THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF CHEMICAL ENGINEERING

SALT-INDUCED CHANGES IN TRANSMISSION OF LINEAR, OPEN-CIRCULAR, AND SUPERCOILED PLASMID DNA

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ABSTRACT

Previous studies have demonstrated that ultrafiltration can be used to effectively purify plasmid DNA, potentially providing an opportunity for the large-scale production of DNA-based vaccines and gene therapy agents. However, the selectivity for the separation of the different DNA isoforms has typically been inadequate.

The objectives of this thesis were to: (1) determine the effect of varying salt concentrations on the transmission of the linear, supercoiled, and open-circular isoforms of plasmid DNA through different ultrafiltration membranes, (2) compare the effects of monovalent (NaCl) and divalent ($MgCl_2$) salts on plasmid transmission, and (3) examine the possibility of using salt concentration to improve the selectivity of isoform separation.

Experiments were performed with UltraCel composite regenerated cellulose membranes having 100 and 1000 kDa nominal molecular weight cutoff along with 100kDa Biomax polyethersulfone membranes. Data were obtained using a 3 kilobase pair plasmid in Tris-EDTA buffer with varying NaCl and $MgCl_2$ salt concentrations. Limited experiments were also performed using a 16.8 kbp plasmid. Sieving coefficients were evaluated using a PicoGreen fluorescence assay. The results show a strong increase in plasmid transmission with increasing salt concentration for all three isoforms. In addition, the critical flux for plasmid transmission decreased with increasing salt concentration for both the monovalent and divalent salts. $MgCl_2$ has a much larger effect on plasmid transmission than NaCl, consistent with the greater increase in ionic strength and the possibility of intramolecular salt bridges. The effect of salt on transmission of the linear plasmid was less pronounced than that on either the open-circular or supercoiled plasmids, which could potentially be exploited for enhanced separations. These

results provide valuable insights into the effects of salt concentration on plasmid ultrafiltration and on the possible optimization of plasmid isoform separations.

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ACKNOWLEDGEMENTS

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Chapter 1

Introduction

Ultrafiltration is used to separate or concentrate biomolecules by applying a pressure gradient across a semipermeable membrane. Molecules that do not permeate through the membrane due to size or charge restrictions are left in the solution known as the retentate, while molecules that pass through the membrane make up what is known as the permeate or filtrate. Ultrafiltration membranes are generally classified based on the nominal molecular weight cut-off, which refers to the molecular weight of a solute that is 90% retained by the membrane.

Ultrafiltration has been used in an industrial setting to purify or concentrate a wide range of protein solutions in both the food industry and in biotechnology [1]. For example, during the production of cheese, whey protein is produced as a byproduct of the curdling process. Ultrafiltration membranes have been employed in the production of cheese to recover additional whey proteins, resulting in an increase in its use as a food supplement as well as a decrease in waste disposal costs. Ultrafiltration is also used in the production of several juices for the removal of suspended solids and colloidal material, which can affect juice quality if not removed during processing. In the fish and poultry industry, waste streams generated during processing tend to have relatively high amounts of valuable proteins that can be recovered via ultrafiltration, improving economic margins and reducing the amount of waste discharged to the environment. Ultrafiltration could potentially be employed in the meat industry to recover colloids, suspended matter, and mineral substances, as suggested by Bohdziewicz et al. [7].

Ultrafiltration is also employed throughout the biopharmaceutical industries for the recovery and purification of high-value biological products such as monoclonal antibodies [8]. This includes concentration of feed streams prior to other purification steps in the downstream process (e.g., chromatography or crystallization) as well as achieving the desired product concentration as part of the final formulation step. Diafiltration is used for buffer exchange and desalting, with the protein retained by the ultrafiltration membrane while the smaller buffer components are washed through the membrane and into the permeate.

There is also interest in the possibility of using ultrafiltration for the purification of plasmid DNA for use in gene therapy applications and as DNA-based vaccines [2-4]. Plasmids are circular, self-replicating, extrachromosomal DNA molecules. They exist in three topological isoforms: supercoiled, linear, and open-circular. Sketches of these isoforms are provided below:

Figure 1-1: Sketch detailing the structure of the supercoiled, open-circular, and linear plasmid isoforms, respectively.

Bacteria cells, generally E. coli, are employed to produce plasmid DNA for bioprocessing applications. E. coli naturally produce supercoiled DNA, also known as circular covalently closed DNA, with the two ends of the DNA chain joined together, but with a “twist” in the DNA helix [5]. However, shear stress during processing can cause breaks in the strands of supercoiled DNA. When one strand is broken, it will unwind to alleviate the helical stress,

forming the open-circular isoform. When both strands are broken, the strands will unwind to generate the linear isoform.

The supercoiled isoform is of greatest interest in therapeutic applications, since this structure is most easily transcribed into mRNA and then protein. For example, in gene therapy applications, foreign plasmid DNA is introduced into the body to replace a malfunctioning gene (e.g., in the treatment of hemophilia) or to produce specific antigens to promote a strong immune response. Extensive research is currently being conducted on gene therapy for a wide range of applications including the treatment of many genetic disorders as well as cancer [6]. The plasmids used in gene therapy and in the development of DNA-based vaccines are generally small, on the order of 3-20 kilobase pairs (kbp). They contain origins of replication appropriate for both the bacterial host and the human patient as well as the gene sequence for the protein of interest. According to the FDA (Considerations for Plasmid DNA Vaccines for Infectious Disease Indications), plasmid DNA needs to have a supercoiled isoform composition of at least 80%. Separation processes must therefore be able to lower the concentrations of the open-circular and linear isoforms to less than 20% of the total plasmid concentration.

Plasmid purification is currently well-established on a laboratory scale, but the purification methods currently used at small-scale are inadequate for large-scale commercial applications due to limited yield and throughput as well as high labor costs [2]. For example, density gradient centrifugation is widely used for lab-scale purification of the supercoiled isoform, but it is labor intensive, has very low throughput, and is relatively expensive [10]. Anion exchange and size exclusion chromatography can both be used for DNA purification, but they do not provide effective separation of the different plasmid DNA isoforms. Anion exchange chromatography also suffers from very low binding capacities due to the large size of the

plasmid DNA [11], while size exclusion chromatography is impractical due to the physical similarities between the different DNA isoforms [12]. Other methods of isoform separation need to be developed to meet the demand for relatively pure (>80%) supercoiled plasmid DNA for therapeutic applications.

Previous work by Latulippe and Zydney [3] has shown that ultrafiltration can be used for the separation of DNA isoforms based on differences in elongational flexibility of the different topological isoforms. The radius of gyration for even a relatively small 3 kbp plasmid falls in the range of 60-80 nm, which is significantly larger than the average pore size of a typical ultrafiltration membrane (between 1 and 15 nm). However, plasmid DNA is still able to permeate through these small pore size ultrafiltration membranes due to the elongation of the plasmid in the converging flow field into the membrane pores [3]. Latulippe and Zydney [14] showed that it is possible to identify ultrafiltration conditions which allow separation of the different plasmid isoforms based on differences in the elongational flexibility of the linear, open-circular, and supercoiled plasmids.

Although most studies of DNA ultrafiltration have been performed using moderate to high salt concentrations, Latulippe and Zydney [15] have also shown that transmission of the supercoiled plasmid during ultrafiltration is a function of the salt concentration due to the effects of both inter- and intra-molecular electrostatic interactions on DNA structure and elongation. The transmission of the supercoiled plasmid increased with increasing NaCl concentrations at filtrate flux values between 20 and 100 μm/s. However, no data are currently available on the effects of different salt concentrations on the transmission of either the linear or open-circular DNA isoforms.

