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Ultrafiltration behavior of bacterial polysaccharides used in vaccines

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ABSTRACT

Ultrafiltration is widely used for the purification of polysaccharide-based vaccines against pneumococci and meningococci, but there is currently no fundamental understanding of the factors controlling the ultrafiltration behavior of these polysaccharides. Experiments were performed using purified pneumococcus polysaccharides (serotypes Pn3, Pn9V, and Pn14) provided by Pfizer. Ultrafiltration data were obtained in a stirred cell using Ultracel™ composite regenerated cellulose and Biomax™ polyethersulfone membranes with different nominal molecular weight cutoffs. Polysaccharide transmission was a strong function of filtrate flux, with data at low bulk concentrations in good agreement with the classical concentration polarization model. Polysaccharide fouling became significant at high filtrate flux during ultrafiltration of more concentrated solutions, consistent with the presence of a critical value for the wall concentration. Polysaccharide transmission was in good agreement with available hydrodynamic models accounting for the presence of a pore size distribution, with the effective size of the polysaccharide determined by dynamic light scattering or size exclusion chromatography. These results provide important insights into the factors controlling the ultrafiltration behavior of bacterial polysaccharides of interest in bioprocessing applications.

1. Introduction

Capsular polysaccharides from *Streptococcus pneumoniae* have been used to produce a number of vaccines against important diseases. For example, Pfizer currently markets a vaccine containing 13 of the most prevalent pneumococcus serotypes while Merck has commercialized a vaccine containing 23 serotypes that provides protection against all of the most frequent pneumococcus pathogens [1,2]. Polysaccharide vaccines are T cell-independent and are thus largely ineffective for children under 2 years of age [3–5]. A much stronger immunogenic response can be generated by chemically coupling the polysaccharide to a highly immunogenic protein like CRM_{197}, a non-toxic cross-reacting mutant of diphtheria toxin [6,7]. The resulting conjugated vaccines are T cell-dependent, making them highly effective for both children and adults, providing individuals with long-term immunological memory [3,8].

The development of multivalent conjugated vaccines involves the covalent coupling of each purified capsular polysaccharide to a carrier protein [3,9]. The conjugation reaction is typically conducted in the presence of excess polysaccharide [4,9], requiring the removal of unreacted (free) polysaccharide from the final product [8]. A number of methods have been explored for purification of the conjugate including size exclusion [3,8], hydrophobic interaction [3,8], and reverse phase [10] chromatography, as well as liquid–liquid extraction [3] and ammonium sulfate precipitation [3]. Although the chromatographic methods do provide reasonably good selectivity, they have very low dynamic binding capacities due to the large size of the polysaccharides/conjugates. Extraction and precipitation are both high throughput purification methods, but it can be difficult to obtain the required resolution between the conjugate and the free polysaccharide.

There has thus been significant interest in the application of ultrafiltration (UF), both for the concentration of various intermediates and for removal of unreacted polysaccharides from the vaccine conjugates. Ultrafiltration is not limited by the large size of the polysaccharides since the separation is driven by convection (with minimal diffusional limitations), but resolution between species with similar size (e.g., the unreacted polysaccharides and the conjugate) can be challenging. Goncalves et al. [11] described the use of tangential flow microfiltration and ultrafiltration for purification of polysaccharide serotype 23 F, although no data were reported for either the filtrate flux or polysaccharide retention. Meacle et al. [9] examined the filtration of several polysaccharides and conjugates through 0.1 and 0.2 μm microfiltration membranes. Significant polysaccharide retention was seen with both membranes, despite the large pore size. Polysaccharide transmission was largely independent of the transmembrane

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pressure, which the authors attributed to the combined effects of membrane fouling and concentration polarization, although no detailed analysis of this behavior was presented. Wen et al. [4] found relatively high retention (greater than 90%) during ultrafiltration of a polysaccharide through a 0.05 μm polysulfone membrane, with the retention increasing with increasing transmembrane pressure. Greater transmission (although still much less than 20%) was obtained in the presence of backpulsing. In contrast, Brou et al. [12] obtained essentially complete transmission of a bacterial exopolysaccharide through a 0.2 μm pore size membrane in a rotating disk filter with no evidence of fouling. Takagi et al. [13] used a 100 kDa ultrafiltration membrane to remove small proteins from a capsular polysaccharide produced by *Haemophilus influenzae* type b. The polysaccharide recovery was only 75%, suggesting significant loss of polysaccharide due to transmission through this relatively small pore size ultrafiltration membrane.

Although these studies clearly demonstrate the potential of using ultrafiltration for purification of polysaccharide-based vaccines, there is considerable discrepancy over the retention characteristics and the key factors controlling the ultrafiltration behavior of these polysaccharides through different pore size membranes. The objective of this study was to obtain quantitative data for the transmission of Pneumococcus serotypes Pn3, Pn9V, and Pn14 through a series of ultrafiltration membranes with different pore size. Data were analyzed using the classical concentration polarization model along with available hydrodynamic models, with the effective size of the different polysaccharides determined from size exclusion chromatography or dynamic light scattering.

