Vaccine 37 (2019) 1087–1093

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Mitigating base-catalysed degradation of periodate-oxidized capsular polysaccharides: Conjugation by reductive amination in acidic media

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ARTICLE INFO

Article history:
Received 25 June 2018
Received in revised form 12 December 2018
Accepted 29 December 2018
Available online 21 January 2019

Keywords:
Polysaccharide
Degradation
Conjugate vaccine
Reductive amination
β-Elimination

ABSTRACT

Reductive amination coupling an aldehyde-containing polysaccharide, generated by periodate oxidation, with the amino groups in protein has been widely used in the synthesis of glycoconjugate vaccines. The conjugation is often achieved under slightly basic conditions via a Schiff's base intermediate followed by its reduction with sodium cyanoborohydride. We observed that oxidized capsular polysaccharides such as *Streptococcus pneumoniae* type 6B (Pn-6B) and *Haemophilus influenzae* type a (HiA) underwent significant degradation during the conjugation in slightly basic media leading to sub-optimal glycoconjugates. Further study on oxidized Pn-3, Pn-6A, Pn-6C, Pn-2 polysaccharides and dextran provided evidence that the degradation is a result of base-catalysed β-elimination. In contrast to HiA, Pn-2, Pn-3, Pn-6B polysaccharides and dextran, oxidized Pn-6A and Pn-6C polysaccharides were stable under basic conditions due to lack of the leaving group at the β-position of the aldehyde. By performing conjugation of oxidized polysaccharides to bovine serum albumin (BSA) in phosphate buffer at pH 6.0, 6.8, 7.2 and 8.0, we concluded that the reductive amination proceeds best in slightly acidic media, particularly with those β-elimination susceptible polysaccharides.

1. Introduction

The glycoconjugate vaccine is a landmark achievement in vaccine development, that effectively induces memorable protective antibodies in infants [1–3]. Since the introduction of glycoconjugate vaccines against *H. influenzae* type b in 1990s, glycoconjugate vaccines for *N. meningitides* group A, C, Y, W135, and *S. pneumoniae* (up to 13 serotypes) have also been introduced as essential vaccines for young children [4]. Although the immunogenicity of a glycoconjugate vaccine largely depends on the individual polysaccharide and the carrier protein, the method of conjugation can also be an important factor [5]. Among many conjugation methods [2,6], only two major ones have been used for the manufacture of commercial conjugate vaccines. One is based on coupling between a polysaccharide cyanate ester [7,8], derived from reaction with cyanobromide or 1-cyano-4-dimethylaminopyridinium tetrafluoroborate, and the lysine ε–amino groups of the carrier protein, leading to a stable *O*-alkylisourea conjugate [9]. This method is used in a currently licensed 10-valent pneumococcal conjugate vaccine, Synflorix® (GlaxoSmithKlein, UK), though for Pn-18C, addition of adipic dihydrazide (ADH) to the polysaccharide cyanate ester was performed, which was then followed by EDC mediated coupling with the carboxylic acid of tetanus toxoid [10]. A similar method is applied to the synthesis of meningococcal A and C polysaccharide-tetanus conjugates in Menactra (A, C, Y, W135) (Sanofi Pasteur, France). The second major conjugation method is reductive amination, which involves periodate oxidation of polysaccharide to generate active aldehydes, followed by Schiff's base formation with the amino groups of protein and its reduction to stable conjugates [11,12]. Currently licensed Prevnar®13, HibTiter, Meningitec™ (Pfizer, USA), and NeisVac-C® (Baxter, USA) are produced based on this technology. Reductive amination is also a key step in the synthesis of Menjugate (GlaxoSmithKlein, UK) [13] and MenAfriVac (Serum Institute of India, India) [14,15].

Although the reductive amination has been widely used in glycoconjugate synthesis because of its simplicity [16], the main shortcoming was its low yield or coupling efficiency. The technology for polysaccharide-protein conjugate vaccine was first introduced by Jennings et al. in early 1980s to prepare group A, B, and C meningococcal polysaccharide-tetanus toxoid conjugates [11]. Since the polysialic acids in meningococcal B and C capsular polysaccharides are acid labile, basic conditions were chosen for the reductive amination. The conjugation between the terminally oxidized polysialic acid and tetanus toxoid was achieved in phosphate buffer at pH 9.0 in the presence of sodium cyanoborohy-

