

Versatile and efficient synthesis of protein–polysaccharide conjugate vaccines using aminooxy reagents and oxime chemistry

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Abstract

Applications of oxime chemistry are described for the efficient bioconjugation of proteins and polysaccharides for the preparation of conjugate vaccines. A number of approaches are described in this manuscript to functionalize proteins and polysaccharides with aminooxy (AO) groups and aldehydes which could then be covalently linked to each other via oxime formation, without the need for reduction. By using limiting numbers of active groups on each component, the extent of inter- and intramolecular crosslinking could be controlled. The approaches described are compatible and complementary to a number of chemistries currently used in conjugate vaccine synthesis. Oxime chemistry can be used to both simplify the synthesis of and increase yields of conjugate vaccines. Mice immunized with pneumococcal type 14 conjugates that were made using oxime chemistry mounted significant anti-polysaccharide immune responses. The primary immune response could be boosted, indicating that the polysaccharide conjugate had characteristics of a T cell dependent antigen.

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1. Introduction

Antibodies to encapsulated bacteria provide protection against disease caused by these organisms. However, capsular polysaccharides are usually poorly immunogenic in infants under two years of age unless they are covalently linked to a protein carrier, a process which converts the polysaccharide from a T cell independent antigen to a T cell dependent antigen. T cell dependent antigens, unlike T cell independent antigens, induce an immune response that undergoes class switching and exhibits immunological memory [1]. Protein conjugates with the capsular polysaccharides of *Haemophilus influenzae b* (Hib) [2], *Neisseria meningitidis* C [3,4] and *Streptococcus pneumoniae* [5] have almost eliminated disease incidence in areas where immunization using these vaccines is widespread.

The synthesis of conjugate vaccines can be a complex and expensive process involving the preparation of the individual components, the chemical activation of the polysaccharide and protein, a conjugation step, and the purification of the conjugate from its components [6,7]. Different vaccine manufacturers employ their own conjugation technologies and these differences among the technologies are based both on practical as well as licensing considerations for the particular chemistries and carrier proteins. Reductive amination is currently used to prepare a number of licensed conjugate vaccines, including Hib, *Neisseria* and *S. pneumoniae*. In this process, the first step involves creating aldehydes on oligo or polysaccharides. Conjugation occurs when aldehydes are subsequently reacted with lysines on proteins to form Schiff base linkages. A disadvantage of reductive amination is that, due to the high pK_a of the ϵ -amino group of lysine, alkaline conditions are needed and the coupling reaction is slow and often inefficient. Another disadvantage is that the Schiff bases formed are reversible and must be reduced to stabilize the linkage, usually with a toxic borohydride or

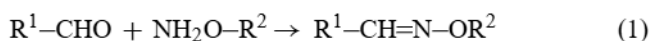
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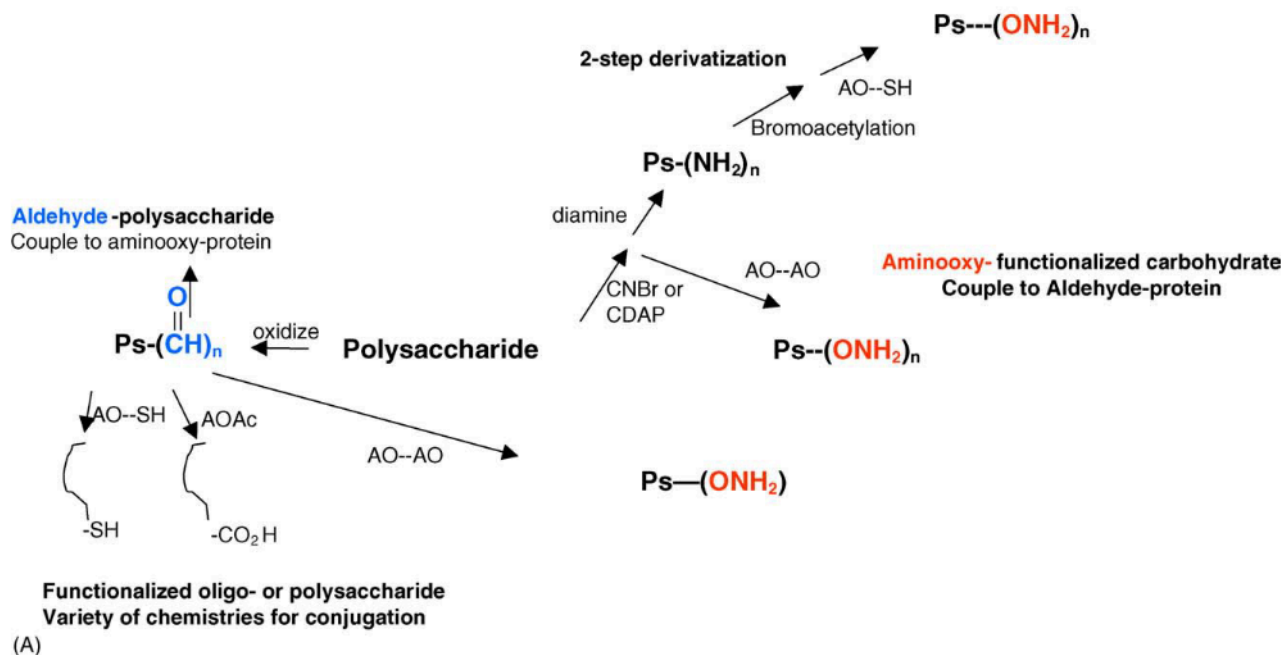
organic borane. Typical reaction times for conjugates made using reductive amination are >24 h [8–12]. Furthermore, pH-sensitive polysaccharides such as the one from *Neisseria meningitidis A* can hydrolyze or deacylate during the alkaline conditions of the coupling step, which can affect their ability to induce protective antibodies.

Here, we use oxime-based chemistry to introduce some novel reagents and approaches for covalently linking proteins to polysaccharides. In this process, aldehyde or ketone

carbonyls are reacted with the highly nucleophilic aminoxy (AO) group to form oximes. Either the protein or the carbohydrate moiety can be functionalized with aminoxy groups. In contrast to reductive amination, which yields an unstable Schiff base, aminoxy groups rapidly condense with aldehydes or ketones to form stable oximes, as indicated in Eq. (1).



Functionalization of oligo- and polysaccharides



Functionalization of Protein

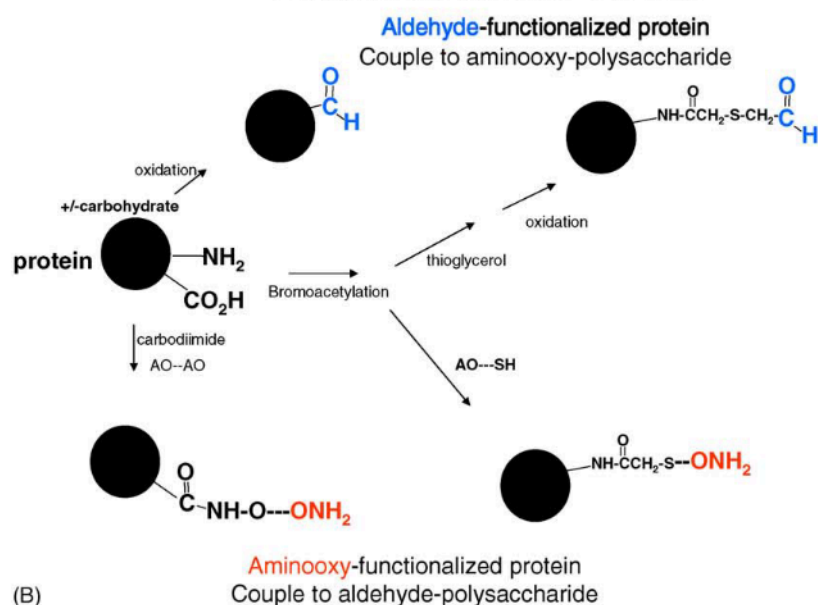


Fig. 1. Synthesis of aldehyde or aminoxy-derivatized proteins and polysaccharides. Schemes indicating methods for functionalizing proteins and polysaccharides with aminoxy and aldehyde groups. (Panel A) Functionalization of oligo- and polysaccharide. (Panel B) Functionalization of proteins.

