunogenic composition or vaccine described herein is superior to a reference vaccine (*e.g.*, PCV13 or PPSV23) when the upper bound of the 95% confidence interval (CI) for the geometric mean titer (GMT) ratio of the immunogenic composition or vaccine over the reference vaccine is at least 1.3 or above, including, *e.g.*, at least 1.4, at least 1.5, at least 2, at least 2.5, at least 3, at least 4, or higher. In some embodiments, an immunogenic composition or vaccine described herein is superior to a reference vaccine (*e.g.*, PCV13 or PPSV23) when the two-sided 95% confidence interval (CI) for the geometric mean titer (GMT) ratio of the immunogenic composition or vaccine over the reference vaccine excludes zero.

[0159] **Susceptible to**: A subject who is "susceptible to" a disease, disorder, or condition is at risk for developing the disease, disorder, or condition. In some embodiments, a subject who

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is susceptible to a disease, disorder, or condition does not display any symptoms of the disease, disorder, or condition. In some embodiments, a subject who is susceptible to a disease, disorder, or condition has not been diagnosed with the disease, disorder, and/or condition. In some embodiments, a subject who is susceptible to a disease, disorder, or condition is a subject who has been exposed to conditions associated with development of the disease, disorder, or condition. In some embodiments, a risk of developing a disease, disorder, and/or condition is a population-based risk (*e.g.*, family members of subjects suffering from the disease, disorder, or condition).

[0160] **Symptoms are reduced**: As used herein, "symptoms are reduced" when one or more symptoms of a particular disease, disorder or condition is reduced in magnitude (*e.g.*, intensity, severity, etc.) and/or frequency, *e.g.*, to a statistically and/or clinically significant or relevant level. For purposes of clarity, a delay in the onset of a particular symptom is considered one form of reducing the frequency of that symptom.

[0161] **Treatment**: As used herein, the term "treatment" (also "treat" or "treating") refers to any administration of a therapy that partially or completely alleviates, ameliorates, relieves, inhibits, delays onset of, reduces severity of, and/or reduces incidence of one or more symptoms, features, and/or causes of a particular disease, disorder, and/or condition. In some embodiments, such treatment may be of a subject who does not exhibit signs of the relevant disease, disorder and/or condition and/or of a subject who exhibits only early signs of the disease, disorder, and/or condition. Alternatively or additionally, such treatment may be of a subject who exhibits one or more established signs of the relevant disease, disorder and/or condition. In some embodiments, treatment may be of a subject who has been diagnosed as suffering from the relevant disease, disorder, and/or condition. In some embodiments, treatment may be of a subject known to have one or more susceptibility factors that are statistically correlated with increased risk of development of the relevant disease, disorder, and/or condition.

[0162] **Vaccination**: As used herein, the term "vaccination" refers to the administration of a composition intended to generate an immune response, for example to a disease-causing agent. For the purposes of the present invention, vaccination can be administered before, during, and/or after exposure to a disease-causing agent, and in certain embodiments, before, during, and/or shortly after exposure to the agent. In some embodiments, vaccination includes multiple

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administrations, appropriately spaced in time, of a vaccinating composition. In some embodiments, vaccination initiates immunization.

Detailed Description

[0163] The present disclosure relates, generally, to compositions, systems, and methods that include novel complexed proteins and polysaccharides, *e.g.*, vaccines of complexed proteins and polysaccharides. Such complexes can be used, *e.g.*, to induce and/or increase an immunoprotective response in subjects at risk of or suffering from pneumococcal infection.

[0164] Previous attempts at preparing a vaccine to protect against pneumococcal infection have had limited efficacy.

[0165] Wyeth's first polyvalent polysaccharide conjugate vaccine, Prevnar 7 (PCV7), was licensed in 2000 in the United States and Europe. GlaxoSmithKline's 10-valent polysaccharide conjugate vaccine Synflorix (PCV10) was licensed in Europe, but not the United States, in 2009. Synflorix was not licensed in the United States because clinical trials did not demonstrate non-inferiority in infants compared to Prevnar 7; also, despite coverage of three additional serotypes compared to Prevnar 7, Synflorix did not formally have coverage of the clinically important *S. pneumoniae* serotype 19A. Also, Synflorix had originally been designed as an 11-valent polysaccharide conjugate vaccine (PCV11), but one *S. pneumoniae* serotype (serotype 3) was removed when clinical trials suggested no efficacy against this serotype, despite immunogenicity.

[0166] Merck's polyvalent polysaccharide conjugate vaccine PCV-15, is still in clinical trials. PCV15 has undergone clinical evaluation in adults, where it appears to be non-inferior to Prevnar 13 in adults. PCV15 also appeared to be non-inferior in toddlers, but then failed to meet non-inferiority in infants, so was returned to formulation. The newly-formulated PCV15 is now being evaluated in adults and infants.

