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SCHREYER HONORS COLLEGE

DEPARTMENT OF CHEMICAL ENGINEERING

ENGINEERING AND ANALYSIS OF BIOMIMETIC MEMBRANES USING THE HIGHLY PERMEABLE OUTER MEMBRANE PROTEIN, OMPF

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ABSTRACT

In this project, a functional biomimetic membrane utilizing the highly permeable and mutation-tolerant *E. coli* Outer Membrane Protein F (OmpF) was developed. OmpF was self-assembled with the block copolymer polybutadiene-polyethylene oxide (PB-PEO) to form two-dimensional crystals using a dialysis technique. The *E. coli* OmpF was overexpressed in an expression strain and purified through a series of centrifugations and chromatographic steps. Once the OmpF was crystallized with the PB-PEO, transmission electron microscopy imaging was utilized to analyze morphology of the single layer. Suitable single layers were then stacked in a layer-by-layer method to produce a functional membrane. This membrane was subsequently characterized to obtain its water permeability and solute rejection data for dyes of various molecular weights and sizes. This experiment indicated that OmpF can be successfully incorporated into a PB-PEO membrane and can be used for separations of molecules with molecular weights greater than 500 Da with high permeability.

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Chapter 1

Introduction

1.1 Problem statement

The purpose of the research done for this thesis was to engineer membranes that are more economical and more efficient in small-molecule separations, important for a range of applications including bioseparations and desalination[1]. With the ever-increasing perils associated with global climate change, more practical and viable removal of small molecules and contaminants from water has become of high interest, especially in areas where drinkable water is scarce[1]. While the world population continues to increase at a rapid pace with limited clean water resources, the percent of people worldwide without access to clean water will only worsen[2]; a focus must be placed now on efficient and affordable clean water recovery from brackish water and saltwater to mitigate the severity of the problem currently and in the future.

The major goal of this project was to engineer, at the laboratory scale, biomimetic membranes based on the highly permeable *E. coli* Outer Membrane Protein F (OmpF). Biological systems, such as the cell membrane, possess high permeability along with high selectivity that is desired in macroscale, industrial systems. They can overcome the trade-off between permeability and selectivity as seen in current commercial membranes. Through characterization of membranes combining block copolymers (BCPs) and OmpF with regard to solute rejections and water permeability, an important step toward production of larger scale membrane separations was taken that can drastically improve the performance of membranes.

1.2 Background

1.2.1 Biological Membranes

In biological cell membranes, a plasma membrane composed of amphiphilic phospholipids controls the separation of the components inside and outside of the cell. This lipid bilayer, which self-assembles due to the combination of the phosphate head group's hydrophilicity and the fatty acid's hydrophobicity, plays an important role, as it allows small molecules to passively diffuse across the membrane down their concentration gradient, as well as keeping out or in larger molecules that cannot penetrate the membrane without the use of specific protein channels or transporters[3]. The cell membrane is remarkable in the fact that the lipid bilayer is so stable yet still dynamic[4]. It is this biological stability, selectivity, and permeability that leads researchers to seek to mimic the mechanisms displayed in the cell membrane[4]. The cell membrane is indubitably effective in its purpose of intracellular and extracellular separations.

Membrane proteins, utilizing both diffusion/passive transport (no energy input) and active transport (energy input required), transport solutes that are impermeable to the lipid bilayer yet still require movement. The three classes of these membrane proteins, displayed in Figure 1 below, are channels, transporters, and pumps[4]. One very vital and relevant example of a class of membrane proteins is aquaporins, whose purpose is to selectively transport water. With a pore size of only about 2.3 Angstroms[4], these aquaporins allow small water molecules to diffuse through the cell membrane one at a time at remarkably high rates (about 10×10^{-14} cm^3/s for a single aquaporin)[5]. Additionally, while maintaining this high permeability, they are selective in excluding other solutes (including protons) from crossing the membrane[4,5]. The charged amino acids on the surface of the channel as well as the small size of the pore underlie this property[4]. It is this impressive specificity of this biological system that has caught the attention of researchers for many years. With a goal to reproduce the mechanisms employed by biological membranes, the field of biomimetic membranes was developed.