The objectives of this thesis were to: (1) evaluate the effects of both NaCl and $MgCl_2$ on the transmission of the linear and open-circular isoforms of 3 and 16.8 kilobase pair (kbp) plasmid through several different ultrafiltration membranes, and (2) determine whether it is possible to enhance the purification of the supercoiled plasmids from the linear and open-circular isoforms by appropriate adjustment of the solution environment.

Chapter 2

Materials and Methods

2.1 Membrane Preparation

Composite regenerated cellulose (UltraCel) and polyethersulfone (Biomax) membranes were obtained from EMD Millipore (Bedford, MA). The UltraCel membranes were available with both 100 kDa and 1000 kDa nominal molecular weight cut-off (MWCO), while the Biomax membranes were used with 100 kDa MWCO.

Membranes were first cut into 25mm discs using a special cutting device available in our laboratory. The membranes were then soaked in a solution containing 10% deionized (DI) water and 90% isopropanol for 45 minutes to fully wet the membrane pore structure. The DI water was obtained from a NANOpure Diamond water purification system (Barnstead Thermolyne Corporation, Dubuque, IA). Membranes were then placed in the base of a 15 mL Amicon stirred cell (EMD Millipore) and flushed with 100 mL of DI water to remove residual isopropanol and any other impurities. Membranes were then flushed with 15 mL of the appropriate buffer solution to be used in the subsequent plasmid ultrafiltration experiment.

2.2 Preparation of Linear / Open-circular Plasmids

3.0 kbp pBluescript supercoiled plasmids (200 µg/mL) and 16.8 kbp pBluescript supercoiled plasmids (250 µg/mL) were purchased in bulk from Aldevron and used as stock

plasmid solutions. 110 µL of each of these solutions were transferred to several micro-centrifuge tubes for enzymatic digestion to form the linear and open-circular isoforms. Linear P-EMP (3 kbp) plasmids were generated using the restriction enzyme BamHI (Invitrogen, 4 u/µg) in 10x NEBuffer 3.1 buffer (New England Biolabs, Inc.). P-EMP open-circular plasmids were generated using the enzyme NtA1WI (New England Biolabs, Inc., 2 u/µg) in 10x CutSmart buffer (New England Biolabs, Inc.). 5 µL of the BamHI enzyme solution was added to 27.5 µL of NEBuffer 3.1, 110 µL of the P-EMP supercoiled plasmid, and 135 µL of DI water. The resulting solutions were gently mixed and incubated at 37°C for three hours. An analogous procedure was used for the P-EMP open-circular isoform with 6.6 µL of NtA1WI enzyme added to 27.5 µL of the 10x CutSmart buffer, 110 µL of plasmid, and 135 µL of DI water. Linear P-FDY (16.8 kbp) plasmids were generated using 2.75 µL of the enzyme PaeR71 (New England Biolabs, 2 u/µg) in 10x NEB buffer, 110 µL of plasmid, and 135 µL of DI water.

The desired isoform was purified from the enzymatic digestion as follows. 1375 µL of Buffer PB (Qiagen) was added to each sample. The resulting solution was mixed and divided equally among four separate spin filters, each of which was centrifuged for 1 min at 2000 rpm. The filtrate was discarded, an additional 750 µL of Buffer PE (Qiagen) was added to each column, and the columns were centrifuged again for 1 min. The top portion of each column was then removed and placed in a clean micro-centrifuge tube. 50 µL of warm TE buffer (at 60 ^{o}C) was then added to each tube and incubated for 5 min. Samples were then centrifuged for 1 min to obtain the purified plasmid solution.

2.3 Agarose Gel Electrophoresis

The integrity and identity of the plasmid isoforms were evaluated by Agarose Gel Electrophoresis (AGE). The gel was prepared by adding 0.36 g agarose powder to 45 mL of TAE buffer to produce a 0.8% agarose solution. The solution was loosely covered and microwaved for 30 s, mixed gently, and then microwaved again for 5-10 s (or until boiling was observed in the flask). The solution was cooled for 15 min, 4.5 µL of nucleic acid staining dye (GelStar) was added, and the gel was poured into a cast and allowed to solidify for 40 min before being placed in the electrophoresis apparatus.

Samples of the linear and open-circular isoforms were prepared by mixing 2 µL of the plasmid solution with 3 µL of loading dye and 13 µL of DI water; 1 µL of the plasmid solution was used for the supercoiled isoform (with an additional 1 µL of DI water). A TrackIt 1kbp DNA ladder (Invitrogen) was used as an internal calibration standard for the AGE.

The samples were loaded into wells, TAE buffer added, and an electric field of 50 V was applied for 90 min, causing the DNA to migrate through the gel. A typical picture of a gel, taken using a FluoroChem E System (ProteinSimple, San Jose, CA), is shown in Figure 2-1. The DNA ladders are in the left- and right-most columns. Lanes 2, 3, and 4 show samples of the supercoiled, open-circular, and linear DNA. The solution of the linear isoform (Lane 4) appears to contain a small amount of supercoiled DNA. The open-circular sample (Lane 3) also contains some undigested supercoiled DNA. The faint band in Lane 2 is probably due to DNA dimers or a small amount of open-circular plasmid. The samples used in this thesis were relatively pure, showing undetectable amounts of the undesired isoforms on the agarose gels.

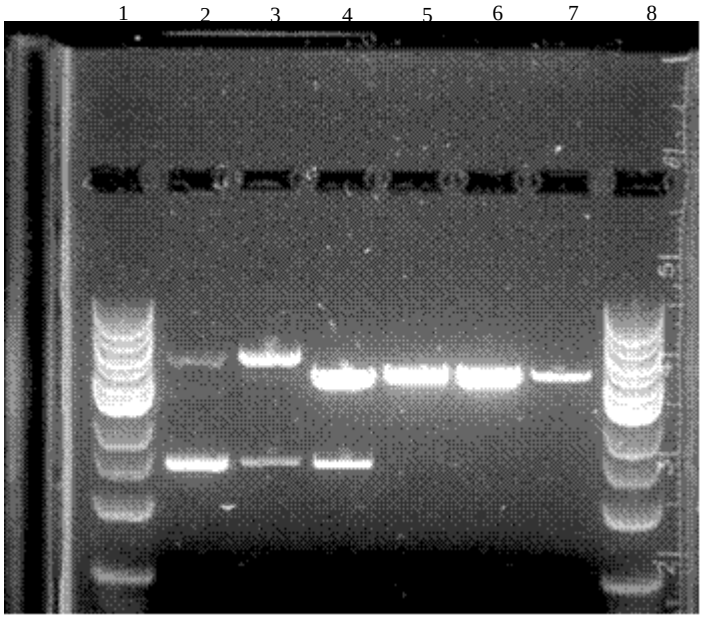

Figure 2-1: Agarose Gel Electrophoresis. Ladder solutions were loaded into lanes 1 and 8. Linear plasmid solutions were loaded into lanes 4, 5, 6 and 7. Lanes 2 and 3 contain the supercoiled and open-circular isoforms.

