THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF CHEMICAL ENGINEERING

THE EFFECTS OF SPERMINE ON PLASMMID DNA TRANSMISSION THROUGH ULTRAFILTRATION MEMBRANES

RACHEL BOLTEN
SPRING 2017

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree in Chemical Engineering
with honors in Chemical Engineering

Reviewed and approved* by the following:

Andrew Zydney
Distinguished Professor of Chemical Engineering
Thesis Supervisor

Michael Janik
Professor of Chemical Engineering
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

ABSTRACT

Although recent studies have demonstrated that ultrafiltration can be an effective method of plasmid DNA purification, the selectivity between the different plasmid isoforms is often below that needed for large-scale production of gene therapy agents. The objectives of this thesis were to evaluate the effects of spermine, a positively-charged polyamine, on the transmission of supercoiled, open-circular, and linear isoforms of plasmid DNA through ultrafiltration membranes and to determine whether the addition of spermine could facilitate isoform separation. Experiments were performed with Biomax 300 kDa ultrafiltration membranes. The linear and open-circular isoforms of the 9.8 kbp (kilo base pair) MDY plasmid were prepared by enzymatic digestion of the supercoiled plasmid. The addition of low concentrations of spermine ($\leq$ 10 μM) increased transmission for all three plasmid isoforms, with the largest increase obtained with the supercoiled isoform. The use of spermine concentrations above 10 μM caused a significant reduction in plasmid transmission, likely due to the inhibition of plasmid elongation. The largest selectivity for the purification of the supercoiled isoform from the open-circular isoform was observed with the addition of 2 μM spermine in a 10 mM NaCl TE buffer. These results suggest that polyamines can be used to control the transmission of plasmid DNA during ultrafiltration and may be able to facilitate the separation of the different DNA isoforms due to the difference effects on isoform transmission.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to thank everyone who helped me complete this thesis for all of their support and encouragement. This project was at times challenging and time consuming, and I appreciate all of the help I received.

I would like to thank Dr. Zydney for all of his guidance and support with the research and writing for this thesis. I really appreciate being able to work in his lab throughout my years as an undergraduate student at Penn State.

I would also like to thank Ying Li for her guidance throughout this project. I am extremely grateful for all of the time she took to teach me important laboratory skills and help me design the best experiments to obtain meaningful results for this thesis.

Finally, I would like to thank my family and friends for always being there for me and believing that I could do a great job with this thesis. I really appreciate them listening to me talk about my thesis on a regular basis and offering encouragement when I needed it.

1. Introduction

Gene therapy is a promising technology for the treatment and prevention of various diseases such as cancer, Alzheimer's, and cystic fibrosis [1]. Gene therapy involves the introduction of one or more functional genes to a patient in order to prevent, treat, or cure genetic defects. In addition, DNA-related vaccines can be developed using appropriate genes from a pathogenic organism to develop immunity against subsequent infection. Therapeutic genes can be delivered using both viral or non-viral vectors [1]. Non-viral vectors like liposomes or polyersomes avoid issues of carcinogenesis and immunogenicity that have been associated with the use of adeno-associated viral delivery [2]. However, successful implementation of non-viral vectors requires large amounts of purified plasmid DNA due to the relatively low rates of successful transfection (uptake of the DNA by the targeted cell line).

Plasmids are covalently closed, double stranded DNA molecules that carry genetic information outside of the chromosome. Naturally occurring plasmids have a higher-order structure called supercoiling, which facilitates transcription of the DNA into RNA followed by translation into proteins. Plasmid DNA isoforms are DNA molecules with the same sequence and number of base pair units, but significantly different structures. During manufacturing, it is very common to find low levels of the open-circular and linear isoforms, which are generated by breaks (or nicks) in one or both strands of the plasmid, respectively. The supercoiled plasmid is the therapeutically desired isomer for gene therapy applications due to its higher gene expression efficiency. Linear and open-circular isoforms are therefore considered product impurities and must be removed before the plasmid can be used in gene therapy applications.

Plasmid molecules are usually 5-20 kbp (kilo base pair) in size, with radius of gyration on the order of 100 nm, which is much larger than the size of typical therapeutic proteins. Similar to many recombinant proteins, however, plasmids are typically produced in *Esherichia coli* by fermentation [3]. One challenge for gene therapy and DNA vaccination is obtaining large amounts of pharmaceutical-grade plasmids consistent in purity, potency, and identity suitable for clinical applications.

While effective laboratory techniques for plasmid purification are well-established, the large quantities of DNA required for gene therapy treatments will likely require totally new strategies for the production and purification of plasmid DNA. For example, plasmid DNA can be purified and different isoforms separated by agarose gel electrophoresis and various chromatographic methods. Chromatographic processes are currently used for large-scale plasmid DNA purification, however, mass transfer limitations and low binding capacities pose challenges for this technology [4]. In contrast, membrane processes are only weakly affected by diffusional limitations and are easily scalable. Several studies have demonstrated the potential of using membrane systems for DNA purification. Ultrafiltration membranes (with pore size between 1 and 50 nm) have been used to remove proteins and RNA impurities from DNA solutions, and microfiltration membranes (with pore size between 100 and 600 nm) have been used to remove cell debris and genomic DNA [5]. In addition, ultrafiltration can be used to separate different DNA isoforms due to the different extensional flexibility of the isoforms in the elongation flow field into the membrane pore [6]. During ultrafiltration, plasmids tend to orientate and elongate in the direction of flow [1], allowing them to pass through pores that have radii that are much smaller than the effective radius of gyration of the plasmid. Therefore, differences in isoform

elongation lead to differences in transmission through the membranes which can in turn be used to separate and purify specific plasmid isoforms.

Kong et al. studied the key parameters that affect the performance of a microfiltration system for DNA purification, including plasmid size, concentration, filtrate flux, membrane pore structure, and formulating buffer [3]. They found that transmission of DNA vectors with 6-116 kbp decreased with increasing DNA size during filtration through 0.22 μm polyvinylidene fluoride PVDF membranes at a fixed flux of 0.2 mL/min/cm^2. The performance of the membrane system was largely unaffected by the initial DNA concentration or the operating flux. This suggests that lower DNA transmission was not caused by adsorption to membrane sites. However, backbone breakage of the large plasmid DNA did occur at high filtrate fluxes (0.8 and 2.3 mL/min/m^2). DNA transmission also increased in the presence of 150 mM NaCl, which is likely due to a reduction in intermolecular repulsion between the charged plasmid and the charged membrane [3].

The effect of consecutive filtration on DNA transmission and integrity was also determined [3]. Plasmid DNA transmission increased with the number of filtration passes. A large increase in transmission occurred for the larger DNA vector tested (72 kb). However, DNA damage increased with consecutive filtration passes. Membrane type also had an effect on DNA transmission. While PVDF membranes have a homogeneous structure (uniform pore size throughout the membrane thickness), PES membranes have an asymmetrical pore structure with a wider diameter at the inlet which decreases through the membrane thickness. A small increase in both transmission and backbone integrity was observed when using the PES membrane [3]. These results suggest that membrane pore geometry is an important factor to consider for DNA filtration.

More recently, Li et al. studied the effects of solution ionic strength on the purification of plasmid DNA isoforms during ultrafiltration [7]. Solution conditions have a large impact on the structure of plasmid DNA due to intramolecular electrostatic interactions between the phosphate groups along the DNA backbone. Therefore, this study aimed to determine whether proper selection of the solution ionic strength and ion type could enhance the use of ultrafiltration for separation of different plasmid isoforms. All three plasmid isoforms studied showed an increase in transmission with increasing solution ionic strength, which was attributed to electrostatic shielding effects. However, the ultrafiltration behavior of each plasmid isoform differed greatly. While transmission of the linear isoform was very weakly dependent on the ionic environment, with only a slight increase in transmission with increasing ionic strength, the transmission of the supercoiled isoform increased significantly with increasing salt concentration [7]. This difference in behavior for the supercoiled isoform was likely due to a change in conformation to a more tightly inter-wound structure associated with the shielding of intramolecular electrostatic interactions.

The changes in plasmid transmission as a result of salt concentration allowed for enhanced selectivity between the linear and supercoiled plasmid isoforms at low ionic strength. In contrast, the selectivity between the supercoiled and open-circular plasmid isoforms was greatest at higher ionic strength. Selectivity is the critical parameter governing the separation performance of a membrane and is calculated as the ratio of the sieving coefficient (equal to the fractional transmission) of the more permeable isoform to the sieving coefficient of the less permeable isoform [7].

$$\psi = \frac{S_1}{S_2} \tag{1}$$

Li et al. suggested that a staged ultrafiltration process could be used to separate the different plasmid isoforms to obtain the desired supercoiled plasmid. A low ionic strength solution would be used initially to remove the linear isoform in the permeate. Then, the supercoiled plasmid would be recovered with a second ultrafiltration using a high ionic strength solution.