[0167] A first protein vaccine based on PspA was evaluated by Sanofi Pasteur. An early clinical trial (published in 2000, PMID: 10699322) was a Phase 1 trial with 5-125 μg recombinant PspA (rPspA), which was associated with a clear rise in antibody levels, antibodies that were reactive with heterologous rPspA molecules, and increased binding of post-immune

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sera to 37 pneumococcal strains expressing a variety of PspA clades and capsular serotypes. Some concerns were raised over a region of PspA that has homology with cardiac myosin, and the program was terminated by Sanofi Pasteur.

[0168] Another protein vaccine based on a combination of the three proteins, PhtD, pneumolysoid, and PcpA, was evaluated by Sanofi Pasteur from 2011 to 2014. The vaccine passed Phase 1 in adults and was then tested in toddlers (12-13 mo) and infants (6 weeks). A study in Bangladesh showed immunogenicity; however, no evidence of impact on *S. pneumoniae* carriage was reported (presented at ISPPD 2014; the study was not powered to detect a small difference in prevalence of carriage). The program was terminated by Sanofi Pasteur.

[0169] A protein vaccine based on the combination of PhtD and pneumolysoid was evaluated by GlaxoSmithKline from 2012 to 2016. The vaccine was deemed safe and immunogenic in all ages (adults, toddlers, infants). However, in two further infant trials conducted in The Gambia and the Navajo Nation in the United States, there was no evidence of clinical efficacy (no impact on *S. pneumoniae* carriage in The Gambia, or on otitis media in the Navajo Nation). The program was terminated by GlaxoSmithKline.

[0170] A protein vaccine GEN-004, based on a combination of three proteins, SP0148, SP1912 and SP2108 adjuvanted with aluminum hydroxide, was developed and evaluated by Genocea Biosciences. GEN-004 was shown to be safe and immunogenic in adults in Phase 1. However, a Phase 2 trial of intentional pneumococcal challenge, conducted in the UK, showed no significant effect on *S. pneumoniae* carriage. The GEN-004 development program was suspended in 2015.

[0171] A live attenuated, engineered *Salmonella* vaccine was developed at Arizona State University. Mutant *Salmonella typhi* strains engineered to express PspA were evaluated in a Phase 1 clinical trial in adults (PMID: 23916987) from 2009 to 2011. Three different strains were evaluated. No significant adverse events were reported, but one patient had a positive blood culture for *Salmonella typhi*. No subjects shed vaccine in stool. There were no differences in pre- or post-vaccination ELISA or ELISPOT results between groups. The vaccine was deemed safe but non-immunogenic, therefore not efficacious, and development was stopped.

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[0172] There are two *S. pneumoniae* vaccines currently available in the U.S. Wyeth-Pfizer's second polyvalent polysaccharide conjugate vaccine, Prevnar 13 (PCV13), was licensed in 2010 in the U.S. and Europe. PCV13 has been approved for the prevention of IPD caused by the 13 serotypes contained in the vaccine in children and for the prevention of pneumonia and IPD in adults. In this vaccine, covalent conjugation of saccharides from 13 pneumococcal serotypes to a diphtheria toxoid mutant (CRM197) protein creates saccharide-protein conjugates, which are capable of inducing a T cell-dependent immune response against one or more of the 13 pneumococcal serotypes represented by the saccharides. [PREVNAR 13 prescribing information, 2017].

[0173] PCV13 did not formally meet non-inferiority criteria compared to PCV7 and was not immunogenic against several serotypes. However, the inclusion of *S. pneumoniae* serotype 19A assisted with licensure of PCV13, since serotype 19A had emerged between 2000-2010 as a major cause of morbidity and mortality and was associated with antibiotic resistance. While infections with *S. pneumoniae* of multidrug-resistant serotypes contained in PCV13 appeared to decrease after approval of this vaccine, an increase of infections with multidrug-resistant serotypes 35B, 23A, 23B and 15B, which are just a few of the close to 100 known serotypes of *S. pneumoniae* not included in PCV13, was noted. Also, PCV13 was reported to have marginal activity against serotype 3, as its prevalence persists in the population [Richter et al, 2014].

[0174] The second vaccine, PPSV23, is a 23-valent polysaccharide vaccine developed by Merck, and is indicated for the prevention of pneumococcal disease in adults greater than 50 years of age, or in persons greater than 2 years of age at increased risk of pneumococcal disease. It is composed of purified capsular polysaccharides from 23 pneumococcal serotypes. While this vaccine has the potential to protect against more serotypes when compared to PCV13, it does not provide protection against the emerging serotypes 35B, 23A and 23B. In addition, PPSV23 elicits a T cell-independent polysaccharide immune response that stimulates mature B-lymphocytes, but not T-lymphocytes. Thus, this vaccine induces an immune response that is neither long-lasting nor anamnestic upon subsequent challenge. PPSV23 is not effective against colonization. In addition, polysaccharide-type vaccines are not used in infants and children less than 2 years of age, because these children respond poorly to T cell-independent antigens [PNEUMOVAX 23 prescribing information, 2017; CDC, 2010]. Data suggest that PPSV23 may

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protect adults and the elderly against IPD; however, no consistent effect has been observed in the prevention of pneumonia [Gruber et al, 2008].

