



Development of a pneumococcal conjugate vaccine based on chemical conjugation of polysaccharide serotype 6B to PspA

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ABSTRACT

The use of conjugate vaccines remains an effective intervention to prevent pneumococcal diseases. In order to expand vaccine coverage, the inclusion of pneumococcal proteins as carriers is a propitious alternative that has been explored over the past few years. In this study, pneumococcal surface protein A (PspA) clade 1, family 1 (PspA1) and clade 3, family 2 (PspA3) were used as carrier proteins for pneumococcal capsular polysaccharide serotype 6B (Ps6B). Employing an improved reductive amination chemistry, 50% of Ps6B was incorporated to each protein, PspA1 and PspA3. The effect of chemical modifications in Ps6B and PspA was assessed by an antigenicity assay and circular dichroism, respectively. Fragmentation and oxidation decreased the antigenicity of Ps6B while conjugation improved antigenicity. In the same manner, introduction of adipic acid dihydrazide (ADH) reduced PspA secondary structure content, which was partially restored after conjugation. Immunization of Ps6B-PspA1 and Ps6B-PspA3 conjugates in mice induced specific IgG antibodies against the Ps6B and the protein; and anti-PspA antibodies had functional activity against two pneumococcal strains with different serotypes. These results suggest that chemical coupling between Ps6B and PspA did not affect antigenic epitopes and support the further development of PspA as a carrier protein in pneumococcal conjugate vaccines to provide broader protection.

1. Introduction

Twenty years after the introduction of the first pneumococcal conjugate vaccine, *Streptococcus pneumoniae* still contributes significantly to morbidity and mortality especially among children, elderly people and immunocompromised individuals [1,2]. The World Health Organization (WHO) estimates that almost half a million children less than 5 years of age die due to pneumococcal infections every year, mainly in low-income countries [3].

S. pneumoniae causes invasive diseases such as bacteremic pneumonia, sepsis and meningitis; and non-invasive diseases like otitis media and sinusitis [4]. Since pneumococcus colonizes the nasopharynx asymptomatically, colonized individuals act as reservoirs, facilitating bacterial transmission in the community [5]. Among different etiologies, *S. pneumoniae* was the leading cause of pneumonia mortality according to a study performed in 2016 [6].

As the most external structure, the pneumococcal capsule prevents bacterial phagocytosis facilitating immune evasion and invasiveness.

For that reason, capsular polysaccharides (CPs) are considered the main pneumococcal virulence factor [7] and are the main constituents of all pneumococcal vaccines licensed until now. The first vaccines against pneumococcus contained exclusively CPs from different serotypes, and presented several limitations, such as low immunogenicity in toddlers and the inability to generate immune memory regardless of age [8]. These barriers were overcome with the emergence of pneumococcal conjugate vaccines in 2000. The chemical coupling of CPs to carrier proteins changed the T-independent nature of polysaccharides to T-dependent antigens.

The first licensed pneumococcal conjugate vaccine (PCV) Prevenar 7 (PCV7) included serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F; all conjugated with a nontoxic mutant of diphtheria toxin (CRM197) [9]. However, some years after its introduction and due to emerging limitations, PCV7 was replaced by two novel conjugate vaccines with an expanded number of serotypes. Synflorix (PCV10) [10] from GlaxoSmithKline, employing different carrier proteins (non-typeable *Haemophilus influenzae* protein D, diphtheria toxoid, and tetanus toxoid) and a different

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conjugation chemistry added serotypes 1, 5 and 7F to those previously included in PCV7; while Prevenar 13 (PCV13) contains the same serotypes as PCV10 plus serotypes 3, 6A, and 19A [11]. In spite of their benefits, such as the reduction of infantile pneumonia, pneumococcal carriage and the achievement of herd immunity, those formulations have a lower efficacy against non-invasive pneumococcal disease (non-IPD) and its extensive use is followed by the replacement of vaccine serotypes for those not included in the formulation [12].

To overcome these limitations, the development of new PCV with expanded valence has been carried out. The PCV15 added serotypes 22F and 33F to the 13 serotypes included in PCV13, all individually conjugated with CRM197. This vaccine was well tolerated and induced IgG and opsonophagocytic activity (OPA) against all vaccine serotypes in infants and adults [13,14]. On the other hand, the PCV20 included 13 serotypes from PCV13 plus seven new serotypes (8, 10A, 11A, 12F, 15B, 22F, and 33F) which are known to cause IPD [15], meningitis [16] and are associated with antibiotic resistance [17]. Results from Phase 1 clinical trials displayed the presence of substantial specific IgG levels with functional activity to all pneumococcal vaccine serotypes [18,19].

Nevertheless, increasing the number of vaccine serotypes in the PCV formulations is complex and expensive since there are at least 98 pneumococcal serotypes [20]. Meanwhile, there is an urgent need to develop new pneumococcal vaccines with a broader coverage, regardless of serotype.

In this sense, several pneumococcal proteins have been studied as vaccine candidates such as the pneumococcal surface protein A (PspA) [21] pneumolysin (Ply) [22,23], pneumococcal surface protein C (PspC) [24], pneumococcal histidine triad proteins (PhtA, PhtB, PhtD and PhtE) [25] and pneumococcal surface adhesin A (PsaA) [26].