Further studies demonstrated that the selectivity increased with increasing filtrate flux [8] due to elongation of the plasmids in the converging flow field entering the membrane pores. For example, the maximum selectivity of $\psi = 9$ for the separation of a 3 kbp and a 16.8 kbp supercoiled plasmid was obtained at a filtrate flux around 70 μm/s. Selectivity decreased at larger fluxes due to an increase in transmission of the larger plasmid. Membrane pore size also had an effect on selectivity. Selectivity was reduced when using membranes with larger pore sizes, as the sieving coefficients for both plasmid sizes increased. However, membranes with very small pore sizes also had low selectivity due to the high retention of both plasmids. A binary mixture of the two plasmids, both at concentrations of 0.25 μg/mL, was accomplished by adjusting the membrane pore size and filtrate flux. Agarose gel electrophoresis (AGE) showed that the filtrate contained only the smaller 3.0 kbp plasmid with no evidence of the larger 16.8 kbp plasmid [8], demonstrating that ultrafiltration can be effectively used for the separation of plasmid isoforms based on size.

Latulippe and Zydney studied the effects of plasmid size, pore size, operating temperature, and solution viscosity on the critical flux for the transmission of a 3 kbp plasmid through composite regenerated cellulose membranes [9]. The critical filtrate flux is defined as the lowest flux at which plasmid transmission is significant. A modified elongation flow model was used to describe the experimental results for filtration of supercoiled plasmids through different pore size membranes:

$$J_{crit} = \left(\frac{n^2 D}{R_G^2}\right)\left(\frac{\varepsilon R_S^3}{r_p^2}\right) \quad (2)$$

where D is the plasmid diffusion coefficient, R_G is the plasmid radius of gyration, ε is the membrane porosity, and r_p is the membrane pore radius [9].

The contributions of DNA elongation, shear deformation, and concentration polarization on observed plasmid transmission were studied. While the sieving coefficient was essentially zero at low values of filtrate flux, it increased significantly at higher filtrate flux. This increase in plasmid transmission with increasing filtrate flux was not due to concentration polarization effects. Rather, the increase was due to the elongation of the large DNA molecules in the converging flow field above the membrane pore, indicating the importance of flow-induced elongation on DNA transmission [9].

One of the challenges in using ultrafiltration for plasmid purification is membrane fouling. Borujeni and Zydney studied membrane fouling as a function of the supercoiled plasmid concentration [6]. As the plasmid concentration increased, the observed sieving coefficient and filtrate flux significantly declined as a result of partial blockage of the membrane pores by individual plasmids. A fouling model was developed based on this pore blockage phenomenon that effectively described both the flux decline and changes in plasmid transmission. Filtrate flux and sieving coefficient remained relatively constant during ultrafiltration of the dilute plasmid solution (0.28×10^{-3}kg/m^3), indicating negligible membrane fouling. However, at plasmid concentrations of 1.2×10^{-3}kg/m^3 and 7.2×10^{-3}kg/m^3 there was a pronounced decline in filtrate flux and sieving coefficient [6]. Additionally, the membranes showed irreversible fouling for both higher concentration solutions. The model accounts for both the partial blockage of membranes pores by plasmids and the rate at which pores are blocked. The probability of a

plasmid blocking a pore increased with increasing plasmid size, likely due to the greater probability of knot formation in longer plasmids [6].

The DNA structure can also be altered by small polyamines such as spermine, which are abundantly found in living cells and are believed to aid in the dense packaging of DNA. Spermine, a small linear cation, is believed to lower the energy of transition, effectively stabilizing and condensing DNA [10]. In addition, spermine may cause electrostatic shielding of the negative charges in DNA molecules. High valence cations like spermine can cause supercoiled plasmid DNA to adopt a tightened plectonemic structure. At higher spermine concentrations, DNA condensation is likely to occur.

Shao et al. found that adding either spermine or spermidine to DNA molecules produced more compact plectonemes in physiological concentrations of monovalent salts, and they promoted plectoneme formation at lower values of torsion [11]. Inside living cells, plectonemic supercoils form the basis of DNA compaction and play a crucial role in the regulation of DNA transcription and replication. DNA supercoiling and its regulation fundamentally impact cellular functions, therefore, experiments to determine the effects of polyamines on DNA supercoiling are of significant interest. The multiple positively charged amine groups can interact electrostatically with the negatively charged phosphates of DNA, and spectroscopy data indicate that polyamines can also bind in the major and minor grooves of DNA to interact with DNA bases as well [11]. Experiments were performed on single DNA molecules in 200 mM KCl and 0, 0.2, 0.5, 0.7, 1, and 2 mM of spermine. Results showed that spermine and spermidine increased plectoneme formation for negative supercoiling and positive supercoiling. In addition, the polyamines shrunk the plectonemic supercoils. These results indicate that polyamines promote tightly wrapped plectonemes [11]. Therefore, natural polyamines stabilize base-pairing,

limit twist, and promote supercoiling, which aids in replication and transcription and is essential for DNA packaging.

Although spermine has been shown to have a significant effect on DNA structure, there have been no studies of the possible effects of this small polyamine on the ultrafiltration behavior of plasmid DNA. In addition, almost all of the available data on the effects of spermine are limited to studies of the supercoiled isoform, with very little information available on the interactions of spermine with either the linear or supercoiled isoforms. The objectives of this thesis were to obtain experimental data for: (1) plasmid transmission through Biomax 300 kDa ultrafiltration membranes as a function of the spermine concentration over a range of filtrate flux, and (2) the effect of spermine on the separation of the open-circular and supercoiled plasmid isoforms. These studies provide the first demonstration that spermine can have a dramatic effect on plasmid ultrafiltration, even at micromolar concentrations, potentially providing opportunities to enhance the use of ultrafiltration for plasmid DNA purification.

2. Materials and Methods

2.1 Solution preparation.

Buffer solutions were prepared by diluting 100× Tris-EDTA (TE) buffer (Fluka) in deionized distilled water to produce a final TE solution containing 10 mM Tris-HCl and 1 mM EDTA. Sodium chloride (Sigma-Aldrich) was added to achieve the desired ionic strength. NaCl was added to the TE buffer at concentrations of 10 and 100 mM NaCl. Solutions of 10 mM spermine in TE buffer with NaCl were prepared and then diluted to the desired final concentration (typically less than 30 μM) before mixing with the plasmid solution.

2.2 Plasmid Preparation

Stock solutions of the supercoiled isoform of the MDY plasmid DNA were obtained from Aldevron (Fargo, ND). The MDY plasmid is 9.8 kbp (kilo base pairs) in length, corresponding to a radius of gyration of approximately 120 nm [9]. The plasmid was digested into the open circular isoform by incubation in the presence of NtA1W1 enzyme and 10× CuSmart buffer at 37°C for three hours to allow for complete digestion. The plasmid was purified by chromatography using the QIAQuick PCR purification kit (Qiagen, CA). To obtain the linear isoform of the plasmid, BamH1 enzyme solution and NEBuffer were used. The sample was once again incubated at 37°C for three hours and purified by chromatography afterwards. Plasmids were stored in the freezer at $-20℃$ until use in ultrafiltration experiments.

2.3 Membrane Preparation

Biomax 300 kD ultrafiltration membranes were obtained from Millipore either as large sheets or as 25 mm disks. The large sheets were cut into small disks using a specially-designed cutting device. Before use in the ultrafiltration experiments, membranes were soaked in 90% isopropyl alcohol for approximately 45 minutes and then flushed with approximately 100 mL of DI water to remove any storage / wetting agents before use. The permeability of each membrane was evaluated by measuring the filtrate flux as a function of applied pressure as described in Section 2.5. Membranes were discarded if the permeability was more than 30% different than the expected value of 35 μm/psi. Membranes were stored in DI water and kept in the refrigerator between experiments.

2.4 Stirred Cell Set-up

A 10 mL stirred cell with a membrane area of 4.1 cm^2 was used for the ultrafiltration experiments. The cell was placed on a magnetic stirrer, operated at a stirring speed of 730 rpm, and connected to a pressure source. The pressure was monitored with a digital differential pressure gauge and adjusted to obtain the desired filtrate flux (equal to the volumetric filtrate flow rate divided by the membrane area). The appropriate feed solution was added to the stirred cell, and the system was allowed to stabilize. Samples of feed and filtrate were collected during each experiment for subsequent analysis.

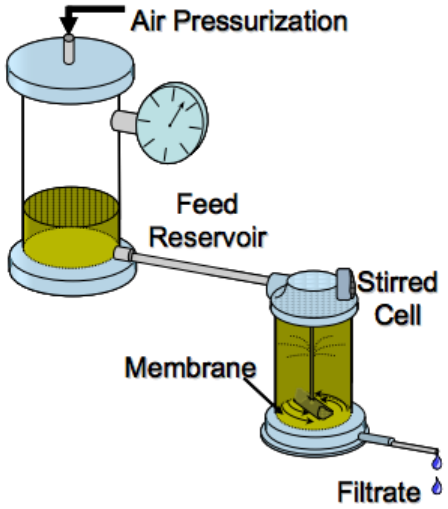

Figure 1. Diagram of stirred cell experiment set-up [13].