[0175] The presently disclosed novel complexed proteins and polysaccharides, *e.g.*, vaccines of complexed proteins and polysaccharides, and combinations thereof represent a substantial advance over the previous attempts at *S. pneumoniae* vaccine development and over the currently available options for immunizing patients against pneumococcal infection. Such novel complexes and vaccines can be used, *e.g.*, to induce and/or increase an immunoprotective response in subjects, such as those at risk of or suffering from pneumococcal infection.

Immunogenic Complexes

[0176] The present disclosure encompasses immunogenic complexes that include one or more polysaccharides and/or polypeptides of *S. pneumoniae*.

[0177] In some embodiments, immunogenic complexes are, or are based on, Multiple Antigen Presenting System (MAPS) complexes. Aspects of the MAPS platform have been previously described in WO2012/155007, the contents of which are herein incorporated by reference in their entirety, and are shown schematically in Figure 1. See also Zhang et al, 2013.

[0178] As described herein, immunogenic complexes of the disclosure include one or more antigenic polypeptides non-covalently complexed with one or more antigenic polysaccharides. In some embodiments, one or more antigenic polypeptides are complexed via affinity interaction with one or more antigenic polysaccharides. In some embodiments, immunogenic complexes of the disclosure include one or more antigenic polypeptides non-covalently complexed with one or more antigenic polysaccharides using one or more affinity molecule/complementary affinity molecule pairs. In some embodiments, an immunogenic complex includes (i) a first affinity molecule described herein conjugated to one or more antigenic polysaccharides, and (ii) a fusion protein that is or comprises a complementary affinity molecule described herein and a polypeptide. In some embodiments, an immunogenic complex includes (i) a plurality of a first affinity molecule described herein conjugated to one or more antigenic polysaccharides, and (ii) a fusion protein that is or comprises a complementary affinity molecule described herein and a polypeptide. Upon association of the first affinity molecule and

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the complementary affinity molecule, the one or more antigenic polypeptides are non-covalently complexed to the one or more antigenic polysaccharides.

[0179] In some embodiments, one or more antigenic polypeptides are complexed via affinity interaction with one antigenic polysaccharide. In some embodiments, immunogenic complexes of the disclosure include one or more antigenic polypeptides non-covalently complexed with one antigenic polysaccharide using one affinity molecule/complementary affinity molecule pair. In some embodiments, immunogenic complexes of the disclosure include one or more antigenic polypeptides non-covalently complexed with one antigenic polysaccharide using one or more affinity molecule/complementary affinity molecule pairs. In some embodiments, each of the affinity molecule/complementary affinity molecule pairs is the same, *e.g.*, biotin/biotin-binding moiety pairs. In some embodiments, an immunogenic complex includes (i) a first affinity molecule described herein conjugated to one antigenic polysaccharide, and (ii) a fusion protein that is or comprises a complementary affinity molecule described herein and a polypeptide. In some embodiments, an immunogenic complex includes (i) a plurality of a first affinity molecule described herein conjugated to one antigenic polysaccharide, and (ii) a fusion protein that is or comprises a complementary affinity molecule described herein and a polypeptide. Upon association of the first affinity molecule and the complementary affinity molecule, the one or more antigenic polypeptides are non-covalently complexed to the one antigenic polysaccharide.

[0180] In some embodiments, the affinity molecule/complementary affinity molecule pair is selected from one or more of biotin/biotin-binding moiety, antibody/antigen, enzyme/substrate, receptor/ligand, metal/metal-binding protein, carbohydrate/carbohydrate binding protein, lipid/lipid-binding protein, and His tag/His tag-binding molecule.

[0181] In some embodiments, the first affinity molecule is biotin (or a derivative or fragment thereof), and the complementary affinity molecule is a moiety, *e.g.*, a biotin-binding protein, or a biotin-binding domain or biotin-binding fragment thereof. In some embodiments, the biotin-binding moiety is rhizavidin, avidin, streptavidin, bradavidin, tamavidin, lentiavidin, zebavidin, NeutrAvidin, CaptAvidin™, or a biotin-binding domain or biotin-binding fragment thereof, or a combination thereof. In some embodiments, the biotin-binding moiety is rhizavidin, or a biotin-binding domain or biotin-binding fragment thereof. In some embodiments, the biotin-

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binding moiety is or comprises a polypeptide of SEQ ID NO:1, or a biotin-binding domain or biotin-binding fragment thereof. In some embodiments, the biotin-binding moiety is or comprises a polypeptide of SEQ ID NO:2, or a biotin-binding domain or biotin-binding fragment thereof.

