Investigation of Electrospun Nanofibers for Separation Applications

Shu-Ting Chen

University of Arkansas, Fayetteville

Follow this and additional works at: https://scholarworks.uark.edu/etd

Part of the Membrane Science Commons, and the Polymer Science Commons

Citation

This Dissertation is brought to you for free and open access by ScholarWorks@UARK. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of ScholarWorks@UARK. For more information, please contact scholar@uark.edu.
Investigation of Electrospun Nanofibers for Separation Applications

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Engineering

by

Shu-Ting Chen
Chung Yuan Christian University
Bachelor of Science in Chemical Engineering, 2012
National Taiwan University of Science and Technology
Master of Science in Chemical Engineering, 2014

December 2020
University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

Xianghong Qian, Ph.D.
Dissertation Director

Ranil Wickramasinghe, Ph.D. Yung Chang, Ph.D.
Committee Member Committee Member

Ed Clausen, Ph.D. Christa Hestekin, Ph.D.
Committee Member Committee Member
Abstract

Electrospun membranes are an attractive alternative to flat sheet membranes as absorbent with numerous advantages like high porosity, large specific surface area and ease of functionalization. This doctoral dissertation focuses on fabricating novel polymeric membrane adsorbents for protein separations and ammonium ion removal. Three distinctly different preparation methods including UV-initiated polymerization, atom transfer radical polymerization, and mixed-matrix formation, have been employed to fabricate the electrospun membranes. Overall, this study aimed to develop electrospun membranes with excellent separation efficiency for application in protein purification and ammonium ion removal.

Chapter 2 details the stepwise development of weak anion exchange membranes and subsequent application in protein purification. The static and dynamic binding capacities of functionalized electrospun PAN and PSf membranes are reported to be ~100 mg/mL and ~200 mg/mL, respectively. Further experimental investigation reveals that surface modification of electrospun membranes by grafting polymeric ligands can enhance protein adsorption due to increased surface area-to-volume ratio.

Chapter 3 details development of responsive electrospun membranes for hydrophobic interaction chromatography (HIC). The responsive HIC ligand explored in the scope of this study leverages the switch between hydrophobic to hydrophilic state depending on salt concentrations. Moreover, experimental results reveal substantial improvement in dynamic binding capacity and recovery by controlling the effects of polymer density and polymer chain length relative to flat-surface membranes.
Chapter 4 details a stepwise development of mixed-matrix membranes fabricated using an electrospinning method for ammonia removal. Moreover, fabrication of novel zeolite embedded electrospun membrane is also provided in detail. In the scope of this study, a maximum binding capacity of ~30 mg/g\textsubscript{zeolite} for the ammonium ions was obtained using the mixed-matrix membrane. Overall, more than 90\% of total ammonia nitrogen (TAN) removal is reported from aquaculture wastewater with high water flux and excellent regeneration using 2 M NaCl solution.

Chapter 5 details a systematic study on the effects of feed conditions on viral clearance for different commercially available media. Experimental results using design of experiment methodology and provide an understanding of the mechanism of adsorption between different commercial products through charge density and porosity.
Acknowledgments

I would like to express my sincere gratitude to my advisor Prof. Xianghong Qian for her motivation, patience, and support for my Ph.D. study. She has given me a good example in terms of academic research and life attitude. Her perceptive suggestions and encouragement promote me to be a confident and independent researcher. Thank you for bringing me into the electrospun membrane and bioseparation research field. Besides my advisor, I would like to thank my dissertation committee: Dr. Ranil Wickramasinghe, Dr. Ed Clausen, Dr. Christa Hestekin, and Dr. Yung Chang for attending my defense, and your comments broaden my perspective. My thank also goes to my colleague, Dr. Pejman Ahmadiannamini, Dr. Anh Vu, and Dr. Zizhao Liu who taught me the excellent experimental skills. I want to thank all the other group members who helped me in my research. Financial support from the National Science Foundation, MAST Center, AstraZeneca, Center for Advanced Surface Engineering (CASE), Arkansas Bait and Ornamental Fish Growers Association, and University of Arkansas (Fayetteville) are gratefully acknowledged. Lastly, I would like to thank my family for supporting my Ph.D. study. Your love always makes me brave to face any challenges.
Table of Contents

Chapter 1. Introduction .................................................................................................................. 1

1.1 Downstream purification ............................................................................................................ 4

   1.1.1 Protein Affinity Chromatography (Protein A) ................................................................. 4

   1.1.2 Ion exchange Chromatography (IEC) .............................................................................. 6

   1.1.3 Hydrophobic interaction chromatography (HIC) ........................................................... 11

   1.1.4 Multi-modal Chromatography (MMC) ............................................................................ 13

1.2 Traditional Packed-bed Chromatography ................................................................................. 14

1.3 Alternative Membrane Chromatography ............................................................................... 15

1.4 Electrospun membrane ............................................................................................................. 17

1.5 Application of electrospun membrane ..................................................................................... 22

   1.5.1 Membrane Chromatography .......................................................................................... 22

      1.5.1.1 Application of Ion Exchange Chromatography .................................................... 24

      1.5.1.2 Application of Hydrophobic Interaction Chromatography (HIC) .................... 27

   1.5.2 Ammonium Removal from Wastewater ......................................................................... 29

1.6 Modification of electrospun membrane ..................................................................................... 35

   1.6.1 UV-initiated Polymerization ........................................................................................... 36

   1.6.2 Atom Transfer Radical Polymerization (ATRP) ............................................................ 40

      1.6.2.1 Surface-initiated Atom Transfer Radical Polymerization (SI-ATRP) ............ 42

   1.6.3 Mixed-Matrix Membranes (MMMs) ................................................................................. 44
Chapter 2. Electrospun Weak Anion-exchange Fibrous Membranes for Protein Purification

Abstract

2.1 Introduction

2.2 Materials and Method

2.2.1 Materials

2.2.2 Methods

2.2.2.1. Fabrication of fibrous membranes

2.2.2.2. UV-initiated polymerization of GMA

2.2.2.3. Membrane morphology

2.2.2.4. Zeta potential

2.2.3. Membrane Binding Capacity Determination

2.2.3.1. Static protein binding capacity measurement

2.2.3.2. Dynamic binding capacity measurement

2.3. Results and Discussion

2.3.1. Membrane characterization

2.3.1.1. Chemical composition of membrane surface
2.3.1.2. Membrane Structure and Morphology ......................................................... 79
2.3.1.3. Zeta potential .............................................................................................. 81
2.3.1.4. Grafting degree............................................................................................ 82
2.3.2. Static and dynamic protein binding capacity tests ........................................... 84
2.4. Conclusions ........................................................................................................ 88
Acknowledgments ....................................................................................................... 88
References .................................................................................................................... 89
Supplementary ............................................................................................................. 93
Chapter 3. High efficiency hydrophobic interaction chromatographic (HIC) membrane
utilizing electrospinning technique ............................................................................. 94
Abstract ....................................................................................................................... 94
3.1 Introduction ......................................................................................................... 95
3.2 Experimental ....................................................................................................... 100
3.2.1 Materials......................................................................................................... 100
3.2.2 Preparation of HIC electrospinning membrane ............................................... 100
3.2.3 Membrane surface modification using surface initiated ATRP ..................... 101
3.2.4 Characterization of the HIC membranes ....................................................... 102
3.2.5 HIC membrane performance ........................................................................ 103
3.2.6 Correlations from Statistical Analysis .......................................................... 104
3.3 Results and discussion ....................................................................................... 106
3.3.1 Physicochemical properties of HIC membranes .............................................. 106
3.3.2 Static binding and mechanism ....................................................................... 111
3.3.3 The statistical analysis of HIC membrane ....................................................... 114
3.3.4 Dynamic binding and mechanism ................................................................. 119
3.4 Conclusions ....................................................................................................... 125
Acknowledgements ............................................................................................... 126
References .............................................................................................................. 127

Chapter 4. High Performance Mix-Matrixed Electrospun Membranes for Ammonium
Removal from Wastewaters .................................................................................. 131