Coupling using oxime chemistry is efficient, and can be effected over a wide pH range. Furthermore, by limiting the number of cross-links between the protein and polysaccharide, control can be exerted over the degree of cross-linking. To facilitate coupling proteins to aminoxy-functionalized polysaccharides, we introduce a method for creating aldehyde-functionalized proteins. A variety of routes are available to functionalize proteins and polysaccharides with the necessary aldehydes and aminoxy groups, some of which are illustrated for polysaccharides in Fig. 1A and for proteins in Fig. 1B. In this article, we describe some of these approaches to prepare conjugate vaccines.

2. Methods

2.1. Reagents

High and medium molecular weight fractions of Dextran T2000 (purchased from Pharmacia), as well as monomeric BSA were prepared as described [13]. Ovalbumin (Grade V) was purchased from Sigma. Diphtheria toxoid (DT) and pneumococcal types 3 and 14 polysaccharides were obtained from GlaxoSmithKline (Rixensart, Belgium). Gp350, an Epstein-Barr viral protein, was made recombinantly in yeast cells, as described in Sarrias et al. [14]. The protein was further purified by passage over a phenyl hydrophobic interaction column (Pharmacia), equilibrated with 0.8 M ammonium sulfate, 25 mM sodium phosphate, pH 7.2. The gp350 eluted isocratically, while a brown contaminant was retained.

Aminoxy acetate (carboxymethoxylamine hemichloride, AOAc), adipic acid dihydrazide (ADH), 1-mercapto-2,3-propanediol (mercaptoglycerol), bromoacetic acid, *N*-ethyl maleimide and 1-methyl-2-pyrrolidinone (NMP) were from Aldrich. Sodium metaperiodate was obtained from Fluka. *N*-succinimidyl bromoacetate (NHS bromoacetate) and sulfo-succinimidylacetate were from Prochem (Rockford, IL). Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) was from Pierce.

Trinitrobenzenesulfonic acid (TNBS) was from Kodak Chemical and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was obtained from Aldrich. Aminoxy-functionalized biotin was obtained from Molecular Probes. Aminoxy reagents were prepared by David Schwartz of Solulink Inc. (Pacific Heights, CA) and were based on 2-(BOC-aminoxy) acetic acid (Bachem). Succinimidyl levulinate was also obtained from Solulink. 1-Cyano-4-dimethylaminopyridine tetrafluoroborate (CDAP) was purchased from Research Organics. Other chemicals were of reagent grade or better.

2.2. Aminoxy addition to acetone

Acetone was made up to an OD 280 nm of 0.85 in solutions at the indicated pH. One hundred fifty microliters of AOAc (0.5 M stock) added to 500 μ l of the acetone solution and the

absorbance at 280 nm was read at 1 min. Percent remaining acetone was determined from the absorbance at 1 min divided by the dilution-adjusted absorbance of the acetone solution alone.

2.3. Aminoxy functionalization of proteins

To test the ability of aminoxy reagents to add to pneumococcal capsular polysaccharides 3 and 14, 1 mg of each was made up to 5 mg/ml in 10 mM sodium acetate, pH 5 and made 10 mM sodium metaperiodate for 10 min, after which 50 μ l of 50% glycerol was added. The polysaccharides were dialyzed against water, made 10 mM sodium acetate, pH 5 and 0.1 mg of aminoxy biotin added. After an overnight reaction, each was dialyzed against water. Carbodiimide-mediated functionalization of BSA with bis(AOAc)tetraethyleneglycol was performed as follows: bis(AOAc)tetraethyleneglycol (85 mg) was made up in 850 μ l of 0.5 M HCl. NaOH of 5 N was added to adjust to a pH $\sim$ 4.5 followed by the addition of 1 ml of monomeric BSA (42.2 mg/ml in saline). The reaction was initiated by the addition of 25 μ l of freshly prepared EDAC (100 mg/ml in water). After approximately 3 h, the solution was dialyzed overnight against saline at 4 °C. The solution was then diafiltered and concentrated with an Amicon Ultra 4 centrifugal device (30 kDa cutoff). Using the BCA assay (Pierce Chemical Co.), the final protein concentration was estimated to be 34 mg/ml BSA.

High- and low-ratio aminoxy-labeled BSA was prepared using a two-step method as follows: monomeric BSA (20 mg/ml in 40 mM HEPES, pH 8) was labeled at either a 68 or a 20 mol/mol ratio of succinimidyl bromoacetate from a 0.2 M stock in NMP. After an overnight reaction at room temperature in the dark, the solutions were dialyzed against saline for 2 days, centrifuged and filtered. Using TNBS [15], it was estimated that the high and low ratio bromoacetylated BSA had an average of 9 and 19 residual amines, respectively. *N*-(AOAc)cysteamine was prepared as follows: 51.5 mg of bis(AOAc)cysteamine was added to a solution of 56 mg TCEP made up in 1.1 ml 1 M sodium carbonate + 586 μ l DMSO + 586 μ l water. After 15 min, TCEP was removed on a 1 cm $\times$ 5 cm Dowex 1 X-8 column (Cl⁻ form), equilibrated with 10 mM bisTris, pH 6. The flow-through was pooled and found to be 22.6 mM thiol, using DTNB [16]. Six and 4 ml of the AOAc-cysteamine solution was added to the solutions of bromoacetylated BSA and the pH adjusted to 8. The reaction was allowed to proceed overnight in the dark and was then dialyzed for 2 days at 4 °C against multiple changes of saline.

2.4. Oxidation and aminoxy functionalization of polysaccharides

Except where indicated, the oxidation protocol for dextran was as follows: a 10 mg/ml dextran solution in 0.1 M sodium acetate, pH 5, was made 10 mM in sodium metaperiodate (from a freshly prepared 0.5 M solution in water)

and incubated in the dark. The standard oxidation time was 10 min. The reaction was then quenched by the addition of 1/10 volume of 50% glycerol and dialyzed against water in the dark. To functionalize with AO groups, the oxidized dextran was incubated with 0.25 M AOAc at pH 5 or with AO-derivatized proteins. Pn14 polysaccharide was also functionalized with aminooxy groups using a two-step method, in which the polysaccharide was activated with CDAP, derivatized with hexanediamine, bromoacetylated [13], reacted with an approximately 10-fold molar excess of AOAc-cysteamine (prepared as described above), and then dialyzed extensively against water. The product contained five AO groups per 100 kDa of polysaccharide. For the experiment described in Section 3.7, AO-Pn14 was prepared by oxidizing the polysaccharide with sodium metaperiodate for 10 min at pH 5, desalted and reacted with 100 mM bis(AOAc)ethylenediamine. The desalted product contained 3.5 aminooxy groups per 100 kDa of polysaccharide.