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https://doi.org/10.1016/j.vaccine.2018.12.072
0264-410X/

dride. Although the Schiff's base can be formed under either basic or acidic conditions, the standard procedure over the next 30 years, albeit with some variations, has followed Jennings' early work by performing the reductive amination under basic conditions. For example, Hib conjugate vaccine was prepared in phosphate buffer at pH 8.0 [12]; pneumococcal glycoconjugates were obtained in sodium bicarbonate buffer at pH 8.0 [17] or in phosphate buffer at pH 7.5 [18]; Group B *Streptococcus* (GBS) capsular polysaccharide-protein conjugates were prepared in phosphate buffer at pH 7.2 [19–21]. However, during our preparation of Pn-6B and HiA polysaccharide-protein conjugates by reductive amination [22], we observed significant degradation of the polysaccharide under basic conditions, which prompted us to investigate how the polysaccharide degradation occurred and how to minimize it in order to improve conjugation. Using Pn-2, 3, 6A, 6B, 6C, and HiA polysaccharides and Dextran T-70 as substrates we were able to establish a plausible mechanism on polysaccharide degradation and consequently improved reductive amination.

2. Experimental

Material: Chemicals, reagents and Dextran T-70 are purchased from Sigma-Aldrich; Pneumococcal polysaccharides type 6A, 6B, and 6C are products from Statens Serum Institute (Denmark), Pneumococcal polysaccharides type 2, and 3 are products from Merck & Co.; *H. influenzae* type a polysaccharide is produced in-house.

Analysis: Agilent Technology 1260 HPLC equipped with either a Superose-6 10/300 or a Superose-12 10/300 column with PBS as eluent was used for SEC-HPLC analysis of polysaccharide degradation and conjugation. ^{1}H NMR spectra of oxidized polysaccharides were recorded in deuterated water on a 500 MHz Varian instrument.

Periodate oxidation of polysaccharide: Polysaccharide was dissolved in 0.1 M sodium acetate buffer at pH 5.7 at 10 mg/mL and 0.15 equivalents of sodium meta-periodate was added. The reaction was kept overnight at room temperature in the dark. The solution was dialyzed against a 10 kDa molecular weight cut off membrane, and oxidized polysaccharide was obtained after lyophilization. The degree of activation was estimated by ^{1}H NMR spectroscopy.

Degradation: Oxidized polysaccharide was dissolved at a concentration of 1 mg/mL in 0.1 M phosphate buffer at pH 6.0, 6.8, 7.2, and 8.0, respectively. The solutions were kept at 37 °C, and aliquots were analyzed by SEC-HPLC to monitor the progressive degradation. The end point of degradation (160 h) was decided when there is no further degradation observed over a period of 24 h.

Conjugation: Oxidized polysaccharides and BSA in a ratio of 1–2:1 (w/w) were dissolved at a concentration of 5–10 mg/mL polysaccharide in 0.1 M phosphate buffer at pH 6.0, 6.8, 7.2, and 8.0. To the solutions were added sodium cyanoborohydride (1:1 w/w to BSA) and the solution was kept at 37 °C for 2 days and aliquots were analyzed by SEC-HPLC to evaluate the conjugation. The