Abstract ................................................................................................................. 131
4.1 Introduction ....................................................................................................... 132
4.2 Material and methods ....................................................................................... 134
4.2.1 Materials ...................................................................................................... 134
4.2.2 Fabrication of Mixed-Matrix Nanofibrous Membranes .................................. 134
4.2.3 Characterization ............................................................................................ 135
4.2.3.1 Particle Size Analysis ............................................................................. 135
4.2.3.2 Membrane Morphology ........................................................................ 136
4.2.4 Performance of Mixed Matrix Membranes .................................................. 136
4.3 Results and discussion ...................................................................................... 137
4.3.1 The Effects of Zeolite Loading on TAN Removal ........................................ 137
4.3.2 The Effects of Filtration Flux on Ammonium Exchange and Removal .......... 141
4.3.3 The Effects of TAN Concentration on Its Removal Capacity ......................... 143
4.3.4 The Effects of Membrane Regeneration on Its Performance ......................... 145
4.3.5 The Effects of Zeolite Type on the Performance of Fabricated Membranes..... 146
4.3.6 Comparison of TAN removal capacity between composite membranes and fibers ........................................................................................................................................ 149
4.4 Conclusion ........................................................................................................... 150
Acknowledgments ........................................................................................................ 151
References .................................................................................................................... 152

Chapter 5. Factors Affecting Robustness of Anion Exchange Chromatography: Selective Retention of Minute Virus of Mice Using Membrane Media ........................................ 153

Abstract ......................................................................................................................... 153
5.1 Introduction ............................................................................................................. 154
5.2 Experimental Methods and Materials ........................................................................ 158
  5.2.1 Experimental Design ......................................................................................... 158
  5.2.2 Materials .......................................................................................................... 159
  5.2.3 Charge Density Measurement .......................................................................... 161
  5.2.4 Membrane Porosity Determination .................................................................. 161
  5.2.5 MVM Production and Purification .................................................................... 162
  5.2.6 MVM Titer Assays ........................................................................................... 163
5.2.6.1 Quantitative PCR (qPCR) ................................................................. 163

5.2.6.2 TCID_{50} assay .................................................................................. 163

5.2.6.3 Large volume plating assay ............................................................... 164

5.2.7 Anion Exchange Membrane Chromatography Runs .............................. 164

5.2.8 Correlations from Statistical Analysis .................................................. 165

5.3 Results and Discussion ............................................................................ 165

5.3.1 The Effects of Feed Condition and BSA Concentration on MVM Clearance ... 165

5.3.2 Comparison The Performances of AEX Membranes for Viral Clearance .... 172

5.3.3 Effect of different mAb types on MVM clearance .................................... 175

5.4 Conclusions ............................................................................................. 179

Acknowledgements ....................................................................................... 179

References .................................................................................................... 180

Chapter 6: Conclusions and Future Direction ............................................... 183

6.1 Conclusions ............................................................................................. 183

6.2 Future direction ......................................................................................... 185
List of published papers generated from this study

[1]
Chapter 1. Introduction

In recent years, the upstream titers in biopharmaceutical industry have increased, which has placed a significant burden on downstream processing. The purification capacity has become a tremendous challenge for the biotechnology industry with rapid progress in upstream production resulting from advanced protein production methods. A total of 79 monoclonal antibodies (mAbs) products have been approved in the US and Europe, and more than 570 mAbs candidates under development in 2019 [2, 3]. The total sales of mAbs products reached $115 billion in 2018 and are predicted to rise to $300 billion in 2025 [3], implying a rapid expansion of the mAb market. The challenge of the manufacturing process comes from the large demand for therapeutic proteins [4]. Meanwhile, regulations enforced by the Food and Drug Administration (FDA) [5] requirements necessitate high-purity products and improved product quality, both of which entail considerable challenges for downstream processing.

The tremendous improvement in upstream production has led to achieving average of >5 g/L in cell titers [6, 7], thereby shifting the pressure to the downstream process [8-10]. Achievable cell densities and product titers have markedly increased in the cell culture process through the development of recombinant technologies. Antibody concentrations of up to 25 g/L can be achieved using a modified perfusion process, differing considerably from the earlier yield of 3–5 g/L [11]; therefore, high product titers from cell concentration cause a burden on the downstream purification. The cost of products shifts from upstream to downstream because of the limited processing capacity and increased material consumption and its corresponding cost [11, 12]. The total cost of bioprocessing has increased gradually by more than 60% because of downstream recovery and purification cost. Hence, the biopharmaceutical industry requires highly productive and operations to reduce costs and improve separation efficiency.
However, reduction of costs by improving downstream processing technologies required considerable effort. Moreover, selectivity, which is an ability of the membrane/resins media to distinguish between sample components, is a challenge in downstream processing and influences the resolution of the polishing steps due to the presence of impurities with similar isoelectric point (pI) and hydrophobicity. One significant characteristic of mAbs is their isoelectric point (pI), normally in the range of 8-9 [13], and molecular weight of mAb is ~150 kDa associated with two heavy chains (~100 kDa and two light chains (~47 kDa). The common contaminants present in downstream processing are host cell protein (HCP), DNA, virus and endotoxins. The detailed of properties of the main impurities show in the Table 1.

Purification methods include utilization of pI, affinity, hydrophobicity, and molecular weight of mAb and contaminants. These properties are used to develop protein operation purification stage. In terms of the downstream protein purification operation, four key factors, namely, resolution, speed, recovery, and capacity, are considered. Resolution refer to that the level of different components in the mixture that can be differentiated in chromatographic separation. The speed represents the time required for purification. The capacity is defined as total amount of targeted molecules can be processed per period of time, and recovery refer to the amount of targeted molecules that could be recovered from the purification processing. The objective of developing and optimizing a downstream processing protocol is to consistently produce high-quality products with sufficient purity while maintaining biological activity and satisfying the regulatory requirements. In the biopharmaceutical industry manufacturers are always looking forward to innovative improvement techniques in the downstream processing sector to solve existing issues in terms of productivity and reach scalability limits.
**Table 1** Physicochemical properties of the main impurities during the production of biopharmaceuticals [11, 14]

<table>
<thead>
<tr>
<th>Class</th>
<th>pI</th>
<th>MW(kDa)</th>
<th>Hydrophobicity</th>
<th>Origin source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host cell proteins (HCPs)</td>
<td>2-11</td>
<td>10-200</td>
<td>Variables</td>
<td>Host cells</td>
</tr>
<tr>
<td>DNA</td>
<td>2-3</td>
<td>90-1000</td>
<td>Low</td>
<td>Host cells</td>
</tr>
<tr>
<td>Virus</td>
<td>4-7.5</td>
<td>200-7200</td>
<td>Variables</td>
<td>Host cells, raw material, contamination</td>
</tr>
<tr>
<td>Endotoxins</td>
<td>1-4</td>
<td>3-40</td>
<td>Variables</td>
<td>Media, contamination</td>
</tr>
</tbody>
</table>

**Figure 1** gives the main purification unit operations in the downstream industry. The goal of clarification is to harvest the targeted product in the solution and remove cell and debris via centrifugation or depth filtration. The subsequent purification steps involve three typical steps including capturing the product based on protein affinity (Protein A) chromatography, followed by two polishing steps. Ion exchange chromatography (IEC) separate the molecules based on their charged ligand. It is used to remove product/process related impurities and viruses. Hydrophobic interaction chromatography (HIC) is used to remove aggregates and degradation products from mAb products. After downstream processing, the HCP and DNA impurity levels should be below 100 ppm and 10 ppb, respectively [15, 16]. Virus particles, which could be introduced into the product stream during mAb production process, should be less than 1 per 10^6 dose when Chinese hamster ovary cells are used [17].

Downstream processing is involved in the separation and optimization of component mixture. In the past decade, chromatography has developed as the backbone of protein purification. Resin particles pore sizes substantially affect the performance of chromatography [18]. Protein A steps were still hindered by high cost. On the other hand, IEC has become common in purification
due to its universal applicability and simplicity. Hydrophobic interaction chromatography (HIC) and multimodal chromatography (MMC) are two strategies used in mAb manufacturing, but both of them are not as prevalent as affinity and IEC. To optimize the purification process, a fundamental understanding of each stage should be deemed as necessary requirement.

![Downstream purification process](image)

**Figure 1** Downstream purification process.

### 1.1 Downstream purification

#### 1.1.1 Protein Affinity Chromatography (Protein A)

Protein A chromatography is a separation method for initial chromatographic purification using a specific stationary ligand to purify an antibody. Protein A chromatography is a recombinant protein ligand obtained from Staphylococcus aureus (SpA) or Escherichia coli [19, 20]. Protein A is an affinity ligand which binds the stationary matrix, a fragment crystallizable (Fc) region of IgG from harvested cell culture (Figure 2). The binding process of Protein A is reversible by switching mobile phase properties, such as pH. Generally, the binding process is carried out in neutral pH (6-8), and the elution process is executed in acidic pH (2.5-4). Selective separation is performed by a specific affinity of a biomacromolecule and the surface of the stationary phase. The target molecules are captured by the stationary matrix on the medium, but other substances in the solution can pass through the column (Figure 3). The target molecules are readily eluted by changing the
pH of the mobile phase. The purity of the final mAb product should be above 95%, and the yield is over 90%.