2.4 Buffer and Plasmid Solution Preparation

Buffer solutions were prepared by diluting 100x concentrated Tris-EDTA buffer (Fluka) with DI water. Sodium chloride (NaCl) and magnesium chloride ($MgCl_2$), both obtained from Sigma Aldrich, were added to achieve the desired salt concentration. All buffer solutions were prefiltered through 0.2 µm pore size membranes (Pall Corporation) to remove any solid particulates from solution. The conductivity and pH of all buffer solutions were measured using a 105APlus Conductivity meter (Thermo Orion) and a 420APlus pH meter (Thermo Orion), respectively. Buffer solutions were generally made in volumes of 0.5 to 1 L and used within two weeks. Concentrated solutions of the desired plasmid solution were added to the buffer to generate 0.25 µg/mL solutions. Solutions were mixed on a rocker before use.

2.5 Stirred Cell Apparatus

All experiments were performed using an Amicon 15mL stirred cell. Membranes were placed in the base of the stirred cell with the shiny-side facing up. The plasmid solution was added, the cap on the stirred cell was screwed in place, and the entire apparatus was connected to an air-pressurized feed reservoir. A schematic of the system is provided below:

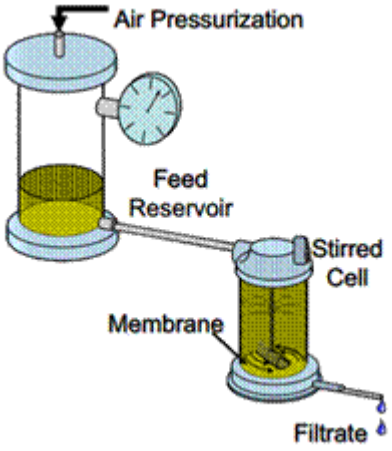

Figure 2-2: Sketch of the stirred cell setup used to perform sieving experiments, taken from [4].

The filtrate flux was set by adjusting the pressure in the feed reservoir. Filtrate samples were collected at each filtrate flux after the system had stabilized, typically after collection of a minimum of 0.2 mL of filtrate. The stirred cell was then opened and a feed sample was taken directly from the stirred cell.

The observed sieving coefficient (S_o) is used as a means of quantifying plasmid transmission through a membrane. It is defined as the ratio of the plasmid concentration in the permeate to the concentration in the feed, as given in equation 2.1.

$$S_0 = \frac{C_{Permeate}}{0.5*[C_{Feed,initial}+C_{Feed,final}]} \quad (2.1)$$

The term in the denominator is the time-average plasmid concentration in the stirred cell, determined from samples taken immediately before and after evaluating the permeate concentration. The plasmid concentrations in the feed and permeate were determined by fluorescence assay as described in Section 2.7.

2.6 Permeability Measurements

The membrane hydraulic permeability, L_P, was determined from data for the filtrate flux (J_V) as a function of the applied pressure (ΔP):

$$L_P = \frac{J_V}{\Delta P} \quad (2.2)$$

The filtrate flux, equal to the volumetric flow rate divided by the membrane area, was calculated from the measured volumetric filtrate flow rate determined by timed collection using a digital scale to evaluate the mass of the collected filtrate samples.

2.7 Sample Analysis

Plasmid concentrations were determined via fluorescence using a GENios FL TECAN microplate reader. 70 µL of each sample were pipetted into a well on a 96-well plate (Black Cliniplate, Thermo Fisher Scientific). Additional wells were filled with calibration standards of known concentrations (0 µg/mL, 0.01 µg/mL, 0.05 µg/mL, 0.1 µg/mL, 0.5 µg/mL). Quant-iT

PicoGreen dsDNA reagent was diluted 200 times with TE buffer (Invitrogen), covered with aluminum foil, and mixed thoroughly on a rocker. 70 µL of this reagent was added to each sample well, the plates were gently shaken for 5 min at 36°C, and the absorbance was measured with the concentration determined by comparison with the calibration standards.

Chapter 3

Results and Discussion

3.1. Plasmid Isoform Analysis

The linear and open-circular plasmid isoforms were generated by enzymatic digestion according to the procedures described in Section 2.2. Purity was qualitatively assessed using agarose gel electrophoresis. Typical results are shown in Figure 3-1 for the open-circular (Lanes 2-5), supercoiled (Lane 6), and linear (Lane 7) isoforms.

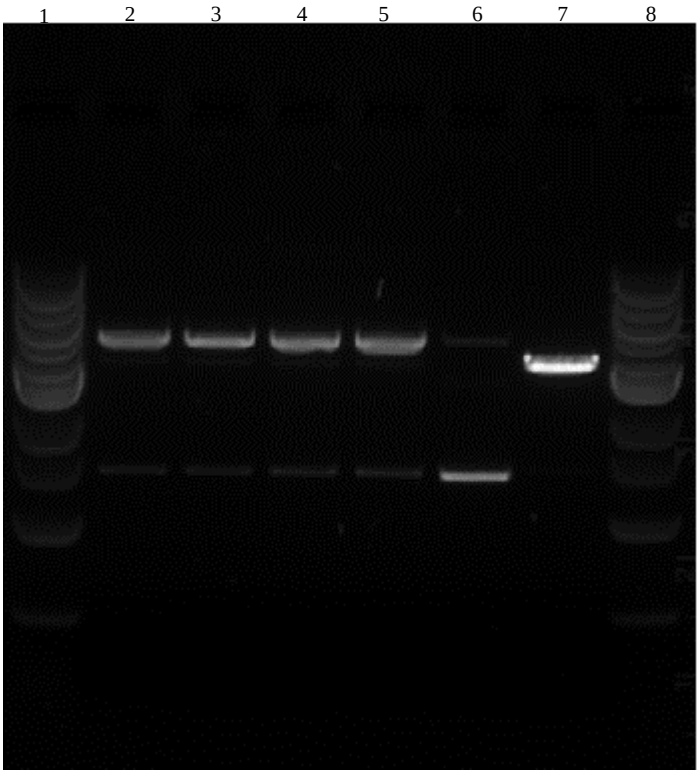

Figure 3-1: Open-circular (lanes 2-5), supercoiled (lane 6), and linear (lane 7) isoforms analyzed via agarose gel electrophoresis.

The open-circular isoform migrates most slowly through the gel and thus appears as a band closest to the top of the image in Figure 3-1; this behavior is consistent with previous results [4]. All of the bands are well-defined, with no measurable impurities or residual amounts of the other isoforms. Agarose gels were also obtained on permeate and feed samples after the ultrafiltration experiment to verify that there were no changes in the isoform identity or size over the course of the run.

3.2. Effect of Salt Concentration on Plasmid Transmission

Typical data for the effect of NaCl concentration on the transmission of the supercoiled isoform through the 100 kDa UltraCel membrane are shown in Figure 3-2. There was no evidence of any plasmid degradation in these experiments, with agarose gels of permeate samples looking identical to that of feed samples. There was also no evidence of any membrane fouling; the permeability evaluate at the end of each experiment was within 10% of the initial permeability. The lack of fouling with this dilute plasmid solution is consistent with results reported elsewhere [17]; fouling is only significant when using more concentrated solutions (well above 1 μg/ml). Plasmid transmission in the solution containing 150 mM NaCl increased with increasing filtrate flux, going from a value of around $S_o = 0.05$ at a flux of 10 μm/s to $S_o > 0.5$ at a flux of 140 μm/s. The increase in transmission with increasing filtrate flux seen in the 150 mM NaCl solution is due to the flow-induced elongation of the supercoiled plasmid as discussed by Latulippe and Zydney [3]. The critical flux for plasmid transmission, determined by extrapolation of the sieving coefficient data to $S_o = 0$, gives $J_{crit} \approx 20 \pm 10$ μm/s.

