THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF CHEMICAL ENGINEERING

EFFECTS OF SOLUTION CONCENTRATION AND IONIC STRENGTH ON THE ULTRAFILTRATION BEHAVIOR OF BACTERIAL POLYSACCHARIDES IN VARYING PORE SIZE MEMBRANES

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ABSTRACT

Bacterial polysaccharides, when coupled to an immunogenic protein, have been shown to increase the immunogenicity of meningitis and pneumonia vaccines. Ultrafiltration with semipermeable membranes is often employed to remove any unreacted polysaccharide from the desired conjugated vaccine. Therefore, understanding the ultrafiltration behavior of these biotherapeutics is essential to the development of vaccines against infectious disease. The overall objective of this thesis was to examine the ultrafiltration behavior of a series of pneumococcus polysaccharide serotypes Pn7F, Pn19A, Pn3, and Pn14, provided by Pfizer, with different physical characteristics. The effective sizes of these polysaccharides were determined by size exclusion chromatography (SEC). Ultrafiltration data were obtained in a stirred cell with Biomax^{TM} polyethersulfone membranes, with samples analyzed using high performance liquid chromatography (HPLC). Data were obtained for a range of membrane pore size, polysaccharide concentrations, and solution ionic strength. The ultrafiltration behavior of Pn7F and Pn19A was largely independent of solution concentration, with the transmission slightly decreasing with increasing polysaccharide concentration. Only Pn19A, the smallest polysaccharide, was able to permeate the 100 kDa pore-size membrane at a high rate, while the other serotypes showed significant fouling at all filtrate flux values. The transmission increased with increasing ionic strength for filtration of the charged serotypes (Pn19A and Pn3) through the 300 kDa membrane. These results provided new insights into the effects of the polysaccharide size and electrical charge on the ultrafiltration characteristics of these important biotherapeutics.

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Section 1

Introduction

Vaccination with pathogenic bacteria capsular polysaccharides has been shown to produce persistent, antibody-mediated immunity against infectious diseases [(1)]. This class of marketed vaccines includes Pfizer's Trumenba®, which prevents meningitis caused by *Neisseria meningitidis* group B, as well as Sanofi's Vivaxim® for immunity toward *Salmonella typhi Vi* [(14, 22)]. Although many infectious diseases can be treated by antibiotics, vaccination both prevents the acquisition and transmission of these infections and eliminates the growing concern with antibiotic resistant bacterial strains. For example, 90 serotypes of the bacterium, *Streptococcus pneumoniae*, or pneumococcus, have been identified, with each serotype encapsulating a different pneumococcal genetic sequence [(12)]. Antibiotic resistance to a small fraction of these serotypes associated with pneumonia led to Merck's 1983 launch of PneumoVax®, the first polysaccharide vaccine. This breakthrough pneumonia treatment contains unconjugated capsular polysaccharide isolated from 23 out of 90 different pneumonia serotypes. Such variety can induce protection against more than 90% of infections caused by these pathogens [(1)].

Polysaccharide vaccines are T-cell-independent; they stimulate B-lymphocytes to produce effective antibodies, but do not induce T-lymphocytes [(22)]. Therefore, they generally fail to elicit optimal antibody responses for sufficient protection of high risk groups, particularly children under two years old, the elderly, and the immunocompromised [(1)]. However, the polysaccharide can be coupled to an immunogenic protein carrier to create a conjugated vaccine, which provides a much stronger immunogenic response [(7)]. Specifically, the carrier protein,

CRM-197, has been chemically coupled to the seven serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) that are most prevalent in children younger than 6, to produce the 7-valent conjugated vaccine known as Pfizer's Prevnar®. This vaccine elicits a T-cell-dependent immune response that leads to enhanced protection. Vaccine immunogenicity increases through the polysaccharide-protein interaction, providing effective immunization of high risk groups and preventing 53,000 pneumonia cases per year [22].

The covalent coupling of purified bacterial capsular polysaccharides to the immunogenic protein is performed with excess polysaccharide. Following the conjugation reaction, unreacted polysaccharides (and protein), which do not provide clinical protection, must be separated from the polysaccharide-protein conjugate [16]. In the case of meningococcal group C polysaccharide, excessive amounts of unconjugated polysaccharide can lead to immunological hyporesponsiveness [9]. Current separation techniques include liquid-liquid extraction, ammonium sulfate precipitation/fractionation, and ultracentrifugation. Chromatographic methods include hydrophobic interaction, gel-filtration, and size-exclusion [16]. Despite chromatography's reasonably good selectivity, the large size of the polysaccharide and conjugates causes very low dynamic binding capacities (mass of polysaccharide that can be bound per unit volume of resin at breakthrough), as only relatively small amounts of product are able to bind to the resin under typical flow conditions. Additionally, extraction and precipitation, although high throughput methods, typically do not provide the desired resolution between the conjugate and the free polysaccharide [7]. Purification through ultracentrifugation is advantageous due to its high fractionation ability without the need for any separation columns or membranes. However, the method is expensive and difficult to scale up, and the separation selectivity can be compromised

due to the polysaccharide's polydispersity, expanded nature in solution, and in some cases, its high charge [8].

Ultrafiltration (UF) has been used to concentrate protein and polysaccharide molecules and change buffer solution compositions. UF can also be used to remove low-molecular weight solutes from solution [16]. There is growing interest in using semipermeable membranes of varying pore size to remove unreacted polysaccharide after the conjugation reactions [7]. The separation is driven by differences in molecular size, with the unreacted polysaccharides ranging from 1,000 to 50,000 Da while the polysaccharide-protein conjugate ranges from 100,000 to 1,000,000 Da. Shape can also contribute to the separation behavior as polysaccharides exist in solution with a variety of conformations (e.g., helical coils), while proteins normally have a defined molecular size and more globular structure. The ultrafiltration behavior of these polysaccharides can vary significantly depending upon their structure / form. For example, Takagi et al. experimented with a relatively small pore size membrane with a 100 kDa nominal molecular weight cutoff (defined as a membrane that is able to retain approximately 90% of a protein with molecular weight of 100 kDa) to remove small proteins from a *Haemophilus influenza type B* capsular polysaccharide and found that some of the small molecular mass polysaccharide was lost in the ultrafiltrate [20]. McMaster patented the claim that a polysaccharide of molecular size 1 Da to 50 kDa can pass through a semi-permeable membrane with a molecular size cutoff of 30 kDa using a diafiltration buffer containing 0.15 M sodium chloride. This patent also claimed that the use of a buffer containing 40% ammonium sulfate can increase the permeability of free polysaccharide through the membrane pores, optimizing the purification of vaccine conjugates [16].

Goncçalves et al. found that tangential flow microfiltration and ultrafiltration with a 30 kDa membrane can be used to recover 89% of pneumococci serotype 23F to meet the vaccine requirements of the World Health Organization. However, their work did not include any information on how the polysaccharide retention varies with filtrate flux [(4)]. Meacle et al. used cross-flow microfiltration to remove 98% of unreacted pneumococcal polysaccharides for conjugate purification and found that membrane fouling increased with increasing flux. The authors used hollow fiber membranes with large pore sizes of 0.1 and 0.2 μm, yet the retained conjugate formed a "cake" layer on the membrane surface. However, the polysaccharide transmission appeared to be largely independent of transmembrane pressure [(17)]. On the other hand, Wen et al. found that retention increased with increasing transmembrane pressure when a pneumoniae serotype was filtered through a 0.05 μm polysulfone membrane. Retention greater than 90% led the the authors to implement backpulsing for improved purification of the vaccine conjugate. Backpulsing was shown to reduce membrane fouling by intermittently forcing permeate back through the membrane and into the retentate in the tangential flow filtration device [(21)]. Complete polysaccharide transmission was observed by Brou et al. during filtration of an exopolysaccharide through a 0.2 μm membrane in a rotating disk filter. The researchers in this case found no evidence of fouling [(2)].

Recently, more in depth experiments have been performed to characterize the retention behavior during polysaccharide ultrafiltration through different pore size membranes. Hadidi et al. analyzed the behavior of Pfizer serotypes Pn3, Pn9V, and Pn14 in stirred cell ultrafiltration experiments over a range of polysaccharide concentrations. At higher concentrations, there was a significant reduction in polysaccharide transmission when the filtrate flux exceeded some critical value, which the authors attributed to membrane fouling [(7)]. Hadidi et al. also explored the effects

of solution conditions, such as ionic strength and pH, on serotype size as determined by size exclusion chromatography (SEC) [6]. The effective size of the charged polysaccharides was shown to increase significantly at low ionic strength due to intramolecular electrostatic interactions between charge groups along the polysaccharide chain.