Among all the pneumococcal proteins studied, PspA stands out. This protein is a virulence factor present on the pneumococcal surface that inhibits deposition of complement activated C3 fragments and, therefore, interferes with phagocytosis [27–29]. According to variations in a sequence divergent region within the N-terminal domain, PspA is grouped into three families that are further divided into six clades. Family 1 includes clades 1 and 2; family 2 comprises clades 3, 4 and 5 and family 3 includes clade 6 [30]. More than 96% of clinical isolates in patients with IPD, belong to families 1 and 2 [31,32].

Immunization with a PspA from a single clade does not protect against the challenge with strains bearing heterologous clades [33]. For this reason, it has been suggested that a combination of PspA from the two most representative families (family 1 and 2) would be the most appropriate choice to protect against a large number of pneumococcal strains [34,35].

In order to increase vaccination coverage, the chemical conjugation of pneumococcal capsular polysaccharides to PspA has been explored, attaining promising results [36–39].

To expand these studies, here, Ps6B was conjugated individually to PspA clades 1 and 3 from family 1 and 2, respectively, using an improved reductive amination method. The humoral immune response induced by these conjugates was evaluated.

2. Material and methods

2.1. *S. pneumoniae* strains and reagents

Polysaccharide serotype 6B (Ps6B) was acquired from American Type Culture Collection (ATCC). Adipic acid dihydrazide (ADH), sodium periodate (NaIO₄) and 1-[3-(dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDAC) were purchased from Sigma-Aldrich. *S. pneumoniae* strains (245/00, TIGR4 and P46) were generously supplied by Instituto Adolfo Lutz (São Paulo, Brazil). The strains were maintained as frozen stocks (–80°C) in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY) plus 10% glycerol until use.

2.2. Polysaccharide activation

Before activation, Ps6B (10 mg/mL) was hydrolyzed with HCl (0.5 M) under agitation at 80 °C for 1 h in a reflux system, followed by neutralization with NaOH to pH 7.5. The hydrolyzed Ps6B was oxidized with NaIO₄ (10 mM) in phosphate buffered saline (PBS), pH 7.5, for 30 min in the dark at room temperature (RT). The reaction was stopped by glycerol (10 eq). Oxidized Ps6B (Ps6Box) was purified from the remaining glycerol and from low-molecular-weight oxidation products through chromatography using Sephadex G-25 gel filtration medium packed in a XK 26/40 column (Pharmacia).

2.3. Protein modification

First, recombinant PspA clade 1 (rPspA1) and clade 3 (rPspA3) were purified according to Carvalho et al. (2011) [40]. In order to improve conjugation efficiency, a spacer of six carbons atoms was introduced to rPspA1 or rPspA3. Both proteins (5 mg/mL in MES buffer (2-(N-morpholino) ethanesulfonic acid) 0.1 M, pH 6.0) were incubated with 3.5 mg/mL EDAC. Then, ADH spacer was added in a ratio of 3:1 (wt:wt). The reaction was incubated for 2 h at RT and then ultra-filtered using a 3 kDa cutoff membrane (Centricon, Millipore).

2.4. Conjugation of Ps6Box to modified recombinant proteins

Ps6Box and modified rPspA1 (mrPspA1) or mrPspA3 10 mg each/mL in PBS (pH 7.2) were incubated at RT for 48 h in the presence of sodium cyanoborohydride (NaBH₃CN) as a reducing agent. A ratio of 20 mol NaBH₃CN per each mol of Ps6B aldehyde was added to the reaction. The reaction was stopped with sodium borohydride (NaBH₄) at a molar ratio of 10 mol per mol of aldehyde in Ps6B and the product was further purified using Superose-12 gel filtration chromatography in a HR 16/50 column (GE Healthcare) using PBS, pH 7.5, as the mobile phase (0.5 mL/min). The percentage of Ps incorporated into the glycoconjugate was calculated dividing the amount of Ps6B contained in the conjugate fraction by the total amount of Ps6B recovered from the column.

2.5. Antigenicity of chemically modified Ps6B

The antigenicity of Ps6Box or Ps6Box conjugated with mrPspA1 or (Ps6B-mrPspA1) or with mrPspA3 (Ps6Box-mrPspA3) was assessed by an inhibition ELISA. At first, standard anti-Ps6B serum (Serum Statens Institute, Denmark) diluted 1:1500 in PBS (pH 7.2) was incubated overnight (ON) at 4 °C with native Ps6B (nPs6B); Ps6Box or Ps6Box-mrPspA1; Ps6Box-PspA3; or a pneumococcal serotype 1 (nPs1) as a negative control, in a concentration range between 0.005 and 500 µg/mL. In parallel, a 96-well Maxisorp plate was coated with nPs6B (5 µg/well) in PBS (pH 7.2), and incubated ON at 4 °C. After that, the plate was washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 1% skim milk for 1 h at 37 °C, followed again by three washes with PBS-T. In the second step, treated anti-Ps6B serum was transferred to each well of nPs6B-coated plate, followed by incubation for 90 min at RT and then the plate was washed three times with PBS-T. Next, polyclonal goat anti-rabbit IgG horseradish peroxidase-conjugated antibody (1:3,000 Sigma-Aldrich) was added, and then incubated at RT for 90 min. The plates were washed three times with PBS-T, and the substrate o-phenylenediamine dihydrochloride (0.5 mg/mL) in citrate buffer 0.1 M (pH 5.0) containing 0.015% of hydrogen peroxide was added to each well and incubated for 20 min in the dark. The enzyme reaction was quenched by adding 4 M H₂SO₄. Absorbance was read at 492 nm on a Multiskan EX ELISA reader (LabSystems-Uniscience). The inhibition percentage was defined by the following formula: 100 - [(Abs A/Abs B) x 100], where Abs A is the absorbance when anti-Ps6B serum was incubated with different polysaccharides/conjugates and Abs B the absorbance when anti-Ps6B serum was added without any treatment.