**Figure 2** Antibody structure.

**Figure 3** Steps of the affinity chromatography process

Although Protein A is known for its attractive performance, it faces several drawbacks, including the high cost of materials and leakings from support, all of which lead to instability. These main challenges propel researchers to conduct extensive studies. Protein A is often used for isolated antibodies. The cost of Protein A resin can reach $8000–$15000 per liter [20, 21]. Therefore, the important requisite for manufacturing is to design a novel and cheap affinity ligand
to reduce costs and avoid ligand leakage. Moreover, due to the harsh regeneration cycle condition, sodium hydroxide is generally used, mAbs tend to denature resulting in the decreased binding capacity, which is major concern [22].

During several cleaning regeneration cycles, the structure of ligand and affinity might be altered. Furthermore, low pH elution (2.5–4) could potentially amend the biological activity of the antibody or cause antibody aggregation. Extensive efforts have been made to develop Protein A and thereby improve its stability and cleaning efficiency. Initially NaOH tolerated stationary phase was investigated. MabSelect™SuRe™ resin is Protein A resin made by GE Healthcare. The alkaline stability NaOH of resins could be 0.1–0.5 M. The resin was tested and found to be viable for 150 cycles in terms of product yield, and impurity clearance was reported [23, 24]. However, a high amount of HCPs was observed in the resin as usage increased.

Amsphere™ is another resin developed by JSR Life Sciences. McCaw et al. reported compared the separation performances of MabSelect™SuRe™ and Amsphere™. The results indicated that in a certain feed condition, Amsphere™ has better compressibility and greater alkaline tolerance than MabSelect™SuRe™ because the binding capacity of Amsphere™ is reduced by less than 10% when 0.1 M NaOH is used as the cleaning solution. In the study, the good ligand stability was proved via resin lifetime. mAbs aggregation is another issue for affinity chromatography[25]. MerckMillipore designed the Eshmuno® Protein A resin, which effectively removes high levels of aggregates from mAb feed; purification yields of mAbs were 79%–89% [24].

1.1.2 Ion exchange Chromatography (IEC)

Ion exchange chromatography is a widely used protein purification technique [26]. It was introduced for bioseparation in the 1960s [27]. The basic principle of IEC is the separation of
proteins through their different charge states. IEX adsorbers are either negatively or positively charged. IEX absorbers make use of the varied isoelectric points (pI) of different substances at a certain pH [28]. The solutes that have negative charges binds to anion exchange (AEX) adsorber when the buffer pH values are above pIs. By contrast, the solutes that have positive charges binds to cation exchange (CEX) adsorber when buffer values are below their pIs. As aforementioned, antibody having a basic pI, cation exchange chromatography can be used as an initial capture step, however, most frequently ion exchange chromatography is applied as a polishing step after the Protein A step. It is ideal for reducing residual DNA, host cell protein (HCP), leached Protein A and viral particles.

The purification mechanism behind the AEX is dominated by Coulombic interactions. Consequently, by selecting an appropriate pH, several contaminants become negatively charged and can be removed by Coulombic interaction with positively charged ligands (typically quaternary ammonium groups) on the resin or membrane. Due to it non-specific nature, the competitive binding of other negatively charged species (eg. HCP, DNA, and virus particles) will occur.

AEX is often operated in the flow-through mode, thus allowing the desired products to flow through, which is an attractive method for removing impurities. AEX resins or membranes make use of the fact that the pIs of many mAbs (pI, ~8-9) is notably higher than those of HCPs and DNA (~4-6) as well as FDA model viruses such as MVM (pI ~6.0) and xMuLV (pI ~6.0) (Table 1) [29-34]. As a result, by choosing an appropriate pH (e.g. pH 7) of the solution condition, these contaminants will be negatively charged and are removed by their electrostatic interaction with the positively charged ligands (typically quaternary amine (-NR₄⁺ where R represents the same or different alkyl groups) and primary amine (-NH₂ groups) functionalized on the resins or
membranes. Generally, a strong ion exchanger (quaternary amine) has a wide operation pH range. The charge on the quaternary amine will remain more or less the same at different pH conditions.

By contrast, a weak ion exchanger (primary amine) has a narrow operation screen. For the primary amine as a weak base, its charge will be affected by the pKb value of the amine group and subsequently the pH of the solution. On the other hand, primary amine (-NH2) is both a hydrogen-bond donor and hydrogen-bond acceptor which enables it to form hydrogen bonding interactions with the afore-mentioned impurities in addition to the electrostatic interaction. On the contrary, quaternary amine (-NR4+) cannot form hydrogen bonds with other functional groups.

The strength of electrostatic interaction depends strongly on the magnitude of the charges on the positively charged ligands and negatively charged impurities. In addition, the conductivity of the feed solution also affects electrostatic interaction significantly as high conductivity (or ionic strength) reduces electrostatic interaction due to the screening effect. As a result, the ligand-impurity interaction based on electrostatic interaction can be significantly affected by the pH and conductivity of the feed solution. Eluting bound substances on a ligand requires increasing the ionic strength or changing the pH values owing to the weakened interaction with ligands. High ionic strength in the buffer interferes with the Coulombic interaction through the shielding effect. Changing the pH value can create a repulsive charge interaction by altering the net charge of the protein. [31, 33].

AEX is also a significant technique for virus clearance. Viral safety is also a critical concern in production of mAbs or other protein therapeutics derived from mammalian cell lines. Viruses could be introduced into the product stream during the production process. Agencies like the U.S. Food and Drug Administration (FDA) offers regulations to guarantee that adequate clearance of virus before product approval. In addition, at least two viral clearance steps with orthogonal
mechanisms are commonly required by European Medicines Agency [29, 30, 32, 33, 35, 36]. Viral clearance steps generally include low pH or detergent viral inactivation after Protein A chromatography, AEX chromatographic polishing step or size-exclusion based virus filtration. Manufacturers must validate adequate clearance of contaminants prior to obtaining regulatory approval for the release of the product. After purification, more than 3-5 logs virus reduction is required compared to that of unprocessed bulk in a single dose equivalent. The pI of a virus is generally below that of a mAb, thus, the virus clearance could be readily performed by AEX adsorber at appropriate pH buffer solution.

The major issue of AEX membrane is their limited binding capacity compared to the packed bed media in equivalent volume. Membrane adsorbers often used in the flow-through mode during the biopharmaceutical production processes [18]. In addition, AEX provides clearance for HCPs but limited clearance for high molecule weight (HMW) aggregates. CEX has been applied for purification processes for many mAbs with pI values ranging from neutral to basic. CEX was often applied in the bind-and-elute mode. The most negatively charged process-related impurities such as DNA, some HCP, leached Protein A and endotoxin are removed in the load and wash fraction. High-capacity CEX chromatography has been developed as an alternative to Protein A chromatography [101-104]. Within the last 10 years, efforts to develop Protein A chromatography have been investigated using less costly non-affinity methods. One typical method uses CEX, followed by AEX and HIC, to significantly reduce purification costs. However, a low volumetric throughput is required to achieve a sufficiently high dynamic binding capacity for a single cycle capture step using the current resin beads, which restricts productivity, the amount of the target protein recovered in the elution step. Therefore, this method has not been widely used. The commonly used AEX and CEX ligands are shown below (Figure 4). Diethylaminoethyl groups
(DEAE), quaternary ammonium (Q), and quaternary aminoethyl (QAE) are often employed as AEXs. Carboxymethyl (CM), methyl sulphonate (S) and sulphopropyl (SP) are often utilized as CEX ligands [37].