Although these studies have begun to identify some of the key factors controlling the ultrafiltration of polysaccharide-based vaccines, there is still considerable uncertainty regarding the effects of the polysaccharide properties on transmission and the degree of fouling. The objective of this thesis was to extend the studies by Hadidi et al. to quantitatively evaluate the ultrafiltration behavior of pneumococcus serotypes Pn7F and Pn19A, in comparison to Pn3 and Pn14, as a function of membrane pore size, polysaccharide concentration, and solution ionic strength. The effective sizes of these serotypes were determined by size exclusion chromatography (SEC). These results provide new insights into the effects of the polysaccharide size and electrical charge on the ultrafiltration characteristics of these important biotherapeutics.

Section 2

Materials and Methods

Solution Preparation

Buffer solutions of varying ionic strength were prepared by dissolving pre-weighed amounts of KCl (BDH Chemicals, BDH) and Bis-Tris (MP Biomedical, 101038) in deionized water from a NANOpure® Diamond water purification system (Barnstead International). The solution pH was measured using a Thermo Orion pH meter and adjusted to a pH of 7 using 1 M HCl as needed. Before experimentation, all buffer solutions, as detailed in Table 1, were prefiltered through 0.2 μm pore size Supor® 200 membranes (Pall Corporation) to remove any insoluble matter.

Table 1. Prepared Buffer Solutions of Varying Ionic Strength

KCl Concentration (mM)	Buffering Agent Concentration (mM)	pH
250	10	7
80	3.2	7
20	0.8	7
5	0.2	7

Purified capsular polysaccharides of the *Streptococcus pneumoniae* bacteria, including the native serotypes Pn7F, Pn19A, Pn3, and Pn14, were provided by Pfizer Inc. (Chesterfield, MO). The structures of these serotypes are illustrated in Figures 1-4. Each serotype generates a unique immunologic response, despite the small differences in saccharide composition. Serotype

Pn3 has the highest charge density, with every other saccharide monomer being a charged glucuronic acid. In contrast, Pn7F and Pn14 are composed of only neutral saccharides. The serotypes were stored at 4 °C to minimize degradation, but were gradually warmed to room temperature (23 ± 2 °C) before use in the filtration experiments. The feed solution was prepared by diluting the polysaccharides in the desired ionic strength buffer in Table 1 to the desired concentration. Prior to experimentation, these solutions were then pre-filtered through 0.2 μm pore size Supor® 200 membranes (Pall Corporation).

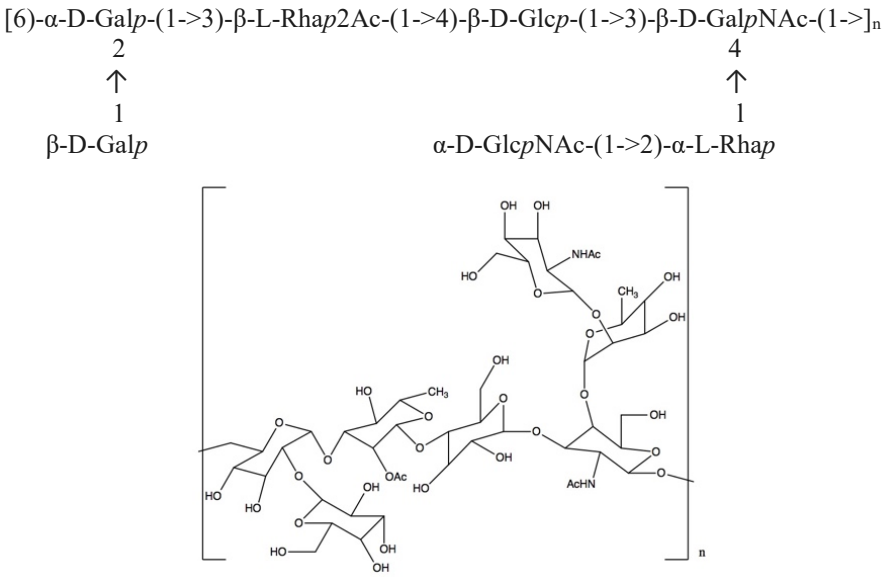


Figure 1. Structure of native polysaccharide serotype Pn7F [18]

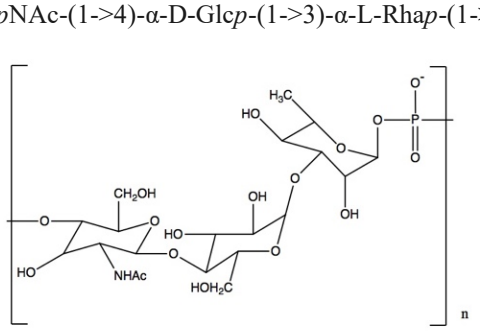


Figure 2. Structure of native polysaccharide serotype Pn19A [11]

[3)-β-D-Glc*p*A-(1->4)- β-D-Glc*p*-(1->]$_n$

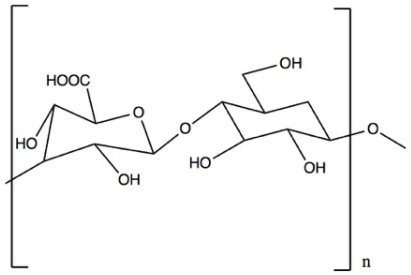


Figure 3. Structure of native polysaccharide serotype Pn3 [15]

[6)-β-D-Gal*p*-(1->4)-]β-D-Glc*p*NAc-(1->3)- β-D-Gal*p*-(1->4)-β-D-Glc*p*-(1->]$_n$

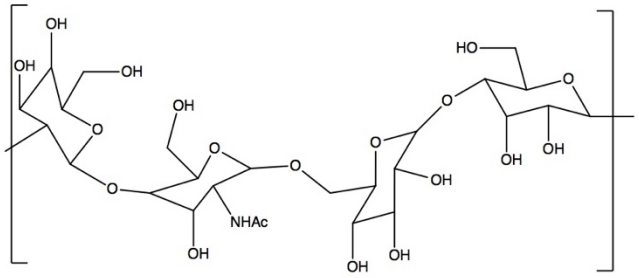


Figure 4. Structure of native polysaccharide serotype Pn14 [10]

Polysaccharide Characterization

The effective sizes of the polysaccharide serotypes were determined by size exclusion chromatography (SEC). An Agilent 1200 series HPLC system (Agilent Technologies, CA) with a PL Aquagel-OH 60 size exclusion column (Agilent Technologies, CA) was used with a running buffer of 250 mM KCl and 10 mM Bis-Tris at pH 7 and a flow rate of 0.8 mL/min. Each serotype sample (0.1 g/L in the running buffer) was injected at a volume of 80 µL and detected by an Agilent 1200 series refractive index detector (RID) at 35 °C. This temperature was

maintained by a column oven. Appendix A includes sample chromatograms of each polysaccharide, with the larger polysaccharides eluting out of the column sooner due to their greater exclusion from the pores of the SEC resin. The molecular weights and elution times of serotypes Pn3, Pn9V, and Pn14 were known from previous research, and therefore, were used to evaluate the effective MW of the other serotypes using the plot of the log of polysaccharide molecular weight (kDa) as a function of elution time shown in Figure 5.