[0182] In some embodiments, the one or more antigenic polysaccharides are, or are derived from Gram-negative bacteria and/or Gram-positive bacteria. In some embodiments, one or more bacterial antigenic polysaccharides are, or are derived from *S. pneumoniae*. In some embodiments, one or more antigenic polysaccharides are, or are derived from one or more pathogens. In some embodiments, one or more antigenic polysaccharides are, or are derived from 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 serotypes or strains of a pathogen. In some embodiments, one or more antigenic polysaccharides are, or are derived from more than 25 serotypes or strains of a pathogen, *e.g.*, 26, 27, 28, 29, 30, 35, 40, 45, or 50 serotypes or strains. In some embodiments, one or more antigenic polysaccharides are, or are derived from more than 60, 70, 80, 90, or 100 serotypes or strains of a pathogen.

[0183] In some embodiments, the one or more antigenic polysaccharides comprise one or more affinity molecules conjugated to the antigenic polysaccharides. In some embodiments, the one or more affinity molecules comprise biotin or biotin derivatives.

[0184] In some embodiments, the antigenic polysaccharides comprise a plurality of affinity molecules conjugated to the antigenic polysaccharides. In some embodiments, the affinity molecules comprise biotin or biotin derivatives.

[0185] In some embodiments, one or more antigenic polypeptides are covalently linked (*e.g.*, fused) to a complementary affinity molecule described herein. In some embodiments a fusion protein comprises one or more antigenic polypeptides and a complementary affinity molecule disclosed herein. In some embodiments, the complementary affinity molecule is or comprises a biotin-binding moiety. In some embodiments, the biotin-binding moiety comprises rhizavidin or a biotin-binding portion thereof.

[0186] In some embodiments, antigenic polysaccharides and/or antigenic polypeptides that may be included in immunogenic complexes are recombinantly or synthetically produced.

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In some embodiments, antigenic polysaccharides and/or antigenic polypeptides that may be included in immunogenic complexes are isolated and/or derived from natural sources. In some embodiments antigenic polysaccharides and/or antigenic polypeptides that may be included in immunogenic complexes are isolated from bacterial cells. Exemplary polysaccharides and/or polypeptides are described below.

Antigenic Polypeptides

[0187] In some embodiments, an immunogenic complex described herein comprises one or more polypeptide antigens. In some embodiments, a polypeptide antigen is a bacterial polypeptide, a fungal polypeptide, and/or a viral polypeptide. In some embodiments, a polypeptide antigen is a polypeptide of, or derived from *S. pneumoniae*. In some embodiments, the one or more polypeptide antigen is a polypeptide of, or derived from, a pathogen other than *S. pneumoniae*. In some embodiments, the one or more polypeptide antigens comprise (i) a polypeptide of, or derived from *S. pneumoniae*, and (ii) a polypeptide of, or derived from, a pathogen other than *S. pneumoniae*. In some embodiments, an immunogenic complex includes one or more of the following *S. pneumoniae* antigenic polypeptides, or portions thereof.

SP0785 Polypeptides

[0188] SP0785 is a conserved hypothetical *S. pneumoniae* protein described in WO 2014/124228. In some embodiments, an SP0785 polypeptide is an efflux transporter protein conserved across *S. pneumoniae* strains. In some embodiments, an SP0785 polypeptide is or comprises a full-length SP0785 polypeptide. For example, in some embodiments, a full-length SP0785 polypeptide has 399 amino acids (38 kDa) and is represented by the amino acid sequence as set forth in SEQ ID NO: 10. Amino acids 1-32 of SEQ ID NO:10 are predicted to be a signal sequence and transmembrane domain of an SP0785 polypeptide (amino acids 1-32 of the full-length protein). In some embodiments, an SP0785 polypeptide includes a portion of an SP0785 polypeptide (*e.g.*, a portion of the SP0785 polypeptide of SEQ ID NO:10, which portion includes at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, or more contiguous amino acids of SEQ ID NO:10). In some embodiments, a portion of an SP0785 polypeptide corresponds to a protein having amino acids 33-399 of the amino acid sequence set forth in SEQ ID NO: 10. In some

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embodiments, an SP0785 polypeptide contains one or more amino acid alterations (*e.g.*, deletion, substitution, and/or insertion) from a naturally-occurring wild-type SP0785 polypeptide sequence. For example, an SP0785 polypeptide may contain an amino acid sequence that is at least 60% or more (*e.g.*, at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 98%) identical to SEQ ID NO:10 or a portion thereof (*e.g.*, at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 50, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, or more consecutive amino acids of the sequence shown in SEQ ID NO:10). Alternatively, an SP0785 polypeptide may contain a portion (*e.g.*, at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 50, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, 300, 350, or 400 consecutive amino acids) of a sequence that is at least 60% or more (*e.g.*, at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 98%) identical to SEQ ID NO:10. A nucleotide sequence encoding an SP0785 polypeptide is provided herein as SEQ ID NO:11.