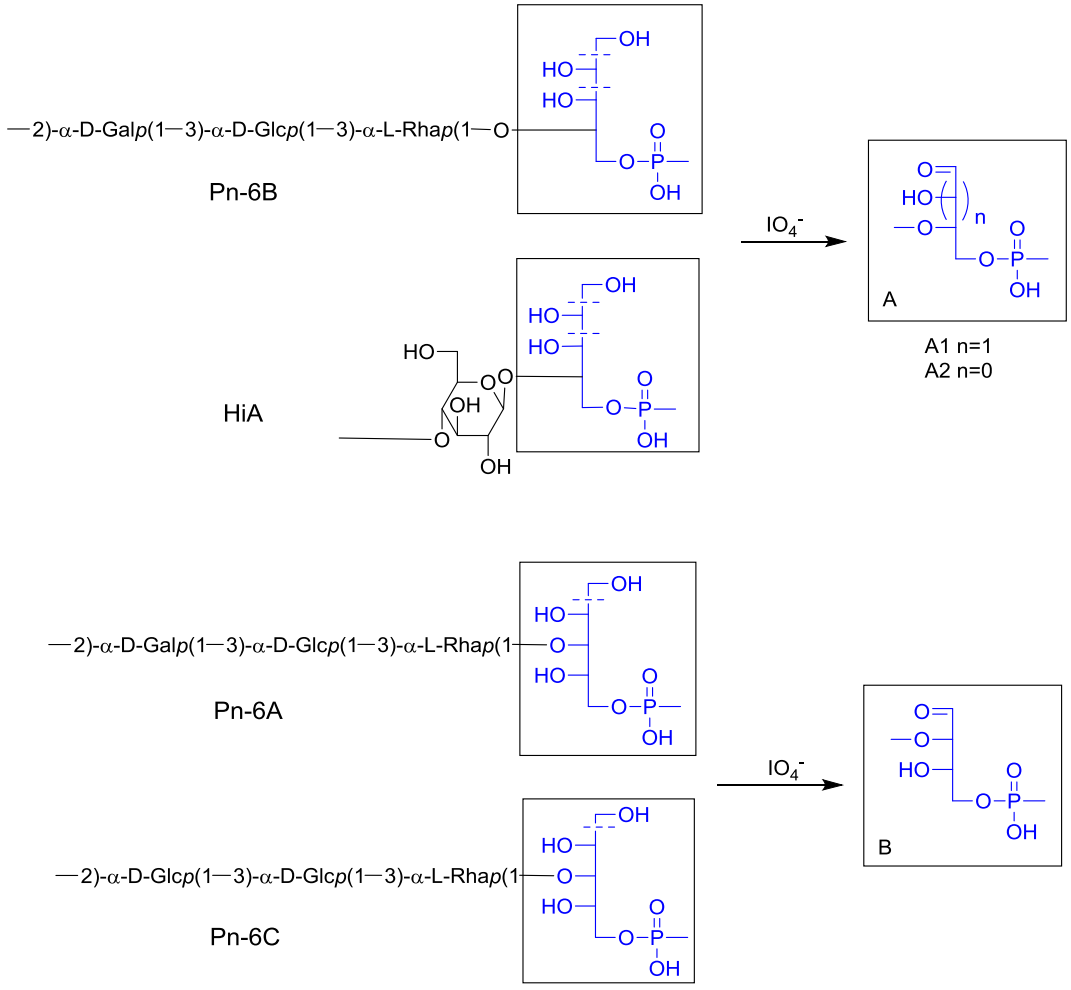
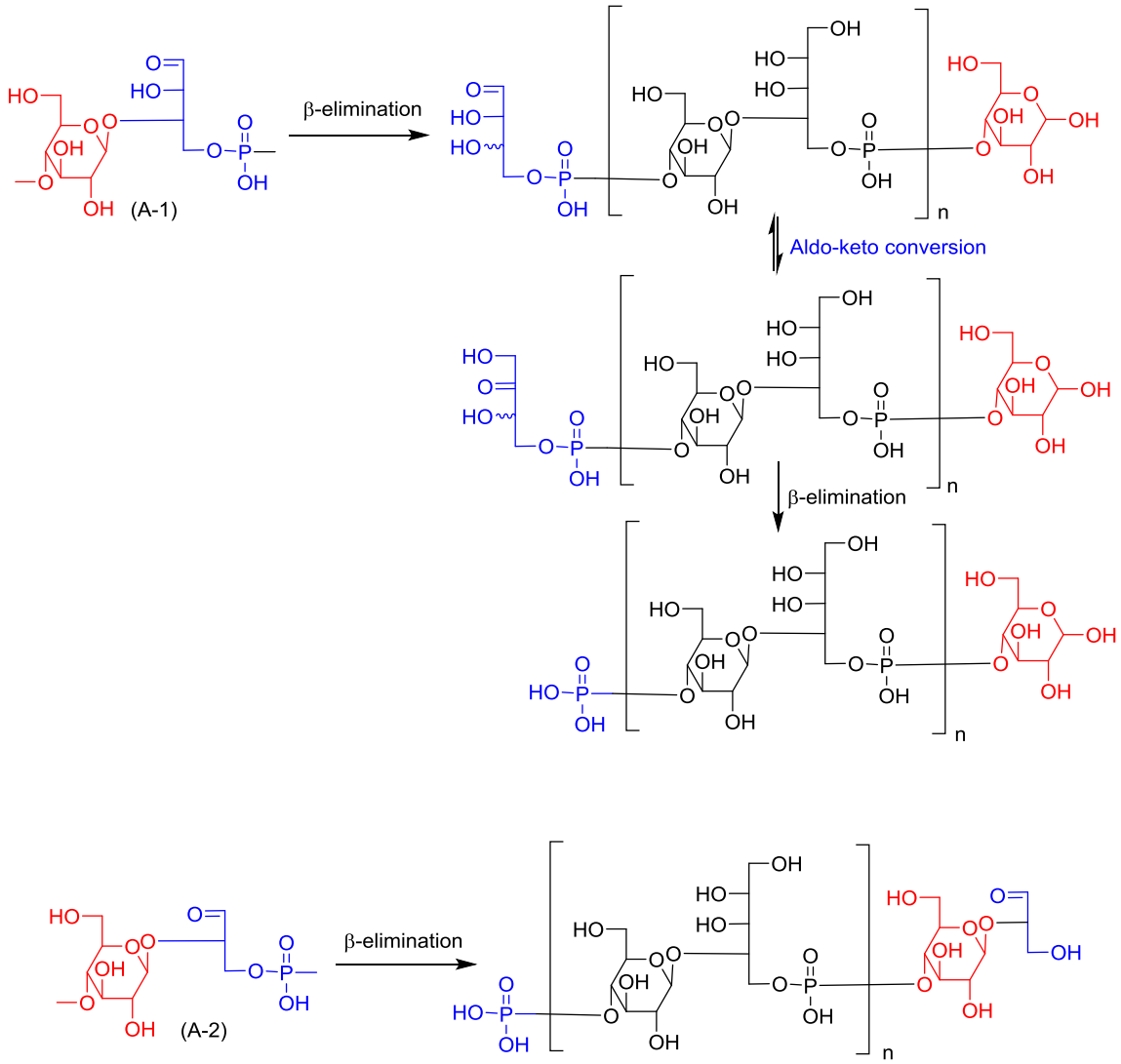