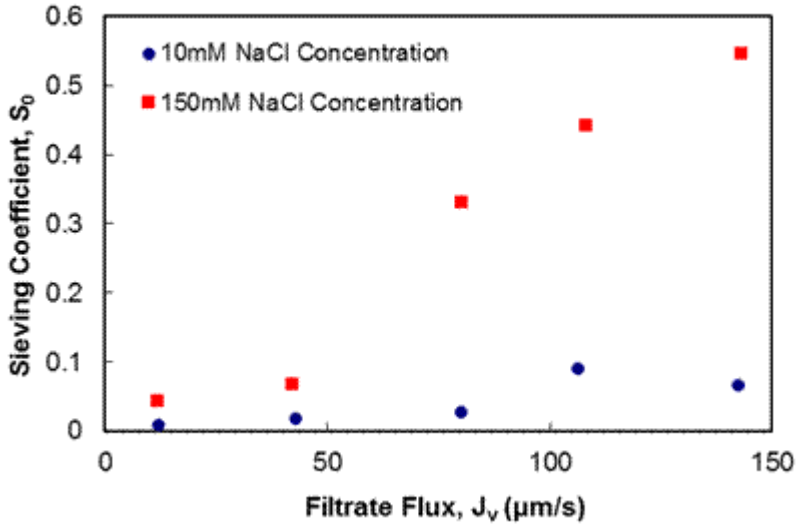

Figure 3-2: Observed sieving coefficient for the supercoiled isoform through a 100 kDa UltraCel membrane as a function of filtrate flux at both low (10 mM) and high (150 mM) NaCl concentrations.

The results in the 10 mM NaCl solution are considerably different. In this case, the transmission of the supercoiled plasmid was nearly independent of the filtrate flux with $S_0 < 0.1$ for filtrate flux values between 10 and 140 µm/s. The net result is that the addition of NaCl causes a large increase in plasmid transmission at high values of the filtrate flux, with the sieving coefficient in the 150 mM NaCl solution being more than five times as large as that in the 10 mM solution at a flux of 145 µm/s.

Corresponding data obtained using a 100 kDa Biomax membrane at 1 and 300 mM NaCl concentrations in TE buffer are shown in Figure 3-3. The sieving coefficients with the Biomax membrane are uniformly larger than those with the UltraCel membrane, even though these membranes have the same molecular weight cutoffs. This behavior is consistent with previous results showing that the Biomax membranes have a larger pore size for a given MWCO [16].

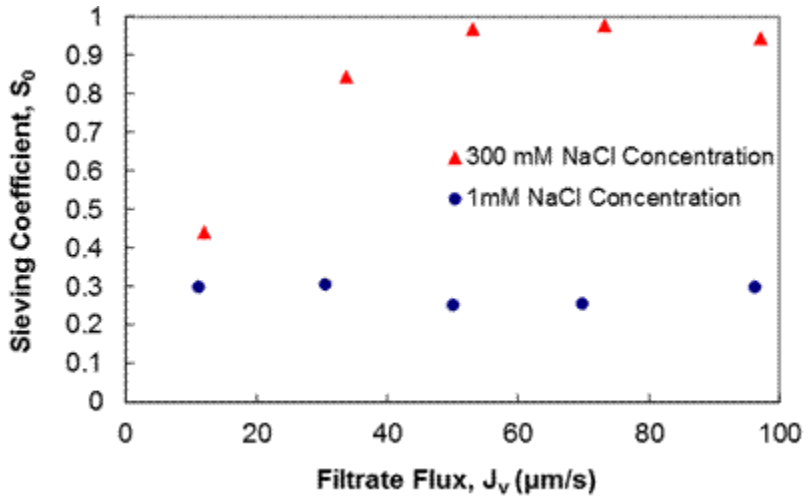

Figure 3-3: Transmission of the supercoiled isoform through a 100kDa Biomax membrane as a function of filtrate flux at 1 and 300 mM NaCl concentrations.

The transmission of the supercoiled plasmid in the solution containing a high NaCl solution increased with increasing filtrate flux, going from a value of around $S_o = 0.45$ at a flux of 10 µm/s to $S_o > 0.95$ at a flux of 50 µm/s. The critical flux for this run would be well below 10 µm/s. The results for the 1 mM NaCl solution are significantly different. Transmission was nearly independent of the filtrate flux with $S_o = 0.25 \pm 0.05$ for filtrate flux values between 10 and 95 µm/s. The origin of this behavior is unclear. The addition of NaCl again causes a large increase in plasmid transmission at high values of the filtrate flux, with the sieving coefficient in the 300 mM NaCl solution being more than three times as large as that in the 1 mM solution at a filtrate flux between 50 and 100 µm/s.

As discussed by Latulippe and Zydney [3], the increase in transmission of the supercoiled plasmid with increasing NaCl concentration is most likely due to conformational changes associated with the shielding of the intramolecular electrostatic interactions between the negatively-charged phosphate groups present in the DNA backbone. Previous studies have shown that increasing solution ionic strength significantly decreases the radius of gyration of

plasmids in solution due to this electrostatic shielding. In addition, the UltraCel and Biomax membranes both have a small negative charge [18], which could lead to electrostatic repulsion between the negatively-charged membranes and the negatively-charged plasmids. This intermolecular repulsion should also be shielded at high ionic strength.

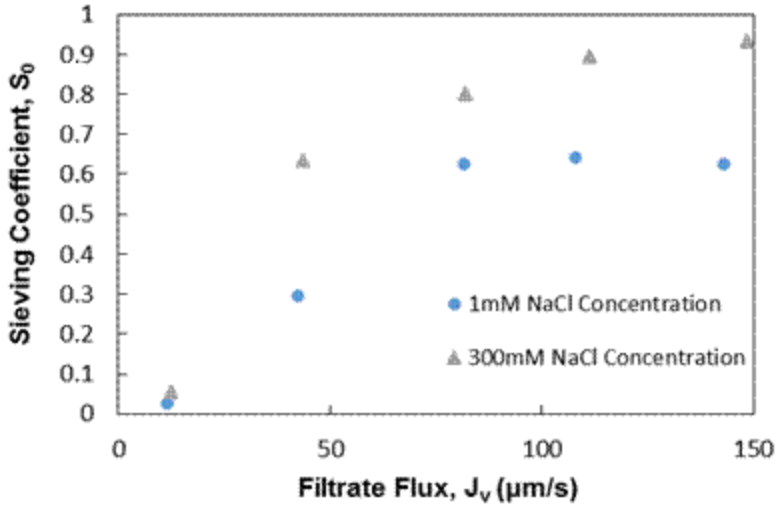

Figure 3-4: Transmission of the linear isoform through a 100 kDa UltraCel membrane as a function of filtrate flux in 1 and 300 mM NaCl solution.

Figure 3-4 shows results for a sieving experiment performed with a 0.25 µg/mL solution of the linear plasmid. Transmission of the linear plasmid is negligible at a filtrate flux of around 12 µm/s at both salt concentrations but then increases with increasing flux. The sieving coefficients in the 300 mM NaCl solution are slightly larger than those in the 1 mM solution, but this effect is quite small over the entire range of filtrate flux. For example, at a flux of around 110 µm/s, S_o increases from 0.64 in the 1 mM NaCl solution to 0.89 in the 300 mM solution, a change of less than 40%. In addition, the sieving coefficients for the linear plasmid at both NaCl concentrations are significantly larger than those for the supercoiled plasmid (Figure 3-3).