2.5. Oxidation of glycoproteins and two-step aldehyde-derivatization of proteins

Ovalbumin was oxidized by preparing a 35 mg/ml solution in 25 mM sodium acetate, pH 5 and adding sodium metaperiodate to a final concentration of 12 mM. Aliquots were quenched at various times by the addition of 1/10 volume 50% glycerol and dialyzed against 10 mM sodium acetate, 150 mM NaCl, pH 5 in the dark. Gp350, at 7 mg/ml in 0.1 M sodium acetate, pH 5, was oxidized in 10 mM metaperiodate for 8 min on ice, in the dark, quenched with glycerol and then diafiltered. Aldehyde-derivatized diphtheria toxoid (DT) was prepared by a two-step method. First, DT (15 mg/ml) was derivatized with a seven-fold molar excess of *N*-succinimidyl bromoacetate at pH 8, followed by a 100-fold molar excess of mercaptoglycerol. Excess reagent was removed by diafiltration using an Amicon Ultra4 (30 kDa cutoff) device. Second, the diol-derivatized protein was oxidized using 10 mM sodium metaperiodate at pH 5 for 10 min, quenched with glycerol, and reagents removed by diafiltration.

2.6. Conjugation of proteins to polysaccharides

Conjugations were performed in sodium acetate buffer at pH 5 in the dark at room temperature, normally at 1 mg protein/mg polysaccharide. Details are given in the figure legends. Monitoring of the conjugation reaction was by SEC HPLC. The aldehyde-diphtheria toxoid was combined with AO-Pn14 and adjusted to pH 5. The final solution contained 5 mg/ml DT, 4.2 mg/ml Pn14 in 75 mM sodium acetate, pH 5. Oxidized gp350 was combined with AO-Pn14 and made pH 5. The final solution contained 3.5 mg/ml gp350, 3.6 mg/ml Pn14 and 0.1 M sodium acetate. In each instance the reaction was carried out overnight in the dark at room temperature. Conjugates were purified by gel filtration on a S400HR column (1 cm × 60 cm) equilibrated with PBS.

2.7. Chromatography and assays

Thiols were determined using DTNB and an extinction coefficient of $13,600 \text{ M}^{-1}$ [16]. Amines were measured using TNBS [18]. Aminooxy groups on polysaccharides were assayed using TNBS, using AOAc as a standard at a wavelength of 500 nm. Unless otherwise indicated, protein concentrations were assayed using either the Coomassie Plus Reagent according to the manufacturer's instruction (Pierce) or by absorbance at 280 nm, using the underivatized protein as a standard. An extinction coefficient of 44,000 and $33,000 \text{ M}^{-1}$ was used for BSA and ovalbumin, respectively. Carbohydrate concentrations were determined using the method of Monsigny et al. [17] with the corresponding polysaccharide as the standard. SDS-PAGE 4–12% NuPage gels were from Invitrogen. HPLC size exclusion chromatography (SEC HPLC) was performed using either a Biosep SEC 3000 column (Phenomenex) or a 1 cm × 30 cm Superose 6 (prep grade, Pharmacia), with detection at 280 nm. Samples were filtered using a Millipore MC (0.45 μ) spin device prior to chromatography. Since monomeric proteins and high molecular weight polysaccharides were used, conjugation was evidenced by a shift in UV area to the void volume. The percent of conjugation was determined from the areas of the monomeric and polymeric UV peaks.

2.8. Immunization and ELISAs

Groups of 5 BALB/c mice were immunized subcutaneously with 1 μ g of conjugated polysaccharide mixed with 1 mg of Alhydrogel. Mice were bled and boosted on day 10 and bled again on day 23. Anti-Pn14 IgG titers in sera were determined by ELISA as described in [13]. To test for biotinylated Pn3 or Pn14 capsular polysaccharide, Nunc Immunosorb plates were coated with streptavidin, washed, and then incubated with the dilutions of the respective polysaccharide starting from 1 μ g/ml in PBS. After an overnight incubation, each was washed, and probed with an anti-Pn3 or Pn14 antibody.

3. Results

3.1. Functionalization of a polysaccharide using aminooxy reagents

Oxidation of carbohydrates with sodium periodate generally requires the presence of vicinal hydroxyls. From their known structures [18], we expected that pneumococcal type 14 but not type 3 would be easily oxidized. Subsequently, type 14 should react with aminooxy reagents, but type 3 should not. To test this hypothesis, pneumococcal types 3 and 14 were incubated with 10 mM sodium metaperiodate at pH 5 for 10 min, quenched with glycerol, dialyzed, and then incubated with an aminooxy biotin reagent. Biotinylation was determined by capture on streptavidin-coated ELISA plates,

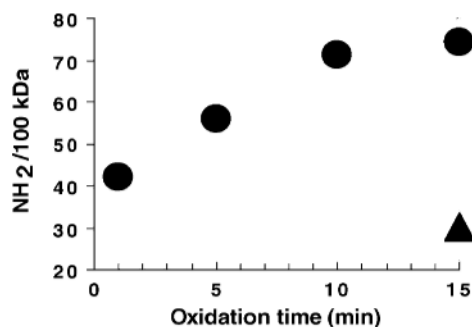


Fig. 2. Reaction of aminoxy or hydrazide with oxidized dextran. T2000 dextran (10 mg/ml, 10 mM NaAc) was oxidized with 10 mM sodium periodate in the dark for 1, 5, 10 and 15 min, quenched by the addition of glycerol and then dialyzed into saline in the dark. A 1 ml aliquot of each was incubated with 0.5 ml 0.5 M aminoxy acetate, pH 5 for 4 h and then dialyzed overnight into saline. To determine the degree of oxime formation, an equal volume of 0.5 M ethylenediamine pH 5, was added, followed by addition of EDAC (freshly prepared at 100 mg/ml in water) to make the solution 7 mg/ml EDAC. Following exhaustive dialysis against saline, each was assayed for dextran (using the resorcinol/sulfuric acid assay) and amines (using TNBS with a glycine standard). For comparison, dextran oxidized for 15 min was also incubated with adipic dihydrazide and assayed for dextran and hydrazide (using TNBS with ADH as the standard). The molar ratio of amines (or hydrazides) per 100 kDa of dextran was calculated. (Circles) amines; (triangle) hydrazides.

followed by detection with anti-Pn3 or -Pn14 antibody. Pn14 but not Pn3 was detected in the capture ELISA, indicating that only the former was conjugated to aminoxy biotin. Thus, the reaction of the aminoxy reagent with the polysaccharide depended on the presence of aldehydes.

While some clinically important polysaccharides contain reactive groups such as carboxyls (e.g. *Neisseria meningitidis* C capsular polysaccharide), many other important polysaccharides contain few reactive chemical groups that can be linked to proteins without some kind of activation or functionalization of the carbohydrate. We initially used the reagent aminoxy acetate (AOAc) to add carboxyl groups to aldehyde groups on polysaccharides via oxime formation. The carboxylated carbohydrate can be further functionalized with a crosslinking reagent [6,19] or directly linked to protein using a water-soluble carbodiimide such as EDAC. As a model high molecular weight polysaccharide that could be easily oxidized, we used T2000 dextran, a β -1-6-glucose polymer. Dextran was oxidized for 1–15 min with sodium periodate and reacted with excess aminoxy acetate at pH 5. To determine the degree of carboxylation of the polymer, the carboxyls were converted to amines using ethylenediamine and EDAC. The amine to dextran ratio was then determined. To compare hydrazone with oxime formation, dextran oxidized for 15 min was also incubated with 0.5 M adipic dihydrazide (ADH).

The degree of amine derivatization increased with oxidation time of the polysaccharide and plateaued at about 75 amines per 100 kDa of polysaccharide (Fig. 2). This is approximately 1 aminoxy per 12 glucose repeat units

and is consistent with the theoretical maximum, based on the starting ratio of 6 mol periodate per mole glucose. The degree of derivatization with ADH was much lower, about 30 hydrazides per 100 kDa of dextran (Fig. 2). This may reflect a less efficient reaction of the hydrazide or, more likely, reversibility of the Schiff base (hydrazone) compared with the more stable oxime product of the aminoxy group with the aldehyde.