2. Materials and methods

2.1. Solution preparation

Buffer solutions were prepared by dissolving pre-weighed amounts of KCl (BDH Chemicals, BDH0258) and Bis–Tris (MP Biomedical, 101038) in deionized water obtained from a NANOpure® Diamond water purification system (Barnstead International). The solution pH was measured using a Thermo Orion pH meter and adjusted using 1 M HCl as required. All buffer solutions were prefiltered through 0.2 μm pore size Supor® 200 membranes (Pall Corporation) prior to use.

Purified capsular polysaccharides of the *S. pneumoniae* bacteria were provided by Pfizer Inc. (Chesterfield, MO), including both the native serotypes Pn3 and Pn9V and activated/capped versions of Pn9V and Pn14, with the latter produced by partial oxidation of the native polysaccharides with periodate followed by reaction with sodium borohydride [14,15]. This process causes partial hydrolysis of the native polysaccharide thus reducing the effective size. The polysaccharides were stored at 4 °C and slowly brought to room temperature (23 ± 2 °C) before use in any of the experiments. The polysaccharides were then diluted to the desired concentration using Bis–Tris buffer (pH 7) with added KCl. The resulting solutions were filtered through 0.22 μm Acrodisc® syringe filters (Pall Corporation) immediately prior to use.

2.2. Polysaccharide characterization

The effective size of the different polysaccharides was determined by both size exclusion chromatography (SEC) and dynamic light scattering (DLS). SEC was performed using an Agilent 1200 series HPLC system (Agilent Technologies, CA) equipped with a PL Aquagel-OH 60 size exclusion column (Agilent Technologies, CA). The running buffer was 250 mM KCl solution buffered with 10 mM Bis–Tris at pH 7 at a flow rate of 0.8 mL/min. Sample detection was performed using a refractive index detector, with the measured retention volume compared with that of narrow molecular weight dextran standards with molecular weights of 505–2,655,000 Da (obtained from American Polymer Standards Corp., Mentor, OH).

Dynamic light scattering data were obtained using a Zetasizer ZS (Malvern Instruments, UK) at a scattering angle of 173° and a temperature of 25 °C. The effective size was determined from the intensity–intensity autocorrelation function, $g^2(\tau)$

$$g^2(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)^2\rangle} \quad (1)$$

where $I(t)$ is the light intensity at time t, and τ is the delay time. The autocorrelation function was fit to a decaying exponential in terms of the diffusion coefficient, D

$$g^2(\tau) = B + \beta\, exp(-2\, D\, q^2\, \tau) \quad (2)$$

where B is the baseline at infinite delay, β is the correction function amplitude at zero delay, and q is the scattering vector. The hydrodynamic radius, R_h, was evaluated from the Stokes–Einstein equation as

$$R_h = \frac{k_B\, T}{6\,\pi\,\mu\, D} \quad (3)$$

where k_B is the Boltzmann constant and T is the absolute temperature.

2.3. Ultrafiltration

Ultrafiltration experiments were performed using Ultracel™ composite regenerated cellulose membranes and Biomax™ polyethersulfone membranes with nominal molecular weight cut-offs (MWCO) between 30 and 1000 kDa, all provided by EMD Millipore (Bedford, MA). Small 25 mm diameter disks were cut from large flat sheets (roll stock) using a specially designed stainless-steel cutting device. Membranes were soaked in 90% (V/V) isopropyl alcohol for 45 min to remove any residual storage agents and ensure thorough wetting of the pore structure. The membranes were then flushed with at least 100 L/m^2 of deionized (DI) water.

Ultrafiltration data were obtained in a 10 mL Amicon 8010 stirred cell (Millipore Corporation, MA). Each membrane was placed at the bottom of the stirred cell on top of a Tyvek support to minimize any deformation of the membrane at high pressures. The stirring speed was set to 1200 rpm using a 461830 digital stroboscope (Extech Instruments, Nashua, NH). The stirred cell was connected to an acrylic feed reservoir that was pressurized with compressed air at 7–70 kPa (corresponding to 1–10 psi) as determined using a digital differential pressure gauge (Omega, CT). All filtration experiments were performed at room temperature (23 ± 2 °C) with samples stored at 4 °C until analysis.

The membrane hydraulic permeability (L_p) was evaluated by measuring the filtrate flux (by timed collection) as a function of the transmembrane pressure using a 250 mM buffered KCl solution at pH 7:

$$L_p = \frac{\mu J_v}{\Delta P} \quad (4)$$

where μ is the solution viscosity, J_v is the filtrate flux, and ΔP is the transmembrane pressure. Polysaccharide ultrafiltration experiments were only performed if the measured hydraulic permeability was within ± 10% of the mean value for that membrane lot.

The stirred cell was then emptied, refilled with the polysaccharide solution, and connected to a pressurized feed reservoir containing additional feed solution. The filtrate flux was controlled using a Masterflex peristaltic pump connected to the outlet tubing