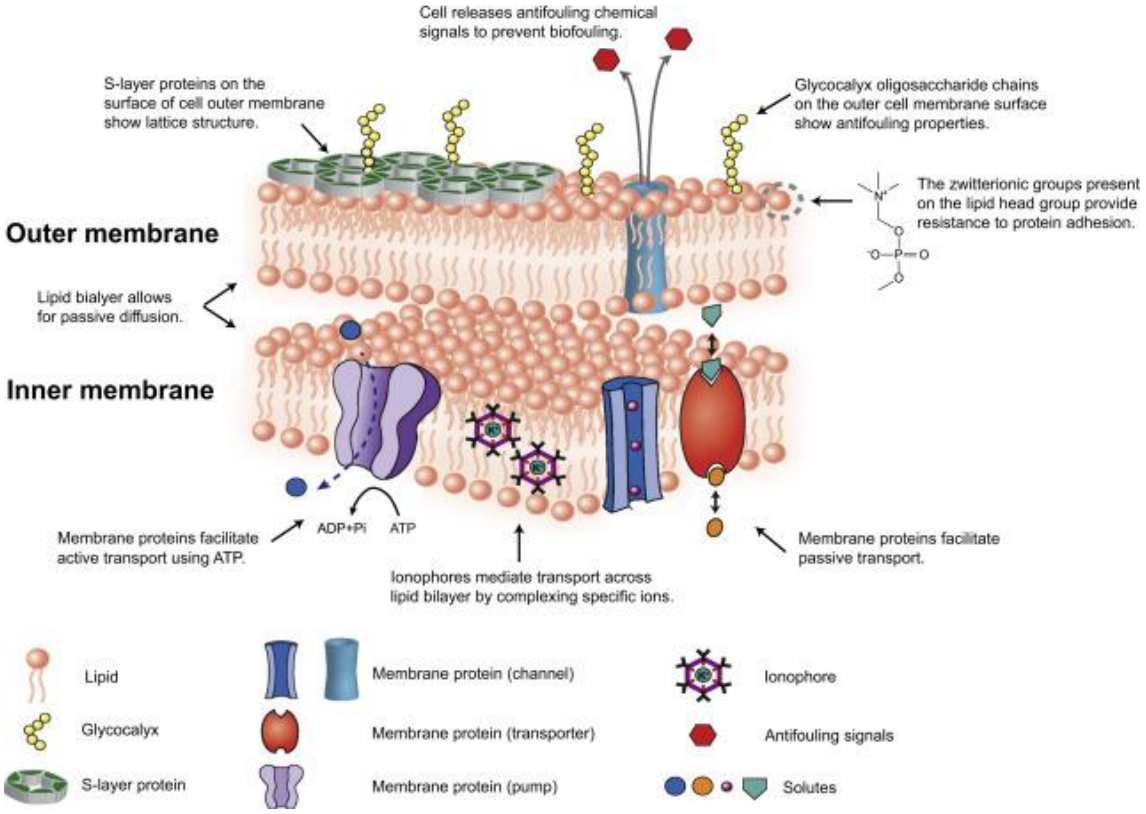

Figure 1. Lipid bilayer with embedded proteins[4]

1.2.2 The Use of Biomimetic Membranes in Separation Processes

Many processes rely on efficient separations, including but certainly not limited to desalination, pharmaceutical separations, sensors, etc [6]. In reverse osmosis (RO) membranes, a large amount of energy is required to overcome a large pressure gradient between the sides of the membrane, pushing solvent across the membrane and accomplishing the molecular separation by exclusion of solutes. While the use of membranes is not a new idea in any way, these traditional RO membranes for small molecule separation are sought to be improved to decrease their associated costs while not losing their efficacy. This is the goal of biomimetic membranes: to deliver a more cost-effective and practical industrial separation mechanism[6]. Additionally, traditional RO membranes often suffer from a lack of uniformity in free volume elements. This lack of uniformity in free volume elements leads to the membrane not separating with perfect accuracy,

which has led to interest in a more efficient method else. In biomimetic membranes, proteins that are produced by cells exhibit a much higher degree of uniformity in their pore sizes, as biological organisms produce proteins with remarkable precision[8]. For this reason, biomimetic membranes offer more effective separations compared to traditional membranes.[8]

While it may be tempting to employ the exact same components of the cell membrane in industrial membranes, amphiphilic phospholipids lose much of their stability when employed on a larger scale. Thus, amphiphilic block-copolymers (BCPs), which are much more stable, are designed to mimic the lipid bilayer in biomimetic membranes[9]. BCPs are of interest because the choice of monomers in the polymer can greatly influence the desired property of a membrane, and in addition, the BCPs can be chemically modified to achieve certain characteristics[9]. However, in order to use these BCPs in biomimetic membranes, a method is needed for effective insertion of proteins into the BCP membrane. Rehydration methods, even with high concentrations of protein, are limited in their abilities to insert high amounts of membrane proteins into polymeric vesicles. A method developed by Kumar et al employing dialysis proved successful in the formation of protein-BCP aggregates that are necessary for effective membrane engineering[7]. In this method, a solution of protein and BCPs are solubilized using a high concentration of detergent, and then the detergent is slowly removed so that the protein inserts itself into BCP aggregates that form[7]. Incorporation of these proteins into the BCP aggregates allowed for a two-dimensional crystal-lattice structure to form, which allows for planar membrane formation with high amounts of inserted proteins.

1.2.3 *E. coli* Outer Membrane Protein F

With a method in place for insertion of protein into BCPs, the logical next step in the preparation of biomimetic membrane is to determination of what channel to insert. For this research, OmpF was chosen for its high stability and its ability to be modified using mutations to specific amino acid residues. The structure of OmpF is shown below in Figure 2. The pore size of wild type OmpF is about 10.8 by 8.0 Å,

which constitutes an ovate structure. However, through mutations, this pore size can be modified and thus used for a range of separations.[8] Wild type OmpF is a trimeric structure, with primarily beta barrels constituting the pore. Its pore is bigger than that of an aquaporin, as it is less selective in the small solutes that are able to be transported.[8] Thus, it is preferred for separations involving molecules such as antibiotics, amino acids, and sugars[8]; the wild type is not effective in desalination as the pore is simply too large. Also, when combined with block copolymers, OmpF, through a dialysis method, assembles into two-dimensional crystal sheets.[8] It remains stable and intact even in varying temperature and different environments, which makes it a viable protein for biomimetic membrane separations.