SP1500 Polypeptides

[0189] SP1500 is a *S. pneumoniae* protein described in WO 2014/124228. In some embodiments, an SP1500 polypeptide is an Amino Acid ABC Transporter, amino acid-binding polypeptide conserved across *S. pneumoniae* strains. In some embodiments, an SP1500 polypeptide is or comprises a full-length SP1500 polypeptide. For example, in some embodiments, a full-length SP1500 polypeptide has 278 amino acids (28 kDa) and is represented by the amino acid sequence as set forth in SEQ ID NO: 12. Amino acids 1-26 of SEQ ID NO:12 are predicted to be a signal sequence of an SP1500 polypeptide (amino acids 1-26 of the full-length protein). In some embodiments, an SP1500 polypeptide includes a portion of an SP1500 polypeptide (*e.g.*, a portion of the SP1500 polypeptide of SEQ ID NO:12, which portion includes at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, or more contiguous amino acids of SEQ ID NO:12). In some embodiments, a portion of an SP1500 polypeptide corresponds to a protein having amino acids 27-278 of the amino acid sequence set forth in SEQ ID NO: 12. In some embodiments, an SP1500 polypeptide contains one or more amino acid alterations (*e.g.*, deletion, substitution, and/or insertion) from a naturally-occurring wild-type SP1500 polypeptide sequence. For example, an SP1500 polypeptide may contain an amino acid sequence that is at least 60% or more (*e.g.*, at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 98%) identical to SEQ ID NO:12

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or a portion thereof (*e.g.*, at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 50, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, or more consecutive amino acids of the sequence shown in SEQ ID NO:12). Alternatively, an SP1500 polypeptide may contain a portion (*e.g.*, at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 45, 50, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250, or more consecutive amino acids) of a sequence that is at least 60% or more (*e.g.*, at least 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 98%) identical to SEQ ID NO:12. A nucleotide sequence encoding an SP1500 polypeptide is provided herein as SEQ ID NO:13.

[0190] In some embodiments, nucleic acid sequences encoding an SP0785 polypeptide (SEQ ID NO:10), an SP1500 polypeptide (SEQ ID NO:12), and a CP1 polypeptide (SEQ ID NO:6) are provided as SEQ ID NOS: 11, 13, and 9, respectively. Due to degeneracy in the genetic code, those of ordinary skill in the art would understand that other DNA sequences (including codon-optimized sequences) could encode these polypeptides, as well as the others disclosed herein.

Fusion Proteins that Include Antigenic Polypeptides

[0191] Antigenic polypeptides described herein can be part of a fusion protein. For example, in some embodiments, an immunogenic complex described herein comprises a fusion protein that is or comprises a complementary affinity molecule and one or more antigenic polypeptides described herein. In some embodiments, a fusion protein of the immunogenic complex has carrier properties. In some embodiments, a fusion protein of the immunogenic complex has antigenic properties. In some embodiments, a fusion protein of the immunogenic complex has carrier properties and antigenic properties.

[0192] In some embodiments, the fusion protein of the immunogenic complex comprises one or more linkers and/or tags, *e.g.*, a histidine tag. In some embodiments, the linker comprises a polypeptide comprising an amino acid sequence of SEQ ID NO:3 (GGGGSSS). In some embodiments, the linker comprises a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:3. In some embodiments, the linker comprises the amino acid sequence AAA. In some embodiments, the fusion protein of the immunogenic complex comprises a first linker

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comprising a polypeptide comprising the amino acid sequence of SEQ ID NO:3 (GGGGSSS), and a second linker comprising the amino acid sequence AAA. In some embodiments, such a linker may be synthesized, or derived from amino acid residues from a restriction site (*e.g.*, a Not I restriction site).

Complementary Affinity Molecules

[0193] In some embodiments, a complementary affinity molecule comprises a biotin-binding moiety. In some embodiments, a fusion protein of the immunogenic complex comprises a biotin-binding moiety, and one or more polypeptide antigens. In some embodiments, a fusion protein comprises a biotin-binding moiety and two or more polypeptide antigens. As used herein, a "biotin-binding moiety" refers to a biotin-binding protein, a biotin-binding fragment thereof, or a biotin-binding domain thereof.

[0194] In some embodiments, MAPS complexes disclosed herein utilize the high affinity (dissociation constant [KD] $\approx 10^{-15}$M) non-covalent binding between biotin and rhizavidin, a biotin-binding protein that has no significant predicted homology with human proteins. Rhizavidin, a naturally occurring dimeric protein in the avidin protein family, was first discovered in *Rhizobium etli*, a symbiotic bacterium of the common bean. Rhizavidin has only a 22% amino acid identity with chicken avidin, a protein commonly found in eggs, but with high conservation of amino acid residues involved in biotin binding. No cross-reactivity to rhizavidin is observed in human serum samples obtained from subjects exposed to avidin [Helppolainen et al, 2007], suggesting that rhizavidin antibodies may not cross-react with chicken avidin. Biotin conjugates have been used in several clinical applications without any reported adverse events [Buller et al, 2014; Paty et al, 2010; Lazzeri et al, 2004].