2.6. Circular dichroism (CD) analysis

The CD spectra were obtained on a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo, Japan) at 20 °C. The measurements were performed at wavelengths from 185 to 260 nm at 0.1 nm intervals in a 0.1-cm-path cell. The spectra represent the averages of five scans, and the data obtained were reported as molar ellipticity (degrees-cm²·dmol⁻¹). A baseline measurement with 10 mM sodium phosphate buffer (pH 7.5) was subtracted from each spectrum. The secondary structure deconvolution analysis was performed with Dichroweb software [41], using the CDSSTR algorithm [42].

2.7. Analytical procedures

Ps6B was quantified by the orcinol-sulfuric method [43], using D-glucose as standard. The extent of oxidation was estimated by the bicinchoninic acid (BCA) colorimetric method [44] with glucose as the standard. The extent of the reaction with ADH was estimated by the trinitrobenzenesulfonic acid (TNBS) method [45] using ADH as standard. The concentrations of rPspA1 and rPspA3 were determined by the BCA method using bovine serum albumin as standard.

2.8. Immunization procedures

BALB/c mice (female, 8 weeks old, six per group) were supplied by the Animal Care Facilities of the Institute Butantan. The animals were immunized subcutaneously (s/c) in a three-dose schedule (at 0, 14, and 28 days) with: Ps6Box-mrPspA1 conjugate (7 µg Ps6B and 10 µg mrPspA1/dose) and Ps6Box-mrPspA3 conjugate (10 µg Ps6B and 10 µg mrPspA3/dose). Ps6B (7 µg or 10 µg) plus mrPspA1 or mrPspA3 at 10 µg (co-administered) and 0.9% saline solution were used as controls. All samples (200 µl per mouse) were prepared in 0.9% saline solution with 125 µg of Al (OH)₃ as the adjuvant. Blood samples were collected at 13, 27, and 42 days via the retro-orbital plexus and sera were kept at -20 °C before use. All *in vivo* experiments were previously approved by the Institute Butantan Animal Use Ethics Committee under license number CEUAIB 1280/14.

2.9. Evaluation of the immune response induced by conjugates

The antibody titers anti-Ps6B and anti-mrPspA1/mrPspA3 were determined by conventional indirect ELISA. ELISA 96-well microtiter plates (NuncMaxiSorp) were coated with 5 µg/well of Ps6B in PBS (pH 7.2) ON at 37 °C or 0.1 µg/well of protein (mrPspA1 or mrPspA3) in PBS (pH 7.2) ON at 4 °C. The plates were washed three times with PBS-T and blocked with PBS and 1% skim milk for 1 h at 37 °C. The plates were washed with PBS-T and incubated with serial dilutions of serum from individual mice in PBS containing (0.5% skim milk; 4 mM EDTA and 0.01% Tween 20) for 90 min at RT and later washed with PBS-T. Peroxidase-conjugated polyclonal goat anti-mouse IgG (1:1,000 Sigma-Aldrich) was then added, and the plates were incubated at RT for 90 min. The plates were washed three times with PBS-T, and the substrate reaction step was carried out as described above. The antibody titer was determined as the log10 of the last serum dilution with an optical density of 0.1.

2.10. Opsonophagocytic assay

This assay was performed as described by Goulart et al. (2011) [46] with modifications. BALB/c mice were injected intraperitoneally (ip) with 10 µg of concanavalin A (ConA, Sigma). After 48 h, animals were euthanized and peritoneal cells were withdrawn using cold PBS.

Bacterial suspensions from *S. pneumoniae* strains (245/00 and TIGR4) were thawed, plated on blood agar plates and incubated for 18 h at 37 °C, under 5% CO₂ tension. A single colony from each strain was harvested and incubated in THY up to 0.4 to 0.5 O.D. at 600 nm which

corresponds to approximately 1 × 10⁸ CFU/mL. Bacterial suspensions were then centrifuged at 4,000×g for 5 min, pellets were washed once with PBS and resuspended in Hanks' buffer (Invitrogen) containing 0.1% gelatin (opsono-buffer).

Aliquots containing 2.5 × 10⁶ CFU were incubated with pooled heat-inactivated immune sera at 1:8 and 1:16 dilutions, for 30 min at 37 °C, in triplicate. Serum from mice treated only with saline was used as a control for all the assays. Then, samples were incubated with 10% fresh normal mouse serum, as a source of complement proteins, diluted in opsono-buffer at 37 °C for 30 min. After that, samples were washed once with PBS and later incubated with 4 × 10⁵ peritoneal cells diluted in opsono-buffer at 37 °C for 45 min with shaking. The reaction was stopped by cooling on ice for 5 min. Ten-fold dilutions of the samples were performed and 10 µl aliquots of each sample were plated on blood agar plates in triplicate. The number of CFU was determined after incubation for 18 h at 37 °C, under 5% CO₂ tension.