1.3 Hypothesis

It was hypothesized that OmpF can be extracted from *E. coli* cells and stably inserted into polybutadiene-polyethylene oxide (PB-PEO) copolymers to form an effective laboratory scale membrane for separation processes that can serve as a baseline for industrial biomimetic membrane separations. Through analysis of the protein purity and structure, transmission electron microscopy (TEM) imaging of the crystalline structure of the protein inserted into PB-PEO, rejection data, and permeability data, the field of biomimetic engineering can move forward in its ability to reduce the energy consumption of separation processes.

Chapter 2

Methods and Materials

This project depended on many preliminary steps in order to obtain pure OmpF protein. Since the goal of the project was to determine if quality membranes with inserted OmpF could be engineered, it was vital that the purity of the WT OmpF used was high. The bulk of the individual work done in this experiment was the purification of OmpF through centrifugation and fast protein liquid chromatography, but all steps of the process for OmpF membrane engineering are included in this paper for a better understanding of the process. The preparation of wild type *E. coli* cells, dialysis insertion of OmpF into PB-PEO copolymers, TEM imaging, and membrane setup and testing were primarily done by graduate students in the laboratory.

2.1 Preparation of Wild type *E. coli* cells

The desired protein, wild type *E. coli* OmpF, was overexpressed in an *E. coli* strain. This strain was streaked and the cells were cultured in Luria-Bertani (LB) media. Ampicillin at a concentration of 50 mg/L ensured that only the desired cells were grown on the plates. They were grown at a temperature of 37 °C and mixing at 300 rpm. When the OD600 reader reached a value between 0.5 and 0.8, isopropyl beta-D-1thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM and the temperature was reduced to about 16 °C. The cells were then grown for an additional 12 hours to maximize the expression of OmpF in the cells, which were then harvested via centrifugation. Wet cells were stored at -80 °C.

2.2 Purification of OmpF protein from cells

2.2.1 Centrifugation

5 grams of the block of WT OmpF cells were dissolved in approximately 10 mL of a buffer of 20 mM Tris buffer at a pH of 8.0 with 0.5 mL DNAse. This solution was use to lyse the cells by disrupting the cell membrane.

5 grams of frozen WT OmpF were obtained from the -80 °C storage freezer. After thawing the solution, the cells were centrifuged in a table-top centrifuge for 15 minutes at 4000g and 4 °C. The purpose of this was to remove all unbroken cells in the solution. The pellet of this centrifugation was discarded. Figure 2, below, displays the initial solution of *E. coli* WT OmpF cells.

Figure 2. Wild Type OmpF cells prior to centrifugation

10% sodium dodecyl sulfate in a Tris buffer was added to the supernatant to reach a final concentration of 0.5%. This solution was then incubated at 4 °C for 20 minutes. After incubation, the solution was placed in the ultracentrifuge at 100,000g and 4 °C for 60 minutes. Special care was taken to make sure that the ultracentrifuge tubes were free of defects (such as scratches), as the pressure in the ultracentrifuge caused breakage at times. The solution following the first round of ultracentrifugation is shown in Figure 3.

Figure 3. OmpF solution after first round of ultracentrifugation

At this point, the OmpF was contained in membranes that are present in the pellet. The pellets were taken from the tubes (supernatant discarded) and combined with 25 mL of a solution of 0.125% n-Octylpolyoxyethylene (Octyl-POE) in 20 mM NaH_2PO_4 at a pH of 7.4. The Octyl-POE is a detergent used to solubilize membrane proteins, but in this case, it was used to wash the pellet to remove loosely bound proteins. A glass homogenizer was used to fully homogenize the pellet in the solution. The homogenized solution was then incubated at 37 °C for 1 hour, followed by another round of ultra-centrifugation at 100,000 g at 4 °C for 1 hour. The protein is now present in the pellet. This solution, before and after centrifugation, is shown below in Figure 4.

Figure 4. 25% Octyl-POE solution and OmpF protein before and after ultra-centrifugation

The supernatant was discarded, and the pellet was then combined with 25 mL of 3% Octyl-POE in 20 mM NaH_2PO_4 solution at a pH of 7.4 (pH checked with a pH meter). Again, the pellet was homogenized in the solution using a glass homogenizer.

The now homogenized solution was incubated at 4 °C overnight. The following morning, the solution was ultra-centrifuged for a third time, for 30 minutes at 100,000g and 4 °C. The OmpF protein was now present in the supernatant, so the pellet was discarded. The supernatant, which should be about 25 mL, was stored at 4 °C until further purified by fast-protein liquid chromatography.