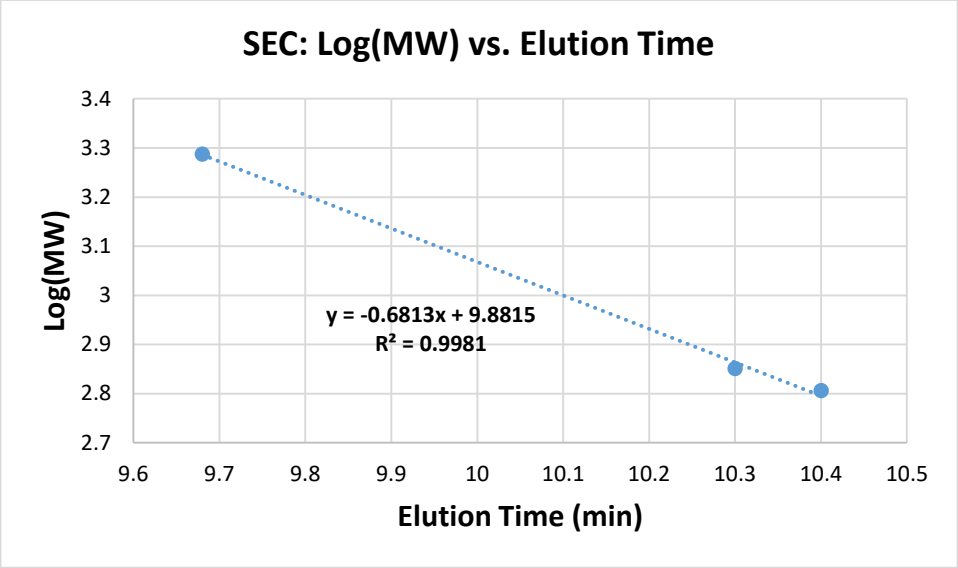


Figure 5. SEC plot of log(MW) vs. elution time of the polysaccharides Pn3, Pn9V, and Pn14 [3, 6]

Ultrafiltration

Dead-end ultrafiltration experiments were performed using Biomax™ polyethersulfone membranes with nominal molecular weight cut-offs (MWCO) of 100 kDa and 300 kDa (EMD Millipore, Bedford, MA). A 25 mm diameter stainless steel cutting device was used to cut small circular membrane disks from a large flat sheet (roll stock). The membranes were then soaked in

90% (V/V) isopropyl alcohol and 10% deionized water for 45 min on a Rocker II shaker (Boekel Scientific). This step removed residual storage agents and ensured complete membrane wetting. The membranes were then rinsed with deionized water before they were placed in the base of a 10 mL Amicon 8010 stirred cell on top of a Tyvek support (Millipore Corporation, MA). The support prevented any deformation or damage to the membrane at high pressures. A 461830 digital stroboscope (Extech Instruments, Nashau, NH) was used to set the stirring speed to a constant 1200 rpm.

The stirred cell was connected to an acrylic feed reservoir pressurized with compressed air at 1-10 psi (7-70 kPa). A digital differential pressure gauge (Omega, CT) was used to evaluate the reservoir pressure. The membranes were initially flushed with at least 80 L/m^2 of deionized water at a pressure of 5 psi. The deionized water was then emptied from the stirred cell and reservoir and replaced with the appropriate buffer to measure the hydraulic membrane permeability (L_p). L_p was evaluated using Equation 1 by measuring the filtrate flux through the membrane by timed collection over a range of transmembrane pressure (from 2 to 5 psi). μ is the solution viscosity, J_v is the filtrate flux, and ΔP is the transmembrane pressure.

$$L_p = \frac{\mu J_v}{\Delta P} \quad (1)$$

Only membranes with permeability within ± 10% of the mean value for that lot were used to perform the polysaccharide ultrafiltration experiments.

After measuring the permeability, the stirred cell was emptied and refilled with the polysaccharide solution. A 120 µL of the initial bulk solution was collected for subsequent analysis of the polysaccharide concentration. The stirred cell was then connected to the feed reservoir, which was filled with any additional solution and maintained at 5 psi for the entire experiment. The filtrate flux was controlled using a Masterflex peristaltic pump that was

connected to the outlet tubing from the stirred cell. Eight samples were periodically collected (after filtration of a minimum of 1 mL at each pressure) as the filtrate flux was increased. This ensured stable operation and wash out of the dead volume beneath the membrane. After all eight samples were collected, the stirred cell was opened and an additional 120 μL sample was obtained to evaluate the final bulk solution concentration.

The observed sieving coefficient was calculated using Equation 2, where C_f and C_b are the polysaccharide concentrations in the filtrate and bulk solutions, respectively.

$$S_o = \frac{C_f}{C_b} \tag{2}$$

Both C_f and C_b were determined using HPLC analysis with the Agilent 1200 series system (Agilent Technologies, CA) equipped with a refractive index detector, as described in the previous section. The polysaccharide concentrations in each filtrate and bulk sample were determined from the area under the HPLC peak using a serotype-specific calibration curve evaluated using data for samples of known serotype concentrations. An S_o value of unity indicates that the filtrate and bulk solutions are in equilibrium, with equal polysaccharide concentrations in each. Conversely, a small S_o value less than 1 indicates that C_b is greater than C_f, and therefore, the polysaccharide had low transmission through the membrane pores.

Section 3

Results and Discussion

Polysaccharide Characterization

Streptococcus pneumoniae derived capsular polysaccharides consist of oligosaccharide repeating units with two to eight monosaccharides or sugars. Table 2 details the monomer composition, charge, charge density, molecular weight, and SEC radius (R_{SEC}) of Pn7F, Pn19A, Pn3, and Pn14.

Table 2. Characterization of Pn7F, Pn19A, Pn3, and Pn14 through serotype structural analysis and SEC

Serotype	Monomers	Charge	Charge Density	Molecular Weight (kDa)	SEC Radius (nm)
Pn7F	Gal, GalNAc, Rha, RhaAc, Glc, GlcNAc	Neutral	0	856	21
Pn19A	Rha, Glc, ManNAc, PO	Negative	1/4	209	11
Pn3	Glc, GlcA	Negative	1/2	1940	31
Pn14	Gal, GlcNAc, Glc	Neutral	0	640	18

Rha=rhamnose; RhaAc=N-acetyl-L-rhamnose; Glc=glucose; GlcNAc=N-acetyl-D-glucosamine; GlcA= glucuronic acid; Gal=galactose; GalNAc=N-acetyl-D-galactosamine; ManNAc=N-acetyl-D-mannosamine

*Note: Pn3 and Pn14 molecular weight data were obtained from previous research [3, 6]

Both Pn7F and Pn14 are neutral and have an expected charge density of zero at pH 7. Pn19A is negatively charged, with a charge density of 1/4, due to the phosphate monomer, which is

ionized and negatively charged at pH 7. Pn3 is also negatively charged, with a charge density of 1/2, due to every other monomer (glucuronic acid - GlcA) being negatively charged at pH 7 due to the negative carboxylic acid group. In terms of size, Pn19A is the smallest polysaccharide (209 kDa), followed by Pn14 (640 kDa), Pn7F (856 kDa), and Pn3 (1940 kDa), with the size of the Pn7F and Pn19A being determined from the SEC calibration (Figure 5) based on the known molecular weights of Pn3 and Pn14. The effective radius (R_{SEC}) of each serotype was estimated using Equation 3, an empirical correlation developed by Granath to calculate the radius of dextran standards with molecular weights of 505-2,655,000 Da [5]. Molecular weight (M_w) is in units of Da and R_{SEC} is in units of nm.

$$R_{SEC} = 3.1 \times 10^{-11}(M_w)^{0.47752} \quad (3)$$

Appendix B includes a sample calculation of R_{SEC} and the molecular weights (Pn7F, Pn19A) based on the linear fit shown in Figure 5, with the values summarized in Table 2.

Pn7F Ultrafiltration Behavior

Figure 6 shows the observed sieving coefficient (S_o), defined as the ratio of the polysaccharide concentration in the permeate solution compared to that in the bulk solution, as a function of filtrate flux for solutions of 0.1 g/L Pn7F in both a high and low ionic strength buffer using a Biomax™ 100 kDa membrane. Note that high sieving coefficients (close to one) would be required for a process designed to remove the free polysaccharide from the larger conjugate.