2.11. Statistical analysis

Statistical analysis was performed using the parametric test one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test. For all comparisons, a *P* value of <0.05 was considered to represent statistical significance.

3. Results

3.1. Polysaccharide activation

Native Ps6B (nPs6B) was hydrolyzed and oxidized in order to allow its coupling to protein. The acid hydrolysis of nPs6B reduced its size (Supplementary Fig. 1A) to approximately 15–20 kDa and a yield >60%. The oxidation reaction resulted in 10 mol aldehyde per mol of polysaccharide, which corresponds to 20% of the repetitive units oxidized. There was no reduction in the molecular weight of Ps6B after oxidation as confirmed by HPLC (Supplementary Fig. 1B) and there were no considerable losses during the activation of Ps6B (>70% yield).

3.2. Conjugation of Ps6Box to rPspA

Both recombinant proteins rPspA1 and rPspA3 were successfully purified (>90% purity). Protein activation with ADH doubled the molar ratio of amino groups. The NH₂:protein (mol:mol) ratio for rPspA1 increased from 4.4 to 10.7 while the NH₂:protein ratio for rPspA3 increased from 5.3 to 9.7. Ps6B-mrPspA conjugates were obtained by reductive amination (Fig. 1) and purified by gel filtration chromatography (Superose 12 prep grade). Approximately, 50% and 60% of Ps6B was incorporated in the conjugate fraction for Ps6B-mrPspA1 and Ps6B-mrPspA3, respectively. The mass ratio of Ps6B:mrPspA obtained in the Ps6B-mrPspA1 and Ps6B-mrPspA3 conjugates were 1.5:1 and 1:1, respectively. The characterization of glycoconjugates by SDS-PAGE confirmed the formation of a high molecular weight macromolecule and Schiff's staining corroborated the incorporation of polysaccharide (Ps) in the conjugate (Supplementary Fig. 2).

3.3. Antigenicity of chemically modified Ps6B

Antigenicity of Ps6Box drastically decreased since the concentration required to achieve 50% inhibition of anti-Ps6B serum was two orders of magnitude higher than the native Ps (Fig. 2). In contrast, the antigenicity of Ps6B improved after conjugation. In this case, the concentration of Ps required to achieve 50% inhibition of anti-Ps6B serum was only one order of magnitude higher than the native Ps. As expected, the negative control, a polysaccharide from pneumococcal serotype 1 (nPs1) did not inhibit the anti-Ps6B serum.

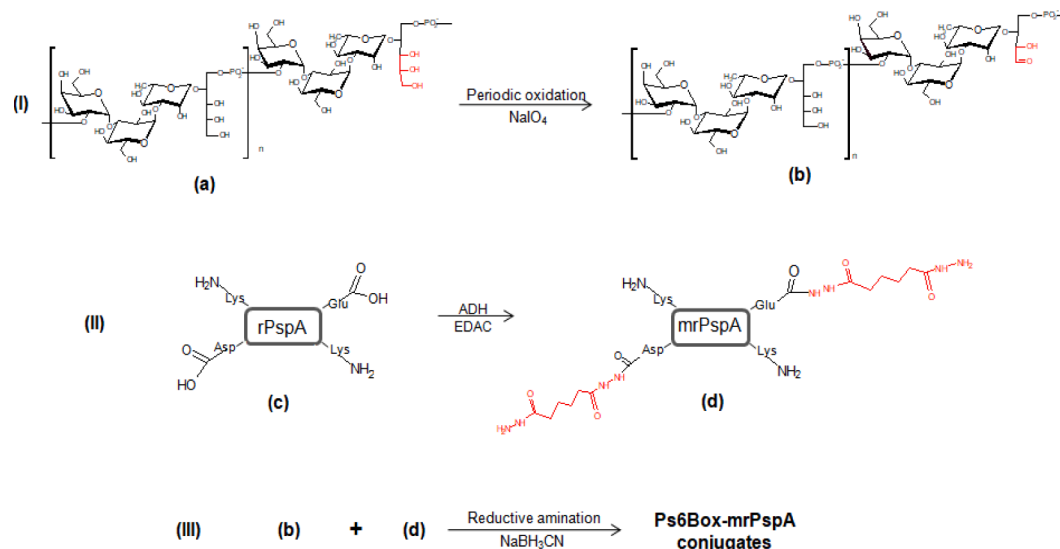


Fig. 1. Schematic representation of conjugation steps. (I) Periodic oxidation: vicinal hydroxyl groups in fragmented Ps6B (a) were oxidized in the presence of NaIO₄ and aldehyde groups were generated in Ps6B (b). (II) Introduction of a spacer in the protein: carboxyl groups in rPspA (c) reacted with adipic acid dihydrazide (ADH) in the presence of EDAC to generate mrPspA (d). (III) Reductive amination: amino groups introduced in the protein through ADH spacer (d) reacted with aldehyde groups in Ps6Box (b) in the presence of NaBH₃CN as a reductive agent to generate Ps6Box-mrPspA conjugates. Reactive groups are highlighted in red.