The relatively small effect of salt concentration on transmission of the linear isoform is further illustrated in Figure 3-5, which shows results for a sieving experiment performed with a 0.25 µg/mL solution of the linear plasmid in both 10 and 150 mM NaCl buffer using a different sample of the 100 kDa UltraCel membrane. The data at the two salt concentrations were statistically identical (within ± 5%) for all values of the filtrate flux between 10-80 µm/s. The results are qualitatively similar to those in Figure 3-4, with the data at the 10 and 150 mM solutions generally lying between the results in the 1 and 300 mM NaCl solutions as expected.

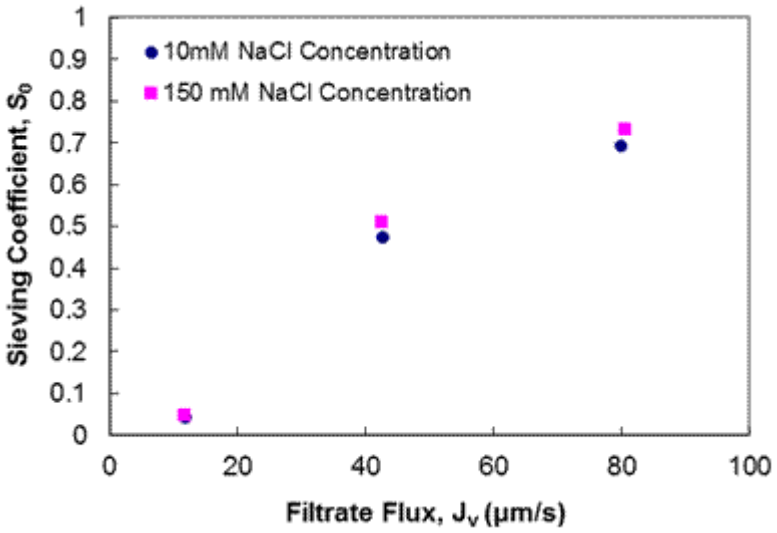

Figure 3-5: Transmission of the linear isoform through a 100 kDa UltraCel membrane as a function of filtrate flux in 10 and 150 mM NaCl solution.

Figure 3-6 shows results for a sieving experiment performed with a 0.25 µg/mL solution of the open-circular isoform through a much larger pore size 1000 kDa UltraCel membrane. The transmission of the open-circular isoform through the 1000 kDa membrane was very low, with $S_o < 0.1$ even at filtrate flux values greater than 110 µm/s. This high degree of retention is due to

the strain in the open-circular isoform, which significantly hinders the flow-induced elongation. In addition, the open-circular plasmid would have to be "pinched" for the ring shape to deform so that it can enter the narrow pores in these membranes.

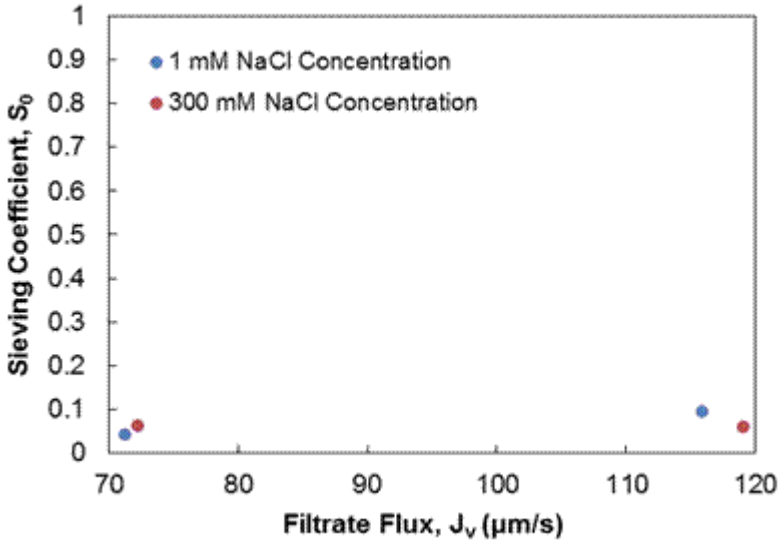

Figure 3-6: Transmission of the open-circular isoform through a 1000 kDa UltraCel membrane as a function of filtrate flux in 1 and 300 mM NaCl solutions.

Corresponding data for transmission of the open-circular plasmid through a Biomax 100 kDa membrane are shown in Figure 3-7. In contrast to the data with the UltraCel 1000 kDa membrane, there was significant transmission of the open-circular isoform through the Biomax membrane, even though the Biomax 100 kDa membrane should have a slightly smaller pore size than the UltraCel 1000 kDa. Note that the UltraCel 1000 kDa membrane had a permeability of 49 µm/s/psi, which is more than twice that of the Biomax 100 kDa (21 µm/s/psi). It is possible that the Biomax 100 kDa membrane used in this experiment was defective, or that there was a leak in the base of the stirred cell, although there was no evidence of this during the experiment. The sieving coefficient for the open-circular plasmid through the Biomax membrane increased

with increasing salt concentration, but was essentially unaffected by filtrate flux. The origin of this behavior is unclear.

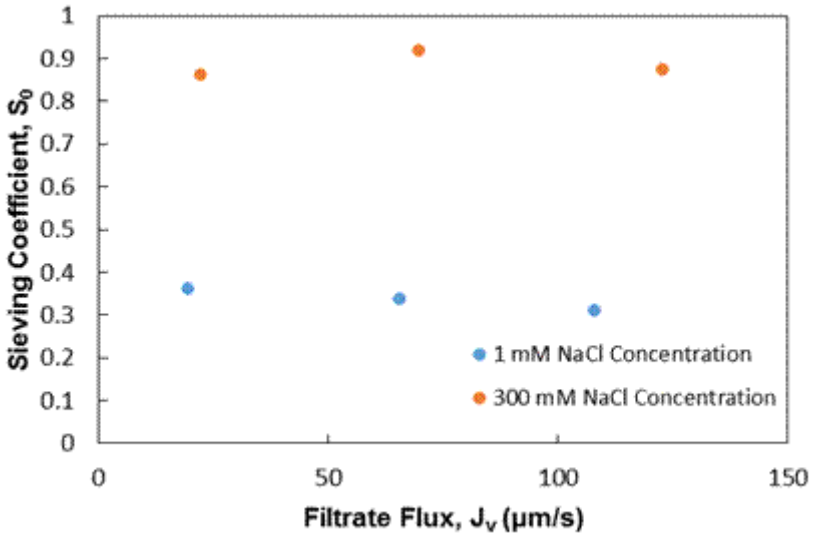

Figure 3-7: Transmission of the open-circular isoform through a 100 kDa Biomax membrane as a function of filtrate flux in a 1 and 300 mM NaCl solution.

3.3. Effects of Divalent Salts on Plasmid Transmission

Additional insights into the effects of salt on plasmid transmission were obtained by performing sieving experiments using TE buffer with different levels of magnesium chloride ($MgCl_2$). Figure 3-8 shows results for a sieving experiment performed with a 0.25 µg/mL solution of the linear isoform through a 100 kDa UltraCel membrane in the presence of either 150 mM NaCl or 40 mM $MgCl_2$. The data in the two salt solutions were essentially identical, even through the concentration (molarity) of the NaCl solution is more than three times higher than that of the $MgCl_2$ solution. The divalent cations in the $MgCl_2$ solutions are much more

effective at shielding the electrostatic repulsion than the monovalent NaCl [13]. This is clearly seen in the definition of the solution ionic strength:

$$I = \frac{1}{2}\sum_{i=1}^{n} C_i z_i^2 . \qquad (2)$$

where C_i is the molar concentration of each ion and z_i is the ion valence. Thus, the ionic strength of the 40 mM $MgCl_2$ solution is actually 120 mM, which is only slightly less than that of the 150 mM NaCl. In addition, the divalent Mg^{2+} has the potential to form salt bridges between the negatively-charged phosphate groups along the DNA backbone, which would likely provide a further reduction in the effective DNA size.