[0195] In some embodiments, the biotin-binding moiety of the fusion protein comprises rhizavidin or a biotin-binding domain or biotin-binding fragment thereof, as further described in WO 2012/155053 the contents of which are herein incorporated by reference in their entirety. In some embodiments, a biotin-binding moiety is or comprises a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to rhizavidin, or a biotin-binding domain or biotin-binding fragment thereof. In some embodiments, the biotin-binding moiety comprises a

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polypeptide of SEQ ID NO:1 or a biotin-binding domain or biotin-binding fragment thereof. In some embodiments, the biotin-binding moiety is or comprises a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:1, or biotin-binding domain or biotin-binding fragment thereof. In some embodiments, the biotin-binding moiety comprises a polypeptide of SEQ ID NO:2 or a biotin-binding domain or biotin-binding fragment thereof. In some embodiments, the biotin-binding moiety is or comprises a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2, or biotin-binding domain or biotin-binding fragment thereof.

[0196] In some embodiments, the fusion protein is or comprises a complementary affinity molecule described herein (*e.g.*, a biotin-binding moiety described herein), and one or more polypeptides of or derived from *S. pneumoniae*. In some embodiments, the fusion protein of the immunogenic complex is CP1, further described in U.S. Provisional Application No. 62/730,199, entitled "Pneumococcal Fusion Protein Vaccines" and filed September 12, 2018 ("'199 application") as well as in a PCT Application entitled "Pneumococcal Fusion Protein Vaccines" and filed September 12, 2019, which claims priority to the '199 application, the contents of each of which are incorporated herein by reference in their entirety. In some embodiments, the fusion protein comprises a complementary affinity molecule described herein (*e.g.*, a biotin-binding moiety described herein) and a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:4 (amino acids 33-399 of *S. pneumoniae* SP0785) or an antigenic fragment thereof and a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:5 (amino acids 27-278 of *S. pneumoniae* SP1500) or an antigenic fragment thereof. In some embodiments, the fusion protein comprises a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO: 6. In some embodiments, the fusion protein is or comprises a complementary affinity molecule described herein (*e.g.*, a biotin-binding moiety described herein), a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SP0785 or an antigenic fragment thereof, and a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SP1500 or an antigenic fragment thereof.

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[0197] In some embodiments, the fusion protein of the immunogenic complex comprises a complementary affinity molecule described herein (*e.g.*, a biotin-binding moiety described herein) and a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:4 (amino acids 33-399 of *S. pneumoniae* SP0785) or an antigenic fragment thereof. In some embodiments, a fusion protein comprises a complementary affinity molecule described herein (*e.g.*, a biotin-binding moiety described herein) and a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:5 (amino acids 27-278 of *S. pneumoniae* SP1500) or an antigenic fragment thereof.

[0198] In some embodiments, the fusion protein of the immunogenic complex comprises a biotin-binding moiety that is or comprises a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:1 (rhizavidin), or biotin-binding fragment thereof. In some embodiments, the fusion protein comprises a biotin-binding moiety that is or comprises a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 (amino acids 45-179 of rhizavidin), or biotin-binding fragment thereof. In some embodiments, the fusion protein comprises a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:4 (amino acids 33-399 of *S. pneumoniae* SP0785) or an antigenic fragment thereof. In some embodiments, the fusion protein comprises a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:5 (amino acids 27-278 of *S. pneumoniae* SP1500) or an antigenic fragment thereof.

[0199] In some embodiments, the fusion protein of the immunogenic complex comprises each of: (a) a biotin-binding moiety that is or comprises a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:1 (rhizavidin), or biotin-binding fragment thereof; (b) a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:4 (amino acids 33-399 of *S. pneumoniae* SP0785) or an antigenic fragment thereof; and (c) a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:5 (amino acids 27-278 of *S.*

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pneumoniae SP1500) or an antigenic fragment thereof. In some embodiments, the fusion protein further comprises one or more linkers.

[0200] In some embodiments, the fusion protein of the immunogenic complex comprises each of: (a) a biotin-binding moiety that is or comprises a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:2 (amino acids 45-179 of rhizavidin), or biotin-binding fragment thereof; (b) a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:4 (amino acids 33-399 of *S. pneumoniae* SP0785) or an antigenic fragment thereof; and (c) a polypeptide comprising an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:5 (amino acids 27-278 of *S. pneumoniae* SP1500) or an antigenic fragment thereof. In some embodiments, the fusion protein further comprises one or more linkers.