We next examined the ability of carbonyls to react with the aminoxy group as a function of pH. Acetone, which has significant absorbance at 280 nm, was combined with an equimolar amount of AOAc at various pH values. Monitoring the reduction in absorbance provided a convenient way to monitor the reaction of the aminoxy group. The extent of oxime formation was similar over the pH range of 3–11, indicating that the reaction can be performed over a broad pH range. At 1 min, the absorbance was reduced by 35% at pH 1 and by about 55% at pH 11. Over 10 min, the absorbance at all pH values approached zero, indicating complete reaction of the ketone. Furthermore, the rapidity of the addition of a small ketone to aminoxy groups provides a convenient way to “cap” residual aminoxy groups.

These experiments show that low molecular weight aminoxy reagents can be used to usefully functionalize polysaccharides for conjugation.

3.2. Conjugation of an aminoxy-derivatized protein to an oxidized polysaccharide

Next we examined the utility of aminoxy reagents for conjugation of proteins to polysaccharides. In our initial work we used a model system of aminoxy-derivatized BSA and oxidized dextran. AO-BSA was prepared by using a water-soluble carbodiimide to mediate coupling of bis(AOAc)tetraethyleneglycol to protein carboxyls. Under the conditions used (described in Methods), the BSA product was heavily derivatized with aminoxy groups and protein aggregates formed as a result of protein crosslinking (Fig. 3, lane 5). Reaction of the purified AO-derivatized protein with TNBS at pH 9.3 gave an intense reddish orange color, characteristic of the aminoxy functionality [20], whereas primary amines react with TNBS to give a yellow color. Dextran was oxidized at pH 5 with 10 mM sodium metaperiodate for 1, 5, 10 and 15 min. The AO-BSA and oxidized dextrans were combined at a 1:1 weight ratio and incubated overnight at pH 5. No gelation was evident, although an increase in viscosity was noticed, indicating that crosslinking was occurring. When examined by SDS-PAGE, none of the protein was able to enter the gel, indicating that all of the protein had been converted to a high molecular weight form (Fig. 3, lanes 1–4). SEC HPLC indicated that all of the monomeric BSA now eluted in the void volume. The high efficiency of the coupling, even at the shortest dextran oxidation time, was probably due to the high level of aminoxy derivatization of the protein. It is worth noting that the conjugates were stable even under the conditions of the electrophoresis. In the absence of oxida-



Fig. 3. SDS-PAGE of AO-BSA + oxidized dextran conjugates. Solutions of T2000 dextran (2 ml at 10 mg/ml, pH 5) were oxidized by the addition of 41 μ l of 0.5 M sodium periodate and quenched 1, 5, 10 and 15 min by the addition of 200 μ l 50% glycerol. Each was exhaustively dialyzed against saline. Carbodiimide mediated addition of bisAO to the BSA is described in Section 2. Conjugates were prepared by combining equal weights of oxidized dextran and AO-BSA at pH 5 (final concentration 4 mg/ml each) and incubating overnight in the dark at room temperature. (Lanes 1–4) BSA–dextran, oxidized 1 min (lane 1), 5 min (lane 2), 10 min (lane 3) and 15 min (lane 4); (lane 5) AO-BSA only.

tion of the polysaccharide, no coupling was evident by SEC HPLC.

All four conjugates were pooled and fractionated on an S400HR gel filtration column. Consistent with the results of the SDS-PAGE gel, virtually all the protein from the gel filtration run was found in the void volume fraction of the eluant.

3.3. Stability of oxime-based conjugates

To test the intermediate-term stability of oxime-based conjugates, we prepared conjugates containing high and low levels of protein–polysaccharide crosslinks. To do this, BSA was functionalized at high and low levels with aminooxy groups and reacted with a low or high ratio of oxidized dextran, respectively. The high ratio AO-BSA conjugate solution was made up at 0.2 mg BSA/mg dextran and the low ratio conjugate at 0.8 mg BSA/mg dextran. After an overnight incubation, the reaction was quenched by making each solu-

tion 0.1% acetone overnight, followed by dialysis into saline. No free BSA was observed in the high ratio conjugate, while the low ratio conjugate contained 17% free protein, as determined by SEC HPLC. The reversibility of the conjugation was examined by incubating each conjugate in 0, 10 or 100 mM in AOAc in 0.1 M sodium acetate, pH 5, at room temperature, for up to two weeks. To further explore stabilization, separate aliquots were reduced with 10 mM sodium cyanoborohydride prior to incubation with 0.1 M AOAc. Each was then analyzed by SEC HPLC to determine the percentage of free and high molecular weight protein. Chromatograms of conjugates prepared with protein containing a high ratio of aminooxy groups were essentially unchanged after two weeks of incubation even in the presence of 0.1 M aminooxy acetate. Chromatograms of conjugates made using protein with a low ratio of aminooxy groups were unchanged up to 10 mM AOAc at 24 h. At 0.1 M AOAc, the percent of unconjugated BSA increased from 17 to 28% at 24 h and to 68% after two weeks. The amount of free BSA in both the high and low ratio conjugates was unchanged in the presence of 0.1 M AOAc when the conjugates were pretreated with sodium cyanoborohydride. The amount of free BSA in both the high and low ratio samples without AOAc was unchanged after two weeks of incubation, indicating that the conjugates were stable in the absence of a powerful competing nucleophile. Furthermore, the SEC chromatograms of the solutions without competing AOAc from day 1 and day 14 were similar, indicating that there was no further polymerization, suggesting that the active groups had been capped.

These data suggest that conjugates containing multiple oxime links are stable for at least two weeks at pH 5 and can be reversed only under forcing conditions. Proteins linked to the polysaccharide by a limited number of oximes are more susceptible to reversal under extreme conditions of a high concentration of a competing nucleophile but are stable under normal buffer conditions.

3.4. Chemical reactivity of aminooxy groups

In order to avoid undesired crosslinking when derivatizing proteins with homobifunctional reagents, it is necessary to use a large excess of the reagent [19]. Due to the limited amounts of our bis-aminooxy reagents on hand, we prepared aminooxy-derivatized proteins using a heterobifunctional reagent, (AOAc)cysteamine which was linked via a thioether linkage to bromoacetylated protein. In order for this method to be successful, the aminooxy group must be much less reactive toward the electrophilic reagent than the thiol. We first confirmed that the thiol group would preferentially react with maleimide and bromoacetate, functionalities which are commonly used in bioconjugation. Solutions of 1 mM mercaptoethanol or AOAc were incubated with 2 mM bromoacetate buffered at pH 8. After 3 min, all of the mercaptoethanol had reacted with the bromoacetate, as no thiol groups were detectable with DTNB. In contrast, the concentration of aminooxy groups, determined using TNBS, was

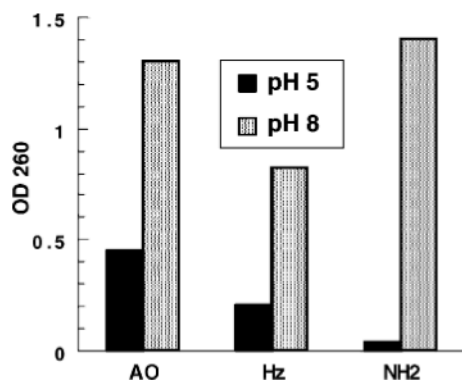


Fig. 4. Reaction of amine, hydrazide and aminoxy functional groups with an NHS ester at pH 5 and 8. Solutions were prepared of 1 mM ethanolamine, hydrazide (as adipic dihydrazide) or aminoxy acetate in 0.1 M sodium acetate, pH 5 or HEPES, pH 8. At $t=0$ 20 μ l/ml of freshly prepared NHS acetate in methanol was added and the absorbance at 260 run read at 30 min. Control solutions without the nucleophile were used to correct the data for hydrolysis at each pH. Each point was done in triplicate.

unchanged after 1 h, indicating low or no reactivity. The reactivity of maleimide with aminoxy and thiol groups was determined by monitoring the absorbance at 301 nm [19]. After 2.5 h of incubation of 3.1 mM *N*-ethylmaleimide with 25 mM AOAc at pH 6.5, less than 7% of the maleimide had reacted. Even after 20 h of incubation, the remaining maleimide was still 55% of the control solution, indicating that the addition reaction was slow at pH 6.5. Under the same conditions but substituting mercaptoethanol for AOAc, the absorbance at 301 nm dropped to zero within 5 min, indicating complete reaction of the maleimide with the thiol.