2.5 Permeability Measurements

Before each ultrafiltration experiment, the permeability of the membrane was evaluated to ensure that the membrane was in adequate condition and that there was no fouling from the plasmid solutions. The filtrate flux was evaluated with a NaCl buffer solution using timed collection for at least three different pressures. Permeability was calculated from the slope of the filtrate flux versus pressure data as:

$$L_p = \frac{J_v}{\Delta P} \tag{3}$$

If the permeability was too low, or dropped significantly during an experiment, the membrane was discarded and a new membrane was used for subsequent experiments.

2.6 Ultrafiltration Experiments

The effects of spermine on DNA transmission were studied by observing the change in the sieving coefficient with / without added spermine at different concentrations. The observed sieving coefficient is calculated as the ratio of the concentration of plasmid in the filtrate solution to the concentration of plasmid in the feed:

$$S_o = \frac{C_{filtrate}}{C_{feed}} \quad (4)$$

Solutions of plasmid DNA and spermine in TE buffer containing NaCl were prepared before each experiment. Typically, solutions were studied in the order of increasing spermine concentration. The desired solution was added to the stirred-cell and the feed tank, and a 200 μL sample was taken directly from the stirred cell before attaching the cap. Pressure was applied to the system using compressed nitrogen to produce the desired filtrate flux. About 0.5 mL of filtrate was collected after the system had stabilized for subsequent analysis of the DNA concentration. After the filtrate was collected, the stirred cell was opened, another 200 μL sample was obtained, the stirred cell was re-sealed, and the filtration was continued. This was repeated at several different fluxes for each solution.

2.7 PicoGreen Analysis

Plasmid concentrations were determined by fluorescence assay using the PicoGreen fluorescent assay. The PicoGreen solution was prepared by diluting 200× Quant-iT PicoGreen dsDNA reagent (Invitrogen) and analytical TE buffer (20×) in DI water. A 70 μL sample was combined with 70 μL of PicoGreen solution in the wells of a 96-well plate. The samples were

shaken for three minutes and the fluorescence intensity of the samples was then measured at 36°C using an excitation wavelength of 485 nm. DNA concentrations were evaluated by comparison of the measured fluorescence with a calibration curve created with samples of known concentrations of DNA (0, 0.01, 0.1, 0.2, and 0.5 µg/mL). An example calibration curve is shown in Figure 2. The sieving coefficient was calculated using the average concentration of the feed evaluated before and after obtaining a permeate sample at each flux.

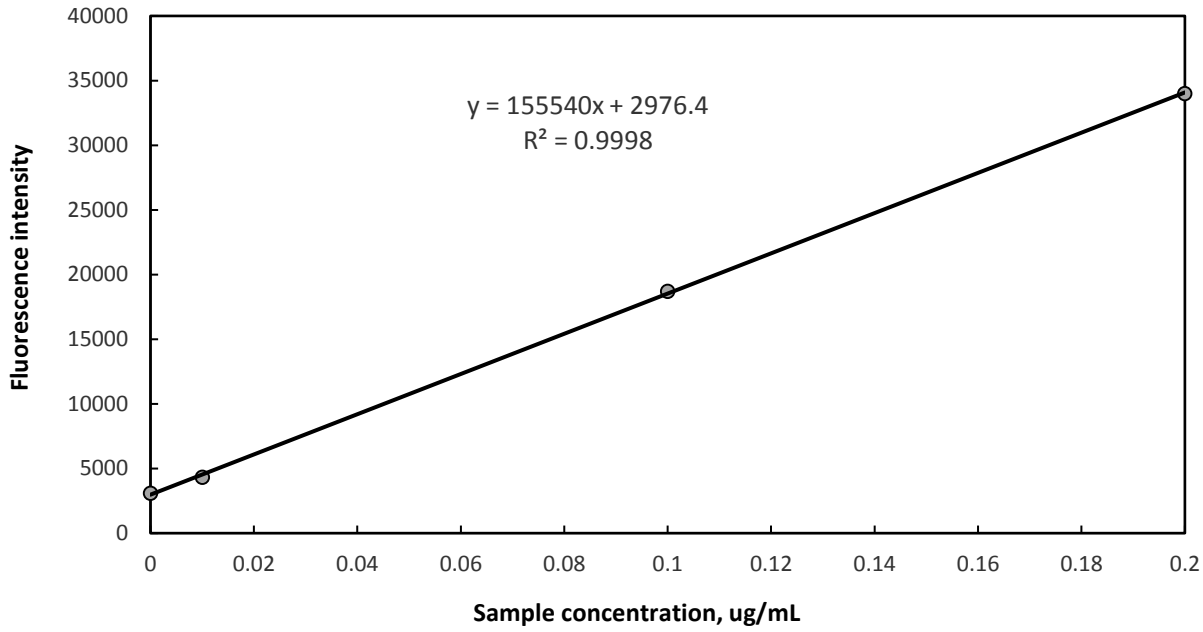

Figure 2. Calibration curve for determining DNA concentration from fluorescence assay.

2.8 Gel Electrophoresis

The purity of digested samples was determined using agarose gel electrophoresis. The gel was prepared by adding 0.36 g of agarose to 45 mL of 1x Tris-acetate-EDTA (TAE) buffer and heating the solution to its boiling point to dissolve the agarose. After cooling for approximately 20 minutes, 10,000x diluted GelStar dye was added and the solution poured into the cast.

Approximately 17.5 µL plasmid samples were added to each lane of the gel, along with a solution containing TrackIt 1 kbp DNA ladder, a mixture of linear DNA of known numbers of base pairs (Invitrogen). The sample solutions contained 1-2 µL of plasmid DNA, 3 µL of dye, and 14 µL of DI water. The gel was placed in TAE buffer and a constant voltage of 50 V was applied for 60 minutes.

3. Results

3.1 Supercoiled Isoform

Figure 3 shows data for the ultrafiltration of 5 different DNA solutions, each containing a different concentration of spermine, over a range of filtrate flux. Spermine was added to solutions of 0.2 μg/mL supercoiled plasmid DNA in 10 mM NaCl at concentrations of 0, 2, 10, 15, and 30 μM. Constant flux sieving experiments were performed at three different fluxes (approximately 30, 50, and 80 μm/s). The addition of spermine initially increased the transmission of supercoiled plasmid DNA up to a concentration of approximately 10 μM of spermine in the 10 mM NaCl buffer. For example, the observed sieving coefficient increased by a factor of about 15 at a flux of 50 μm/s in the presence of 10 μM spermine compared to no spermine, and by a factor of almost 10 at a flux of 80 μm/s and 10 μM spermine. However, at 15 μM of spermine and above, there was a large decrease in transmission through the ultrafiltration membrane, with the sieving coefficient decreasing to essentially zero in the 30 μM spermine solution.

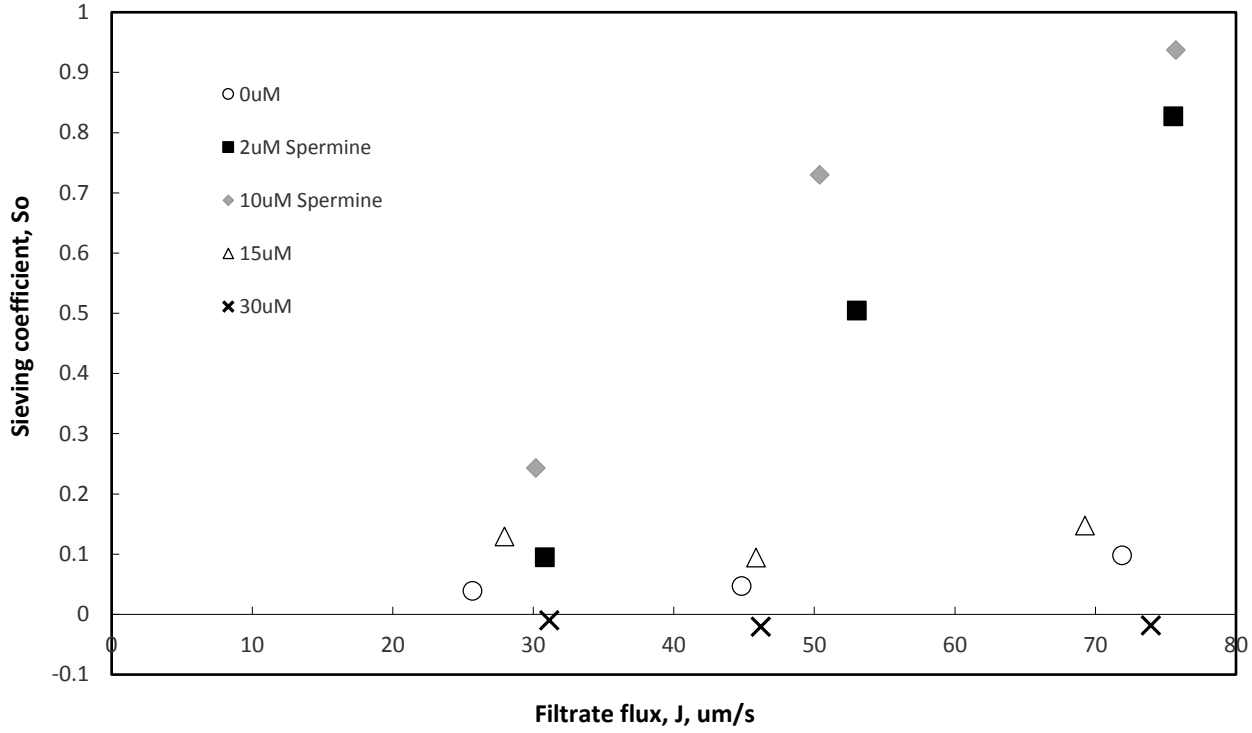

Figure 3. Sieving coefficient as a function of filtration for supercoiled isoforms in 10 mM NaCl TE buffer with different spermine concentrations.