[0201] In some embodiments, the fusion protein of the immunogenic complex is or comprises a CP1 fusion protein. In some embodiments, a CP1 fusion protein comprises (a) a biotin-binding moiety that is or comprises a polypeptide having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to the sequence of SEQ ID NO:1 (rhizavidin) or to the sequence of SEQ ID NO:2 (amino acids 45-179 of rhizavidin), or biotin-binding fragment thereof; (b) a first polypeptide linker comprising the amino acid sequence of SEQ ID NO:3 (GGGGSSS); (c) an SP0785 polypeptide described herein; (d) a second polypeptide linker comprising the amino acid sequence AAA; and (e) an SP1500 polypeptide described herein. In some embodiments, such a CP1 fusion protein may further comprise a detection or purification tag, *e.g.*, a His tag. In some such embodiments, a CP1 fusion protein comprises an SP1500 polypeptide between a biotin-binding moiety and an SP0785 polypeptide. In some embodiments, a CP1 fusion protein may comprise an SP0785 polypeptide between a biotin-binding moiety and a SP1500 polypeptide. In some embodiments, an SP0785 polypeptide included in a fusion protein of an immunogenic complex is or comprises an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:4 (amino acids 33-399 of *S. pneumoniae* SP0785) or an antigenic fragment thereof. In some embodiments, an SP1500 polypeptide included in a fusion protein of an immunogenic complex is or comprises an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%,

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97%, 98%, 99%, or 100% identity to the sequence of SEQ ID NO:5 (amino acids 27-278 of *S. pneumoniae* SP1500) or an antigenic fragment thereof. In some embodiments, the fusion protein comprises an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to the sequence SEQ ID NO:6. In some embodiments, the fusion protein comprises the amino acid sequence SEQ ID NO:6. In some embodiments, the fusion protein consists of the amino acid sequence SEQ ID NO:6.

Antigenic Polysaccharides

[0202] In some embodiments, an immunogenic complex described herein includes one or more *S. pneumoniae* polysaccharides. In some embodiments, an immunogenic complex described herein includes one *S. pneumoniae* polysaccharide. Capsular polysaccharides are used to distinguish serotypes of *S. pneumoniae*. There are at least 97 distinct serotypes of *S. pneumoniae* polysaccharides, each having a different chemical structure. Serotype designations as used herein are designations according to Danish nomenclature [Kauffmann et al, 1960; Geno et al, 2015]. (N.B. Serotype 20 in U.S. nomenclature is referred to as serotype 20A in Danish nomenclature.)

[0203] In some embodiments, an immunogenic complex includes one or more *S. pneumoniae* capsular polysaccharides from, or derived from, one or more *S. pneumoniae* serotypes selected from 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10C, 10F, 11A, 11B, 11C, 11D, 11E, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20A, 20B, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33C, 33D, 33E, 33F, 34, 35A, 35B, 35C, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, and 48.

[0204] In some embodiments, an immunogenic complex includes one or more *S. pneumoniae* capsular polysaccharides from, or derived from, one or more *S. pneumoniae* serotypes selected from 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F. In some embodiments, an immunogenic complex includes one or more *S. pneumoniae* capsular polysaccharides from, or derived from, one or more *S.*

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pneumoniae serotypes selected from 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F. In some embodiments, an immunogenic complex includes one or more *S. pneumoniae* capsular polysaccharides from, or derived from, one or more *S. pneumoniae* serotypes selected from 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B.

[0205] In some embodiments, an immunogenic complex includes one *S. pneumoniae* capsular polysaccharide from, or derived from, one *S. pneumoniae* serotype. In some embodiments, an immunogenic complex includes one *S. pneumoniae* capsular polysaccharide from, or derived from, one *S. pneumoniae* serotype selected from 1, 2, 3, 4, 5, 6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H, 7A, 7B, 7C, 7F, 8, 9A, 9L, 9N, 9V, 10A, 10B, 10C, 10F, 11A, 11B, 11C, 11D, 11E, 11F, 12A, 12B, 12F, 13, 14, 15A, 15B, 15C, 15F, 16A, 16F, 17A, 17F, 18A, 18B, 18C, 18F, 19A, 19B, 19C, 19F, 20A, 20B, 21, 22A, 22F, 23A, 23B, 23F, 24A, 24B, 24F, 25A, 25F, 27, 28A, 28F, 29, 31, 32A, 32F, 33A, 33B, 33C, 33D, 33E, 33F, 34, 35A, 35B, 35C, 35F, 36, 37, 38, 39, 40, 41A, 41F, 42, 43, 44, 45, 46, 47A, 47F, and 48. In some embodiments, an immunogenic complex includes one *S. pneumoniae* capsular polysaccharide from, or derived from, one *S. pneumoniae* serotype selected from 1, 2, 3, 4, 5, 6A, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20B, 22F, 23F, and 33F. In some embodiments, an immunogenic complex includes one *S. pneumoniae* capsular polysaccharide from, or derived from, one *S. pneumoniae* serotype selected from 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 22F, 23F, and 33F. In some embodiments, an immunogenic complex includes one *S. pneumoniae* capsular polysaccharide from, or derived from, one *S. pneumoniae* serotype selected from 2, 8, 9N, 10A, 11A, 12F, 15B, 17F, and 20B.