2.2.2 Fast Protein Liquid Chromatography

Prior to fast protein liquid chromatography (FPLC), a series of buffer solutions were made to be used in the procedure. These solutions were 100 mL of 5 mM NaH_2PO_4, 3 mM NaN_3, with 1% Octyl-POE at a pH of 7.6 (buffer 1), 100 mL of 50 mM NaH_2PO_4, 3 mM NaH_2PO_4 with 1% Octyl-POE at a pH of 7.6 (buffer 2), 5 mM NaH_2PO_4, 3 mM NaN_3, 30 mM ethylenediaminetetraacetic acid (EDTA), and 100 mM NaCl at a pH of 7.6 (buffer 4), and 500 mL of 10 mM Tris, 100 mM NaCl with 1.2% octyl-beta-glucoside detergent at a pH of 8.0 (gel filtration buffer). All solutions were filtered via vacuum filtration to remove any particles.

The anion-exchange column was used first. With an FPLC machine (GE AKTA Pure 150), the DEAE FF anion exchange column, which was stored in 20% ethanol, was first equilibrated with buffer 2 at a flowrate of 2 mL/min, which was finished when the pH meter stabilized at a pH of a 7.6 and conductivity of 7-8 ms/cm. The column was then fully equilibrated with buffer 1. The protein was then fed into the column. Then, buffer 1 was used so that the UV-280 reading was stable at 0. Buffer 4 was then put through the column to elute the OmpF. The OmpF was tracked through the UV-280 monitor, and the fractions that were in the peak of the UV-280 line were collected. The tracking of the protein via the UV-280 line is shown in Figure 5.

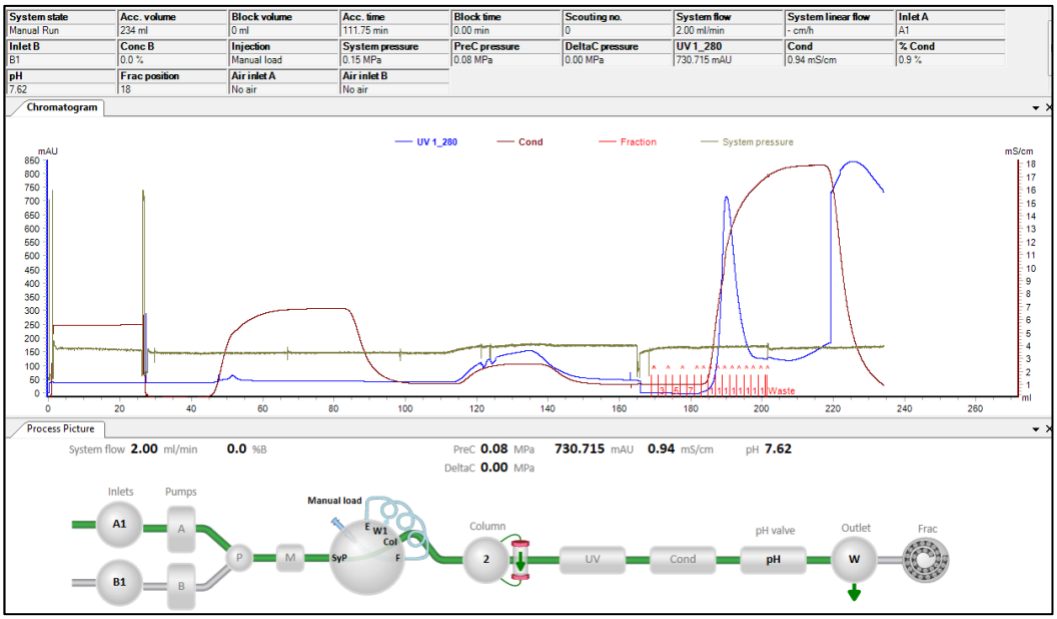

Figure 5. Anion exchange curve, showing peak UV-280 fractions where OmpF was present

After the fractions with OmpF were collected, they were concentrated to a volume of about 2 mL with ultra-centrifugal filter units. A Superose 12 size exclusion column was equilibrated with gel filtration buffer (described above). The concentrated OmpF purified from the anion exchange column was then put through the size exclusion column and eluted using the gel filtration buffer. The protein was again monitored via UV-280, shown in Figure 6.