Figure 4 Structure of ligands, (a) quaternary ammonium (Q), (b) diethylaminoethyl groups (DEAE), (c) quaternary aminoethyl (QAE), (d) sulphopropyl, (e) methyl sulphonate (S), and (f) carboxymethyl (CM).
1.1.3 Hydrophobic interaction chromatography (HIC)

Hydrophobic interaction chromatography is another established protein purification method. In 1972, the HIC was first reported for bioseparation [38]. The separation principle of HIC is based on the difference in the hydrophobicities of the desired product and the ligand. “Salt-in” and “salt-out” play important roles in HIC. Hydrophobicity can be enhanced by increasing the presence of salt concentration in the buffer [39]. A high salt concentration is favorable for hydrophobic interactions between adsorbents and proteins. Typically, a single salt is used to enhance adsorption ability. Salt types and concentrations are protein- and adsorbent-system dependent, respectively [40, 41]. The optimal salt concentration varies considerably due to the interaction strengths of adsorbents and proteins. However, the concentration of salt should be lower than that of precipitated proteins. Generally, the optimal ammonium sulfate concentration is 0.75 to 2 M, whereas that of sodium chloride is 1 to 4 M [42].

The effects of salt on electrostatic and hydrophobic interactions have been investigated. Melander and Horvath reported an equation that represents the linear relationship between capacity and salt molality at a sufficiently high salt concentration. However, the equation cannot be fitted in a wide range of salt concentrations[40]. Subsequently, Machold et al. proposed a simple equation that represents the relationship between binding capacity and ionic strength. The models were validated for a considerable number of proteins and sorbents with different hydrophobicities and ionic strengths [43].

The salt type effects on the adsorption and elution of proteins follow the Hofmeister series [44]. The direct Hofmeister series for anions follows the order \( \text{PO}_4^{3-} > \text{SO}_4^{2-} > \text{CH}_3\text{COO}^- > \text{Cl}^- > \text{Br}^- > \text{NO}_3^- > \Gamma > \text{ClO}_4^- > \text{SCN}^- \). For cations, the order follows \( \text{NH}_4^+ > \text{K}^+ > \text{Na}^+ > \text{Li}^+ > \text{Mg}^{2+} > \text{Ca}^{2+} \). Ions on the left side are called “kosmotropic salt,” which promotes hydrophobic interactions
and shows the “salt-out” effect. Ions on the right side are called “chaotropic salt,” which interrupts hydrophobic interactions and shows the “salt-in” effect [45]. However, this rule is in conflict in some cases. Nfor et al. reported that monovalent salts showed stronger salting-out properties than did multivalent salts at low salt concentrations. Their results revealed that the order of the effects of salts on retention time followed the Hofmeister series[46].

HIC can be carried out by two operation modes, flow-through and bind-and-elute modes. Aggregates are removed in the flow-through mode. Large proteins bind more strongly than small proteins, and aggregates are considerably better than monomers due to their multipoint attachment. In binding and elution applications, targeted proteins bind to hydrophobic ligands in high ionic strength buffers and elute in low ionic strength buffers. High ionic strength buffer increase the aqueous surface tension, thereby resulting in favorable hydrophobic interactions [47-50]. On the contrary, low salt concentrations reduce the surface tension, leading to the dissociation of proteins and ligands. The distinctive binding under high salt and elution in low salt enables HIC to be applied in a polishing step after the IEX and affinity steps. The disadvantages of traditional HIC chromatography are its low capacity, low recovery and high concentration of salt required during the process. The capacity for these HIC ligands is generally below 40 mg/mL, which is significantly lower than the capacity of protein A, ion-exchange, and mixed mode-based resins (> 100 mg/mL).

A growing interest has developed over the past 10 years in the development of membrane for HIC in bind-and-elute mode. Membrane-based HIC offers all the advantages of membrane adsorbers, including dynamic binding capacities independent of flow rates, high throughputs, and easy scale-up. However, conventional hydrophobic ligands tend to denature proteins, thereby resulting in irreversible protein binding and aggregation. At present, thermoresponsive polymers
have been considered due to their biocompatibility. Thermo-responsive polymers exhibit both hydrophobic and hydrophilic residues, and the hydrophobic-to-hydrophilic transition can be tuned by adjusting environmental conditions such as the temperature or salt concentration. High resolution and high recovery of protein separations could be expected because the binding and elution of the proteins is based on the conformational change of the thermo-responsive ligands.

1.1.4 Multi-modal Chromatography (MMC)

The design purpose of the MMC media was to fit specific aims such as high salt concentration operation environment, pH-tunable hydrophobicity, and target biologics capture in the feed stream [51, 52]. MMC offers an alternative to a series of unimodal chromatography steps, including van der Waals, electrostatic, and hydrogen-bonding interactions. The interactions can be obtained from the ligand, spacer, or matrix. MMC has been developed to separate mAbs [53-56], glycosylated proteins [57], and vaccines [58, 59].

HIC and IEX are two commonly steps for purification in a bioseparation application. Most of the ligands design of HIC and IEX adsorber is built on hydrophobic and Coulombic interaction, which typically contains at least one hydrophobic moiety (butyl, phenyl, and hexyl groups) and one ion moiety. The ion moieties are divided into strong (sulfonic and quaternary amine groups) and weak (carboxyl and amine groups) ligands. The hydrophobicity intensity is a critical point to the ligand selection, which requires determination of a suitable and reasonable balance between the hydrophobicity binding ability and the sufficient hydrophilicity of adsorber materials to be wetted by feed solutions.

When the MMC media contains strong IEX groups, it retains the charges over a wide pH range, on the other hand, weak IEX groups are used in charge induction chromatography. The hydrocarbaryl amine is the most popular type of multimodal ligands for protein purification. Among
commercially available products, hexylamine- and phenylpropylamine-hypercel types resin are example of this type of ligand, where the amine is the charged group and hydrocarbyl is the hydrophobic end.

Heterocyclic compound are another exclusive multimodal ligand, and with its own specific aromaticity/hydrophobicity and dissociation properties [60]. The commercial MEP Hypercel is developed with this principle. It does not have any charge, and the adsorption can be achieved by hydrophobic interaction [61]. The lower pH value (~4) conditions mobile phase is used to release the bound protein, which is adsorbed by the ligand due to the ligand charge is altered to positive and repel the protein carrying the equivalent charge. In addition, the hydrogen group around the ionic group enables the protein binding at higher salt concentrations. In these types of multiligand, CaptoTM MMC and CaptoTM Adhere resin are two typical and representative commercial products [51].

Typically, the MMC media provide an efficient binding ability on a wide range of salt concentration because of the interaction between hydrogen bonding and hydrophobic and Coulombic interactions. The protein release is through tuning the salt concentration and pH. Thus, multimodal media potentially provides a new option to replace the protein A resin. Additionally, reporting a novel multimodal ligand was prepared, which has up to 48 mg/ml IgG binding capacity. For comparison, the elution condition of this multimodal ligand with mild pH [62]. Thus, the development of multimodal ligands is major advancement for protein purification.

1.2 Traditional Packed-bed Chromatography

Packed-bed chromatography is the dominant technique in the biopharmaceutical industry. However, poor mass transfer and back pressure are inherent limitations of packed bed
chromatography. The mass transfer in traditional chromatographic media is controlled by the inner pore diffusion of the particles. Large impurities, such as viruses, cannot easily penetrate the pores, resulting in mass transfer resistance with limited surface area to volume ratio and hence, reduced column capacity.

Most large molecules can only bind to the bead surface and require long residence time to bind with ligands. Increasing packed-bed height and decreasing flow rate are employed as solutions to the aforementioned issues. Furthermore, disadvantages associated with conventional packed-bed chromatography comprise the high-pressure drop across a column bed, which could be due to media deformation or blockage [63] and the low throughput, which is defined as the amount of feed steam passing through a process. The slow pore diffusion often leads to degradation of protein product. Apart from their yield and economy requirements, large columns can lead to a large dead volume and resin compression. These conditions, in turn, cause unpredictable fluid distribution and pressure drop. Adsorber membrane, which the ligands are used to a microporous support material, offer the possibility of overcoming this limitation.

1.3 Alternative Membrane Chromatography

Membranes have been extensively explored to develop a sustainable process for protein purification applications and hence, have become an important part of biotechnology industrial processes in last few decades. Membrane adsorbers were discovered in the 1980s to overcome the diffusional limitation of packed-bed chromatography. The high operational and material costs of packed-bed chromatography have motivated researchers to find optimal solutions.

Membrane chromatography alleviates several of the major disadvantages associated with conventional packed-bed chromatography, including the high-pressure drop across a column bed.
Convective flow carries solutes to the active binding site close to the pore. Hence, the diffusional limitation is reduced, and the throughput is significantly increased. Moreover, the pressure drop across the membrane devices tends to be lower in membrane chromatography than in packed-bed chromatography because of the considerably shorter flow paths in the former. Buffer consumption is also reduced because of the small membrane unit, which has minimal operational dead volume leading to less buffer storage requirement facilities with improved waste management. Time costs are likewise lessened.