This experiment was repeated several times, as the results showed a dependence on the type and condition of the membrane used. Throughout each experiment, transmission increased at low spermine concentration and decreased at higher spermine concentration. For the current batch of membranes, the transmission of plasmids was relatively similar with low or no concentration of spermine, whereas a rapid drop of the sieving coefficient was observed during the experiment at high spermine concentration. Experiments were also performed with Biomax 100 kDa membranes and UltraCel membranes. Results of these experiments can be seen in section 3.6. Transmission through both of these membranes was very low and the effects of spermine less pronounced, therefore, Biomax 300 kDa ultrafiltation membranes were used for the majority of this project. Transmission also depended upon the solutions reaching equilibrium.

When solutions were prepared and the spermine and DNA allowed to equilibrate, results similar to those shown in Figure 2 were obtained. In contrast, results varied and transmission was less affected by the addition of spermine when the solutions were used less than thirty minutes after they were prepared, most likely due to inadequate equilibrium between the plasmids and spermine.

The effect of spermine concentration on transmission of the supercoiled DNA is shown more explicitly in Figure 4. The data were all obtained at a constant filtrate flux of approximately 50 μm/s (corresponding to a transmembrane pressure of approximately 9.7 kPa = 1.4 psi) using solutions with spermine concentrations of 0, 2, 6, 10, 15, and 30 μM. The DNA transmission increases from $S_o = 0.05$ in the absence of spermine to $S_o \approx 0.8$ for spermine concentrations between 2 and 10 μM. However, there is a significant reduction in DNA transmission at higher spermine concentrations, with the observed sieving coefficient of the MDY plasmid in the 15 and 30 μM spermine solutions being less than 0.004.

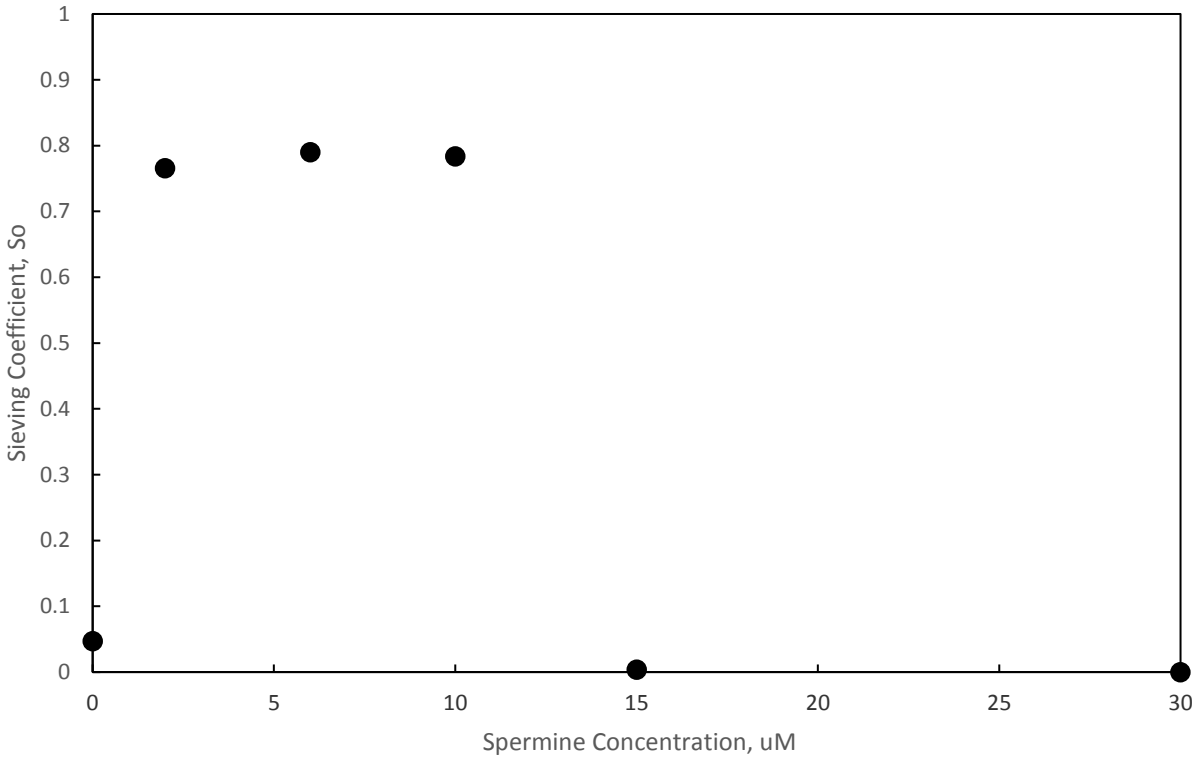

Figure 4. Sieving coefficient as a function of spermine concentration at a filtrate flux of 50 µm/s.

3.2 Linear Isoform

Corresponding data for the linear isoform of the MDY plasmid DNA are shown in Figure 5. Similar to the results with the supercoiled plasmid, transmission of the linear isoform increased (although only slightly) in the presence of 2 and 10 µM solutions of spermine. For example, the sieving coefficient at a flux of approximately 30 µm/s increased from $S_o = 0.56$ in the absence of spermine to $S_o = 0.73$ in the 2 µM solution. Again, transmission of the plasmid in solutions containing 15 or 30 µM spermine was negligible, with sieving coefficients less than 0.1

over the entire range of filtrate flux.

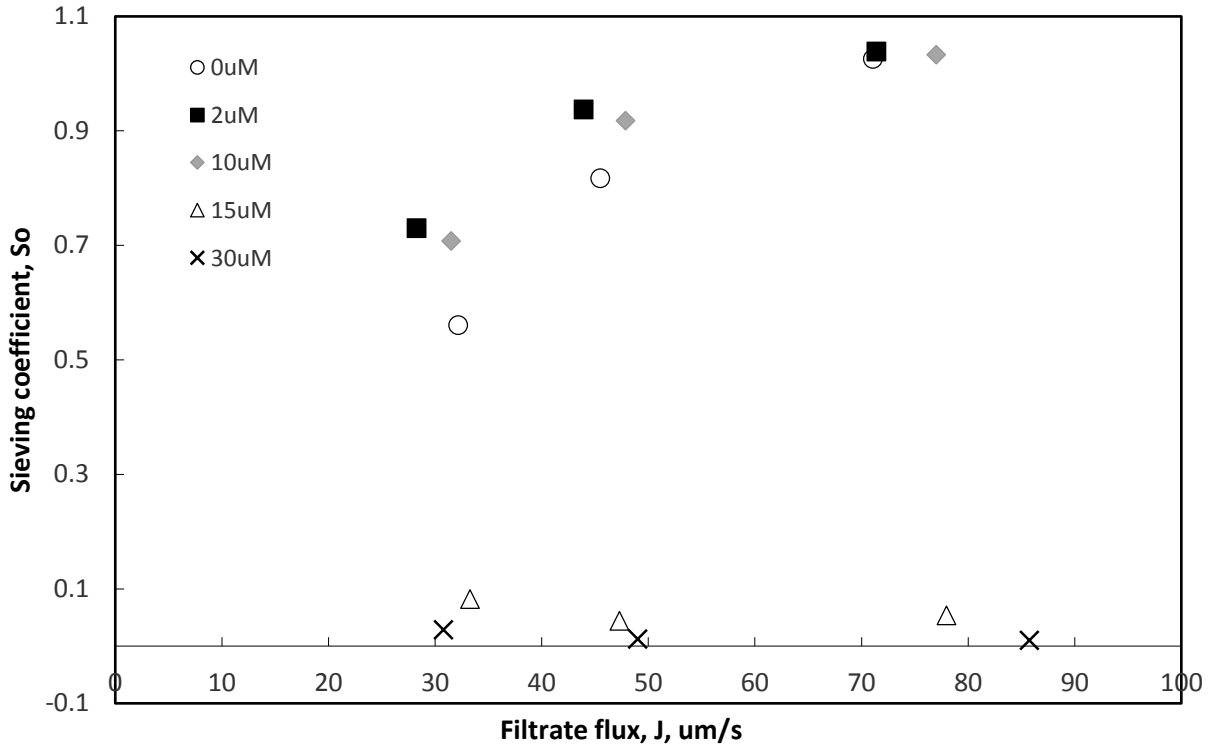

Figure 5. Sieving coefficient for linear plasmids as a function of the filtration flux in 10 mM NaCl TE buffer with different concentrations of spermine.