Methods of Isolating and Purifying Polysaccharides

[0206] In some embodiments, the disclosure provides methods of purifying one or more polysaccharides described herein from one or more cellular components of bacteria. In some embodiments, methods comprise purifying capsular polysaccharides from one or more cellular components of bacteria.

[0207] In some embodiments, the bacteria are Gram-negative. In some embodiments, the bacteria are Gram-positive. In some embodiments, the bacteria are *S. pneumoniae*.

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[0208] In some embodiments, the cellular components include protein. In some embodiments, the cellular proteins include nucleic acid. In some embodiments, the cellular components include lipids. In some embodiments, the cellular components include polysaccharides. In some embodiments, the cellular components are part of a lysate.

[0209] In some embodiments, the polysaccharide purification processes incorporate a series of ethanol precipitations, washes of crude polysaccharide preparations with ethanol, diethyl ether, and/or acetone, and drying under vacuum to furnish purified products. In some embodiments, a phenol extraction step is incorporated for polysaccharide purifications. In some embodiments the purification process employs a CTAB (cetyltrimethyl ammonium bromide) precipitation step in addition to using ethanol and phenol precipitation steps.

[0210] Figure 3 depicts exemplary structures and chemical information for *S. pneumoniae* capsular polysaccharides included in immunogenic complexes of the invention. All structures are from European Pharmacopoeia 9.0.

Methods of Biotinylating Polysaccharides

[0211] In some embodiments, the disclosure provides methods of biotinylating one or more polysaccharides described herein. In some embodiments, the method comprises reacting purified polysaccharides with 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) for activation of hydroxyl groups in the polysaccharides followed by the addition of amine PEG biotin under conditions that result in covalent linkage of biotin to the polysaccharides. In some embodiments, the desired level of biotinylation is achieved by varying the ratio of CDAP to polysaccharide. In some embodiments, the biotinylated polysaccharides are purified by filtration to remove process residuals such as unreacted biotin, dimethylaminopyridine, acetonitrile, cyanide and unreacted glycine. In some embodiments, the level of polysaccharide biotinylation described herein is optimized to reduce the amount of accessible biotin following MAPS complexation.

Manufacture of Immunogenic Complexes

[0212] The present disclosure includes methods for manufacturing immunogenic complexes described herein. In some embodiments, a method of manufacturing immunogenic complexes comprises complexing at least one biotinylated polysaccharide with at least one

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biotin-binding fusion protein. In some embodiments, the fusion protein comprises an amino acid sequence having at least 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO:6.

[0213] In some embodiments, the average (*e.g.*, the mean) protein (*e.g.*, antigenic protein) to polysaccharide ratio of a plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average protein to polysaccharide ratio of a plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average protein:PS ratios are chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0214] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 1. In some embodiments, the average ratio of CP1

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protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 1 in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may

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comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0215] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 2. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio

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of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 2 in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0216] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 3. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is

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approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 3 in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0217] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 4. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae*

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serotype 4 in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 4 in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0218] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 5. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein

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to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 5 in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0219] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 6A. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 1:1

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(w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6A in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0220] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 6B. In some embodiments, the average ratio of

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CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 6B in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the

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invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0221] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 7F. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio

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of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 7F in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0222] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 8. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is

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approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 8 in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0223] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 9N. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae*

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serotype 9N in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9N in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0224] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 9V. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 1:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein

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to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 9V in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0225] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 10A. In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 1:1, 1.5:1, 2:1, 2.5:1, 3:1, 3.5:1, 4:1, 4.5:1, 5:1, 5.5:1, 6:1, 6.5:1, 7:1,7.5:1, 8:1, 8.5:1, 9:1, 9.5:1, or 10:1 (weight/weight [w/w]). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 1:1

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(w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 2:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 3:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 4:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 5:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 6:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 7:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 8:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 9:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is approximately 10:1 (w/w). In some embodiments, the average ratio of CP1 protein to capsular polysaccharide from or derived from *S. pneumoniae* serotype 10A in the plurality of immunogenic complexes is chosen to enhance the polysaccharide immunogenicity potential (carrier function) and/or to elicit protection against, or to inhibit, pneumococcal colonization by any pneumococcus (independent of polysaccharide serotype) through a protein-specific immune response. Immunogenic compositions and vaccines of the invention may comprise mixtures of immunogenic complexes with different average protein to polysaccharide ratios.

[0226] In some embodiments, a vaccine or immunogenic composition comprises a plurality of immunogenic complexes comprising a CP1 protein and a capsular polysaccharide, from or derived from *S. pneumoniae* serotype 11A. In some embodiments, the average ratio of

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