The relatively easy scalability of membrane chromatography provides additional advantages. A manufacturer provides membrane modules of varying sizes, and these devices can easily be inserted into current processes to achieve the required rapid scale-up or scale-down. Membrane module generally scale linearly, and membrane adsorbers are usually single-use devices.

However, although membrane chromatography has greater potential than packed-bed chromatography, membrane chromatography has some limitations that need to be overcome. The main limitations are mainly poor inlet flow distribution, large membrane pore size distribution, lower binding capacity. [64]. In case of bind-and-elute operational mode, low binding capacity still remains the main disadvantage [65]. The low binding capacity could be attributed to the lower surface-to-volume ratio and flow distribution problems. The available commercial membrane adsorbers are commonly flat sheet membranes which are limited due to low surface area to volume ratio owing to a limited ligand density [66]. There is a great possibility to improve the limitation of membrane chromatography if the current drawback of low ligand density can be resolved. It will be further discussed in chapter 1.5.1. Recently, an outlook on more interesting forays such as
3D printing and nanotechnology has gained interest. To develop superior high-performance materials, attention to nano-sized materials has increased.

1.4 Electrospun membrane

Electrospinning is by far the most effective technique for fabricating continuous fibers of up to several nanometers in diameter. Nanofibers are a unique class of nanomaterials, which possess excellent separation application relevant properties due to their nanometer diameter and large specific area. They exhibit excellent mechanical properties and are easily modified. By changing the electrospinning parameters and the nature of the polymer solution, electrospinning can be used to produce different morphologies.

The method can be applied to synthetic and natural polymers, polymer alloys, as well as to metals and ceramics [67]. Fibers with complex architectures, such as core-shell fibers, helix fibers, porous fibers, and hollow fibers, can be produced by special electrospinning methods. The fiber structure can be a single layer or multi-layered. Electrospinning involves the application of a high electric field to produce nanofibers from a charged polymer solution or melt. Cautious control of operating conditions and solution parameters could ultimately lead to the production of smooth, defect-free nonwoven nanofiber membranes with highly porous structures.

Electrospinning is a highly versatile technique because the surface topology, fiber morphology and orientation depend on nature of the casting solution and operating conditions. The rheology of the polymer solutions is critical to the fiber formation process; therefore, the properties of the solution directly affect the fiber properties. Fiber properties are also affected by operating conditions, such as applied voltage, solution flow rate, and tip collector distance. Environmental
conditions such as temperature and humidity in the electrospinning chamber will also change the fiber morphology. **Table 2** lists the controlling parameters on electrospinning.

**Table 2** Parameter effect on the fiber morphology.

<table>
<thead>
<tr>
<th>Solution Parameters</th>
<th>Processing Parameters</th>
<th>Ambient Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymer Concentration</td>
<td>Applied Voltage</td>
<td>Humidity</td>
</tr>
<tr>
<td>Viscosity</td>
<td>Tip to Collector Distance</td>
<td>Temperature</td>
</tr>
<tr>
<td>Conductivity</td>
<td>Injection Rate</td>
<td></td>
</tr>
<tr>
<td>Surface Tension</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In contrast to traditional methods of manufacturing phase inversion membranes, the electrospinning membrane results in the relatively uniform pore size distribution of the membrane which has high pore interconnectivity and significantly higher porosity, usually about 80% [68, 69]. As a result, electrospun fibers have received extensive attention in separation processes owing to those advantageous properties.

However, choosing an appropriate electrospinning condition could be critical to achieve optimum membrane efficiency. The electrospun fiber diameter is ascribed to the polymer concentration. It was found that pore sizes and porosity of electrospun polyurethane membranes increased with increasing fiber diameters [70]. It is well-known that the fiber diameter, pore size and porosity of electrospun membrane can lead to different separation efficiency. For example, microfiltration membranes with three reported nominal pore sizes (0.2, 0.45, 1.0 μm) were modified to be a strong cation-exchange adsorber. A broad pore-size distribution leads to inefficient utilization of the membrane because pores of different sizes have different solute residence times and capacities.
The results indicated that with decreasing pore size, static and dynamic binding capacity for proteins was increased. The surface area to volume of the small pore size membranes would increase leading to more initiation sites, and, thus, more chains for protein binding [71]. Specifically, the large number of binding sites and adsorption area is contributed to enhance the separation efficiency. Considering this, a porous structure with a high charge density was selected as an adsorbent, which could provide high static binding capacity.

Selection of appropriate porosity is also regarded as an impact factor for specific applications. The improper porosity of the adsorbent may result in loss of separation performance due to difficult accessibility of solute [72]. Chiu et al. reported that electrospun polyacrylonitrile ion-exchange membranes exhibited higher protein binding capacity than the commercial membrane due to the higher porosity and ligand capacity. The smaller pores with the high porosity could strengthen the ligand density further enhance the binding site [73].

Among the existing nanostructures, three-dimensional (3D) nanostructures are currently of particular interest because of their unique applications. Electrospinning is being extensively studied and applied in the industrial processes because of the unique advantages of electrospun membranes, such as high porosity, excellent mechanical properties, the possibility of incorporating different additives, and a high surface-to-volume ratio [74, 75]. Several literature reports on electrospinning propose that this technology with its inherent advantageous properties could pave the future way for the development of methods for bioengineering, environmental protection, sensors, and catalysis [76-79]. The importance of electrospun membranes has been extensively studied and emphasized in biological engineering due to their biocompatibility and biodegradability [80].
Electrospun scaffolds can be tailored by different purposes such as enhancement of the interaction between cells and scaffolds and drug delivery to the target site [81, 82]. Besides biological applications, the electrospun membrane is used as an affinity membrane to protect the environment [83]. Furthermore, the electrospun membrane can be employed in the production of high surface area chemical and biological nanosensors [84]. Researchers emphasized that the sensor demonstrated enhanced capacities. In addition, ultra-fine electrospun nanofiber scaffolds have also been used to prepare nanotubes [85].

Table 3 Characteristic properties of various membrane.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Average pore size (um)</th>
<th>Porosity (%)</th>
<th>Fiber Diameter (nm)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial RC*</td>
<td>0.1</td>
<td>59.3</td>
<td>-</td>
<td>This study</td>
</tr>
<tr>
<td>Commercial PVDF*</td>
<td>0.28±0.01</td>
<td>62</td>
<td>-</td>
<td>[86]</td>
</tr>
<tr>
<td>Commercial PES*</td>
<td>0.8</td>
<td>64</td>
<td>-</td>
<td>Chapter 5</td>
</tr>
<tr>
<td>RC electrospun membrane</td>
<td>3.24±0.27</td>
<td>85.7</td>
<td>330-440</td>
<td>This study</td>
</tr>
<tr>
<td>Psf electrospun PAN</td>
<td>7.02±0.94</td>
<td>87.3</td>
<td>1250 ±230</td>
<td>This study</td>
</tr>
<tr>
<td>electrospun PVDF</td>
<td>1.63±0.40</td>
<td>79.2</td>
<td>150-340</td>
<td>This study</td>
</tr>
<tr>
<td>electrospun PVDF</td>
<td>0.31±0.02</td>
<td>79</td>
<td>150±57</td>
<td>[86]</td>
</tr>
</tbody>
</table>

* Flat sheet membrane; RC: regenerated cellulose membrane; PVDF: polyvinylidene fluoride; Psf: polysulfone; and PES: polyethersulfone

To verify the high porosity of electrospun membrane, a series of evaluation tests were performed on flat sheet and electrospun membranes, including pore size, porosity, and fiber diameter. Detailed measurement information is provided in the chapter 5. In Table 3, it can be seen that the average pore size and porosity of the flat sheet membrane is less than the electrospun
membrane, which is consistent with the literature. Consequently, the electrospun fibers have a relatively large surface to volume ratio.

On the other hand, in the case of electrospun membranes, higher diameter of pores is, are less tightly arranged, which leads to a relatively large average pore size and porosity of the electrospun membrane. Due to the tightly packed nature of the fiber, the average pore size could be relatively small, but both porosity and pore size is significant. Therefore, in terms of the of the membrane characteristics, a high surface to volume ratio is expected to provide more usable modification area to improve the binding capacity. However, it is well known from the literature that the increase in adsorption capacity is due to the increase in ligand density, which in turn, would lead to shrinking the membrane pores size. Under the adsorption separation mechanism of macromolecules, the increase of the interception rate leads to a decreased recovery [63]. The effect of fiber diameter on the protein binding and recovery will discuss in the section 3.3.4.