Fig. 1. Oxidation of Pn-6B and HiA polysaccharides forms intermediates (A1 and A2), which are susceptible for β-elimination, whereas, the oxidation intermediate B from Pn-6A and Pn-6C polysaccharides does not undergo β-elimination.

final conjugates were purified by preparative HPLC on a Superose-6 column with PBS as eluent. The fractions containing conjugates were concentrated using a centrifugal filter unit (10 K).

3. Results and discussion

The degradation of oxidized Pn-6B and HiA polysaccharides during conjugation and even in PBS led to a hypothesis that the common subunit, a 4-*O*-glycosyl and 5-*O*-phosphate linked ribitol (see Fig. 1) could be responsible. Similar to the preferred oxidation sites on terminal sialic acid in polysialic acids and GBS polysaccharides, the diols (1,2- and 2,3-) of ribitol in Pn-6B and HiA polysaccharides are more susceptible to periodate oxidation and as a result the ribitol is converted to glycerol aldehyde or erythrose intermediates with substitution at the β-position (see structure A in Fig. 1). On the other hand, when ribitol in the Pn-6A and 6C polysaccharides is oxidized the intermediate is an erythrose substituted by α-*O*-glycose and γ-*O*-phosphate (see structure B in Fig. 1). The periodate oxidation in sodium acetate buffer at pH 5.7 does not affect the polysaccharide size as evidenced by SEC-HPLC analysis. Unlike oxidized Pn-6B and HiA polysaccharides, no degradation in oxidized Pn-6A and 6C polysaccharides was observed when kept in PBS at 37 °C. Since it is known that oxidized polysaccharide may degrade under basic conditions [23] and one of the mechanisms is β-elimination. The fact that oxidized Pn-6A and Pn-6C were stable but not the Pn-6B and HiA suggests that the aldehyde intermediates (structure A in Fig. 1) from Pn-6B and HiA polysaccharides likely underwent a β-elimination/degradation. In the case of oxidized HiA polysaccharide as shown in Scheme 1, the β-elimination can take place in both glycerol aldehyde (A1) and erythrose (A2) intermediates resulting in smaller polysaccharides. Initially, we explored this β-elimination/degradation as a method of depolymerisation to generate saccharides with a terminal aldehyde suitable for conjugation. However, the ^{1}H NMR study on degraded HiA polysaccharide showed that the characteristic signal (at c.a. δ 5.2 ppm) of the hemiacetal proton derived from the aldehyde of oxidized HiA polysaccharide became depleted as indicated by the change of its relative integration to H-2 signal of the glucose (δ 3.4 ppm) from 19% to 8%. Since the aldehyde could be converted to ketone through an aldose-ketose tautomerization under basic conditions [24], and the ketone cannot be easily characterized by ^{1}H NMR analysis, nevertheless, we attempted conjugation of the degraded polysaccharide to BSA, which failed to yield descent conjugate. These observations suggest that the terminal aldehyde/ketone may no longer present after degradation due to a combination of aldose-ketose conversion and β-elimination. A plausible degradation mechanism is illustrated in Scheme 1. Periodate oxidation could produce two intermediates, A1 and A2, and



Scheme 1. A proposed degradation mechanism by β-elimination. Functional aldehyde/ketone groups from intermediate A1 were eliminated.