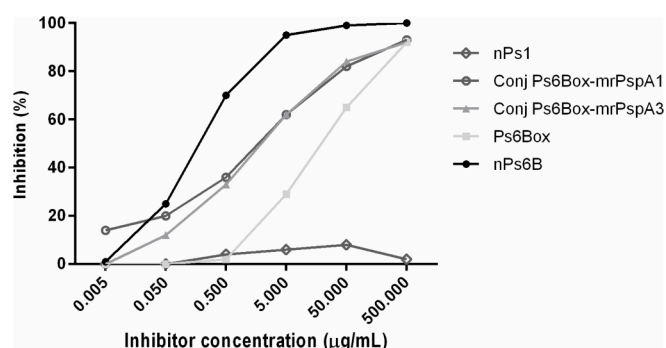


Fig. 2. Antigenicity of chemically modified Ps6B measured by inhibition ELISA. Polyclonal specific serum against the capsular polysaccharide of *S. pneumoniae* serotype 6B (Ps6B) was initially incubated with Ps6Box or Ps6Box conjugates (conj) prepared with modified recombinant (mr) PspA1 (Ps6B-mrPspA1) or with mrPspA3 (Ps6B-mrPspA3). Additionally, serum was incubated with native Ps6B (nPs6B) as a positive control and with native pneumococcal polysaccharide serotype 1 (nPs1) as a negative control. Secondly, the previously treated polyclonal anti-Ps6B specific serum was set to recognize nPs6B by ELISA.

3.4. Circular dichroism analysis of PspA1 and PspA3

The effect of Ps6B conjugation on mrPspA secondary structure was analyzed by comparing the deconvoluted CD spectra of conjugates and controls (native and mrPspA1 or mrPspA3). As shown in Fig. 3 and Supplementary Fig. 3, there was a predominance of alpha-helical structures in rPspA1 and rPspA3 (83% and 87%, respectively). After the introduction of the ADH spacer, the alpha-helix content of PspA1 decreased drastically to 33% (mrPspA1). In the case of PspA3, the change was more subtle, from 87% (nPsA3) to 65% (mrPspA3). However, the conjugation process seemed to partially restore the content of alpha-helix, in PspA it increased to 60% and in PspA3 it returned to the same level observed for the native recombinant protein. Reductions in alpha-helix content were accompanied by proportional increases in random-coil content.

3.5. Immunogenicity of conjugates

The anti-Ps6B and anti-PspA IgG titers induced by the conjugate and coadministered antigens were measured by ELISA and are shown in Fig. 4. Since Ps6B is one of the less immunogenic among pneumococcal polysaccharides [47] and it exhibits a low immunogenicity in murine models [48] in immunization protocols, polysaccharide doses of 7 μg and 10 μg for Ps6B-mrPspA1 and Ps6B-mrPspA3 conjugates were used, respectively. Anti-Ps6B IgG antibody titers are shown in Fig. 4A. There were no significant differences between the groups immunized with Ps6B conjugates and free Ps6B (co-administered with protein). Mice immunized with Ps6B-mrPspA1 conjugate induced anti-Ps6B titers significantly higher than the control group. On the other hand, high titers of anti-PspA IgG antibodies were generated by conjugates and free PspA (co-administered). Although there were no significant differences between conjugated and co-administered PspA, both groups induced significantly higher IgG titers than the control group (Fig. 4B).

3.6. Opsonophagocytic activity

The functionality of anti-PspA antibodies was evaluated by their opsonophagocytic activity (Fig. 5). The protective immunogenicity of anti-PspA antibodies was tested using pneumococcal strains bearing a PspA homologous to the conjugate (either PspA1 or PspA3) and a heterologous polysaccharide (no-Ps6B). As expected, sera of mice immunized with co-administered Ps6B plus PspA1 or PspA3 were more efficient in reducing the number of CFU when compared to the negative-control group. The conjugation of the protein to Ps6B did not result in loss of opsonophagocytic activity of the anti-PspA1/anti-PspA3 antibodies. However, anti-Ps6B antibodies did not show an effective functional activity when a pneumococcal serotype 6B strain carrying a heterologous PspA to the conjugate was employed (Supplementary Fig. 4).

4. Discussion

The development of new pneumococcal vaccines with the potential to provide broad serotype coverage is essential to overcome the limitations of PCV. In light of this, the conjugation of capsular polysaccharides with conserved pneumococcal proteins that could confer a