Moreover, one needs to understand the fundamental difference between distinct materials before selecting the most appropriate electrospun fibers for specific applications. Various polymers and their corresponding fiber diameters for the electrospinning process are listed in Table 4. In general, particular application and material characteristics should be considered while selecting the material. For example, regenerated cellulose membrane (RC), polylactic acid (PLA), and chitin membrane exhibit biocompatibility and high hydrophilicity which could prevent non-specific binding for use in biological separations. In terms of water treatment, due to the complexity of the feed stream, base materials require high solvent resistance, mechanical properties, and durability. Among available material, polyvinylidene fluoride (PVDF), polysulfones (PSf), and polyethersulfone (PES) membranes are widely used for wastewater treatment.
Therefore, only if developing the electrospinning membrane with appropriate pore size, fiber diameter, and high porosity can truly effectively improve the separation efficiency because of specific separation principles and demands. Combining their unique interconnected fiber structure [87], electrospun membranes serve as a platform for bio separation materials and water treatment tools and are thus promising alternatives to overcome the aforementioned challenges.

Table 4 Various polymers and their corresponding fiber diameters for the electrospinning process.

<table>
<thead>
<tr>
<th>Materials</th>
<th>Molecular weight (Da)</th>
<th>Solvent</th>
<th>Diameter (nm)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose acetate</td>
<td>30,000</td>
<td>Dichloromethane (DCM)/Methanol</td>
<td>1030 ± 310</td>
<td>[88]</td>
</tr>
<tr>
<td>Chitosan</td>
<td>112,000</td>
<td>Trifluoroacetic acid (TFA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>25,430</td>
<td>Water</td>
<td>170-260</td>
<td>[89]</td>
</tr>
<tr>
<td>PVA</td>
<td>150,000</td>
<td>Water</td>
<td>90-200</td>
<td>[90]</td>
</tr>
<tr>
<td>PAA</td>
<td>450,000</td>
<td>Water</td>
<td>100</td>
<td>[91]</td>
</tr>
<tr>
<td>PEI</td>
<td>215,000</td>
<td>Methanol</td>
<td>460-1200</td>
<td>[92]</td>
</tr>
<tr>
<td>PCL</td>
<td>80,000</td>
<td>Tetrahydrofuran (THF)/Dimethylformamide (DMF)</td>
<td>250-700</td>
<td>[93]</td>
</tr>
<tr>
<td>PAN</td>
<td>50,000</td>
<td>Dimethylacetamide (DMAc)</td>
<td>200-400</td>
<td>[94]</td>
</tr>
<tr>
<td>PSF</td>
<td>26,000</td>
<td>Pyridine</td>
<td>1000-2000</td>
<td>[95]</td>
</tr>
<tr>
<td>PES</td>
<td>55,000</td>
<td>Dimethylformamide (DMF)/Toulene (1:1, v/v)</td>
<td>450-2000</td>
<td>[96]</td>
</tr>
<tr>
<td>PBT</td>
<td></td>
<td>Dichloromethane (DCM)/Trifluoroacetic acid (TFA)</td>
<td>420 ± 70</td>
<td>[97]</td>
</tr>
<tr>
<td>PVDF</td>
<td>30,000-32,000</td>
<td>Dimethylacetamide (DMAc)</td>
<td>120</td>
<td>[98]</td>
</tr>
<tr>
<td>PET</td>
<td></td>
<td>Trifluoroacetic acid (TFA)</td>
<td>200-600</td>
<td>[99]</td>
</tr>
</tbody>
</table>

1.5 Application of electrospun membrane

1.5.1 Membrane Chromatography

The demand for high-quality proteins is growing owing to its critical function in biotechnology. A high-purity product is a prerequisite for practical applications. Among a variety
of protein purification methods, chromatography is a common technique employed in the biopharmaceutical industry owing to its high separation efficiency.

Recently, growing interest has developed in employing adsorptive membranes in bind-and-elute mode due to an increase in membrane binding capacity [101, 102]. With the improvement of the membrane substrate for higher surface area and the introduction of polymer ligands, high binding capacity membranes have been developed to compete with protein A resins. Although the binding capacity of membrane chromatography has been improved, the membrane binding capacity is still lower than resin-based chromatography.

A main disadvantage of membrane comes from its low ligand density compared to that of resins [103]. To improve the drawbacks of general flat sheet membrane chromatography, it is necessary to start with low ligand density. Many studied have reported that increasing the density of ligands can effectively improve the adsorption capacity of proteins [63, 103-105]. However, the higher density of ligands on the flat membrane could lead to too high interception rate, hence, reduce the recovery. In addition, higher ligand density could lead to inaccessibility of large molecules to some of the binding sites.

Electrospun membranes can effectively improve and solve the limitation of flat sheet membranes. Electrospun membranes provide high porosity and high specific surface area. The high specific surface area provides room to graft more ligands. Simultaneously, the high ligand density will not affect the pore size of the membrane. Furthermore, high porosity will not block large particles entering the binding sites. In this scope, electrospun membranes provide advantages of a large specific surface area compared to the flat membranes. Up to now, the nanofibrous membrane with a large surface-to-volume ratio and high porosity is attracting attention owing to its capability of enhancing the binding capacity and reducing the pressure drop.
1.5.1.1 Application of Ion Exchange Chromatography

The inherent limitation of anion exchange membranes is binding capacity with low ligand density. Currently, most of the commercially available IEX membranes are flat surface membranes (Table 5). For example, Mustang Q XT from Pall corporation is made of a PES membrane. However, due to the inherent limitations of flat membranes, many scientists have begun to study the use of electrospun membrane membranes to develop high-adsorption IEX membranes. A protein purification study reported that an electrospun membrane could enhance separation efficiency.

In 2017, Rajeshe et al. reported a novel cellulose graft-polypropionic acid CEX nanofiber membrane adsorbers for the purification of positively charged therapeutic proteins. The membranes showed a great lysozyme dynamic binding capacity with a residence time of less than 6 s [106]. Ma et al. revealed that the electrospun PES membranes were functionalized with ligands to be used as affinity membranes.

High selectivity of specific IgG and low non-specific protein binding can be obtained with using the PES affinity membrane. Moreover, the PES affinity membrane had a comparable IgG binding capacity compared with other reported affinity membranes. Furthermore, lower operating pressure drop was observed due to its larger pore size [97]. In addition, the cellulose acetate nanofibers were functionalized by diethylaminoethyl (DEAE) to be an AEX adsorber. This membrane adsorber displayed the highest static binding capacity of 40.0 mg/g for bovine serum albumin (BSA), compared to 33.5 mg/g, 14.5 mg/g, and 15.5 mg/g for the functionalized commercial membrane, cellulose microfiber medium, and cotton balls, respectively. The dynamic adsorption of BSA, at 10% breakthrough, was even higher than a commercial membrane that had the same ligand type [107].
Cellulose-graft-polypropionic acid CEX nanofiber membrane adsorbers were developed for the purification of positively charged therapeutic proteins. With uniform coating of polyacid nanolayers along the individual nanofibers, the membranes demonstrated abundant cationic polyacid binding sites and inherent large surface area and open porous structure. The static adsorption capacity of lysozymes is up to ~1600 mg/g of nanofibers and 10% dynamic binding capacity of ~500 mg/g of nanofibers [106].

Schneiderman et al. reported that the membrane adsorption media based on electrospun carbon nanofibers functionalized with a weak acid cation-exchange ligand showed high protein binding capacity. The capacity value was over 200 mg/g adsorbent as lysozyme is a model protein. Simultaneously, the membrane demonstrated higher operating flow rates and lower pressure drops due to the large pore size (10-15 um) and thin nanofibers (300 nm)[108].
According to aforementioned discussion, the electrospun membranes are able to improve protein binding capacity. It can be seen that there is great potential for electrospun membranes to be used in biological separation. Our work will be contributed significantly to the future industrial development to create a superior product via the electrospinning technology.
1.5.1.2 Application of Hydrophobic Interaction Chromatography (HIC)

Currently, there has been increased interest in using thermoresponsive polymers to be ligand for HIC membrane. Poly(N-isopropylacrylamide) (PNIPAM) and PVCL are the two most common types of thermoresponsive polymers that have been investigated. The thermoresponsive polymer poly(N-vinylcaprolactam) (PVCL) exhibits a relatively low critical solution temperature (LCST) in between 30 °C to 50 °C [109, 110]. This temperature range is directly related to molecular weight of polymer and polymer concentration.