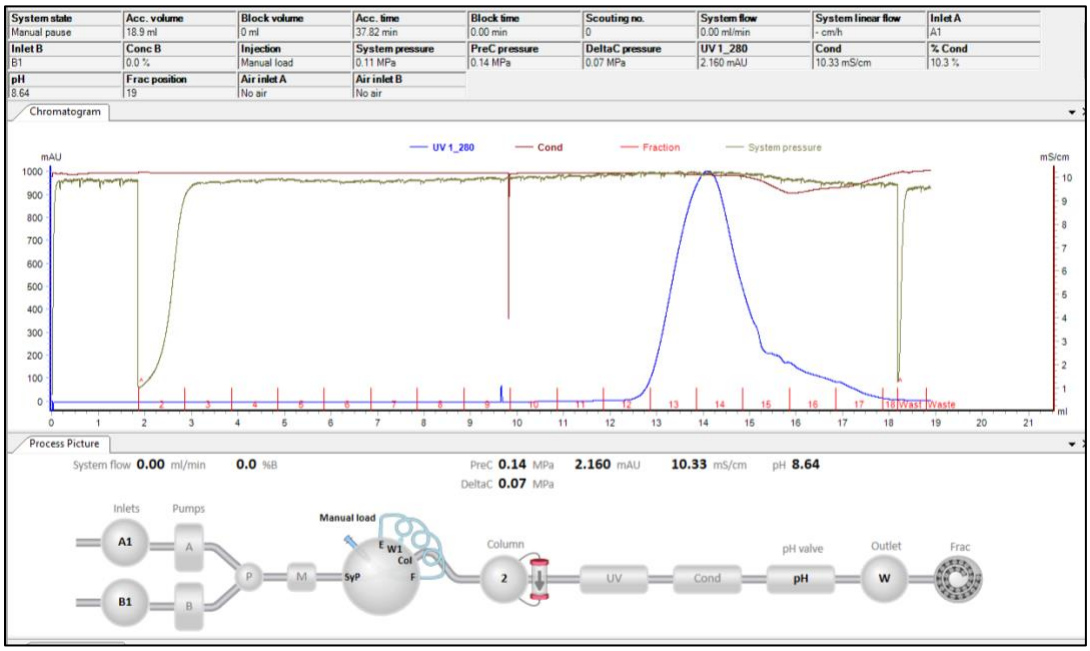

Figure 6. Size exclusion curve, showing peak UV-280 fractions where OmpF was present

The purity of the protein solutions was tested via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the concentration was tested via a Bradford essay. The most concentrated sample (and purest) was used for construction of two-dimensional OmpF crystals

2.3 Dialysis insertion of OmpF into PB-PEO copolymers to form crystal sheets

A 5 mg/mL stock solution of PB-PEO in detergent solution was prepared by combining the PB-PEO copolymer with 20 mM HEPES, 10 mM $MgCl_2$, 3 mM NaN_3, 100 mM NaCl with 4% (weight/volume) Octyl-POE (This is the dialysis buffer.). The OmpF solution was combined with this solution in a polymer to protein mass ratio of 0.15 to 0.6 in a 60 µL dialysis button for a final OmpF protein concentration of 1 mg/mL. Using dialysis membranes of size 12-14 kDa, the buttons were covered. The dialysis buttons were placed in a container with the dialysis buffer of 4% Octyl-POE solution. This solution was switched twice per day with lower and lower Octyl-POE (detergent) concentrations each time. This allowed for slow

removal of the detergent in the dialysis button. Eventually, a dialysis buffer free of all Octyl-POE was used three times to remove any remaining detergent in the buttons. This caused the OmpF and PB-PEO to co-crystallize in the dialysis button.

2.4 TEM analysis of 2D sheets

The OmpF and PB-PEO crystals needed to be confirmed via microscopic analysis in order to ensure that 1.) the crystals were indeed formed through the dialysis, and 2.) that the crystals were suitable for membrane fabrication. The crystallized protein-polymer solution was adsorbed on a carbon-coated transmission election microscopy (TEM) grid and stained with a solution of 0.75% (weight/volume) uranyl formate. TEM images were then obtained using the FEI Tecnai G2 Spirit BioTwin Microscope in the Millennium Science Complex at The Pennsylvania State University in University Park, PA.

2.5. Membrane fabrication using a layer-by-layer method

The procedure to assemble the macro-scale membrane for rejection and permeability tests was based on a previous paper[11]. In this method, a support membrane consisting of a 50 nm pore size polycarbonate (PC) membrane and a 50 nm pore size polyethersulfone (PES) membrane was used. This support membrane was negatively charged using UV ozone for thirty seconds, in order for the necessary layers to adhere to the membrane. In a stirred cell apparatus, the layers were accumulated through successive additions of roughly 13 µg of OmpF crystals and a buffer consisting of 40 mM polyethylenimine, 35 mM $CaCl_2$, and 0.5 M NaCl at a pH of 5.5. In order to cross-link the individual layers to each other, after a desired number of layers are added, the membrane was kept overnight in roughly 10 mL of a solution of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride.

2.6 Rejection and permeability test

The membranes were tested for their water permeability and solute rejection. A stirred cell apparatus with the membrane inside and continued stirring was assembled. To test the water permeability, pure water flowed through the cell. For about two minutes, the permeate was collected and weighed. The pure water permeability could then be calculated. Then solutions of specific dyes were flowed through the membrane at a pressure of 5 psi. Filtration continued until about 20 grams of permeate was collected, and then 1 mL of permeate and 1 mL of retentate were collected and tested for their concentration. From this, rejection data could be analyzed to see the selectivity of the membrane. In order to do this, a range of molecular weight dyes were used as shown below in Table 1.