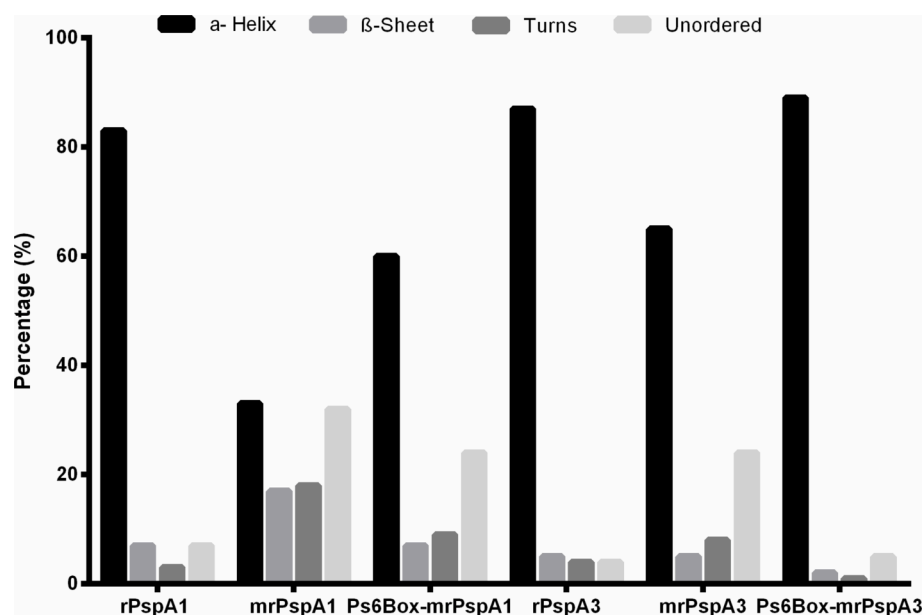


Fig. 3. Secondary structure analysis of rPspA1 and rPspA3 after derivatization and conjugation. Circular dichroism was employed to evaluate the secondary structure of recombinant proteins (rPspA1/rPspA3) after the introduction of ADH spacer (mrPspA1/mrPspA3) and after their conjugation to Ps6B (Ps6Box-mrPspA1/Ps6Box-mrPspA3). CD spectra were measured at 20 °C as described in Materials and Methods. Secondary structure deconvolution analysis was performed with Dichroweb software, using the CDSSTR algorithm.

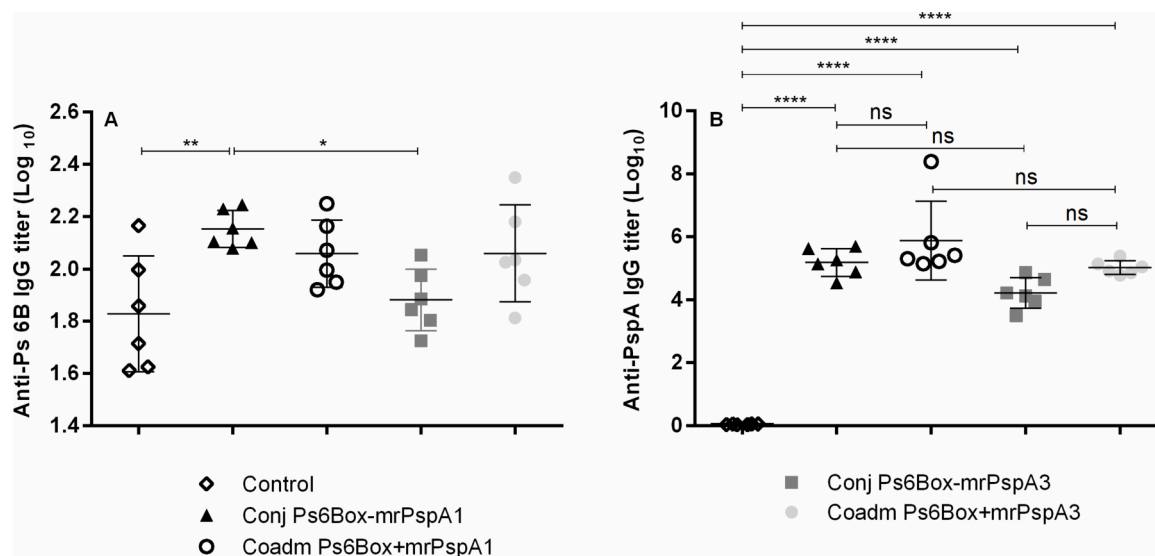


Fig. 4. Production of specific IgG anti-Ps6B and anti-PspA. IgG titers of individual serum samples after a three-dose immunization schedule in mice ($n = 6$) were determined by ELISA. Mice were immunized with the following immunogens: oxidized Ps6B (Ps6Box) conjugated (Conj) with modified recombinant protein (mr) PspA1 (Conj Ps6Box-mrPspA1) [Ps6Box (7 μ g) and mrPspA1 (10 μ g)] (black triangles); co-administered (Coadm) Ps6Box (7 μ g) plus mrPspA1 (10 μ g) (empty circles); Conj Ps6Box-mrPspA3 [(Ps6Box (10 μ g) and mrPspA3 (10 μ g)] (dark grey squares); Coadm Ps6Box (10 μ g) plus mrPspA3 (10 μ g) (light grey circles) and saline as control (empty diamonds). Serum samples were analyzed by ELISA and results were compared by one-way ANOVA with Tukey's multiple-comparison test. (A) Anti-Ps6B IgG titer; (B) Anti-PspA IgG titer. The bars represent the means $\pm$ standard errors of the means (SEM), and asterisks indicate statistically significant differences ($P < 0.05$ *; $P < 0.01$ **; $P < 0.0001$ ****); ns, non-significant differences.

serotype independent protection is a promising strategy. In this study, conjugates of *S. pneumoniae* capsular polysaccharide serotype 6B (Ps6B) to Pneumococcal surface protein A (PspA) clades 1 and 3 (family 1 and 2, respectively) were synthesized by reductive amination. Both glyco-conjugates were immunogenic in mice, inducing functional antibodies able to promote opsonophagocytosis of pneumococcus independent of serotype.

Initially, the size of Ps6B was reduced by acid hydrolysis and then vicinal hydroxyl groups were oxidized to aldehydes. The weakest bond in Ps6B prone to be cleaved in acidic conditions is the phosphodiester linkage D-ribitol-(5-PO₄-2)- α -D-galp [49], confirmed previously by ¹H and ³¹P NMR analysis [50].