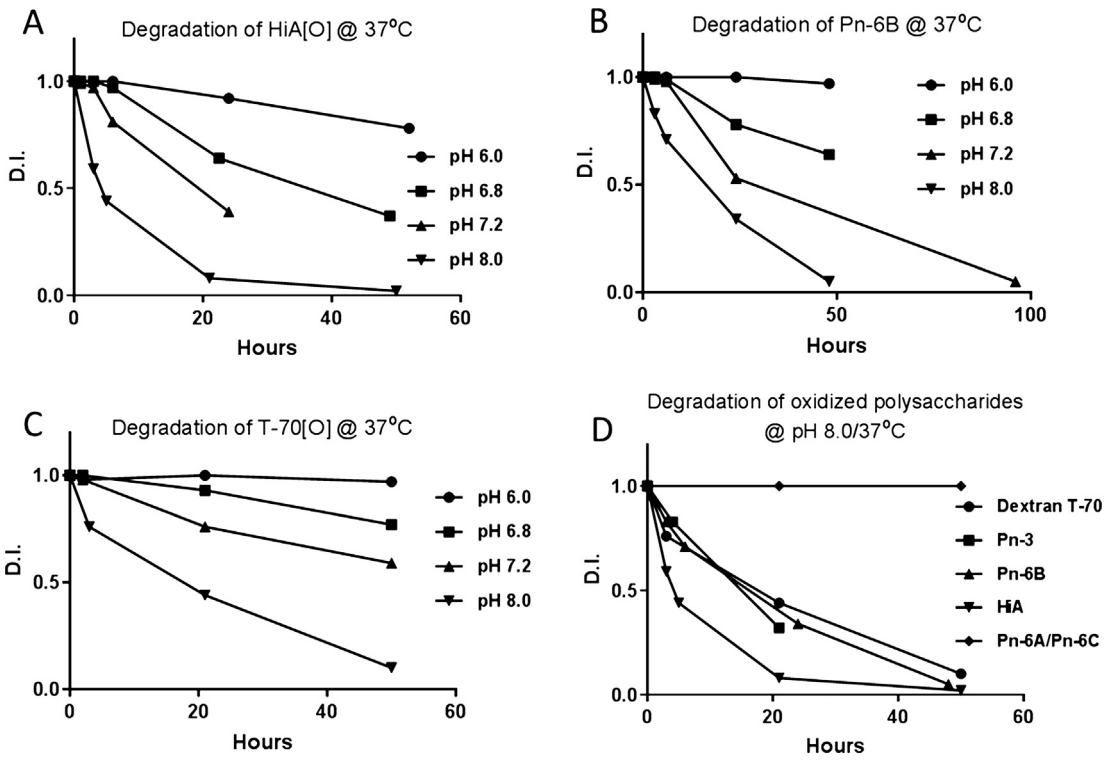


Fig. 2. Degradation of oxidized polysaccharides under 0.1 M sodium phosphate buffer with various pH. A: oxidized HiA degradation (top left); B: oxidized Pn-6B degradation (top right); C: oxidized Dextran T-70 degradation (bottom left); and D: comparison of degradation of oxidized polysaccharides (Dextran T-70, Pn-3, 6A, 6B, 6C, and HiA) at pH 8.0 (bottom right). D.I. was obtained using Eq. (1), and the retention time after 160 h on a Superpose 12 10/300 or Superpose 6 10/300 (Pn-3) column was used as an endpoint for D.I. calculation.