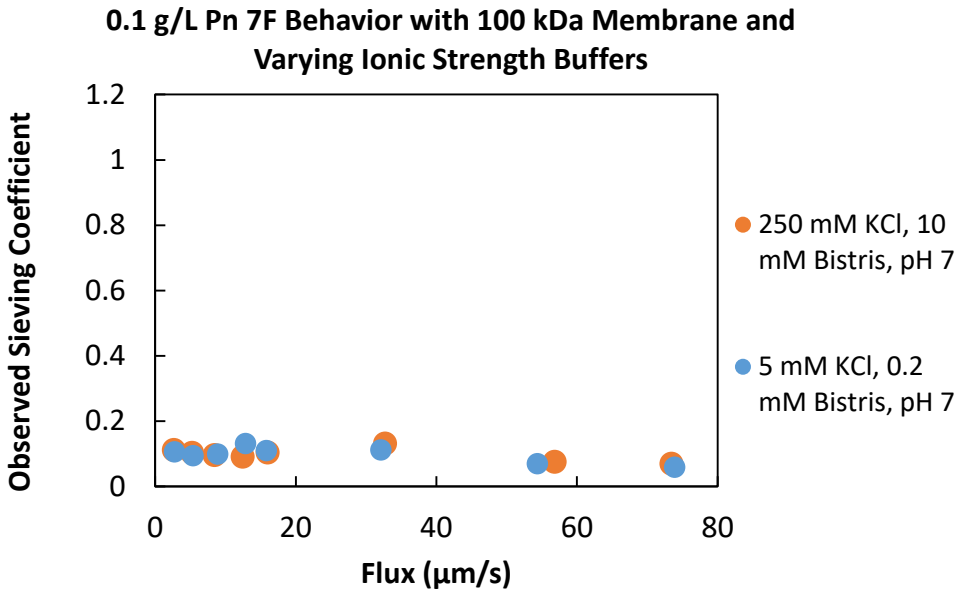


Figure 6. Observed sieving coefficient vs. filtrate flux for the ultrafiltration of 0.1 g/L solutions of Pn7F through a Biomax™ 100 kDa membrane in Bis-tris buffer at pH 7 with 5 and 250 mM ionic strength

The experiments were performed with a dilute solution (0.1 g/L Pn7F) to minimize the extent of fouling. The transmission of Pn7F through the 100 kDa membrane was quite low over the entire range of filtrate flux, with S_o around 0.1. This is consistent with the large size of the Pn7F (R_{SEC} = 21 nm) compared to the effective pore size of the 100 kDa membrane (<10 nm). In addition, the measured membrane permeability following the experiment was as much as 60-80% smaller than the initial permeability, suggesting some significant fouling (or pore blockage) during the ultrafiltration. The sieving coefficients in the low and high ionic strength solutions were essentially identical, consistent with Pn7F being a neutral polysaccharide serotype.

Ultrafiltration experiments with Pn7F were thus repeated with a larger pore-size membrane (Biomax™ 300 kDa), with data obtained at pH 7 in the low ionic strength buffer solution (5 mM KCl, 0.2 mM Bistris). Results at different bulk Pn7F concentrations are shown in Figure 7.

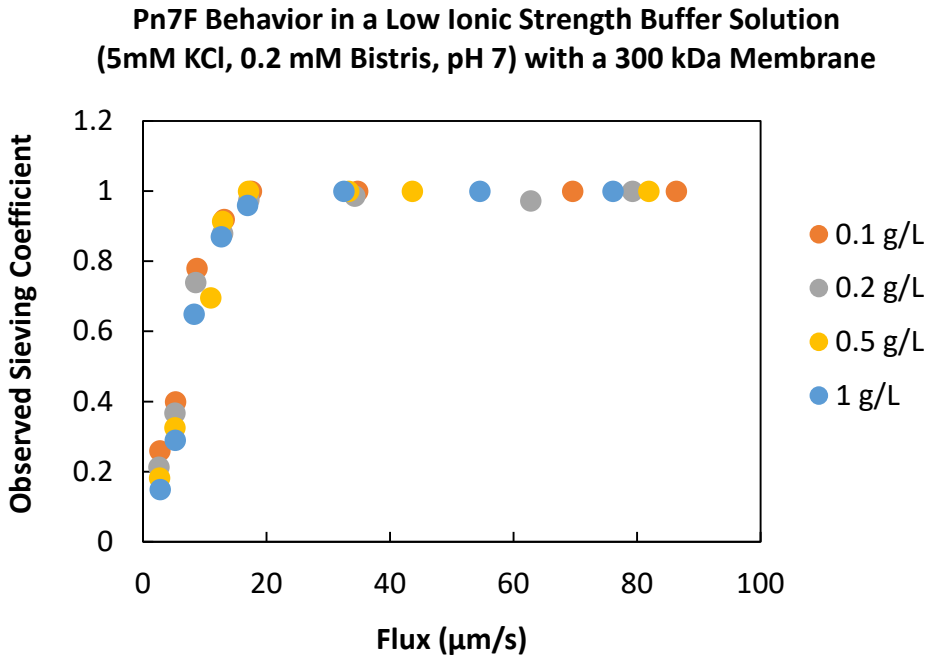


Figure 7. Observed sieving coefficient vs. filtrate flux for the ultrafiltration of solutions of varying concentrations of Pn7F through a Biomax^{TM} 300 kDa membrane in Bis-tris buffer at pH 7 with 5 mM ionic strength

The data at the four different Pn7F concentrations were essentially identical, with S_o increasing with increasing filtrate flux. For example, the transmission of the 0.1 g/L Pn7F solution increased from an S_o value of 0.26 at 2.70 µm/s to an S_o value of 0.92 at 13.14 µm/s. This behavior is likely due to concentration polarization effects in the stirred cell, with the concentration of the partially retained Pn7F accumulating on the upstream membrane surface [(7)]. This phenomenon has been discussed in some detail by Hadidi et al. [(7)]. Alternatively, the increase in transmission could be due to flow-induced elongation of the polysaccharide, allowing for increased permeation at higher filtrate flux values. This behavior has been seen previously during ultrafiltration of plasmid DNA [(13)], although there is no direct evidence that this type of elongation can also occur with these capsular polysaccharides.

To determine if the Pn7F behavior through the Biomax™ 300 kDa membrane is affected by ionic strength, a similar series of experiments to those in Figure 6 were performed with a high ionic strength buffer solution (250 mM KCl, 10 mM Bistris) with the results shown in Figure 8.

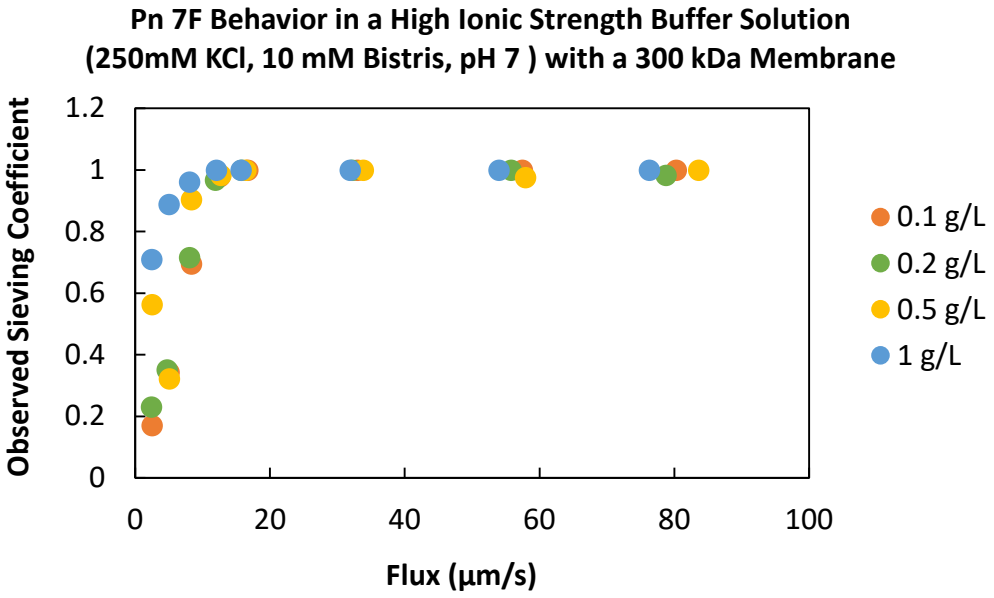


Figure 8. Observed sieving coefficient vs. filtrate flux for the ultrafiltration of solutions of varying concentrations of Pn7F through a Biomax™ 300 kDa membrane in Bis-tris buffer at pH 7 with 250 mM ionic strength

The Pn7F transmission in the high ionic strength buffer solution followed the same general trends as that seen in the low ionic strength buffer solution, consistent with the absence of any fixed charge groups on the Pn7F serotype. For example, at a flux of about 17 µm/s, both the 5 mM and 250 mM ionic strength solutions of 1 g/L Pn7F gave $S_o \approx 1$, while the values at a flux of 2 µm/s was around $S_o \approx 0.2$ for both solutions.