The optimal size of polysaccharide in conjugate vaccines has not

been defined. Some studies indicate that high molecular weight polysaccharides induce greater antibody levels [51], and smaller oligosaccharides are less immunogenic than medium molecular mass oligomers [52]. In the case of 6B, it has been shown that oligosaccharides with molecular mass between 10–30 kDa and 30–100 kDa preserve their antigenicity, however, oligosaccharides between 1 and 10 kDa have a significant reduction in antigenicity [53], indicating that a critical number of repetitive units in Ps is required to guarantee its antigenic capacity. In this work, oligosaccharides of Ps6B between 15 and 20 kDa were obtained by HCl hydrolysis, and after oxidation reaction no decrease in Ps6B molecular size was observed while antigenicity of Ps6Box decreased strikingly.

Traditionally, two alternatives have been used for the synthesis of

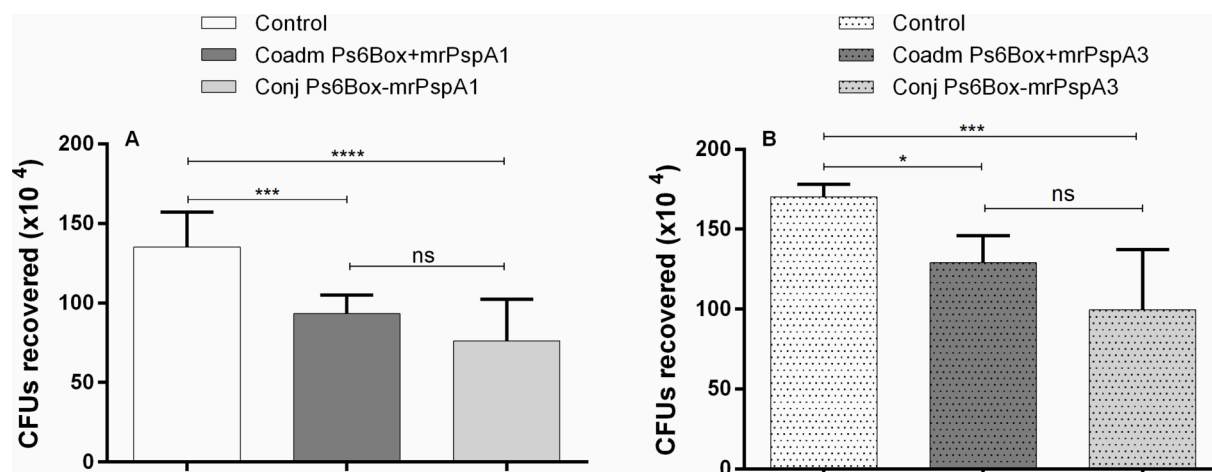


Fig. 5. Opsonophagocytic activity mediated by specific anti-PspA antibodies. (A) Pneumococcal strain 245/00 (PspA clade 1, serotype 14) was used to evaluate the opsonic activity of anti-PspA1 antibodies and (B) pneumococcal strain TIGR4 (PspA clade 3, serotype 4) was used to determine the opsonic activity of anti-PspA3 antibodies. Pneumococcal strains 245/00 and TIGR4 were incubated with pooled sera from mice immunized with conjugates: Conj Ps6Box-mrPspA1 or Conj Ps6Box-mrPspA3, respectively. Sera from mice immunized with saline or with Ps6B co-administered with rPspA1 or PspA3 (Coadm Ps6Box + mrPspA1 and Coadm Ps6Box + mrPspA3) were used as controls. The opsonized pneumococci were incubated with peritoneal-elicited macrophages and plated on blood agar plates. The numbers of CFU recovered after 20 h were compared by one-way ANOVA with Tukey's multiple-comparison test. The bars represent the means $\pm$ standard errors of the means (SEM). Asterisks indicate statistically significant differences ($P < 0.05$ *; $P < 0.001$ ***; $P < 0.0001$ ****); ns, non-significant difference.

conjugate vaccines: one based on the random activation of hydroxyl or carboxyl groups present along the polysaccharide (native or fragmented), followed by conjugation; the second, based on activation of oligosaccharide terminal groups and then covalent linkage to the carrier protein. These conjugation alternatives define the type of conjugate to be obtained: a macromolecular network between the polysaccharide and the protein in the first case, and a radial structure with the oligosaccharide linked by a single end to the protein that occupies a central position, in the second case [54]. Due to the Ps6B fragmentation and oxidation pattern, the conjugates obtained in this study were of macromolecular network type.

Regarding the conjugation methods, there are several procedures reported in the literature; one of the most frequently used in conjugate vaccines is reductive amination. This method is relatively simple, consisting in periodic oxidation of Ps, the aldehyde groups generated then react with the amine groups in the protein [55,56]. The reaction passes through the formation of an intermediate imine or Schiff's base which is subsequently reduced by sodium cyanoborohydride to form a secondary amine [57,58]. In order to improve conjugation efficiency, the introduction of spacers in Ps or protein is recommended, since it increases reactivity, reduces steric hindrance and allows higher yielding [59]. Here, the bifunctional spacer ADH was introduced in the protein (PspA1/PspA3) in a step prior to conjugation, and successfully enabled the incorporation of at least 50% of Ps6B after the conjugation reaction.