Overall transmission of linear plasmid was high, with S_o reaching about 1 at a filtrate flux of 80 μm/s for all solutions with ≤10 μM spermine. This high transmission was due to linear plasmids' elongation flexibility which allows the DNA to pass through the small membrane pores quite easily at high filtrate flux.

3.3 Open-circular Isoform

Sieving experiments were also carried out for DNA in the open-circular isoform, with spermine added to solutions of the open-circular plasmid DNA in Tris-EDTA at a plasmid

concentration of 0.15 µg/mL. Results are shown in Figure 6. Again, the addition of 10 µM spermine caused the greatest increase in transmission of the open circular plasmid DNA, though the overall transmission of the open-circular plasmid remained relatively low in comparison to the other DNA isoforms. In this case, the maximum transmission was below 0.3 even at high filtrate flux in the presence of the 10 µM spermine. Retention with the other spermine concentrations was even greater, with $S_o < 0.1$ over the entire range of filtrate flux.

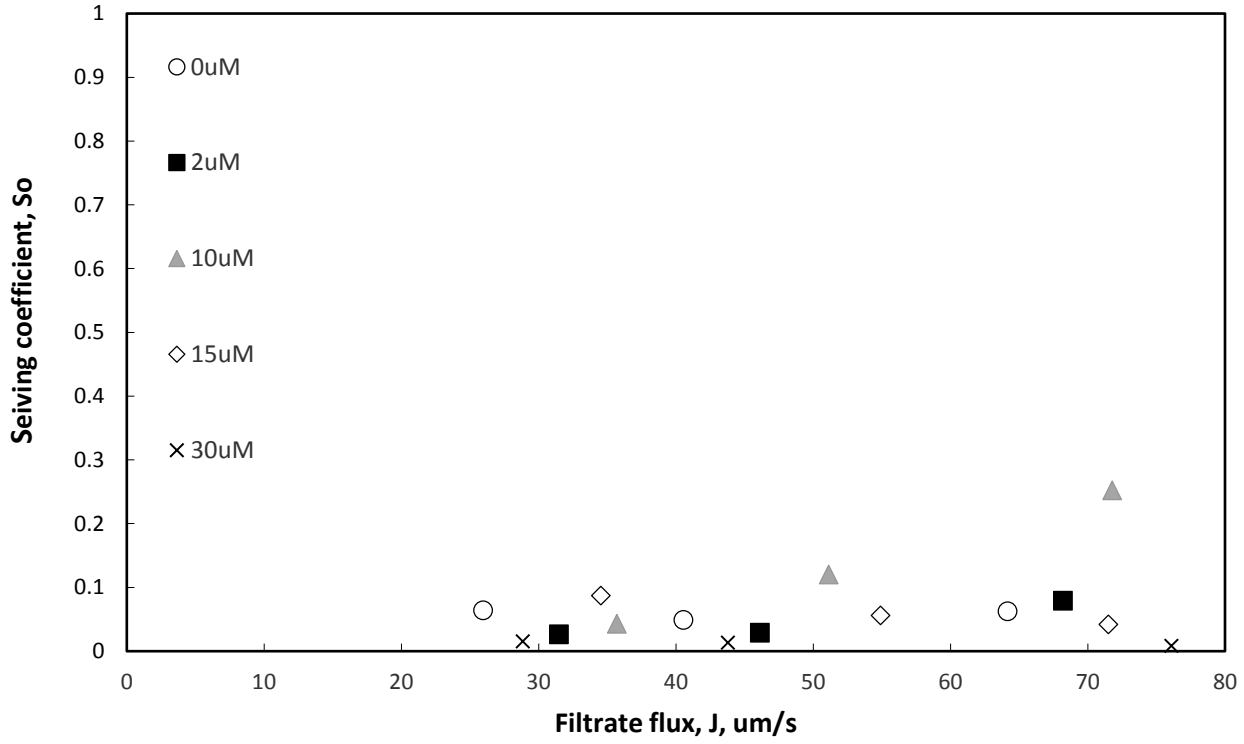

Figure 6. Sieving coefficient for open-circular plasmid in 10 mM NaCl TE buffer at different spermine concentrations.

3.4 Effect of Buffer Solution Concentration

In addition to the experiments conducted with plasmid solutions in 10 mM NaCl TE buffer, data were also obtained with the supercoiled and open-circular isoforms in 100 mM NaCl

TE buffer solution to determine the effects of salt / electrostatic interactions on plasmid transmission. Figure 7 shows the observed sieving coefficient over a range of filtrate flux and spermine concentrations for the open-circular isoform. Spermine did not have a significant effect on the sieving coefficient at the higher salt concentration, and there was no drastic reduction in plasmid transmission in the presence of 30 μM spermine as observed previously for the experiments with 10 mM NaCl. This suggests that the higher ionic strength buffer solution reduces the interactions between the negatively-charged DNA and the positively charged spermine.

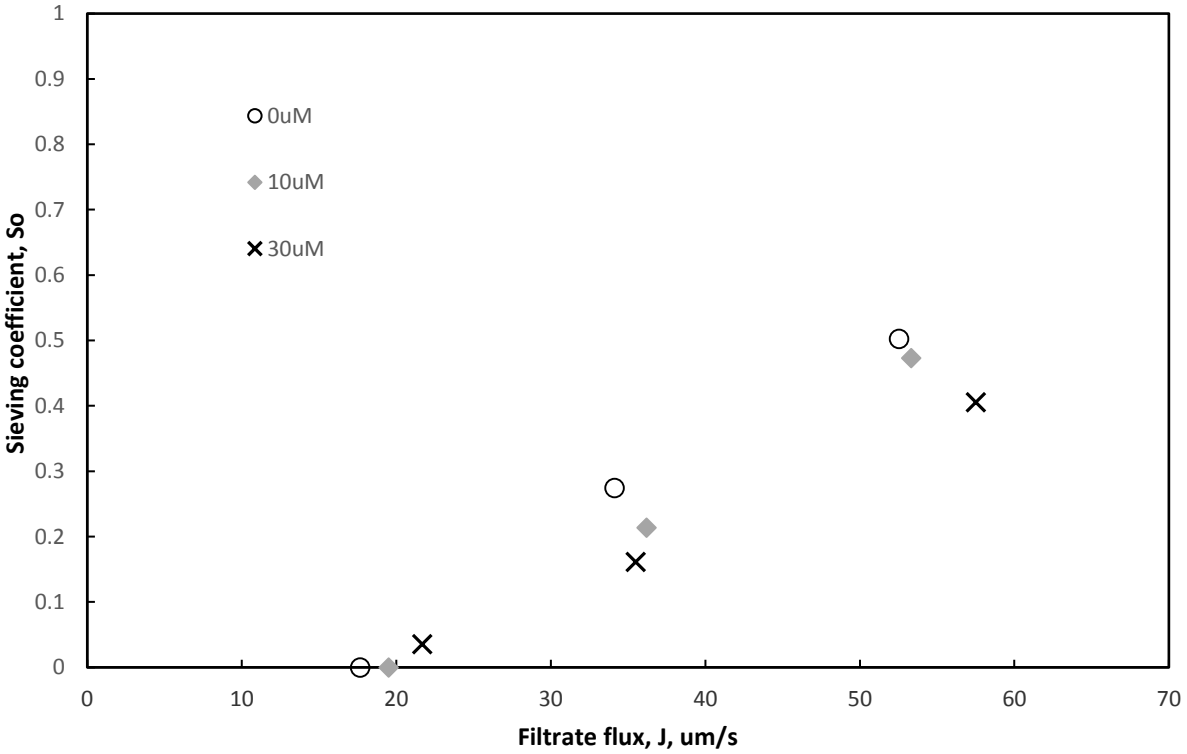

Figure 7. Sieving coefficient for open-circular plasmid in 100 mM NaCl TE buffer as a function of filtrate flux at different spermine concentrations.

Corresponding data for the supercoiled isoform is shown in Figure 8. In this case, S_o was greater than 0.8 throughout the entire range of fluxes at spermine concentrations of 0, 10, and 30 μM. However, the addition of 200 μM spermine caused a dramatic reduction in the sieving coefficient with $S_o < 0.08$ over the entire range of filtrate flux. These data suggest that high concentrations of spermine lead to high degrees of DNA retention, but the critical spermine concentration for this reduction in DNA transmission increases with increasing ionic strength of the buffer solution.