We also examined the relative reactivity of the aminoxy group compared with other common amine nucleophiles. The pK_a of aminoxy groups is in the range of 5–6. Hydrazides have a pK_a of ~ 2 and are unprotonated at pH 5, while primary amines have a pK_a > 9 . Thus, aminoxy and hydrazide, but not amine functional groups, should be nucleophilic at low pH. Adamczyk et al. [21] examined the relative reactivity of amines and aminoxy groups with *N*-hydroxysuccinimide esters as a function of pH. We directly assayed the ability of aminoxy (as AOAc), hydrazide (as adipic dihydrazide), and amine (as ethanolamine) functionalities to aminolyze NHS acetate esters at pH 5 and 8. The reaction was monitored by following the increase in absorbance at 260 run due to the release of *N*-hydroxysuccinimide [22]. Control solutions of the ester at pH 5 and 8 were used to correct for hydrolysis. As indicated in Fig. 4, at pH 5 the order of reactivity was $AO > Hz \gg NH_2$, whereas at pH 8, the aminolysis reaction was more rapid and the order of reactivity was $AO = NH_2 > Hz$. Although hydrazides react with NHS esters over a broader pH range than amines, hydrazides appear to be less nucleophilic than amines in this reaction (Fig. 4). Our data indicate that aminoxy functional groups are more reactive with NHS esters than hydrazides under acidic conditions.

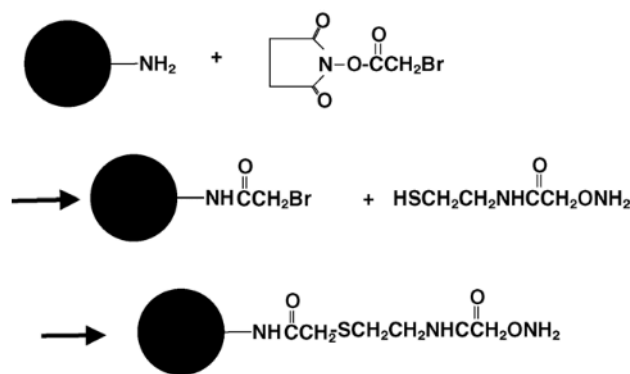


Fig. 5. Scheme showing two-step synthesis of aminoxy-derivatized protein. A limited number of amines on the protein are bromoacetylated and then reacted with AOAc(cysteamine).

3.5. Two-step synthesis of aminoxy-derivatized proteins

Having confirmed the preferential reactivity of the thiol over the aminoxy group in adding to bromoacetate or maleimide, we proceeded with the synthetic scheme for the two-step synthesis of AO-derivatized proteins illustrated in Fig. 5. In contrast to the carbodiimide coupling method, which modifies carboxyl groups, this scheme converts primary amines to aminoxy groups via a spacer. The degree of bromoacetylation can easily be controlled by, for example, varying the derivatization ratio. The addition of the thiol reagent to the bromoacetate group is very efficient and only a small excess needs to be used to obtain complete reaction [23,24]. Using this method, we observed minimal crosslinking of the AO-protein, as determined by SEC HPLC.

The derivatization reaction can be performed as a “one-pot” reaction. Due to the high efficiency of bromoacetylation with NHS bromoacetate, there is only a small amount of free bromoacetate left in solution. A small molar excess (1.5 mol/mol) of thiol-aminoxy reagent over the total amount of NHS bromoacetate is added to the reaction mixture and the free reagents removed only after this step. This one-pot process eliminates the need to purify the intermediate bromoacetylated protein [23,24]. This same two-step method can be used to convert amino-functionalized polysaccharides to aminoxy-functionalized polysaccharides (see below).

In order to illustrate the use of the two-step method, BSA was first bromoacetylated at ratios of 0–40 mol of NHS bromoacetate per mol of BSA. Under the conditions used, approximately 50% of the bromoacetate labeled the protein in each reaction (Fig. 6A). Each product was then reacted with a 1.5 molar excess of AOAc-cysteamine to functionalize with AO groups.

The series of AO-derivatized BSA was incubated overnight with oxidized dextran of medium molecular weight at pH 5 and then analyzed by SEC HPLC. This fraction eluted in the included volume but before the BSA, thus allowing for lower levels of protein conjugation to be monitored. As expected, the extent of conjugation increased as the number

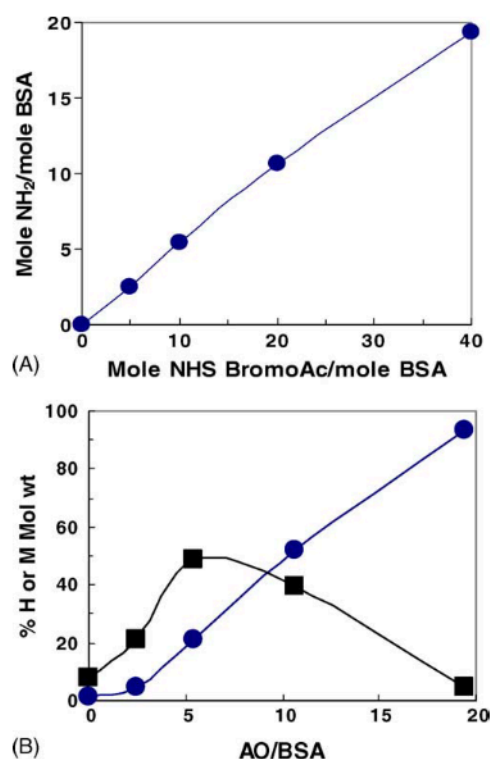


Fig. 6. Two-step synthesis of AO-BSA and conjugation to oxidized dextran. (A) Bromoacetylation of BSA. BSA was bromoacetylated by reacting 0–40 $\times$ molar excess of NHS bromoacetate as described in Section 2. The degree of labeling was determined by assaying for free amines using the TNBS assay with BSA as the standard. (B) Conjugation of aminooxy-derivatized BSA to oxidized dextran. The various aminooxy-derivatized BSA were reacted with an equal weight of oxidized dextran and analyzed by SEC HPLC. The high molecular weight fraction (H) is the percent that eluted in the void volume of a Superose 6 column (circles). The medium molecular weight fraction (M) is the percent that eluted between the void volume and the monomeric protein peak (squares).

of functional groups on the protein increased (Fig. 6B), as indicated by the decreased area of the free protein and the increased area of material eluting earlier. Additionally, as the number of aminooxy groups increased, a larger percentage of the protein was in the void volume, suggesting that there was also more interchain crosslinking, leading to higher molecular weight complexes. Thus, control over the conjugation can be effected by limiting the number of active groups and the conjugation conditions.