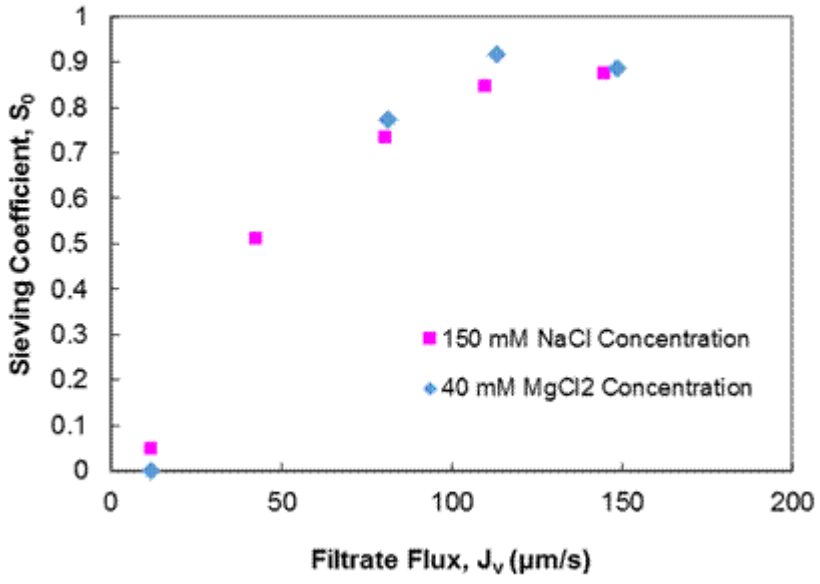

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Figure 3-8: Transmission of the linear isoform through a 100 kDa UltraCel membrane in 40 mM $MgCl_2$ and 150 mM NaCl TE buffer solutions.

Figure 3-9 compares the transmission behavior of the linear and supercoiled isoforms though a 100 kDa UltraCel membrane in a TE buffer containing 40 mM $MgCl_2$. The transmission of both isoforms was negligible at filtrate flux below 40 µm/s. The sieving coefficients increase with increasing filtrate flux, with significantly greater transmission of the linear isoform relative to that of the supercoiled isoform at high values of J_v. The selectivity of the separation, defined as the ratio of the sieving coefficient for the two isoforms, increases to nearly 4 at J_v = 80 µm/s before decreasing slightly at the very highest flux values.

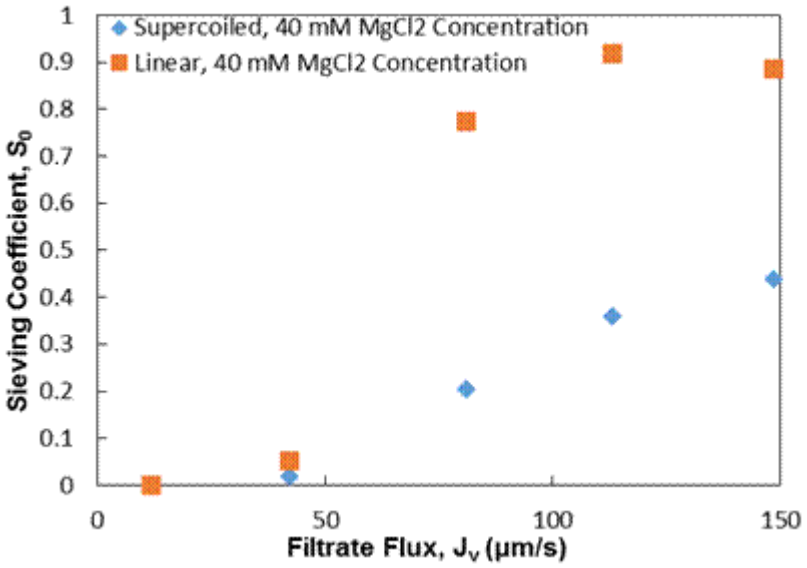

Figure 3-9: Transmission of linear and supercoiled isoforms through a 100 kDa UltraCel membrane in a 40 mM $MgCl_2$ TE buffer solution.

3.4. Behavior of Linear FDY plasmid

Limited experiments were also performed using the larger 16.8 kbp FDY plasmid. Figure 3-10 shows results using 1, 10 and 150 mM NaCl solutions using the UltraCel 100 kDa membrane. The sieving coefficient increases with increasing filtrate flux at all three salt concentrations, consistent with the flow-induced elongation of the large plasmid. The data in the

150 mM solution actually lie somewhat above those for the 3 kbp P-EMP plasmid. Previous studies have shown that plasmid transmission has only a very weak dependence on plasmid size; the larger plasmids elongate more easily with the net result that the transmission is nearly independent of plasmid size. However, the sieving coefficient for the linear 16.8 kbp FDY plasmid is a stronger function of the salt concentration than that seen with the 3 kbp P-EMP plasmid. This could be due to the greater intramolecular electrostatic interactions for the larger plasmid, although additional data would be needed to confirm this behavior.

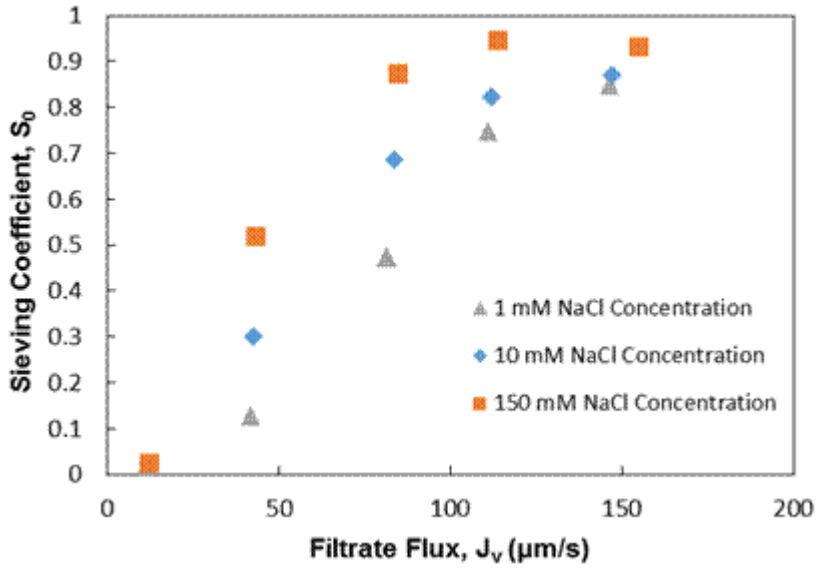

Figure 3-10: Transmission of linear 16.8 kbp FDY plasmid in solutions with 1, 10 and 150 mM NaCl through a 100 kDa UltraCel membrane.