The ultrafiltration behavior for Pn7F can be examined in more detail by estimating the mean pore size (r_p) of the Biomax™ 300 kDa membrane. The membrane hydraulic permeability (L_p) was evaluated experimentally from data for the filtrate flux over a range of transmembrane

pressures giving L_p = 5.4 x 10^{-12} m for the Biomax™ 300 kDa membrane. The permeability can be related to the pore size using the Hagen-Poiseuille equation (Equation 4) where ε represents the membrane porosity (ε ≈ 0.5) and δ represents the thickness of the skin layer of the asymmetric membrane ($\delta \approx 1$ μm).

$$L_p = \frac{\varepsilon r_p^2}{8\delta} \quad (4)$$

This equation assumes that the membrane is composed of a uniform array of cylindrical pores [6]; thus the calculated pore size should be thought of as a flow-weighted mean value for the membrane. Appendix B includes a sample calculation of r_p. The calculated value of r_p for the Biomax™ 300 kDa membrane is r_p = 9.3 nm, which is slightly less than half the size of the Pn7F polysaccharide. Thus, even with a broad pore size distribution, we would expect the Biomax™ 300 kDa membrane to have high retention of the Pn7F, consistent with the data at very low values of the filtrate flux. However, at high filtrate flux, the Pn7F concentration at the membrane surface can be many times that in the bulk solution; thus, even if only a small fraction of the polysaccharide is able to pass through the membrane, the observed sieving coefficient can approach a value of one under these conditions.

Pn19A Ultrafiltration Behavior

The ultrafiltration behavior of a negatively charged polysaccharide serotype was explored using Pn19A, in which one out of every 4 monomers is negatively charged. Experimental data for the observed sieving coefficient obtained with 0.1, 0.2, 0.5, and 1 g/L solutions of Pn19A, buffered with Bis-tris at pH 7 with both low (5 mM) and high (250 mM) KCl concentrations are shown in Figures 9 and 10, respectively.

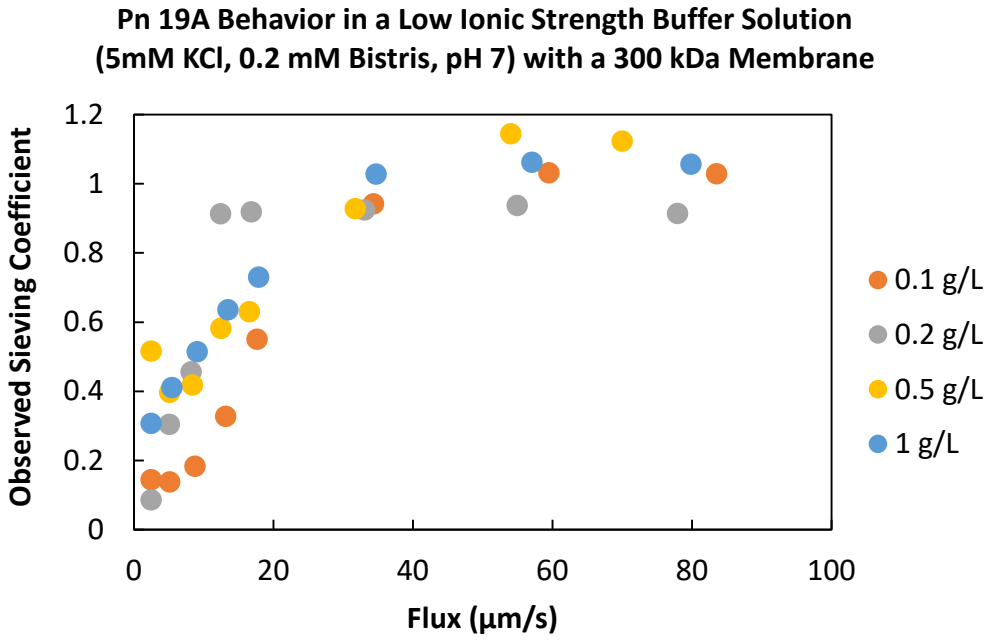


Figure 9. Observed sieving coefficient vs. filtrate flux for the ultrafiltration of solutions of varying concentrations of Pn19A through a Biomax™ 300 kDa membrane in Bis-tris buffer at pH 7 with 5 mM ionic strength

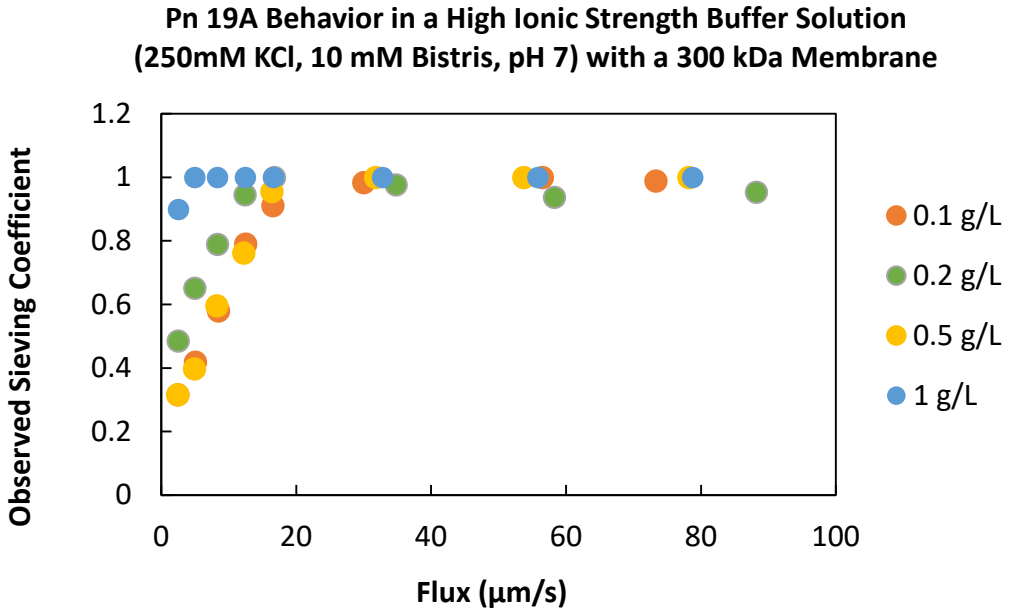


Figure 10. Observed sieving coefficient vs. filtrate flux for the ultrafiltration of solutions of varying concentrations of Pn19A through a Biomax™ 300 kDa membrane in Bis-tris buffer at pH 7 with 250 mM ionic strength

The results in the low and high ionic strength solutions of Pn19A follow the same general ultrafiltration behavior trend as seen with Pn7F, with no obvious dependence on the polysaccharid concentration (although with considerably more scatter in the data) and with the sieving coefficient increasing with increasing filtrate flux. However, when comparing Figures 9 and 10, the high ionic strength Pn19A solutions showed higher transmission than the low ionic strength solutions. For example, the observed sieving coefficient in the 5 mM KCl solution reached $S_o \approx 1$ around 40 μm/s, while that same sieving coeffiecient was achieved at a flux of 20 μm/s in the 250 mM solution. This behavior is likely due to an increase in the effective hydrodynamic radius of Pn19A at low ionic strength arising from the intramolecular electrostatic repulsion between the negatively charged phosphate monomers in the Pn19A. Thus, the dependence on solution ionic strength seen in Figures 9 and 10 is directly due to the underlying molecular composition of the Pn19A polysaccharide.

Given that only a slight reduction in membrane permeability (10-20%) was observed with the 300 kDa pore-size membrane, experiments were performed with a dilute, 0.1 g/L solution of the Pn19A using the smaller, 100 kDa pore-size membrane. Figure 11 shows the results of these experiments at four different ionic strength solutions made with 5, 20, 80, and 250 mM KCl concentrations. The data confirm the increase in transmission of the charged polysaccharides at high KCl concentrations. For example, at a flux of about 50 μm/s, the observed sieving coefficient for the Pn19A in the 80 and 250 mM KCl solutions was essentially equal to one while $S_o = 0.32$ in the 20 mM solution and only $S_o = 0.08$ in the 5 mM solution. Thus, decreasing the ionic strength caused more than a 10-fold reduction in transmission of the Pn19A polysaccharide through the Biomax™ 100 kDa membrane under these conditions. Note that the Pn19A transmission through the 100 kDa membrane (Figure 11) is uniformly smaller than that through

the larger pore-size 300 kDa membrane as expected. For example, a sieving coefficient of approximately 1 was obtained at a flux of 20 μm/s through the 300 kDa membrane, but this required a flux of nearly 50 μm/s when using the 100 kDa membrane.