3.6. Aldehyde-functionalized proteins conjugated to aminooxy polysaccharides

In the previous examples, polysaccharides containing aldehydes were linked with aminooxy-derivatized proteins. We next looked at the reverse linkage model, in which aldehydes were created on the protein component and reacted with aminooxy-functionalized polysaccharide. Aminooxy groups can be added to carbohydrates by a variety of approaches. For example, bis(AOAc)ethylenediamine can be added to oxidized polysaccharides. Alternatively, bis-aminooxy reagents

can be added to polysaccharides activated with CDAP, as has been described for diamines [13]. Both these methods require a large excess of the bisAO reagent in order to minimize crosslinking. However, since we had only limited amounts of bis-aminooxy reagent, we generally opted for a multi-step method similar to the two-step procedure used to label proteins. In this protocol, the polysaccharide was activated with CDAP and derivatized with hexanediamine, bromoacetylated [13] and subsequently reacted with a small molar excess of AOAc-cysteamine. Again, if limiting amounts of reagents are used, the derivatization can be performed without purifying the intermediates.

3.7. Conjugation of a model glycoprotein to an aminooxy-functionalized polysaccharide

Ovalbumin (OVA), used as a model glycoprotein, was oxidized with 10 mM sodium periodate for 0–15 min at pH 5. The details of the preparations are given in Fig. 7. Each OVA(ox) was incubated overnight at pH 5 with AO-

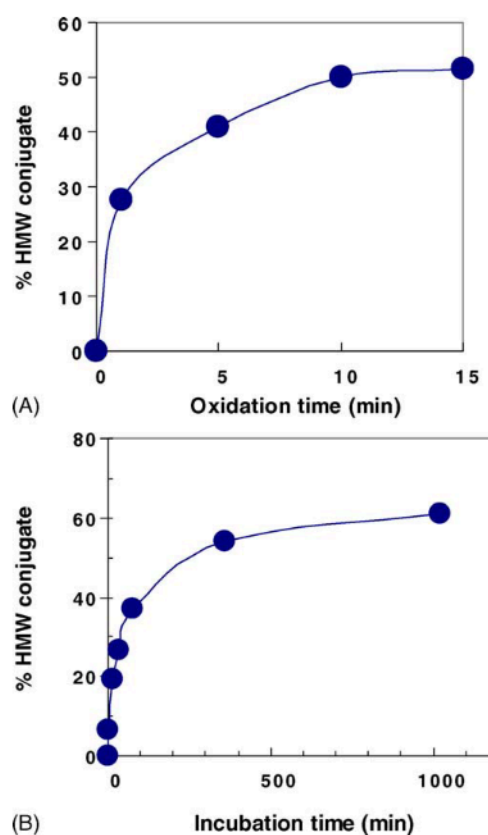


Fig. 7. Reaction of oxidized ovalbumin with AO-Pn14. (A) Conjugation of increasingly oxidized OVA with AO-Pn14. OVA, oxidized for 0–15 min with 10 mM sodium periodate at pH 5, was incubated with AO-Pn14 polysaccharide. Final concentrations were 2.9 mg/ml OVA and 2.2 mg/ml Pn14 in 110 mM sodium acetate, pH 5. After 18 h of incubation in the dark at room temperature, the samples were assayed by gel filtration on a Superose 6 column. (B) Kinetics of conjugation of oxidized OVA with AO-Pn14. OVA, oxidized for 10 min, was incubated with AO-Pn14. At the indicated times, aliquots were assayed by gel filtration on a Superose 6 column.

derivatized Pn14 (1.3 mg OVA/mg Pn14) and then analyzed by SEC HPLC. A progressive increase in the percent of the area of the high molecular mass and corresponding decrease in the area of the OVA peak was observed for increasingly oxidized glycoprotein (data not shown). Under the conditions used, maximum conjugation occurred with OVA oxidized for 10 min (7A). Several control experiments were performed—OVA(ox)+polysaccharide; OVA+AO-polysaccharide, and OVA(ox) alone. Only the combination of OVA(ox)+AO-polysaccharide yielded protein eluting in the void volume. In the absence of AO-polysaccharide, the OVA(ox) remained monomeric, as determined by SDS-PAGE and SEC HPLC. The lack of polymerization of the protein in the absence of AO-polysaccharide indicated that under the conjugation conditions used, the oxidized protein did not aggregate.

In a separate experiment, the conjugation kinetics of the oxidized OVA with AO-Pn14 was examined. OVA was oxidized for 10 min and then incubated at pH 5 with AO-Pn14. The reaction was monitored by SEC HPLC. There was a progressive increase in the percent of high molecular weight conjugate. And the reaction was essentially complete in 6 h (Fig. 7B). Overcrosslinking and gelling is often a problem in the preparation of protein–polysaccharide conjugates. To determine whether the conjugates became insoluble during the kinetic study, the total areas of the conjugated and unconjugated peaks were determined. The sum of the areas was found to be unchanged as the conjugation progressed, indicating that the conjugate had remained soluble during the conjugation process and that the resulting conjugates could pass through the 0.45 μ m spin filters used prior to chromatography.

3.8. Aldehyde-functionalized proteins

Proteins commonly used in conjugate vaccines, such as tetanus toxoid, diphtheria toxoid or CRM197, do not contain carbohydrate and so cannot be oxidized to produce aldehydes. We therefore devised a simple method for functionalizing these proteins with aldehydes. In this approach, the protein is first derivatized with a diol which is subsequently oxidized to produce an aldehyde-functionalized protein. Initially, glyceric acid was linked directly to protein amines using carbodiimide, to form an amide-linked 1,2-diol. However, since this risked crosslinking the protein, we found it more convenient to use a two-step protocol. The protein was first bromoacetylated and then reacted with 1-mercapto-2,3-propanediol (mercaptoglycerol). In each case, mild oxidation of the diol with sodium periodate yielded an aldehyde-derivatized protein. This scheme is shown in Fig. 8.

Unlike the BSA labeled using carbodiimide, the protein labeled in this two-step protocol remained monomeric, as determined by SEC HPLC. Aldehyde-derivatized proteins can be used for Schiff base or hydrazone-based conjugates, as well as the oxime formation described here (see below). Several control experiments were performed. Only BSA func-

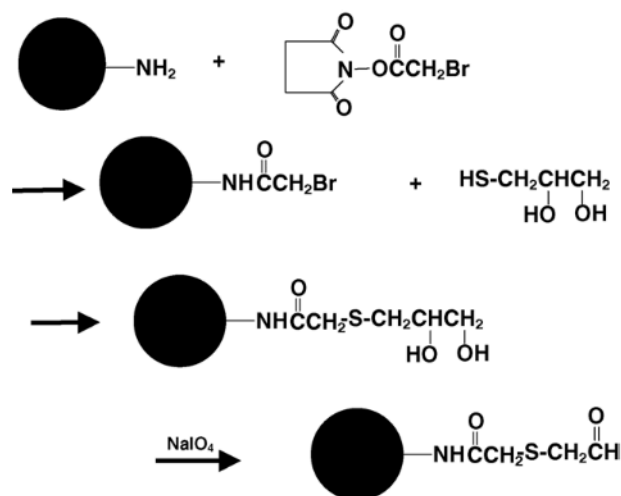


Fig. 8. Scheme showing two-step synthesis of aldehyde-derivatized proteins. A limited number of amines on the protein are bromoacetylated and then reacted with a two-fold molar excess of mercaptoglycerol. Following diafiltration, the diol is oxidized with 10 mM sodium metaperiodate at pH 5 for 10 min to create the aldehydes.

tionalized with mercaptoglycerol and subsequently oxidized formed a high molecular weight conjugate with AO-dextran. Neither bromoacetylated BSA nor bromoacetylated BSA reacted with mercaptoethanol, when treated with sodium metaperiodate, coupled to AO-dextran. Combining of the aldehyde-functionalized BSA with AO-Pn14 yielded a high molecular weight conjugate, with about 80% of the protein coupled, as determined by SEC HPLC.