Table 1. Dyes used for rejection tests and their molar masses

Dye	Molar Mass (g/mol)
Chrysoidine G (CG)	212.26
Methyl orange (MO)	304.02
Crystal violet (CV)	372.55
Rhodamine B isothiocyanate (RITC)	500.627
Methyl blue (MB)	753.84
Rose bengal (RB)	971.68
Fluorescent dextran (FD)	3000

Chapter 3

Results

3.1 OmpF purity and concentration

The concentration of the OmpF in each fraction obtained from the size exclusion column was analyzed via Bradford assay. To do this, an absorbance vs. concentration standard curve was made using known concentrations of protein and tested against absorbance at a wavelength of 595 nm. An example of a standard curve is shown in Figure 7.

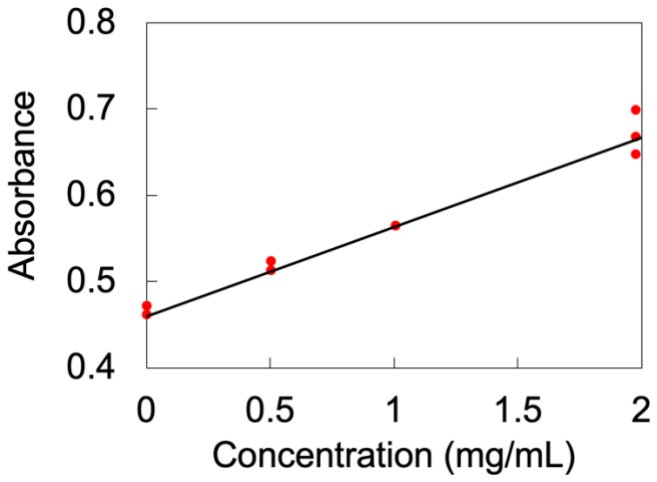

Figure 7. Bradford assay absorbance vs. concentration standards

This allowed a sample of unknown OmpF concentration to be read with an absorbance and correlated to a concentration of the OmpF.

The purity of the OmpF was analyzed via an SDS-PAGE gel. The results of one such purification are shown in Figure 8. The image shows the increase in purity of the OmpF throughout the purification process. A single, solid blue stain at the end of the process signals the purity of the OmpF obtained.

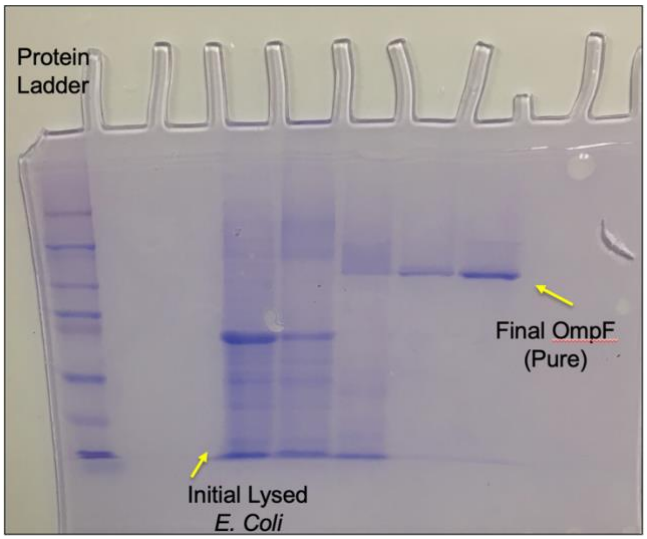

Figure 8. SDS-PAGE showing advancement in protein purity

3.2 TEM results

After the OmpF and PB-PEO aggregates were crystallized, it was necessary to check the morphology of the 2D layers in order to verify its suitability for membrane fabrication. It also indicates the success of the OmpF purification and protein-polymer crystallization steps. The key was to look for a single layer of packed and dense well-ordered arrays that would be acceptable for membrane fabrication. The following image, shown in Figure 9 and taken by Yu-Ming Tu, displays exceptional two-dimensional sheets as observed through the TEM. Note that the scale bar shown on the graph is 100 nm.

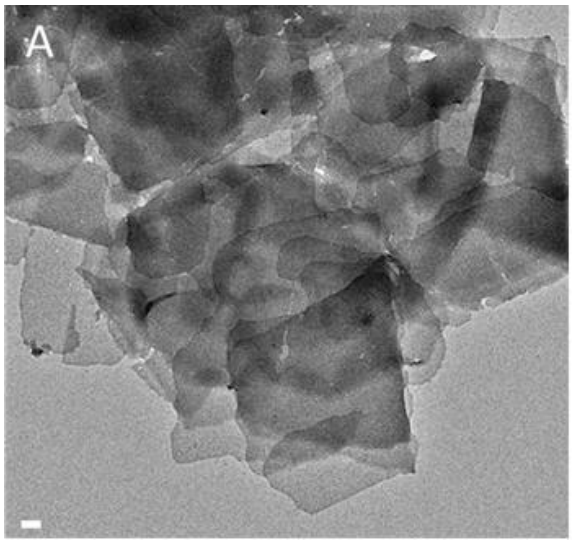

Figure 9. TEM image of 2D sheets of OmpF/PB-PEO crystals formed after dialysis. Scale bar in lower left corner is 100 nm in length.