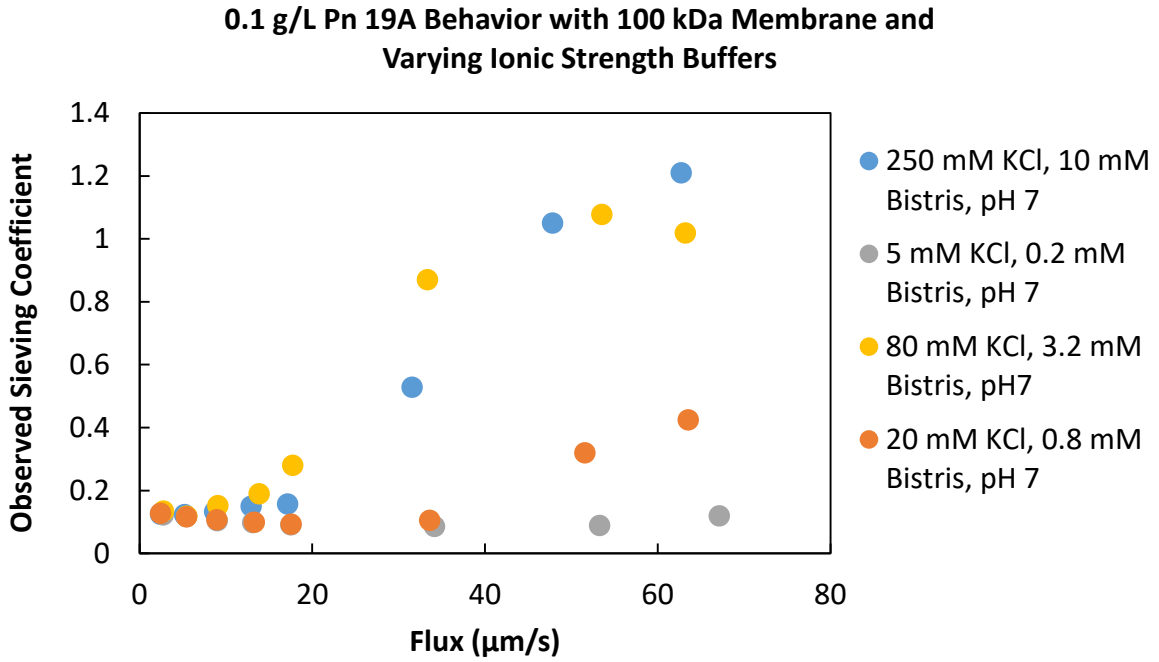


Figure 11. Observed sieving coefficient vs. filtrate flux for the ultrafiltration of solutions of 0.1 g/L Pn19A through a Biomax™ 100 kDa membrane in Bis-tris buffer at pH 7 with 5, 20, 80, and 250 mM KCl concentrations

Pn3 Ultrafiltration Behavior

The ultrafiltration behavior of an additional negatively charged polysaccharide, Pn3, was also examined to provide additional insights into the effects of polysaccharide size and electrical charge on the ultrafiltration characteristics. Figure 12 shows the behavior of a 0.1 g/L Pn3 solution (pH 7) through a 300 kDa membrane, using KCl concentrations of 5 and 250 mM.

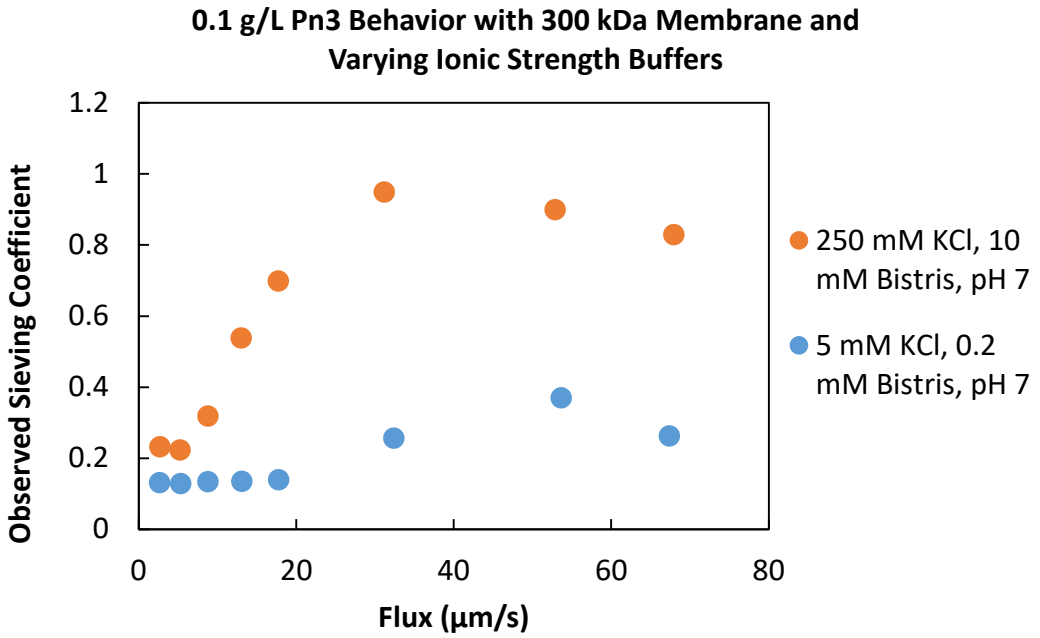


Figure 12. Observed sieving coefficient vs. filtrate flux for the ultrafiltration of solutions of 0.1 g/L Pn3 through a Biomax^{TM} 300 kDa membrane in Bis-tris buffer at pH 7 with 5 and 250 mM KCl concentrations

Similar to the results with Pn19A, the transmission of Pn3 through the 300 kDa membrane was much greater at high ionic strength compared to that at low ionic strength. For example, at a flux of about 30 µm/s, the sieving coefficient in the 250 mM KCl solution reached $S_o \approx 1$, while the sieving coefficient in the 5 mM KCl was only $S_o = 0.26$. The slight decline in Pn3 transmission at very high filtrate flux for the 250 mM solution may well be due to protein fouling under these conditions; a similar effect may have occurred with the single data point at a flux above 60 µm/s with the 5 mM solution.

Pn14 Ultrafiltration Behavior

A limited number of experiments were also performed with the uncharged Pn14 polysaccharide. No significant transmission was observed with the Biomax™ 100 kDa membrane ($S_o < 0.1$). Results at low and high ionic strength with the Biomax™ 300 kDa pore-sized membranes are shown in Figure 13.

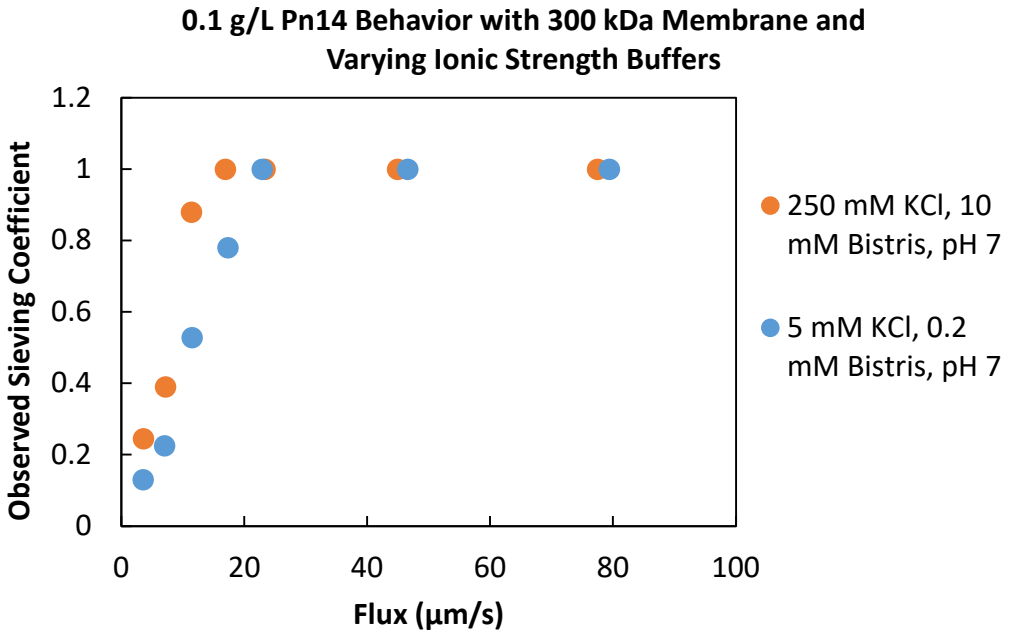


Figure 13. Observed sieving coefficient vs. filtrate flux for the ultrafiltration of 0.1 g/L solutions of Pn14 through a Biomax™ 300 kDa membrane in bistris buffer at pH 7 with 5 and 250 mM KCl concentrations

Pn14 transmission through the 300 kDa membrane was slightly higher in the 250 mM KCl solution, although this may simply reflect the inherent variability in results between different membrane samples. The observed sieving coefficients for Pn14 were similar to those for the 0.1 g/L solution of Pn7F shown previously in Figures 7 and 8. For example, the transmission of both Pn7F and Pn14 was essentially 100% at $J_v \approx 20$ µm/s, consistent with the very similar R_{SEC} for these serotypes (R_{SEC}= 21 nm for Pn7F and R_{SEC}= 18 nm for Pn14).