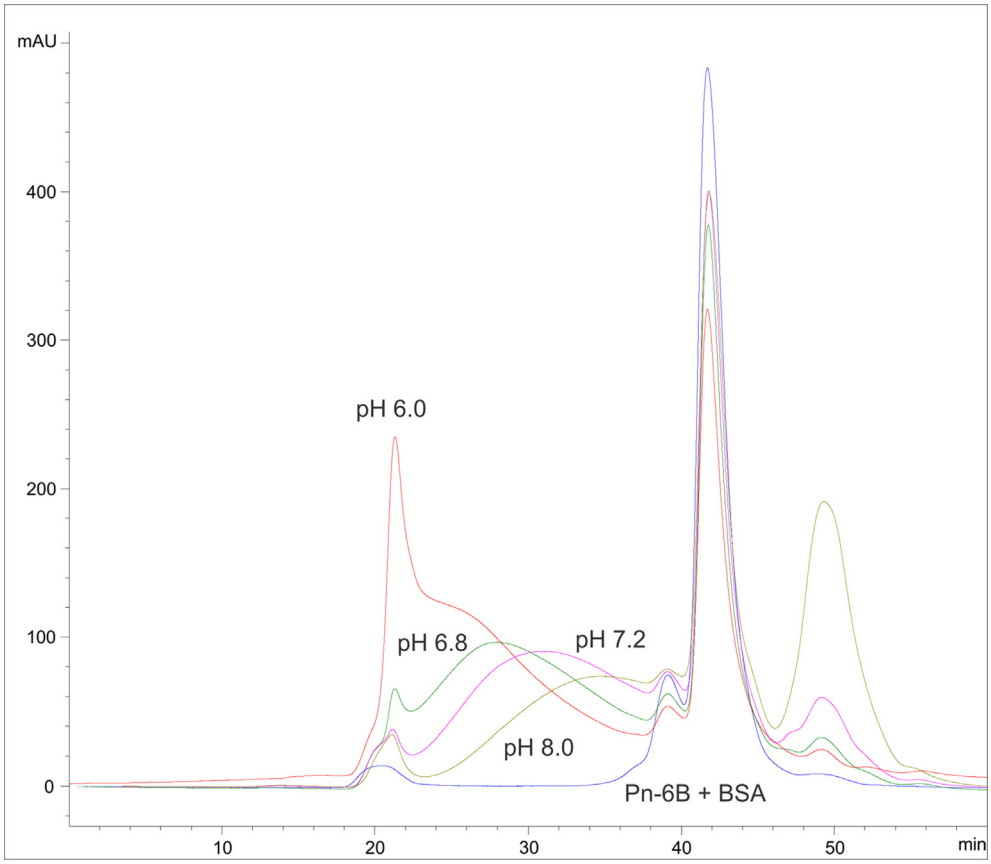


Fig. 3. The conjugation of oxidized Pn-6B to BSA after 40 h at 37 °C analysed by SEC-HPLC (at UV 214 nm) on a Superose 6 10/300GL column with PBS as eluent at 0.4 mL/min. Best conjugation was obtained at pH 6.0, higher pH led to degradation of oxidized polysaccharide by β-elimination.

β-elimination can follow with respective leaving groups, *O*-glycose in A1 and *O*-phosphate in A2 leading to polysaccharide degradation. After the *O*-glycosyl β-elimination from A1, an aldose-ketose conversion leads to subsequent *O*-phosphate β-elimination, and as a result the functional aldehyde/ketone groups were eliminated (see Scheme 1).