The antigenicity assay was developed to determine the capacity of chemically modified Ps6B of being recognized by a reference serum before immunization, to define whether or not antigenic epitopes were affected. Curiously, antigenicity of conjugated Ps6B increased when compared to Ps6Box, probably as a result of conformational rearrangement of epitopes in Ps6B after conjugation. In a similar way, analysis of PspA secondary structure by circular dichroism revealed a decline in α -helical content after the introduction of ADH spacer which was partially restored after conjugation. A reduction in PspA α -helical content after blocking lysine ϵ -amino groups prior to Ps coupling by a different conjugation method has been observed [38]. Interestingly, unlike the present study, they still observed a decrease in α -helical content after conjugation.

Even though PspA secondary structure was altered, the capacity to induce antibodies was not affected. mrPspA, either free or covalently linked to Ps6B, induced specific high antibody titers, equivalent among

different groups. In contrast, specific anti-Ps6B antibody titers were low.

Ps6B is poorly immunogenic in mice [48]. To explore some aspects of immunological memory, Balb/c mice were immunized with the commercial vaccine Prevenar-7 and 26 weeks later received a booster with Ps. The number of Ps6B specific antibody secreting cells was similar to the control group, unlike Ps4 and Ps14 which induced high levels of these cells [60]. Chu et al. (2000) [61] evaluated the adjuvant effect of CpG oligodeoxynucleotides in BALB/c mice, using 5 μ g of Ps 6B conjugated to CRM197 without adjuvant. In this condition, the production of 6B-specific IgG antibody was not observed (even IgG1 responses were not detected). A more efficient animal model in inducing high avidity index antibodies against Ps6B was observed when New Zealand white rabbits were used [53].

In addition, Ps6B and Ps23F have shown to be the least immunogenic of all pneumococcal polysaccharides in humans [47,62]. Due to the low immunogenicity of serotype 6B, a doubled dosage (4 μ g) with respect to the other serotypes is employed in the commercial conjugate vaccines Prevenar-7 and Prevenar-13. In this work, doses of conjugated Ps6B ranged between 7 and 10 μ g. However, immunization of 15 μ g of conjugated Ps6B in Balb/c mice was not able to raise the specific anti-Ps6B antibody titer [38]. Ps6B was chosen in this study because soon after PCV10 implementation in the Brazilian national immunization program, serotype 6B was still isolated from IPD cases [63,64].

Beyond measurement of antibody levels against the antigen of interest, assessment of the protective capacity of antibodies is of major interest. For pneumococcal conjugate vaccines, complement-dependent opsono-phagocytosis mediated by antibodies reactive with the capsular polysaccharides is the gold standard of protection against pneumococcal disease [65]. In this study, the opsono-phagocytic activity of anti-Ps6B antibodies was not evaluated due to the low antibody titers observed.

A correlate of protection for antibodies against pneumococcal proteins has not been established so far, due to the lack of a protein vaccine formulation for use in humans. Nevertheless, serum of mice immunized with conjugates of pneumococcal Ps and PspA develops opsono-phagocytic activity towards pneumococcal strains bearing homologous PspA and heterologous polysaccharide [37–39].

When evaluating the opsonophagocytic capacity of anti-PspA antibodies, we observed that serum from mice immunized with either free or conjugated mrPspA significantly reduced the number of colony-forming units recovered compared to the control group (Fig. 5). This behavior

was similar for both PspA1 and PspA3, indicating that chemical modifications as well as coupling to Ps6B did not modify the PspA epitopes responsible for antigen recognition. Anti-Ps6B antibodies did not show functional opsonophagocytic activity, likely due to their low concentration in mouse serum. Since the antigenicity of Ps6B did not decrease after chemical conjugation to PspA, the weak anti-Ps6B antibody response induced following immunization of conjugates is a consequence of the intrinsically low immunogenicity of Ps6B [47,62], especially in murine models [48].

In summary, we synthesized conjugates of pneumococcal polysaccharide serotype 6B to PspA from different families by an improved reductive amination method. Conjugates were characterized physico-chemically and immunologically. The antigenicity assay allowed tracking how chemical modifications affected the antigenic properties of Ps6B. Both antigens, Ps6B and PspA when chemically modified prior to conjugation showed signs of structural perturbation, since Ps6B antigenicity was markedly reduced and PspA alpha-helical content also diminished. Interestingly, after conjugation the antigenicity of Ps6B increased and PspA alpha-helical content was partially restored. Specific anti-Ps6B and anti-PspA IgG antibody titers were induced by conjugates in Balb/c mice, although anti-Ps6B antibodies did not attain high levels and did not induce functional activity. Furthermore, anti-PspA antibodies showed protective capacity in an opsonophagocytic assay employing pneumococcal strains bearing homologous PspA (clades 1 and 3) and heterologous polysaccharide.

Our results demonstrate that conjugation of Ps6B to PspA does not affect the immunogenicity and that Ps6B-PspA conjugates induced protection against two pneumococcal strains with different serotypes. These findings reinforce the potential use of PspA as a carrier protein in PCV in order to achieve a broad protection with a reduced number of Ps antigens.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.micpath.2021.105092>.

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Author contribution statement

LESL designed and performed the experiments, initiated and supervised the study. JCC and LESL purified recombinant proteins. EC and LESL performed circular dichroism analysis. LESL performed polysaccharide modification and conjugation steps and immunological assays. LESL wrote the manuscript with the help of the other authors. All authors discussed the data and revised and approved the manuscript.

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