Observed Sieving Coefficient as a Function of Polysaccharide Radius

To determine if the ultrafiltration behavior of size-based retention applied to the dilute polysaccharide solutions (0.1 g/L) of Pn7F, Pn19A, Pn3, and Pn14, the observed sieving coefficients through the 300 kDa membrane were plotted as a function of R_{SEC} at constant flux (≈12 µm/s). The results were analyzed under the conditions of both high and low ionic strength, as shown in Figures 14 and 15, respectively.

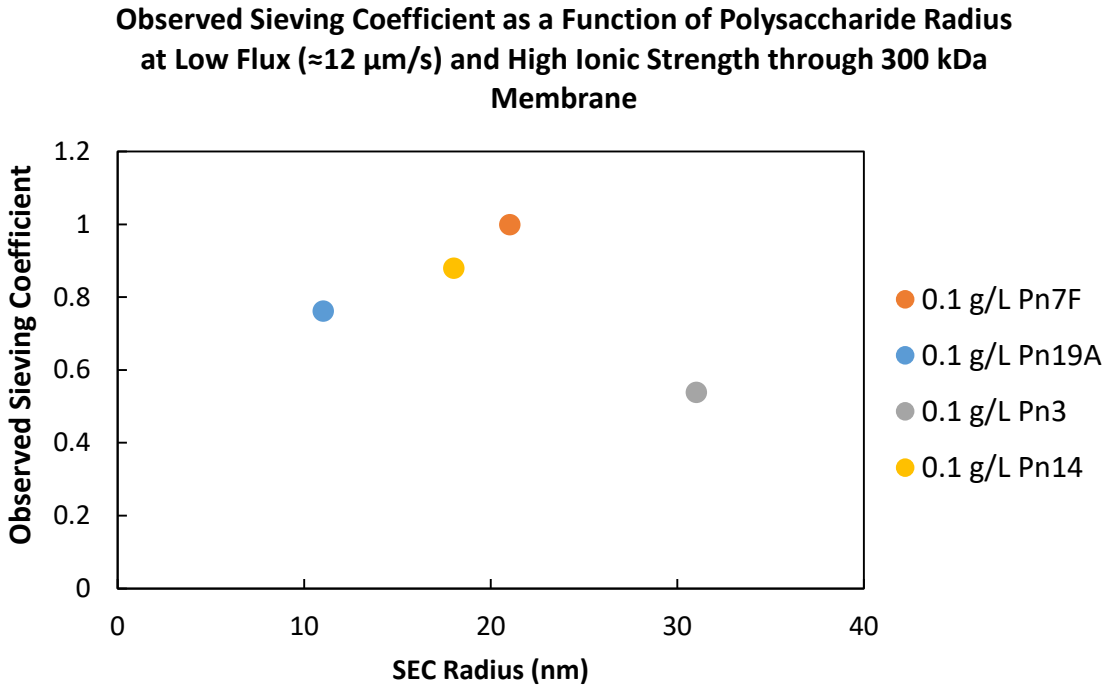


Figure 14. Observed sieving coefficient vs. R_{SEC} for the ultrafiltration of 0.1 g/L solutions of Pn7F, Pn19A, Pn3, and Pn14 through a Biomax™ 300 kDa membrane at low flux (≈12 µm/s) in Bis-tris buffer (pH 7, 250 mM KCl)

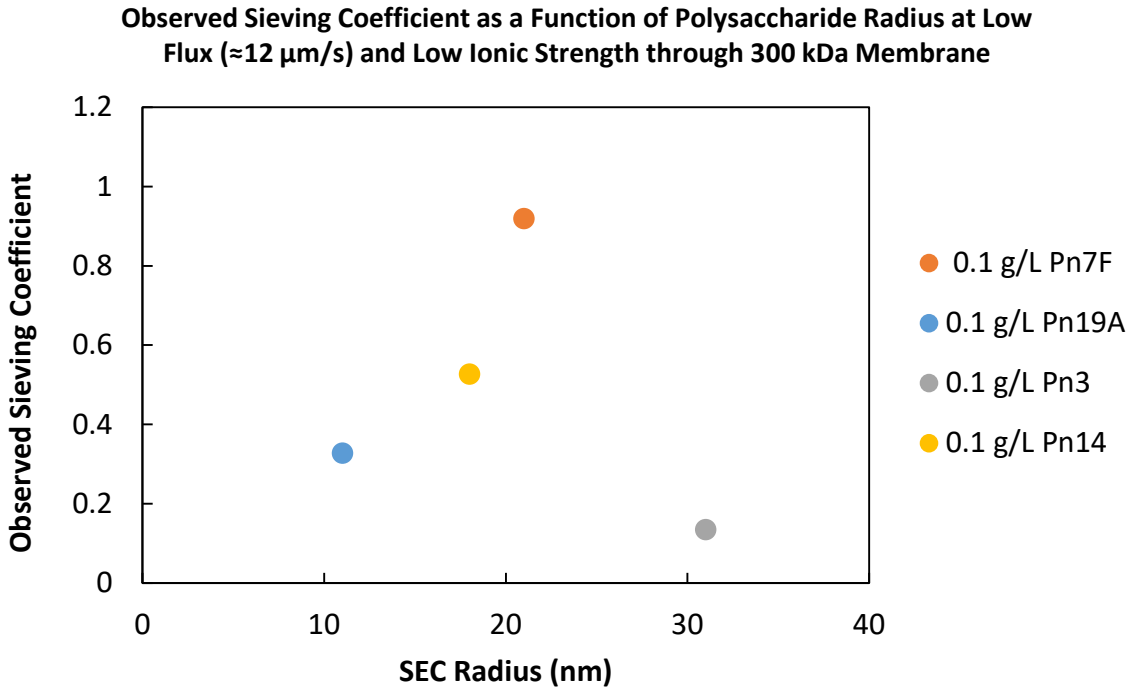


Figure 15. Observed sieving coefficient vs. R_{SEC} for the ultrafiltration of 0.1 g/L solutions of Pn7F, Pn19A, Pn3, and Pn14 through a Biomax^{TM} 300 kDa membrane at low flux (≈12 µm/s) in Bis-tris buffer (pH 7, 5 mM KCl)

Under high ionic strength conditions (Figure 14), size-based retention applied to the charged polysaccharides, Pn19A and Pn3, with the smaller polysaccharide (Pn19A) having a larger observed sieving coefficient. This behavior agrees with the fact that transmission is directly correlated to polysaccharide size. On the other hand, the neutral polysaccharides, Pn7F and Pn14, did not follow this behavior, as their S_o values were larger than that of Pn19A. At this low flux (≈12 µm/s), Pn7F and Pn14 behaved independently of the amount of KCl in solution. As described previously, the concentration of these neutral polysaccharides at the membrane surface could have been many times that in the bulk solution, and therefore, high S_o values were observed. Based on this result, the correlation between S_o and R_{SEC} was not as strong as expected.

The low ionic strength data of Figure 15 shows the charged polysaccharides having the smallest S_o values due to the electrostatic repulsion of the negatively charged phosphate monomers of Pn19A and glucuronic acid monomers of Pn3. The neutral polysaccharides behaved oppositely of what was expected, with Pn7F having higher transmission than Pn14. Even though Pn7F has the larger, branched structure, the two polysaccharides are close in hydrodynamic radius, and therefore, the result could have been due to experimental error. Similar to the results of the high ionic strength polysaccharide solutions, a direct correlation between S_o and R_{SEC} could not be concluded for low ionic strength, polysaccharide ultrafiltration behavior.