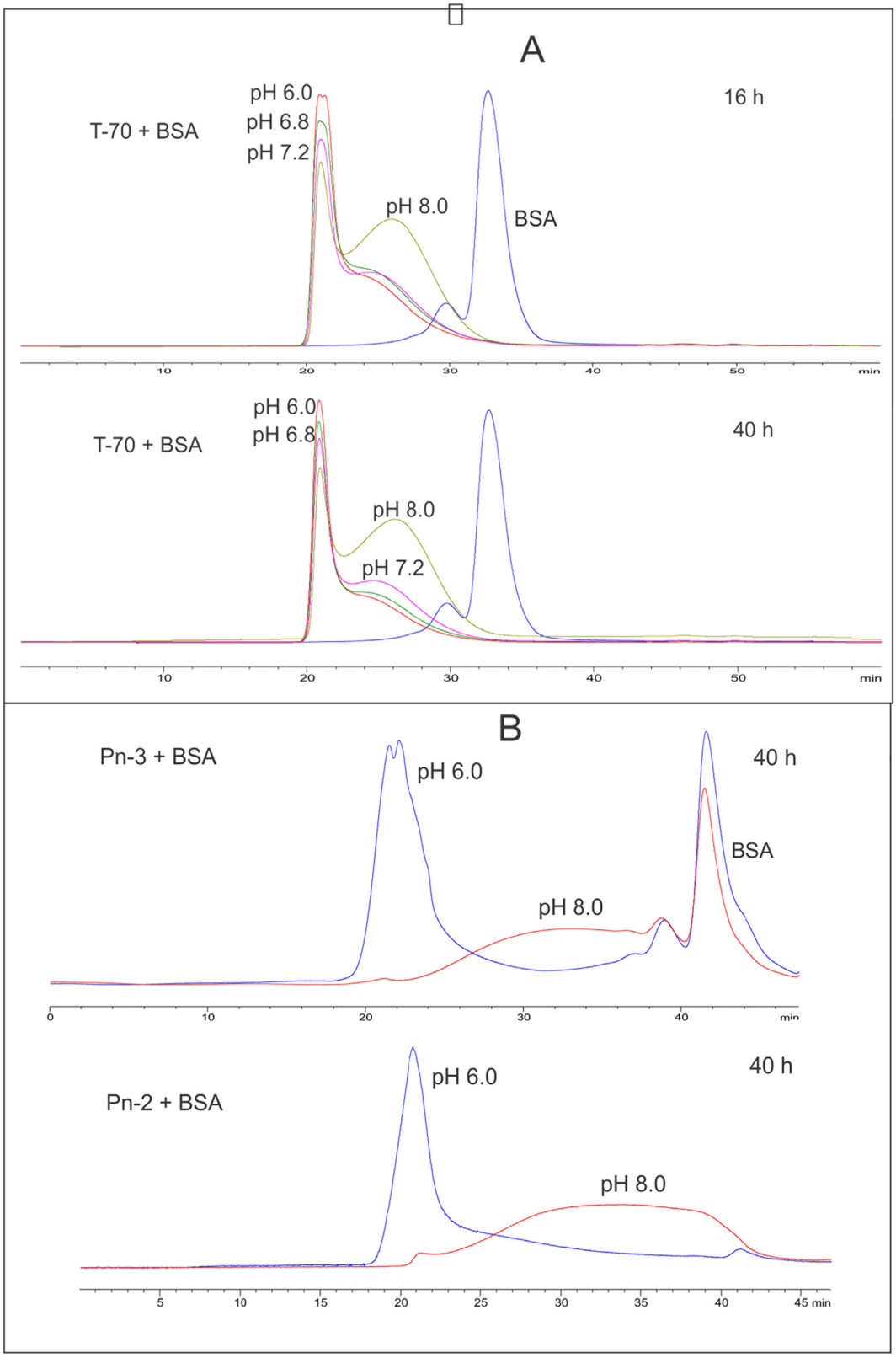


Fig. 4. Conjugation of oxidized Dextran T-70, Pn-2, and Pn-3 to BSA in 0.1 M phosphate buffer. A: T-70 and BSA monitored by SEC-HPLC (at UV 214 nm) on a Superose 12 10/300GL column with PBS as eluent at 0.4 mL/min; B: Pn-2/Pn-3 and BSA monitored by SEC-HPLC on a Superose 6 10/300GL column at UV 254 (Pn-2) or with a refractive index detector (Pn-3), respectively. Conjugation of T-70 and Pn-2 to BSA was very efficient at pH 6.0 but significant degradation for all three glycoconjugates occurred at pH 8.0.

The β-elimination under basic conditions described here (see Scheme 1) is likely followed by a Michael addition of hydroxyl group, a mechanism similar to what we previously reported [25]. Base-catalyzed β-elimination can also release the saccharides on serine and threonine from *O*-glycoproteins [26]. The β-elimination rate depends on both the basicity and the leaving group. The stronger the base, the faster the reaction goes, and a better leaving group such as a phosphate in comparison to an *O*-alkyl group will facilitate the β-elimination [27].

In order to quantify the relative degradation rate, degradation index (D.I.) is introduced. We define the degradation of polysaccharide by D.I. using following equation:

$$\text{Degradation Index(D.I)} = \frac{T_{end} - T}{T_{end} - T_0} \quad (1)$$

T_{end} retention time at the end of degradation
T_0 retention time prior to degradation
T retention time

Although the correlation between the retention time on SEC-HPLC and the molecular size is not linear, Eq. (1) can be used to represent the degree of degradation in a relative scale. Prior to degradation the D.I. equals one and the D.I. becomes zero when the degradation/β-elimination was complete after 160 h at 37 °C.

We systematically examined the degradation of oxidized Pn-6B, HiA polysaccharides and Dextran T-70 in parallel under various conditions. Oxidized polysaccharides were dissolved in 0.1 M sodium phosphate buffer at pH 6.0, 6.8, 7.2 and 8.0 at a concentration of 1 mg/mL, and the degradation at 37 °C was monitored by SEC-HPLC. All three polysaccharides degraded at pH 6.8, 7.2 and 8.0, and the rate of degradation depended on the pH, a characteristic of base-catalysed β-elimination (see Fig. 2A, B and C). The rate of degradation of oxidized HiA and Pn-6B polysaccharides was much faster, a reflection of the β-substituted *O*-phosphate present in their polysaccharide backbone. Oxidized Dextran T-70 was significantly degraded, particularly at pH 8.0.