3.5. Isoform Separation by Manipulation of Salt Concentration

Although the data in Figure 3-9 clearly show that it is possible to obtain separation between the supercoiled and linear isoforms in a solution containing 40 mM $MgCl_2$, the greatest selectivity between these isoforms was obtained using much more dilute salt solutions. Figure 3-

11 shows results from two separate sieving experiments, each performed with 0.25 µg/mL plasmid solutions in 10 mM NaCl TE buffer using a 100 kDa UltraCel membrane; one with the linear isoform and one with the supercoiled isoform. The transmission of the supercoiled isoform was significantly reduced at the low salt concentration, with the sieving coefficient remaining below 0.1 out to a filtrate flux of nearly 150 µm/s. In contrast, the transmission of the linear isoform increased to S_o = 0.7 at a flux of 80 µm/s and remained at approximately this value for filtrate flux up to J_v = 150 µm/s. The data at J_v = 80 µm/s show a selectivity much greater than 10-fold, which should be sufficient to obtain a high degree of purification of the desired supercoiled isoform.

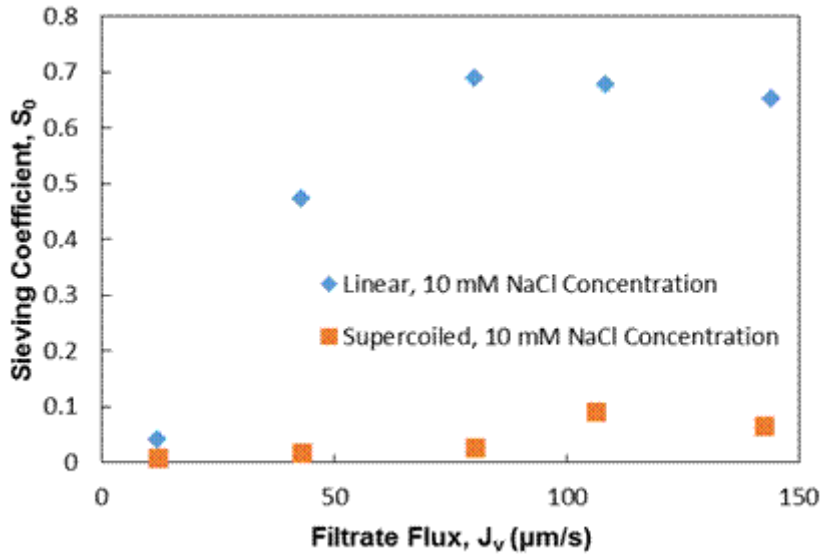

Figure 3-11: Transmission of the linear and supercoiled isoforms through a 100 kDa UltraCel membrane in a 10 mM NaCl TE buffer solution.

Figure 3-12 shows results from similar sieving experiments, each performed with 0.25 µg/mL plasmid solutions but in a 150 mM NaCl TE buffer using a 100 kDa UltraCel membrane; one with the linear isoform and one with the supercoiled isoform. The transmission of the linear

isoform was greater than that of the supercoiled isoform at all conditions, although the magnitude of the difference in sieving coefficients was much smaller than that seen in the low salt concentration solution (Figure 3-11). The transmission of the supercoiled isoform in the 150 mM NaCl increased to $S_o = 0.5$ at a filtrate flux of about $J_v = 150$ µm/s, which is much greater than that seen in Figure 3-11. The sieving coefficient of the linear isoform increased to nearly $S_o = 0.9$ at the highest flux, with a very similar dependence as that seen in the 10 mM NaCl solution.

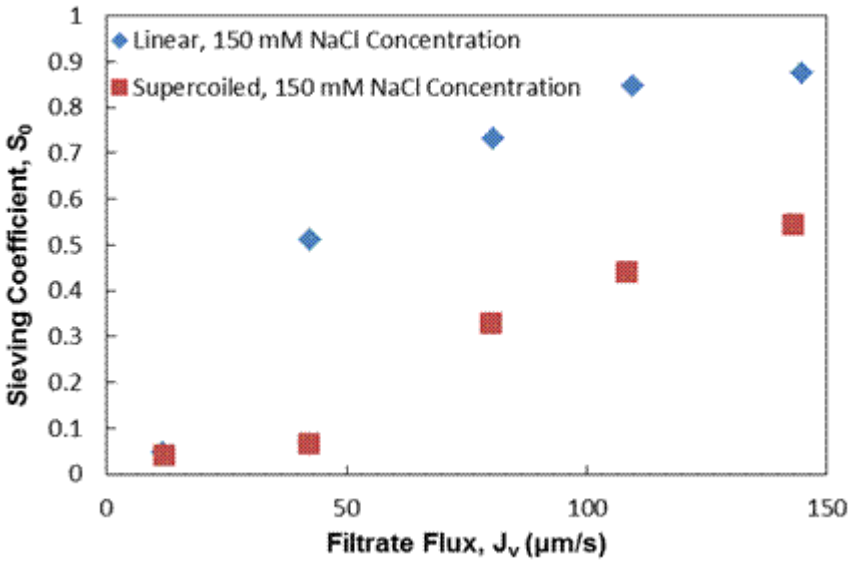

Figure 3-12: Transmission of the linear and supercoiled isoforms through a 100 kDa UltraCel membrane in a 150 mM NaCl TE buffer solution.

In order to confirm the separation behavior, a series of experiments were performed using binary mixtures of the supercoiled and linear isoforms in TE buffer with 10 mM NaCl. Data were obtained at filtrate fluxes of 60, 80, and 100 µm/s based on the results in Figure 3-11. Samples from the feed and filtrate solutions were analyzed by agarose gel electrophoresis with

results shown in Figure 3-13. The first and last lanes show the DNA ladders. Even though the feed contained approximately equal amounts of the supercoiled and linear isoforms, the band for the supercoiled isoform (lower band) was significantly less pronounced in all samples. This might reflect differences in binding of the dye to the two isoforms. The filtrate samples at all three filtrate flux contain only the linear isoform; the bands for the supercoiled isoform were completely absent at the resolution of the gel. The brightness of the band for the linear isoform in the filtrate samples increased with increasing filtrate flux (going from band 3 to band 5 to band 7), with the results at the highest flux (J_v = 100 μm/s) showing the same brightness in the feed and permeate samples (consistent with nearly complete transmission of the linear DNA under these conditions).

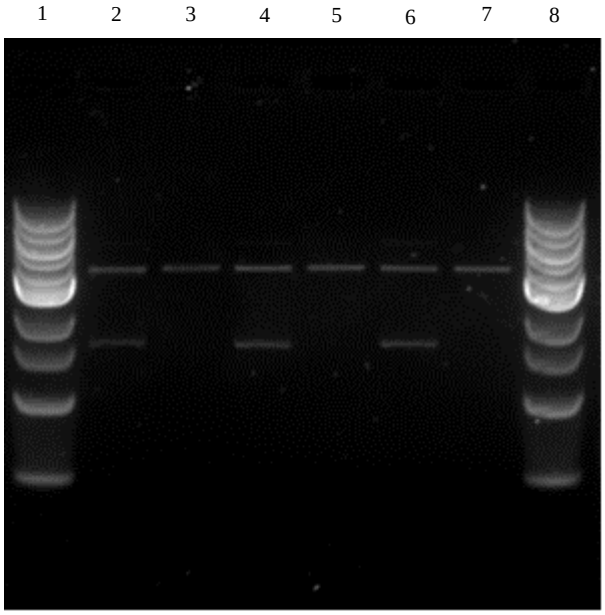

Figure 3-13: Agarose gel electrophoresis for ultrafiltration of a mixture of the linear and supercoiled isoforms through a 100 kDa UltraCel membrane using TE buffer containing 10 mM NaCl. Lanes 2 and 3 are feed and filtrate samples, respectively, at 60 μm/s. Lanes 4 and 5 are the feed and filtrate samples at 80 μm/s, and lanes 6 and 7 are samples at 100 μm/s.