Section 4

Conclusions and Recommendations

Ultrafiltration is often a critical step in the purification of bacterial polysaccharides for the development of vaccines with persistent and effective antibody-mediated immune responses. The experimental studies conducted in this thesis provide one of the first quantitative analyses of the effects of the polysaccharide properties on the ultrafiltration behavior. Data were obtained with Pneumococcus serotypes Pn7F, Pn19A, Pn3, and Pn14 in a stirred cell ultrafiltration device using membranes with different pore size and over a range of polysaccharide concentrations and solution ionic strength. In addition, the effective sizes of the different polysaccharides were evaluated using size exclusion chromatography.

Results with Pn7F and Pn14, both of which were neutral polysaccharides, showed only a weak dependence on the polysaccharide concentration or the solution ionic strength, with the latter consistent with the absence of any significant electrostatic interactions for these neutral molecules. However, polysaccharide transmission through the Biomax^{TM} 300 kDa membrane increased significantly with increasing filtrate flux at all conditions. This increase in transmission was likely due to concentration polarization effects associated with the build-up of a more concentrated region of retained polysaccharides near the membrane surface, as reported previously by Hadidi et al.

The 3 largest polysaccharides (Pn3, Pn7F, and Pn14) were all completely retained by the Biomax^{TM} 100 kDa pore-size membrane (r_p = 6 nm) even at high filtrate flux. However, the smallest polysaccharide, Pn19A, which has a molecular weight of 209 kDa and an effective hydrodynamic radius of 11 nm, was able to pass through the pores of the 100 kDa membrane with high transmission achieved at very high filtrate flux (> 50 μm/s). The transmission of

Pn19A through the 100 kDa membrane was much lower than that through the 300 kDa membrane as expected, reflecting the size-based retention of the polysaccharides.

The ultrafiltration behavior of the charged polysaccharide serotypes (Pn19A and Pn3) was also a strong function of the solution ionic strength, with much greater transmission seen at high salt concentrations. For example, the observed sieving coefficient for Pn19A decreased by more than 10-fold as the KCl concentration was reduced from 80 to 5 mM, indicating that it was possible to move from a membrane that was completely non-retentive to Pn19A ($S_o \approx 1$) to a membrane that has very low transmission ($S_o < 0.1$) simply by reducing the ionic strength of the solution. This increase in retention at low ionic strength is likely due to intramolecular electrostatic interactions between the negatively-charged carboxylic acid groups of the glucuronic acid monomer (GlcA) present in the Pn19A and Pn3 serotypes. When analyzing the dependence of S_o on R_{SEC} for the four polysaccharide serotypes, the correlation was not as strong as expected, specifically under high ionic strength solution conditions.

Additional insights into the ultrafiltration behavior of these polysaccharide serotypes could potentially be obtained by analysis of the observed sieving coefficient data using the concentration polarization model to specifically evaluate the actual sieving coefficient, S_a, and the bulk mass transfer coefficient, k_m. Note that S_a is equal to the ratio of the solute concentration in the filtrate solution to the solute concentration in the solution immediately adjacent to the membrane and provides a measure of the true retention characteristics of the membrane, independent of the filtrate flux. The S_a values could then be analyzed using available hydrodynamic models for membrane transport that account for the partitioning and transport of solutes as a function of the solute and pore size.

All of the values for the effective polysaccharide size (R_{SEC}) were determined at high ionic strength. It would be very interesting to try to perform additional measurements over a range of solution conditions to estimate the effective size of the polysaccharides as a function of the solution ionic strength. These data could potentially be used to develop a model correlation for the polysaccharide transmission through different pore size ultrafiltration membranes as a function of the effective polysaccharide size (determined by SEC) and the effective membrane pore size (determined from the measured values of the membrane permeability).

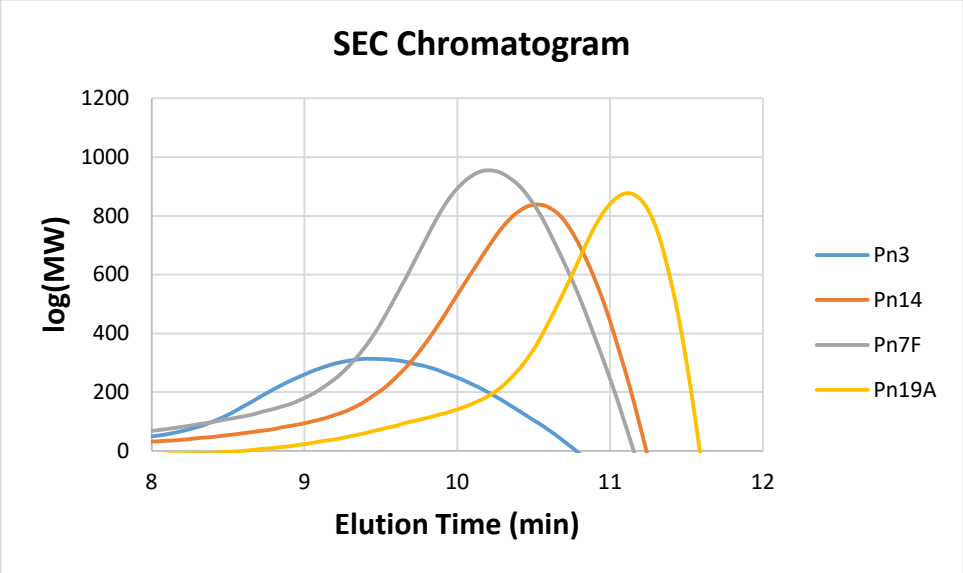
It would also be interesting to perform additional characterization of the different polysaccharide serotypes, e.g., through measurements of the viscosity or viscoelastic properties of the polysaccharide solutions as a function of the polysaccharide concentration and the solution ionic strength. A highly viscous or viscoelastic polysaccharide would likely have very different mass transfer characteristics in the stirred cell, and this could also have an impact on membrane fouling.

Future experiments might also examine the effects of solution pH and the presence of divalent cations (e.g., Ca^{+2} or Mg^{+2}) on both the physical properties and ultrafiltration behavior of the different polysaccharides. Decreasing pH, i.e., increasing the concentration of H^{+} ions, should lead to protonation of some of the carboxylic acid groups on the charged polysaccharides, altering the magnitude of the electrostatic interactions. The divalent cations may also participate in “salt-bridging”, would could significantly alter the structure of the polysaccharides. However, the polysaccharide serotypes may by unstable under some of these conditions, so considerable care must be taken in interpreting the data and accounting for the possibility of polysaccharide degradation (e.g., hydrolysis).

It would also be interesting to expand these studies to a wider range of polysaccharides with different molecular weight and charge density. Due to the limited availability of polysaccharides supplied by Pfizer, we were only able to study two neutral and two highly charged polysaccharide serotypes (with one out of two and one out of 4 charged saccharide monomers). Experiments with a more weakly charged polysaccharide could provide additional support for some of the current conclusions regarding the effects of solution ionic strength on the ultrafiltration behavior. These data could be complemented by studies with membranes having larger effective pore size and with different surface properties (e.g., cellulose versus the polyethersulfone Biomax^{TM} membranes) to provide a deeper understanding of the effects of the membrane properties on the ultrafiltration behavior of these important biotherapeutics.

Appendix A

SEC Chromatogram



Polysaccharide Serotype	Elution Time (min)
Pn3	9.68
Pn7F	10.2
Pn14	10.4
Pn19A	11.1

Appendix B

Sample Calculations

Molecular Weight of Pn7F

Linear Fit Equation of Figure 5: $y = -0.6813x + 9.8815$

Pn7F Elution Time (x): *10.2 min*

$y = (-0.6813*10.2) + 9.8815 = 2.93 = log(MW)$

$MW = 10^{2.94} =$ ***856 kDa***

R_{SEC} of Pn7F

*M_W in Da

$R_{SEC} = 3.1*10^{-11} * (M_W)^{0.47752} = 3.1*10^{-11} * (856{,}000)^{0.47752} =$ ***21 nm***

300 kDa Membrane Mean Pore Size (L_p)

$$L_p = \frac{\varepsilon r_p^2}{8\delta}$$

$5.4*10^{-12}\ m = (0.5 * (r_p)^2) \div (8 * (1 * 10^{-6})\ m)$

$r_p =$ ***9.3 nm***

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- Optimized sunscreen formula aesthetics by screening emollients for whitening and skin feel
- Conducted an interested scientist study to assess consumer aesthetic perception of 6 sunscreen prototypes for lead formulation direction
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