Based on the mechanistic analysis and the degradation observed from oxidized Dextran T-70, it is reasonable to expect that a similar *O*-glycosyl β-elimination/degradation could happen to other oxidized polysaccharides with 1–2, 1–4 and 1–6 interglycosyl linkages. It is important to highlight this concern because reduction of the polysaccharide size in glycoconjugates may disrupt conformational epitopes and render the separation of conjugates from unconjugated polysaccharide difficult due to overlap in their molecular size. Oxidized side chains in branched polysaccharides can also undergo β-elimination resulting in loss of conjugation sites and ultimately poor conjugation yield. Structural analysis suggests that, in addition to Pn-6B, polysaccharides such as Pn-1, 3, 7F, 9V, 14, 18C, 19A, 19F and 23F in Prevnar-13 as well as Pn-2 and 17F could be susceptible to base catalyzed β-elimination after periodate oxidation. For example, treatment of oxidized Pn-3 polysaccharide in phosphate buffer at pH 8.0 at 37 °C led to degradation. A comparison of its D.I. to other polysaccharides at pH 8.0 is shown in Fig. 2D. The observation confirms that the β-elimination in oxidized polysaccharide is more common than we previously realized. Since Pn-17F polysaccharide has a 2-*O*-glycosyl and a 1-*O*-phosphate arabinitol subunit which is similar to the 4-*O*-glycosyl and 5-*O*-phosphate substituted ribitol in Pn-6B polysaccharide, the oxidized Pn-17F could degrade readily via β-elimination under basic conditions.

Essentially, the reductive amination conjugation is competing with the β-elimination. Therefore, one may hope that the β-elimination may not be as detrimental as suggested here to glycoconjugation. To answer the question we performed reductive amination in parallel using oxidized Dextran T-70, HiA, Pn-6B, Pn-3 and Pn2 polysaccharides and BSA in 0.1 M phosphate buffer at various pH conditions. The conjugation was monitored by SEC-HPLC analysis, and they all followed the same pattern. The conjugation of oxidized polysaccharides to BSA was pH-dependent as shown in Figs. 3 and 4. Degradation was observed, as expected, at pH 8.0, 7.2, and 6.8 for Pn-6B conjugates, but not significantly at pH 6.0, which resulted in decreased conjugate molecular size and produced small non-conjugatable saccharides as indicated by the new peak detected at 50 min in SEC-HPLC (see Fig. 3). Similarly, noticeable degradation was also observed at pH 8.0 and 7.2 for Dextran T-70 conjugates (Fig. 4A). Again, degradation was observed from conjugation of Pn-2 and Pn-3 to BSA at pH 8.0 but not at pH 6.0 (Fig. 4B) that further confirms the importance of pH of a buffer to glycoconjugation. Clearly, basic conditions favor β-elimination over reductive amination. We speculate that the significant degradation of Pn-6B during conjugation may be one of the reasons for its relatively poor immunogenicity, thus twice the amount of Pn-6B-CRM conjugate formulated in Prevnar-13 [28]. It should be noted that sodium borohydride capping, which reduces unconjugated aldehyde in the polysaccharide, essentially stops further β-elimination/degradation.

In conclusion, despite the wide use of reductive amination as a method of glycoconjugation for over thirty years, we are still learning how to improve the reaction. The observed low efficiency and poor yield with reductive amination are likely due to the loss of active aldehyde groups by a base catalyzed β-elimination. In order to achieve the best possible conjugation, a buffer with proper pH is critical. The work described here provided evidence that glycoconjugates can be synthesized more effectively in slightly acidic conditions (pH 6.0), though this may not be necessary to the glycocojugation of polysaccharides that are not susceptible to β-elimination. Additionally, this improved conjugation can also facilitate the purification of glycoconjugate vaccines.

Author contribution

W.Z. observed the degradation during glycoconjugation, elucidated its mechanism, and optimized reductive amination conditions with the technical assistance from D.W. W.Z. interpreted the data and wrote the manuscript. A.C. involved in various discussions and provided suggestions and critical comments.

Acknowledgements

The authors thank Dr. Harry Jennings for his suggestions and constructive comments. W.Z. is very grateful to Dr. Jennings for his mentorship over many years. We also thank Perry Fleming and Frank St. Michael for providing HiA polysaccharide.

Conflict of interest

The authors declare no conflict of interest.

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