3.9. Comparison of oxime versus hydrazone crosslinking

Hydrazone formation has been used in reductive amination [6,25]. The reported examples used oxidized polysaccharides and ADH-labeled protein and the described reactions are markedly more efficient and rapid than reductive amination with underivatized protein. To compare the relative efficiency of coupling using hydrazones and oximes, we prepared a closely matched set of hydrazide and aminoxy-derivatized dextran, each with approximately the same number of functional groups. Hydrazide-dextran was prepared by linking ADH to CDAP-activated dextran [23]. AO-dextran was made using the multi-step protocol described above. The two derivatized polysaccharides differed only in the pendant functional group. Following an overnight incubation of hydrazide-dextran or AO-dextran with aldehyde-derivatized BSA the conjugates were analyzed by SEC HPLC (Fig. 9). Both dextrans condensed with aldehyde-functionalized protein, but the high molecular weight area peak was about twice as large for the oxime conjugate as for the hydrazone conjugate (44% versus 22%). The total area (conjugated + unconjugated) for each preparation was approximately the same, indicating similar total masses of protein and minimal loss due to overcrosslinking. The absorbance contribution from derivatized dextrans was negli-

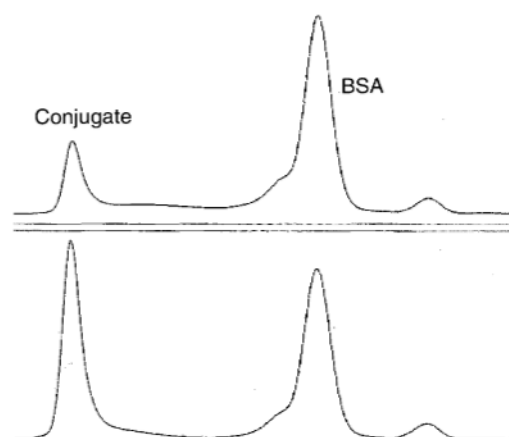


Fig. 9. SEC HPLC of aldehyde-BSA + hydrazide-dextran (upper panel) or aminoxy-dextran (lower panel). BSA, functionalized using the two-step method and containing approximately 5 mol aldehydes per mol BSA was incubated overnight with hydrazide-dextran or AO-dextran, containing 12 and 10 functional groups per 100 kDa polysaccharide, at pH 5. The final solutions were 7.5 mg/ml BSA and 5 mg/ml dextran. The extent of conjugation was assayed by SEC HPLC using a Superose 6 column, monitoring at 220 nm. Aldehyde-BSA + hydrazide-dextran (upper panel) or aminoxy-dextran (lower panel).

gible (not shown). In this experiment, suboptimal conditions were used in order to distinguish the reactivity of the two functionalities. When more optimal conditions are used (e.g. a higher concentration of reagents or higher functional group density), complete conjugation of the protein was easily obtained with both the hydrazide and aminoxy dextrans.

3.10. Oxime conjugates based on ketones

In order to evaluate the suitability of the condensation of ketones with aminoxy groups for the synthesis of conjugate vaccines, we prepared ketone-derivatized BSA by reaction of monomeric BSA with the NHS ester of levulinate. The ketone-derivatized protein (approximately 24 levulinates/BSA) was then reacted with either amino-dextran or AO-dextran at pH 6.5. The higher pH was used because at pH 5 the levulinate-BSA began to come out of solution. After 3 days at room temperature, each was analyzed by SEC HPLC. Less than 1% of the protein in the amino-dextran solution eluted in the void volume, whereas 55% of the protein in the AO-dextran solution eluted in the void volume (data not shown). While conjugation conditions were not further optimized (e.g. increasing concentrations, increasing AO functionalization of the polysaccharide), this experiment illustrates the feasibility of using ketones instead of aldehydes for oxime-based conjugates, thereby avoiding the need to perform an oxidative step.

3.11. Immunogenicity of protein–polysaccharide oxime-based conjugates

In order to study the immunogenicity of oxime-based conjugate vaccines, two conjugates of pneumococcal type 14

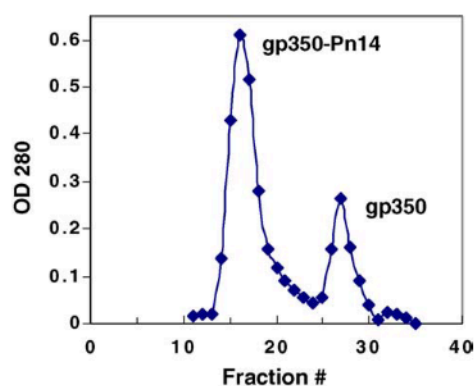


Fig. 10. Gel filtration of a conjugate of oxidized gp350 to AO-Pn14. Gp350 was oxidized with sodium periodate, conjugated to AO-Pn14 and fractionated on a 1 cm × 60 cm S400HR gel filtration column, equilibrated with PBS. One milliliter fractions were collected and the absorbance at 280 nm read. Details are given in Section 2.

polysaccharide were prepared. For the first conjugate, recombinant gp350, a glycosylated Epstein-Barr viral protein that binds to the primate complement receptor [14], was purified from yeast cells. Small-scale experiments, varying the oxidation time of the glycoprotein and the protein/polysaccharide concentrations with analysis by SEC HPLC were performed to determine the conditions which yielded the maximum amount of soluble conjugate. These conditions were duplicated at the 10 mg scale (see Section 2) and the conjugated product fractionated by gel filtration, with about 70% of the protein recovered in the high molecular weight fraction (Fig. 10). Recovery of the polysaccharide was at least 75%, even after an unknown amount of the conjugate was used for analysis. The conjugated protein was more effective than the protein alone in competing labeled gp350 from the complement receptor (G Sen, manuscript in preparation), indicating that the complement receptor binding domain of the conjugated gp350 was intact and multivalent. Previous attempts in our lab to prepare this conjugate using thioether conjugation methodology [26] had resulted in products with only low yields and moderate binding activity.

For the second conjugate, diphtheria toxoid, derivatized using the two-step method with approximately five mercaptoglycerol groups per mole DT, was oxidized, reacted with AO-Pn14 (the same lot of AO-Pn14 was used as used to prepare the gp350 conjugate) and fractionated by gel filtration (data not shown). The purified gp350 and DT pneumococcal conjugates each had a ratio of approximately 0.8 mg protein per mg of Pn14.

Mice were immunized twice with 1 µg polysaccharide of the DT- or gp350-Pn14 conjugates, which had been adsorbed to the aluminum hydroxide adjuvant, Alhydrogel. Gp350 does not bind to mouse complement receptor and so, in this system, the protein acted as a simple carrier protein. Both gp350 and the DT conjugates induced high anti-Pn14 antibody titers that increased after the second immunization (Fig. 11). The increase with boosting was statistically signifi-

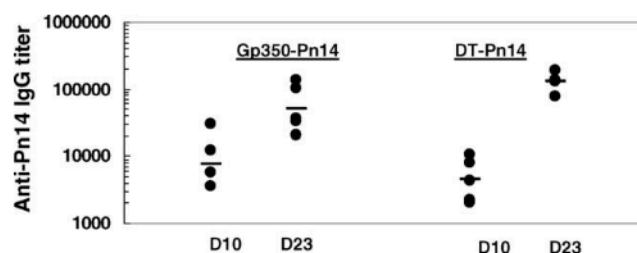


Fig. 11. Immunogenicity of Pn14 conjugates. Groups of 4BALB/c mice were immunized on days 0 and 11 with 1 μ g of the polysaccharide as a conjugate, adsorbed to Alhydrogel. Mice were bled on days 10 and 23 and individual sera were assayed by ELISA for anti-Pn14 IgG antibody titers.

cant for each conjugate ($p = 0.05$ for the gp350 conjugate and $p = 0.001$ for the DT conjugate). When mice were immunized with Pn14 alone, the polysaccharide induced low anti-Pn14 IgG antibody titers and no boosting was observed (data not shown).