When the temperature exceeds the LCST, hydrophobic nature of the hydrocarbon chain causes the chain to collapse onto each other to form hydrophobic globular conformation, thus promoting protein binding. In contrast, when the temperature is below the LCST, the polymer swells, thus promoting protein desorption [111, 112]. LCST transition of thermoresponsive polymer is strongly dependent on temperature, pH, salt types, and ionic strength.

Common practice is to vary the ionic strength during the LCST transition instead of increasing the temperature for protein purification since the LCST of thermoresponsive polymers exceeds the operating temperature, which usually is room temperature during the manufacturing step. With an increase in ionic strength, the LCST of the polymer decreases due to enhancement in the surface tension promoting hydrophobic interactions.

Different salt types play a dominant role in LCST transition of polymer, which attributed to entropically driven hydrophobic interaction of salt ions [113, 114]. The advantages of using PVCL are its biocompatibility and low toxicity. Herein, PNIPAM generates a low-molecular-weight amine during hydrolysis, which is toxic to biological systems; whereas, PVCL does not generate any low-molecular-weight amine during hydrolysis because the amide bond is located within the cyclic moiety [115-117].
Gutiérrez-Villarreal et al. used UV grafting to graft N-vinylcaprolactam (PVCL) onto the surface of a polylactic acid film [118]. A poly(N-isopropylacrylamide) copolymer was grafted on filter paper to prepare a composite membrane for performing temperature-responsive hydrophobic interaction membrane chromatography. The results revealed that a high temperature promoted protein binding and a low temperature favored protein release [48]. Liu et al. reported that an increase in hydrophobicity correlated with the hydrophilic-to-hydrophobic transition of PVCL ligands and that the binding capacity of bovine serum albumin to PVCL ligands increased. The results revealed that the best protein binding capacity was achieved using sodium sulfate; however, the recovery was low because of the irreversible binding and aggregation of proteins [115].

Liu et al [47] referred that even though the thermoresponsive polymers offer the potential for improving capacity and recovery of HIC membrane adsorber, the binding capacity and recovery of flat sheet membranes grafted with PVCL were much lower than commercially available HIC membrane adsorber (Table 6). This was probably due to the low specific area of flat membrane, which limited the number of grafted ligands [119]. By functionalizing thermoresponsive ligands to an electrospun membrane, a relatively high recovery and binding capacity are expected when the protein binding and elution are based on the conformational changes of the thermoresponsive ligands and high specific surface area of the electrospun membrane. In addition to ion exchange and affinity chromatography, HIC, which purifies proteins on the basis of surface hydrophobicity with minimum structural damage, high selectivity and high recovery, has been established as an indispensable and powerful tool for protein separation.
Table 6 List of commercially available HIC membrane adsorbers

<table>
<thead>
<tr>
<th>Company</th>
<th>Membrane adsorber</th>
<th>Ligand</th>
<th>Pore size (um)</th>
<th>Membrane material</th>
<th>Membrane volume (mL)</th>
<th>Mean Binding capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GE Healthcare Life Sciences</td>
<td>ReadyToProcess Adsorber Phen Nano</td>
<td>Phenyl</td>
<td>3-5</td>
<td>regenerated cellulose</td>
<td>3</td>
<td>&gt;0.27 mg/cm² with 1 mg/mL bovine blood gamma globulin in 50 mM potassium phosphate, pH 7.5, 0.9 M (NH₄)₂SO₄</td>
</tr>
<tr>
<td>Sartorius AG</td>
<td>Sartobind® Phenyl nano</td>
<td>Phenyl</td>
<td>3-5</td>
<td>Stabilized reinforced cellulose</td>
<td>3</td>
<td>44 mg (typical dynamic binding capacity of 10% per unit) in gamma globulin from bovine blood</td>
</tr>
</tbody>
</table>

1.5.2 Ammonium Removal from Wastewater

In addition to biological applications, electrospun scaffold materials are widely used in environmental engineering. Ammonia is a commonly found contaminant in wastewater and the surrounding environment [120]. Excessive emissions of ammonia (NH₃) contribute significantly to fine particulate matter formation, eutrophication of ecosystems, acidification of soils, alteration of the global greenhouse balance and odor problems [121]. Depending on temperature and pH, ammonia, which exists in the aqueous phase, can be either unionized (NH₃) or ionized (NH₄⁺). The reversible reaction in aqueous solutions is as follows: NH₃ + H⁺ ↔ NH₄⁺ with a pH value of 9.2 under typical conditions.

If the pH value of an ammonia containing solution is less than 9.2, the reaction tends to produce ammonium ions. With the increase in pH by one unit, the amount of unionized ammonia increases approximately 10 times, representing a significant increase in toxicity for aquatic life. Ammonia or total ammonia nitrogen (TAN), typically referring to the summation of NH₃ and NH₄⁺, has been widely described in aquatic chemistry [122, 123].
Algal overgrowth is engendered by elevated TAN discharge into streams and rivers. This would damage the ecological balance as well as engender eutrophication. Furthermore, the hazards caused by the discharge of ammonia nitrogen into water bodies are as follows: (i) ammonia nitrogen causes increased usage of chlorine in the process of sterilization when water supply disinfection and industrial water resources are used. (ii) Ammonia nitrogen could be corrosive to certain metals, especially copper. (iii) Ammonia nitrogen in water can promote the reproduction of microorganisms in water pipelines and water equipment, leading to clogging pipes and affecting the thermal conversion efficiency due to the formation of biological fouling. Consequently, ammonium removal is considered an important environmental issue to improve water quality.

Many different treatment methods for ammonia removal such as air-stripping, biological treatment, and ion-exchange [124-129] have been developed. Air stripping is suitable to treat alkaline aqueous solutions. However, to achieve higher ammonia removal efficiency following air-stripping, adjustment of the pH of the solution to a relatively high level (>10) is required. Subsequently, the solution should be adjusted to a neutral pH after ammonia removal. The disadvantages of this method are high cost and high maintenance owing to the use of an excessive amount of chemicals and equipment. Meanwhile, biological treatment entails ammonium ion removal using nitrification–denitrification processes executed using microorganisms [130, 131]. Reduced environmental harm along with increased economic benefits can be accomplished by recycling and reuse of wastewater.

Owing to such adverse effects of ammonia, several advanced techniques have been developed in last few years for its removal. Generally, ion exchange is a common method employed to remove harmful chemical materials and ammonium from wastewater because the ion exchange adsorbents typically exhibit high ammonium selectivity. Zeolites as porous crystalline
silicon aluminate and have a negatively charged lattice have been explored for reducing ammonia content in water. As crystalline silicon aluminate has regular cavities within its three-dimensional structure, zeolite has a good ammonia adsorption and ion exchange ability. Numerous zeolites have recently been successfully employed for the removal of ammonium [107, 132-136]. A previous study stated that zeolite 13X had good ammonium removal as it exhibited more than 90% removal at initial NH$_4^+$ concentrations of nearly 25 ppm [136].

\[\text{Na}_{86}(\text{AlO}_2)_{86}(\text{SiO}_2)_{106}\cdot x\text{H}_2\text{O}\] represents the general zeolite 13X formula. In addition to Na$^+$, it contains other cations including Ca$^{2+}$ and K$^+$ along with Mg$^{2+}$. The higher ion exchange capacity reported for zeolite 13X relative to other zeolites, such as Aqua-Multalite, Zeolite of Gordes-Manisa, and zeolite 4A, rendered it more appropriate for applications such as treating the wastewater derived from a hen slaughterhouse [130].

Zeolites have various additional applications, for example, in industrial and slaughterhouse wastewater treatment [136-141] and drinking water treatment [138, 139, 142]. However, the principal concern noted for zeolites the use of is that its particles can gradually leached into water, and the NH$_4^+$ adsorption can be limited by the metal ions. Leaching an extensive load of zeolite into water may produce heavy metals, engendering environmental pollution. Nevertheless, practitioners in the relevant field have developed mixed-matrix membranes (MMMs), which entail incorporating zeolite into membranes to retard the leaching of zeolite into water.