On the TEM, the Fast Fourier Transformation (FFT) could be observed of an image. With high quality OmpF and PB-PEO sheets, a characteristic FFT could be clearly seen, which is shown in Figure 10. The presence of these spots, which are known as first-order spots, indicate a densely packed 2D array, which was the goal of the dialysis and necessary for the membrane sheets prior to macroscale fabrication.

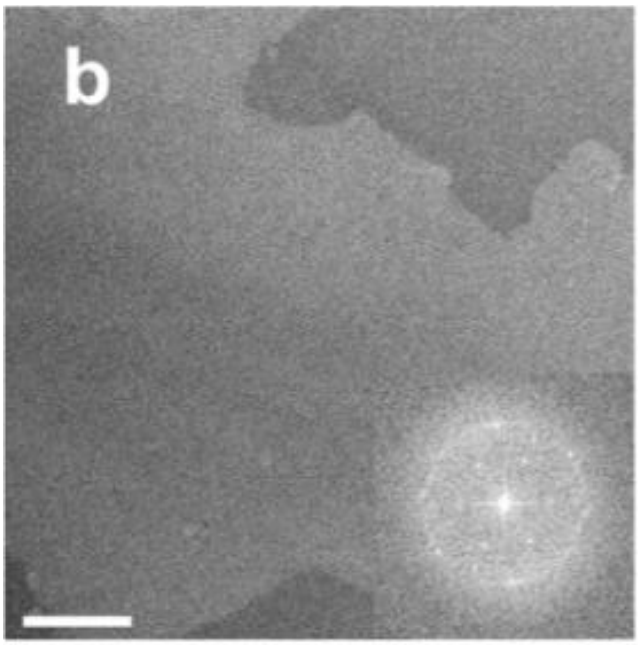

Figure 10. FFT image as observed in TEM of a highly packed 2D OmpF/PB-PEO array. Scale bar in lower left corner is 100 nm in length.

3.3 Rejection and permeability results

Perhaps the most important part of this experiment was being able to characterize the macroscale membranes made based on their rejection and permeability. This data provides the practical information needed for later separation processes. In this experiment, various dyes of increasing molecular weights were filtered through the membrane using a stirred cell apparatus. The permeate and the retentate were then sampled and UV analysis was performed to determine their concentrations. From the comparison of these, an apparent rejection coefficient could be calculated (This apparent rejection coefficient was later corrected for better concentration polarization effects, but that is outside the scope of this paper.).

Table 2 below shows the rejection data for the various dyes through a 6-layer membrane consisting of OmpF and PB-PEO crystallized through the dialysis method. These data were gathered by Yu-Ming Tu.

Table 2. Rejection data for OmpF and PB-PEO membranes

Dye & Molecular Weight (Da)	Membrane 1 Rejection	Membrane 2 Rejection	Membrane 3 Rejection	Average Rejection
CG: 212.26	0.418	0.450	0.500	0.456
MO: 304.02	0.285	0.189	0.151	0.208
CV: 372.55	0.494	0.644	0.604	0.581
RITC: 500.627	0.809	0.854	0.884	0.849
MB: 753.84	0.974	0.956	0.971	0.967
RB: 971.68	0.977	0.972	0.984	0.984
FD: 3000	0.955	0.925	0.910	0.910

To better visualize the relationship between molecular weight of the dye and the average rejection coefficient for the membrane, Figure 11 shows a graph of these two variables and their trends. From this graph, it is clear that for molecular weights above about 500 Da, the membrane rejects a majority of the solute molecules. This cutoff can be used for processes that require a separation around this molecular weight. For instance, as expected from the pore size of the OmpF, this membrane is unable to retain small salts, such as sodium chloride, as the membrane does not reject significant amounts of low molecular weight dyes. Thus, the wild type OmpF would be unsuitable for desalination purposes, but the pore size could potentially be modified to get a more selective membrane[8]. However, this current membrane may be suitable for purification of larger biomolecules.