4. Discussion

Oximes, formed by the condensation of carbonyl and aminoxy functionalities, have been used to covalently join polypeptides [27–30], to prepare multiple antigenic peptides [31–36] and to label and crosslink oligonucleotides [37–39]. Aminoxy PEG has been used to pegylate proteins [40]. Oxime chemistry has also been used to link synthetic oligosaccharides to proteins for diagnostic reagents [41–49]. A phase 1 clinical trial has been conducted with a polyoxime malaria vaccine, without adverse affects [50].

Oxime chemistry has great potential in bioconjugation and aminoxy reagents have been used to replace hydrazide compounds in conjugation reactions, as described in the above references. For this work, we introduced two useful aminoxy reagents starting from BOC-AOAc, a bisAO reagent and a mercapto-AO reagent. A number of other routes to aminoxy reagents have been described [51–54], including AO-maleimide [55] and AO-dithiopyridine heterobifunctional reagents [56]. These reagents would similarly be useful for the synthesis of conjugate vaccines using oxime chemistry.

There are two major models of glycoconjugates [6]. In the “matrix” or “lattice” model, multiple reactive sites exist on the carbohydrate, allowing for the possibility of extensive inter- and intra-polymer–protein crosslinking. At the other extreme are “neoglycoproteins” in which there is only one reactive site on the carbohydrate polymer and no crosslinking of the protein. Both models can yield effective vaccines and are found in currently licensed conjugate vaccines. Each approach has its advantages and disadvantages. For example, it is usually easier to make a defined conjugate using the neoglycoprotein approach [57–59], especially when synthetic glycopolymers are used [60]. However, this approach often involves considerable effort to prepare suitable oligosaccha-

rides. Also, in preparing oligosaccharides from polysaccharides, there is the risk that both structural and conformational epitopes may be lost [61].

Due to the difficulty of linking large numbers of oligosaccharides to a protein, neoglycoproteins tend to have lower levels of carbohydrate loading than lattice conjugates [8,58]. In contrast, in the lattice model the protein is typically linked to a high molecular weight polysaccharide and there is usually more mass of carbohydrate per protein than is found in neoglycoproteins. Lattice structures may also be formed with oligosaccharides containing multiple aldehydes as, for example, results from Hib PRP oxidation [62]. Linking of proteins to high molecular weight polysaccharides, as opposed to linking oligosaccharides to proteins, may be advantageous in that it allows for higher carbohydrate loading. For some polysaccharides, conjugates of high molecular weight polysaccharides have been reported to be more immunogenic and more protective than ones made with low molecular polysaccharides [63]. Low protein:carbohydrate ratios may be especially desirable when multiple capsular serotypes are used in a single vaccination, as high protein levels have been reported to inhibit antibody responses to the carbohydrate [64,65].

The oxime chemistry presented here allows for the synthesis of conjugates following either the matrix or the neoglycoprotein approach. The specific conjugation strategy for a given polysaccharide will, of course, depend on the chemical structure of that polysaccharide. The approaches described allow for great flexibility in terms of the coupling strategy, as indicated in Fig. 1. It is evident that an aminoxy functional group can be substituted for amines in conjugations currently performed using reductive amination. This would include virtually all capsular polysaccharides of current therapeutic interest, including serotypes of pneumonaiae, *N. meningitidis*, group *B streptococci*, Hib and *S. aureus*. Similarly, a carbohydrate which can be activated by any of the chemistries currently employed in clinical vaccines, such as CNBr or CDAP, can be functionalized with an aminoxy group and subsequently linked to an aldehyde-containing protein.

Oxime chemistry is highly efficient and allows the conjugate to be prepared under milder conditions than are required for most conjugation protocols. As a general rule, alkaline conditions are needed to activate carbohydrates or to couple proteins to them. For example, polysaccharides are typically activated with CDAP at pH 9–10, although the extreme alkaline exposure is relatively brief (3–6 min). The conjugation of the protein to the CDAP-activated polysaccharide is at a lower pH, usually pH 8–9, but extends for several hours, which may be detrimental to pH sensitive polysaccharides. For most reductive amination protocols the process of creation of aldehydes on the carbohydrate polymer is relatively gentle, but the conjugation step is usually at pH 8 for >24 h [6,9–12]. Using oxime chemistry allows one to select the appropriate activation chemistry in order to minimize any deleterious effect on the carbohydrate epitopes. For example, polysaccharides

could be activated with CNBr or CDAP and functionalized with aminoxy groups. In other cases, periodate oxidation, performed at pH ~5 would be more appropriate. In this work, we used pH 5 for conjugation, but oxime formation occurs readily over a very broad pH range. Thus, the coupling step, which is generally much slower than the activation step, can be performed at a pH optimal for carbohydrate stability.

Our data suggest that oxime-based conjugate vaccines should be stable, due both to the inherent stability of the oxime bond and the multiplicity of linkages between the protein and the polysaccharide. We found that a highly crosslinked BSA–dextran conjugate was stable for at least two weeks, even in the presence of high concentrations (0.1 M) of competing aminoxy ligand, and a lightly crosslinked conjugate was stable in the presence a competing 10 mM aminoxy ligand. However, if necessary, for example with neoglycoproteins in which there is a single linkage point of an oligosaccharide, the oxime could be reduced to a stable *N,O*-substituted hydroxylamine by reduction with sodium cyanoborohydride [66]. Another consideration in preparing a clinically useful vaccine is the inactivation or capping of active groups. Aminoxy groups can easily be capped by the addition of a low molecular weight aldehyde or ketone such as acetone. Residual aldehydes can be capped by the addition of a low molecular weight aminoxy reagent such as aminoxy acetate.

We observed that an oxidized model glycoprotein, ovalbumin, could be linked efficiently with an AO-functionalized, high molecular weight polysaccharide. However, since most proteins used in conjugate vaccines are of bacterial origin and are not glycosylated, we devised a facile method to create aldehyde-derivatized proteins, employing a simple two-step method to functionalize proteins with 1,2 diols, that could be oxidized to aldehydes under mild conditions. An alternative one-step method involves the addition glyceric acid, a diol, via an amide bond, but an NHS ester of glyceric acid is not commercially available at this time. The two-step method adds a spacer, and the diol, mercaptoglycerol, was added via a thioether linkage to bromoacetylated protein. In each case, the aldehyde was created by mild oxidation of the diol. We found that only limited numbers of aldehydes (approximately five per protein) were needed in order to achieve good conjugation yields with AO-functionalized high molecular weight polysaccharides. We have found it most convenient to prepare a batch of 1,2 diol-labeled protein, using the two-step procedure, without purifying the intermediates, and oxidizing the diol as needed.

Proteins with N-terminal serine or threonine (e.g. 1,2-amino alcohols) can be readily oxidized to an aldehyde [67]. Since carrier proteins relevant to the target disease are being considered for conjugate vaccines [68–70] it is important to preserve critical epitopes on the protein. Genetic engineering of proteins with N-terminal serine or threonines could be used for site-specific crosslinking to an AO-derivatized polysaccharide, allowing maximum preservation of carrier protein epitopes.

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