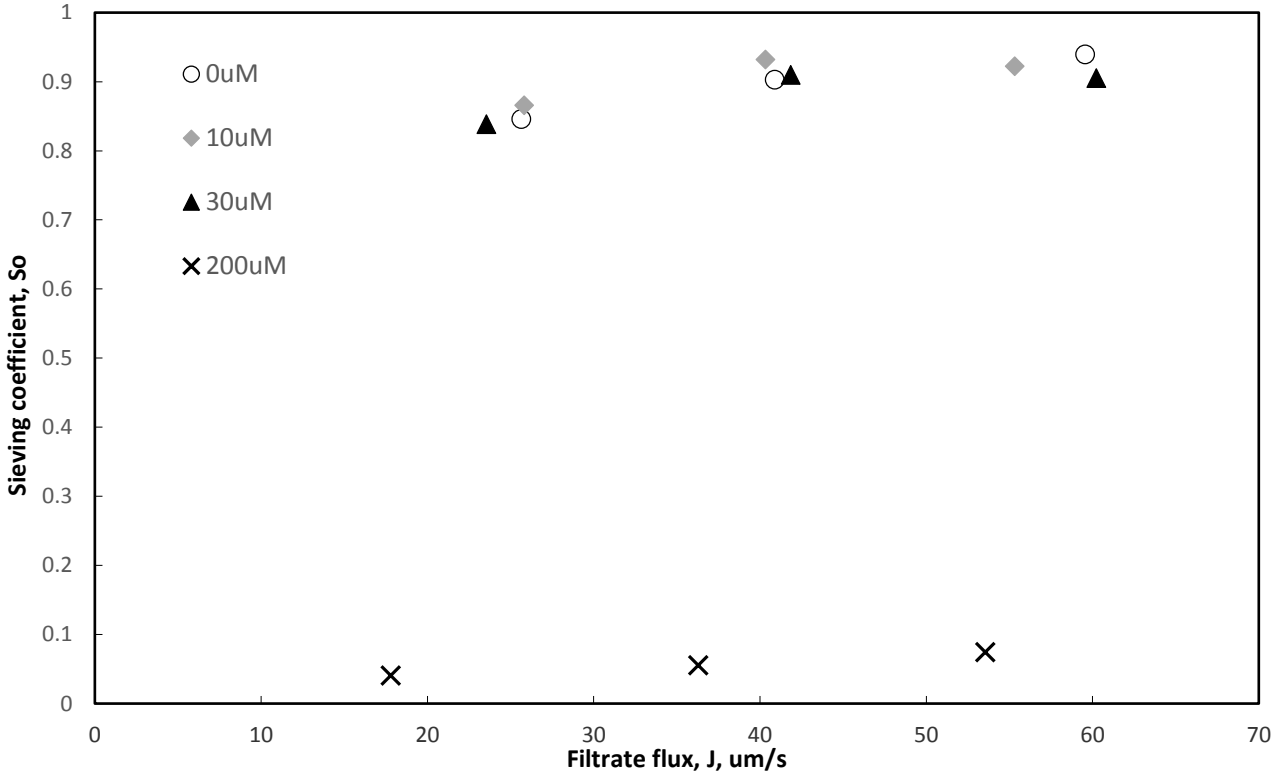

Figure 8. Sieving coefficient for supercoiled plasmid in 100 mM NaCl TE buffer.

Additional insights into the effects of electrostatic interactions on the sieving behavior of the supercoiled isoform were obtained by performing an experiments using 10 μM spermine in a 1 mM NaCl TE buffer. Spermine had a relatively small effect on plasmid transmission at this

low salt concentration, with the addition of 2 µM spermine only increasing the sieving coefficient from $S_o = 0.26$ without spermine to $S_o = 0.34$ at a filtrate flux of 80 µm/s. Figure 9 compares the transmission of the supercoiled isoform in the different ionic strength solutions at a spermine concentration of 10 µM. The sieving coefficient at low filtrate flux increases dramatically with increasing NaCl concentration, going from $S_o \approx 0.01$ to more than $S_o = 0.8$ as the NaCl concentration increases from 1 to 100 mM at a flux around 30 µm/s.

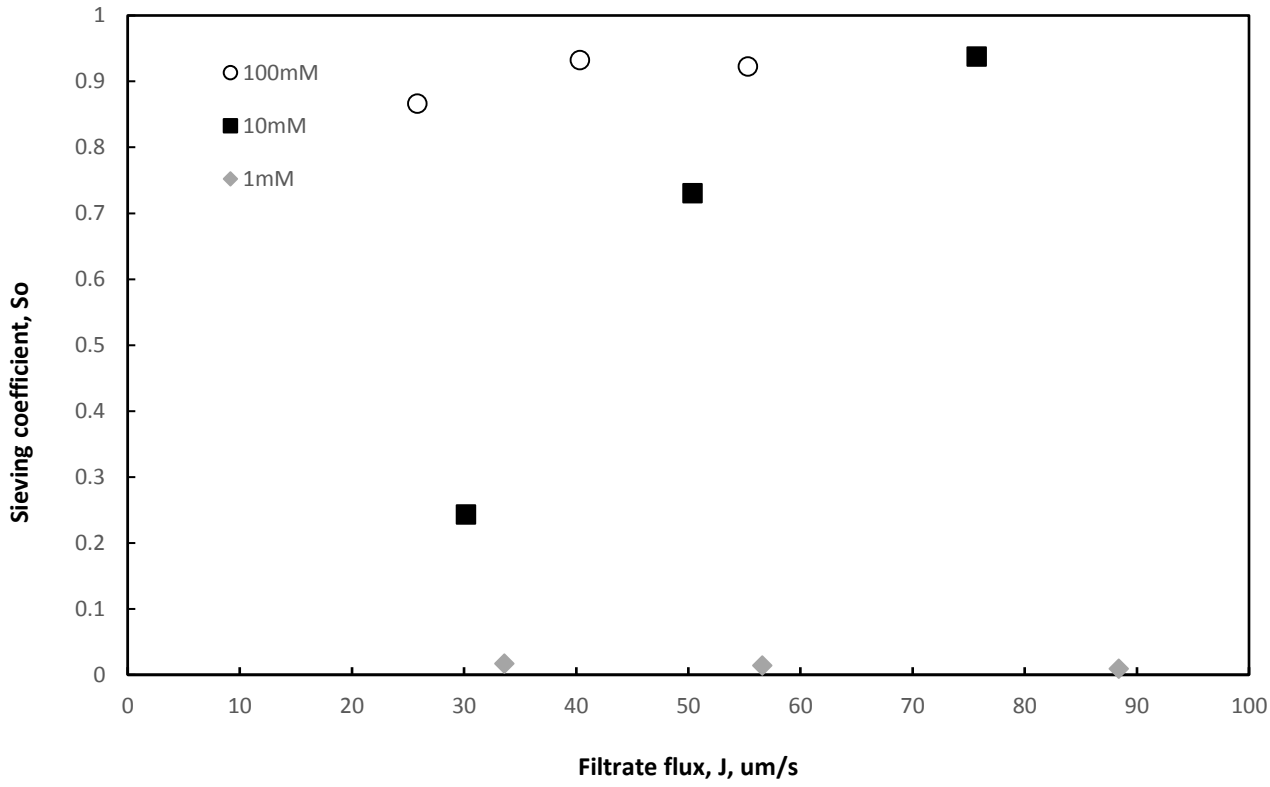

Figure 9. Sieving coefficient for supercoiled plasmids in 10 µM spermine using 1, 10, and 100 mM NaCl in TE buffer.

3.5 Isoform Selectivity

The different effects of spermine on the transmission of the different isoforms of DNA could potentially be used to enhance the effectiveness of the DNA separation. Figure 10 shows

the selectivity for the separation between the supercoiled and open circle isoforms at a flux of about 50 μm/s as a function of the spermine concentration. The selectivity was calculated as the ratio of the sieving coefficient of the supercoiled isoform to that of the open-circular isoform using data obtained in separate experiments using each of the purified isoforms. The largest selectivity occurred at a spermine concentration of 2 μM in the 10 mM NaCl TE buffer. The reduction in the selectivity at high spermine concentrations was due to the significant reduction in the transmission of the supercoiled plasmid under these conditions.