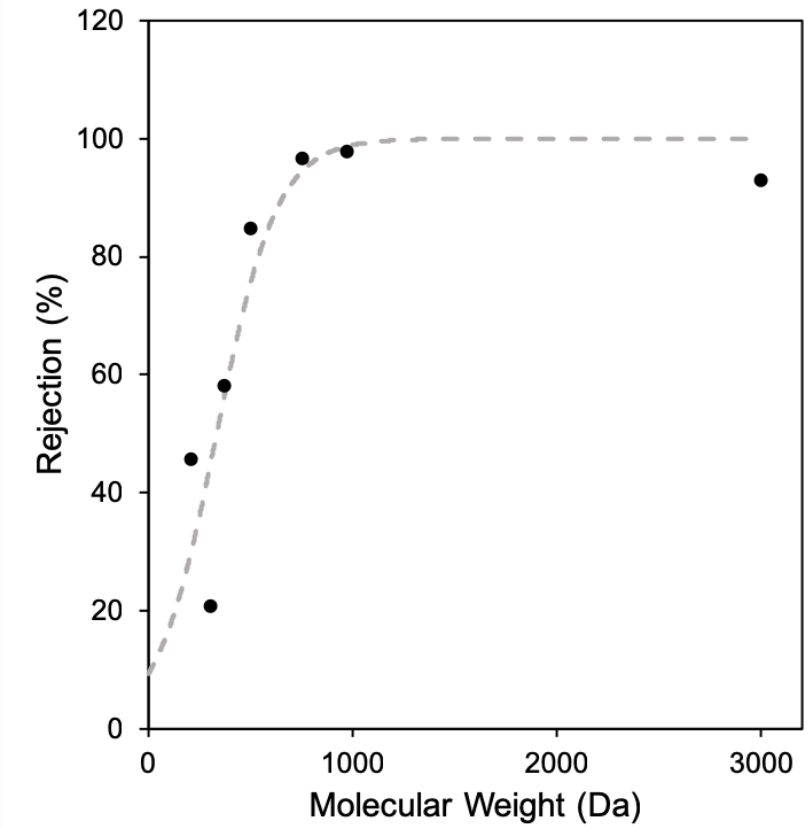

Figure 11. Average rejection based on molecular weight

In addition to rejection data, permeability data were taken. While rejection is important, the desired solution must also flow through the membrane at a reasonable rate for efficient separations. The pure water permeability of the OmpF membrane was compared to commercial membranes of a similar molecular weight cut-off; which is shown in Figure 13. Note that the permeability is given in units of $L/m^2/hr/bar$. This is abbreviated as LMH/bar. From these data (provided by Yu-Ming Tu), it is clear that the permeability through the membrane is much higher than that for similar commercial membranes[10,12].

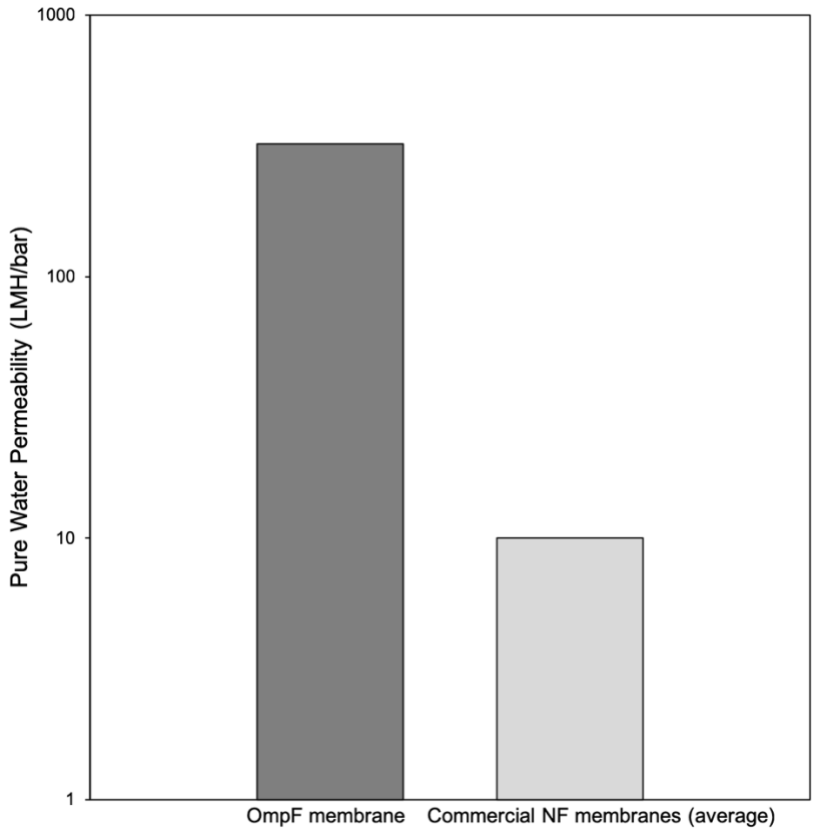

Figure 12. Comparison of permeability to commercial membranes

Chapter 4

Summary and Conclusions

The results of this project indicate the success of engineering a functional biomimetic membrane out of crystallized OmpF and PB-PEO. The OmpF that was purified (as confirmed by the SDS-PAGE gel) was able to be successfully crystallized through a dialysis process in a single layer with PB-PEO block copolymers. This structure was confirmed via TEM imaging. The rejection and permeability data obtained indicates that the Wild Type OmpF and PB-PEO membrane would be useful in separations of molecules greater than 500 Da. Additionally, the common trade-off between permeability and rejection was minimal for the biomimetic membrane produced, as the membrane's permeability was higher than similar macroscale membranes.

Chapter 5

Future Work

Since OmpF is a mutation-tolerant protein, the pore size of the protein can be adjusted to suit different various goals, which has been explored by other researchers in the lab. More mutations can be explored in the future to see the stability and functionality of the various protein-embedded membranes. Perhaps a pore size could be attained for membranes using mutated OmpF for rejection of small solutes (such as salts like NaCl) yet still maintain stability in a biomimetic membrane. Additionally, other block-copolymers could be explored besides PB-PEO to analyze possible enhanced macroscale stability that better mimics the phospholipid bilayer of the cell membrane. More dyes may also be used in the rejection and permeability tests to more accurately predict the molecular weight cut off.

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