Figure 3-14 compares results for the low salt experiment (10 mM NaCl at J_v = 80 μm/s) with data obtained using 150 mM NaCl at J_v = 50 μm/s. The feed samples show stronger bands for the linear and supercoiled plasmids (relative to Figure 3-11), although the band for the linear isoform is again somewhat brighter. There is also a faint band above that for the linear isoform, which likely represents trace amounts of the open-circular isoform or DNA dimers. The filtrate samples for the two experiments show negligible amounts of the supercoiled isoform, consistent with the very low transmission of the supercoiled plasmid under these conditions seen in Figure 3-12. There was reasonably high transmission of the linear isoform, although the degree of separation in the 150 mM NaCl solution appeared to be slightly less than that in the 10 mM solution (Figure 3-13). It was not possible to quantify these differences based on the images in the agarose gels.

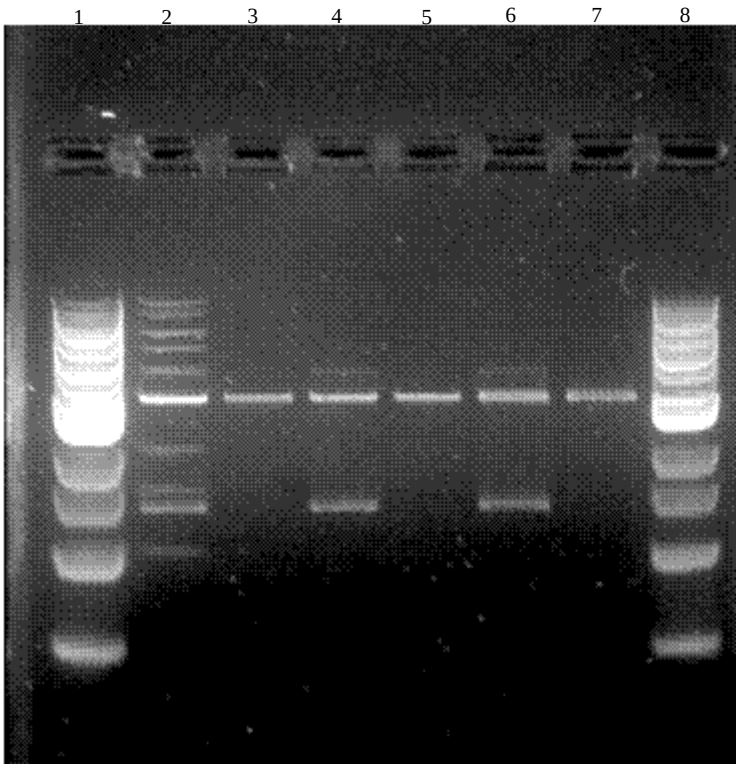

Figure 3-14: Agarose gel electrophoresis for ultrafiltration of a mixture of the linear and supercoiled isoforms through a 100 kDa UltraCel membrane using TE buffer containing 10 mM NaCl. Lanes 1, 2 and 8 are DNA ladders. Lanes 4 and 5 are feed and filtrate samples, respectively, for the 10 mM NaCl solution at a filtrate flux of 80 µm/s. Lanes 6 and 7 are the feed and filtrate samples for the 150 mM NaCl solution at a flux of 50 µm/s. Lane 3 contains the same filtrate sample as lane 5.

Chapter 4

Conclusions

The overall objective of this thesis was to examine the possibility of increasing the effectiveness of using ultrafiltration for the separation of different plasmid isoforms by manipulating the salt concentration and / or composition. Plasmid transmission generally increased with increasing filtrate flux due to the flow-induced elongation of the large plasmids in the converging flow field into the membrane pores. The plasmid sieving coefficient under certain conditions was independent of the filtrate flux (e.g., the supercoiled plasmid through the 100 kDa Biomax membrane at 1 mM NaCl). Additional experimental studies would be required to identify the origin of this unusual behavior.

The transmission of the different isoforms also increased with increasing NaCl concentration, which is likely due to electrostatic shielding of the intra-molecular electrostatic interactions between the negatively-charged phosphate groups on the DNA backbone. An even greater effect was seen using the divalent salt $MgCl_2$, consistent with the greater contribution of the divalent cation to the solution ionic strength. It is also possible that the Mg^{+2} could participate in salt bridging interactions between the phosphate groups.

The effect of salt on plasmid transmission was also found to be different for the different plasmid isoforms. The transmission of the linear isoform was relatively independent of solution ionic strength, particularly for ultrafiltration of the 3 kbp plasmid. Data for the 16.8 kbp plasmid showed a somewhat stronger dependence on the salt concentration, with the sieving coefficient

increasing slightly with increasing solution ionic strength. The transmission of the supercoiled isoform showed a much greater dependence on the salt concentration. For example, the sieving coefficient of the supercoiled plasmid in a 150 mM NaCl solution was more than five times as large as that in a 10 mM solution at a filtrate flux of 145 μm/s. Results with the open-circular plasmid were somewhat less clear. The open-circular plasmid was nearly completely retained by the 100 and 1000 kDa UltraCel membranes at all salt concentrations, but the transmission of the open-circular plasmid through the Biomax 100 kDa increased by around a factor of 3 as the salt concentration was increased from 1 to 300 mM NaCl. Additional experiments will be needed to fully understand the effects of NaCl on the sieving coefficients of the open-circular plasmid.

The different effects of salt concentration on the transmission of the different plasmid isoforms provide an opportunity to enhance the selectivity of the isoform separation. A series of experiments were performed to separate binary mixtures of the linear and supercoiled isoforms, with the solution conditions and the filtrate flux chosen based on results with the individual (pure) isoforms. Samples of the feed and filtrate solutions were examined by agarose gel electrophoresis to determine the relative transmission of the two isoforms. Ultrafiltration experiments performed using the 100 kDa UltraCel membrane showed nearly complete separation of the two isoforms; permeate samples obtained at both 10 and 150 mM NaCl showed no measurable levels of the supercoiled plasmid with nearly complete transmission of the linear isoform.

Although the results obtained in this thesis showed good separation between the linear and supercoiled isoforms at both low and high salt concentrations, it is possible that the effects of different salts could be more pronounced for other plasmid separations. For example, data obtained with the 16.8 kbp plasmid showed a much larger dependence of the sieving coefficient

of the linear plasmid on the NaCl concentration than that seen with the 3.0 kbp plasmid. In addition, the sieving coefficient of the open-circular plasmid was also a function of the NaCl concentration, which could provide opportunities for enhancing the separation of the supercoiled and open-circular isoforms; this separation is considerably more difficult than the separation between the linear and supercoiled plasmids.

One of the advantages of using membranes for the separation of plasmid isoforms is that the separation can be easily scaled up to accommodate larger flow rates by simply increasing the membrane area. The filtrate flux used for the plasmid separations reported in this thesis were between 50 and 100 μm/s, which are very similar to, and in most cases larger than, the flux values currently used in large-scale separations of therapeutic proteins. Note that a 100 m^2 membrane module, which is typical of large commercial installations, would provide a volumetric flow rate of more than 30,000 L/hr at these values of the filtrate flux, which is sufficient to handle even the largest projected processes for the production of plasmid DNA. Thus, the membrane systems examined in this work have the potential to provide a cost-effective means for the purification of plasmid DNA for gene therapy and DNA-based vaccines on an industrial scale.

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