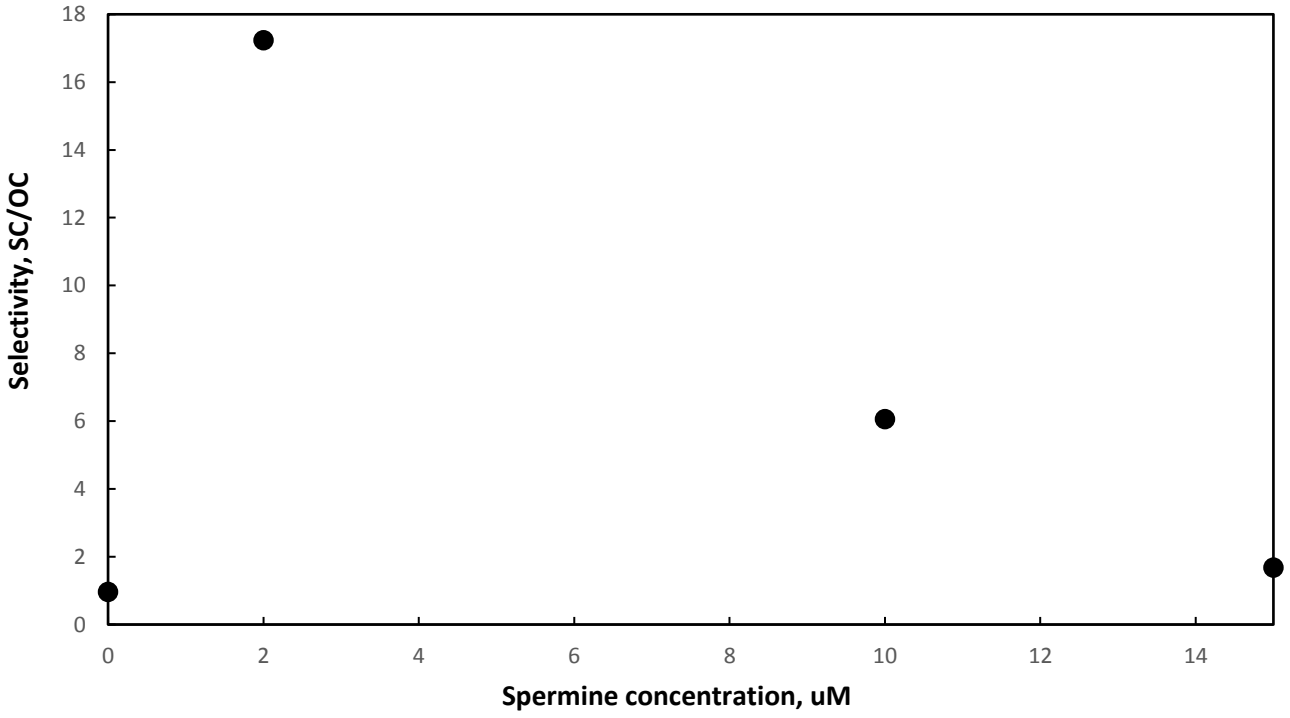

Figure 10. Selectivity for the separation between the supercoiled and open-circular plasmid isoforms at a flux of about 50 μm/s.

3.6 Other experiments

Several other experiments were performed in an effort to identify the optimal conditions for plasmid isoform separation. Figure 11 shows the sieving coefficient as a function of filtrate flux for the supercoiled FDY plasmid at different spermine concentrations.

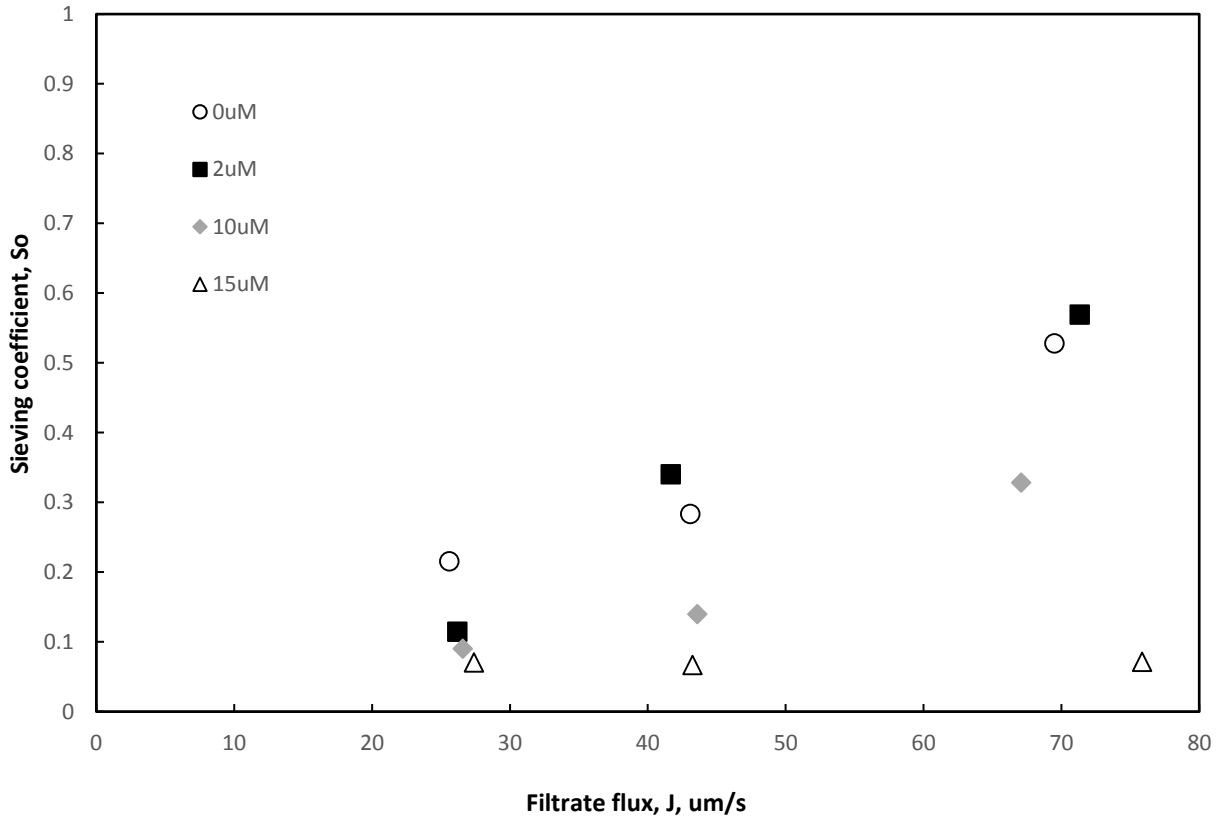

Figure 11. Sieving coefficient of supercoiled FDY plasmid in 10 mM NaCl TE buffer.

Similar to the behavior of the supercoiled MDY, the transmission of the FDY plasmid decreased significantly at high spermine concentrations, with the sieving coefficient in the presence of 15 μM spermine remaining below 0.1 over the entire range of filtrate flux.

Limited sieving experiments were also performed with Biomax 100 kDa membranes. Data obtained with the supercoiled MDY plasmid in 10 mM NaCl, however, showed negligible

transmission at all fluxes and spermine concentrations ($S_o < 0.01$) through this smaller pore size membrane. Similarly, sieving experiments with the supercoiled MDY plasmid in 10 mM NaCl were also performed with the Ultracel 300 kDa membrane as shown in Figure 12. The results were similar to the data obtained with the Biomax 300 kDa membrane, with a low concentration of spermine (2 µM) causing a small increase in plasmid transmission, while higher spermine concentrations significantly reduced the transmission. The largest increase in sieving coefficient was observed at a filtrate flux of 80 µm/s, with S_o increasing from 0.02 in the absence of spermine to $S_o = 0.26$ in the presence of the 2 µM spermine.

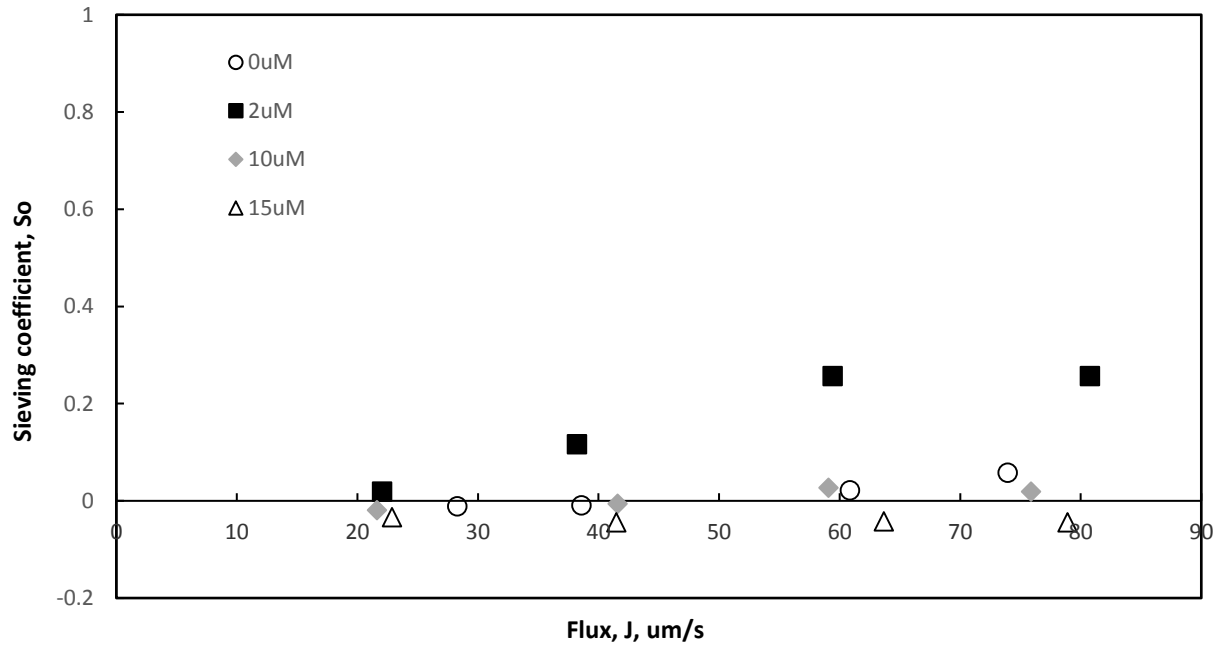

Figure 12. Sieving coefficient of the supercoiled MDY plasmid in 10 mM NaCl TE buffer through the Ultracel 300 kDa membrane at different spermine concentrations.

4. Conclusions

This thesis reported the first experimental results showing the effects of spermine on the transmission of different plasmid DNA isoforms through small pore size ultrafiltration membranes. The addition of spermine at low concentrations enhanced the transmission of all of the DNA isoforms, most likely due to electrostatic shielding of the negative charges in the DNA molecules and / or the change in DNA conformation to a tightened plectonemic structure. This "tighter" structure would likely facilitate the elongation of the plasmid DNA in the flow entering the pores. This would also explain why the largest increase in sieving coefficient was observed with the supercoiled plasmid isoform, since neither the open-circular nor linear isoforms adopt this plectonemic structure.

The addition of 10 μM spermine increased the observed sieving coefficient of the supercoiled plasmid by a factor of about 15 at a filtrate flux of 50 μm/s. A smaller increase was observed for the linear MDY plasmids, with the sieving coefficient increasing from $S_o = 0.56$ in the absence of spermine to $S_o = 0.73$ in the presence of 2 μM spermine at a flux of approximately 30 μm/s. Overall, transmission of the open-circular isoform was the lowest, which is expected due to the lack of elongational flexibility in the structure of the open-circular isoform.

In contrast, spermine concentrations above 10 μM actually caused a very large decrease in DNA transmission when using a 10 mM NaCl TE buffer, which is likely due to DNA condensation at higher salt concentrations. Compaction of DNA can occur when the negative charge density of DNA is neutralized by multivalent cations, such as spermine. The highly compacted / condensed DNA will be unable to elongate in the flow field, and since the DNA size is larger than the membrane pore size, it will be highly retained by the membrane. The results suggest that there is a critical concentration of spermine above which transmission is inhibited.

In the case of a 10 mM NaCl solution, this critical spermine concentration was about 10 µM. The critical spermine concentration was also a function of the ionic strength of the buffer solution. For example, a sharp drop in plasmid transmission was not observed until a 200 µM spermine concentration when using a 100 mM NaCl concentration. In contrast, the plasmid transmission dropped significantly with the addition of only 6 µM spermine in the 1 mM NaCl solution. This suggests that the shielding effects of monovalent ions compete with the binding of spermine.

The different effects of spermine on the ultrafiltration behavior for the different isoforms provided opportunities to enhance the selectivity for the separation of the supercoiled isoform from the linear and open-circular isoforms, both of which are considered process impurities in the purification of DNA therapeutics. The maximum selectivity was achieved when adding 2 µm spermine to a buffer solution with 10 mM NaCl at a filtrate flux of 50 µm/s. The selectivity for the separation of the supercoiled isoform from the open-circular isoform approximately 17 at these conditions, which is sufficient for a fairly high resolution separation of these isoforms.

The data suggest that adding 2 µM of spermine to a mixture of supercoiled and linear plasmid DNA and conducting a sieving experiment at a filtrate flux of approximately 50 µm/s could facilitate the separation of the two isoforms. Future experiments could be used to perform this separation using ultrafiltration and the conditions identified by this thesis. In a diafiltration experiment, supercoiled plasmids would be collected in the filtrate since they would be selectively transmitted through the membrane, and the open-circular plasmids would be retained in the feed. AGE imaging could be used to then determine the purity of each plasmid. Both plasmids should be relatively pure based on the high selectivity for supercoiled versus linear plasmids. Additionally, future work could investigate using spermine to condense DNA when

purifying DNA from cell lysis. Cell lysis is a procedure to produce plasmid DNA for gene therapy, and involves breaking the cell membrane to expose the DNA. Condensation of the DNA molecules by spermine during this process could therefore be used to enhance the production of plasmid DNA.

BIBLIOGRAPHY

[1] Prazeres, D.M.F., Ferreira, G.N.M., Monteiro, G.A., Cooney, C.L., and J. Cabral, Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks, *Trends in Biotechnology*, 17 (1999) 169-174.

[2] Yin, H., Kanasty, R.L., Eltoukhy, A.A., Vegas, A.J., Dorkin, R., and D.G. Anderson, Non-viral vectors for gene-based therapy, *Nature Reviews Genetics*, 15 (2014) 541-555.

[3] Kong, S., Titchener-Hooker, N., and M.S. Levey, Plasmid DNA processing for gene therapy and vaccination: Studies on the membrane sterilization and filtration step, *Journal of Membrane Science*, 280 (2006) 824-831.

[4] Latulippe, D.R., and A.L Zydney, Elongational flow model for transmission of supercoiled plasmid DNA during membrane ultrafiltration, *Journal of Membrane Science*, 329 (2009) 201-208.

[5] Prazeres, D.M.F., and G.N.M Monteiro, Design of flowsheets for the recovery and purification of plasmids for gene therapy and DNA vaccination, *Chemical Engineering and Processing: Process Intensification*, 43 (2004) 609-624.

[6] Borujeni, E.E., and A.L. Zydney, Membrane fouling during ultrafiltration of plasmid DNA through semipermeable membranes, *Journal of Membrane Science*, 450 (2014) 189-196.

[7] Li, Y., Currie, D., and A.L. Zydney, Enhanced purification of plasmid DNA isoforms by exploiting ionic strength effects during ultrafiltration, *Biotechnology and Bioengineering*, 113 (2015) 783-789.

[8] Li, Y., Butler, N., and A.L. Zydney, Size-based separation of supercoiled DNA using ultrafiltration, *Journal of Colloid and Interface Science,* 472 (2016) 195-201.

[9] Latulippe, D.R., and A.L. Zydney, Separation of plasmid DNA isoforms by highly converging flow through small membrane pores, *Journal of Colloid and Interface Science*, 357 (2011) 548-553.

[10] Vijayanathan, V., Thomas, T., Shirahata, A., and T.J. Thomas, DNA condensation by polyamines: a laser light scattering study of structural effects, *Biochemistry*, 40 (2001) 13644-13651.

[11] Shao, Q., Goyal, S., Finzi, L., and D. Dunlap, Physiological levels of salt and polyamines favor writhe and limit twist in DNA, *Macromolecules*, 45 (2012) 3188-3196.

[12] Latulippe, D.R., Ph.D. Thesis, Department of Chemical Engineering, The Pennsylvania State University (2010).

[13] Latulippe, D.R., Ager, K., and A.L. Zydney, Flux-dependent transmission of supercoiled plasmid DNA through ultrafiltration membranes, *Journal of Membrane Science,* 294 (2007) 169-177.

[14] Sato, Y.T., et al., Folding transition into loosely collapsed state in plasmid DNA as revealed by single-molecule observation, *Federation of European Biochemical Societies*, 579 (2005) 3095-3099.

[15] Toma, A.C., de Frutos, M., Livolant, F., and E. Raspaud, DNA condensed by protamine: a "short" or "long" polycation behavior, *Biomacromolecules,* 10 (2009) 2129-2134.

ACADEMIC VITA

Academic Vita of Rachel M. Bolten
rob5305@psu.edu
rachmbolten@gmail.com

Education

B.S. Chemical Engineering with honors (Spring 2017)
Minor in Economics

Thesis Title: The effects of spermine on DNA transmission through ultrafiltration membranes
Thesis Supervisor: Andrew Zydney

Work Experience

PPG Industries, Cranberry Township, PA
Environmental Health & Safety Intern, Summer 2016

- Completed high level Process Safety Management applicability studies at two architectural coatings plants in preparation for upcoming safety audits and upgraded safety training data in a new web-based training management system
- Studied OSHA Process Safety Management requirements and participated in conferences between OSHA and ACC representatives

PPG Industries, Huron, OH
Process Engineering Intern, Summer 2015

- Led a regional study on the "thindown tanks" at seven different architectural coating plants in North America, collecting data at each site and defining how to use PPG proprietary software to model the thindown process of paint-making
- Made recommendations to modify existing tanks in order to improve the quality of mixing at various plants, reduce cycle time, and improve business results

Involvement

Member, Penn State Lyrical Line Dance Company 2014-Present

- Treasurer, 2016-2017

Member, Society of Women Engineers 2013-Present
Member, American Institute of Chemical Engineers 2013-Present
Member, Tau Beta Pi Honor Society 2016-Present
Learning Assistant, Physics (Electricity and Magnetism) 2014
Member, THON 2015 Hospitality Committee 2015
Member, THON 2016 Merchandise Committee 2016
Member, THON 2017 Hospitality Committee 2017

Awards

President's Freshman Award, 2014
Penn State Provost Award, 2014