Zeolite membranes were applied to MMMs, which demonstrated the highest normalized ammonium removal capacity [131]. However, the capacity was limited due to the limited zeolite contact area of the membrane. Even with a high zeolite content in the casting solution, the zeolite in the membrane was reduced after the wet NIPS process.
In the last few years, our previous studies were focused on several approaches to fabricate such mixed-matrix membranes, including a wet phase inversion process and pore filling of commercially available UF membranes. Such efforts resulted in improved ammonium removal, but a significant improvement is still needed. The ammonium removal of mixed matrix membranes fabricated using phase inversion method was about 65% TAN removal, which was obtained for 50% zeolite based on polymer concentration was embedded in the 15% PSf doping solution. Further investigation revealed that with an increase in zeolite loading within the membrane moiety using a variety of membranes, higher the ammonium removal capacity could be achieved. However, high concentrations of zeolite particles present within the membrane led to a significant decrease in water flux.

Therefore, in another aspect of the study, we fabricated a pore-filled membrane by using zeolite 13X particles filling in the macro voids of 30 kD commercial PES membrane support structure from the nonwoven side. Although the results show an excellent enhancement of TAN removal (85%) and high flux (490 LMH/bar), the zeolite particles were mostly lost during regeneration.

Subsequently, the membrane surface was capped by interfacial polymerization (IP capped) and layer-by-layer deposition of polyelectrolyte (PEM-capped) after the zeolite filled by pressure through. The IP capped indeed protected the zeolite particle packed in the macropore of the UF membrane, but a corresponding decline in flux was observed, and it TAN removal reduced ~65% because of the partial occlusion of the particles and thus reduced accessibility of the zeolite particles.

Accordingly, the PEM-capped membranes were developed to effectively enhance the TAN removal attributing to the high porosity of the PEM layer. The ammonium removal capacity of the
PEM-pore filled membrane was found to be 85% TAN removal with flux 32 LMH/bar. Although such pore filling methods helped in achieving higher zeolite loading within the membrane, the ammonium removal efficacy dropped significantly due to the partial occlusion of the particles and thus reduced accessibility of the zeolite particles. Thus, the electrospinning technology is employed to improve the ammonium removal efficacy because of their high porosity and a superior number of continuously connected channels.

To date, only a few studies have concluded the incorporation of electrospinning with zeolite for ammonia removal; however, many studies have been reported success in the use of electrospun zeolite membranes for removing metals and dye [143-147]. For example, a chitosan/polyvinyl alcohol/zeolite electrospun composite nanofibrous membrane adsorbed methyl orange. Most of the dye could be adsorbed within a few minutes and the adsorption capacity (~153 mg/g) was obtained. The adsorption could be described by the Freundlich model [143].

More applications have been reported regarding using zeolites as metal ion adsorbents [147-149]. The electrospun chitosan/polyvinyl alcohol (PVA)/zeolite nanofibrous composite membrane was fabricated. Moreover, the Langmuir isotherm was used for identifying the Cr$^{6+}$, Fe$^{3+}$, and Ni$^{2+}$ ions adsorption on a nanofibrous membrane [148]. Different zeolite types have specific effects on the adsorption performance.

The ion exchange capability of electrospun cellulose acetate (CA) fibers containing zeolite A nanoparticles have been reported. The membrane was employed to ion exchange Na$^+$ with Cu$^{2+}$ and Pb$^{2+}$. A composite Linde Type A (LTA) zeolite membrane was able to exchange more Pb$^{2+}$ than LTA nanoparticles incorporated into fibers [150]. The use of electrospun polyvinyl alcohol (PVA)/NaX nanozeolite nanocomposite nanofibers for the removal of Ni$^{2+}$ and Cd$^{2+}$ ions from aqueous solutions has been developed. The adsorption results indicated that the capacity of an
affinity nanofibrous adsorbent for Cd$^{2+}$ ions sorption was higher than that for Ni$^{2+}$ ions sorption due to the higher diffusivity of cadmium ions into nanofibers in comparison to that of nickel ions [151].

It is worth noting that the mechanical properties and surface properties will be changed when different amounts of zeolite are embedded into the electrospun membrane. The electrospun lignin-zeolite composite nanofiber membranes were characterized in terms of morphology, mechanical properties, hydrophilicity, permeation, and particulate separation performance. The results revealed that significant differences were found in membrane surface properties and membrane mechanical properties because of the addition of inorganic particles. The tensile strength, tensile modulus, hydrophilicity, permeability, and separation factor of the membranes were improved by adding zeolite particles. Yet, the excess of zeolite nanoparticles led to a weakening of mechanical properties, permeability, and particulate retention capability [145].

According to the above discussion many valuable examples of the incorporation of zeolite into electrospun membranes have been mentioned. The electrospun membrane can effectively cause the targeted molecule to rapidly approach the adsorbent, which can also increase adsorption capacity. Moreover, the high porosity of electrospun membrane can enhance throughput. The type of zeolite, ion concentration in the aqueous solution, and amount of embedded zeolite affects the adsorption performance. Therefore, understanding the zeolite electrospun membrane will contribute to the development of the membrane adsorber. Further, our group has been constantly involved in further research work in this aspect to investigate alternative fabrication methods for practical fabrication of zeolite loaded membranes with optimized loading density and ammonium removal efficiency.
1.6 Modification of electrospun membrane

The versatility and simplicity of electrospinning enables diverse applications. Membrane functionalization can effectively regulate the membrane properties. Depending on the functional groups introduced on the membrane surface, the membrane can be employed for different applications, such as heavy metal removal [142, 147, 152-154], antifouling and antibacterial treatment [155, 156], protein separation [157], gas separation [158], and wound dressing [159].

The common membrane modification methods are physical coating, plasma treatment, layer-by-layer assembly, self-assembly, interfacial polymerization, and polymer grafting. Among surface modification methods, polymer grafting exhibits enormous advantages. The stability of polymer grafting is superior to that of physical coating and self-assembly because polymer grafting involves the application of a chemical treatment to the membrane surface through a covalent polymer. This property is critical for developing bioseparation membranes to prevent ligand leakage. Moreover, complex separation can be achieved by introducing a common functional group, such as hydroxyl or amine group, by chemical treatment.

The membrane surface properties can be regulated by a specific monomer. In addition, the polymer chain length and density can be controlled by the polymerization time and initiation condition. Numerous studies have reported that the structure of a polymer plays a critical role in the membrane properties. Therefore, most membrane adsorbers, especially those used in biopharmaceutical downstream processing, are made by polymer grafting.

Two common modification approaches used in polymer grafting are “grafting from” and “grafting to” [160]. In the case of “grafting to,” the monomer or polymer is synthesized prior to grafting to the membrane surface. The characteristics of “grafting to” are short reaction time and uniform functional chain length. Conversely, “grafting from” directly grows the polymer on the
membrane surface, similar to UV-initiated polymerization, plasma treatment, and atom transfer radical polymerization, which tailors the grafting density and chain length of polymer. In this section, we focus on using grafting procedure including UV-initiated polymerization, atom transfer radical polymerization, and the mixed-matrix method.

1.6.1 UV-initiated Polymerization

UV-initiated polymerization has been widely applied in many membrane surface modification fields such as heavy metal removal [161], development of anti-fouling membranes [162, 163], protein separation [164], and surface hydrophilicity enhancement [165]. The free radical polymerization is a UV light treatment for abstracting hydrogen atoms on the membrane surface. Currently, there are two types of UV-initiated polymerization methods, (i) the free radicals can be generated on the polymer backbone with monomers, and (ii) the cathodic based photosensitive polymerization based on functional epoxides. For example, polyethersulfone (PES) and polysulfone (PSf) membranes are sensitive to UV light to generate the functional group [166]. The polymer backbone of PES and PSf is cracked to generate the free radicals followed by polymerization with monomers [167].

Scheme 1 UV-initiated grafting mechanism of PSf.
In several previous studies, self UV-initiated polymerization was broadly used on the PES membrane surface [168]. XueLi et al. reported a polysulfone ultrafiltration membrane surface with N-(3-tert-butyl-2-hydroxy-5-methylbenzyl) acrylamide (MBHBA) grafted via UV-initiated polymerization, which demonstrated outstanding antibacterial properties against Escherichia coli. It is worth noting that the membrane pore size was decreased slightly after surface modification [168]. Additionally, Homayoonfal et al. revealed the feasibility of the UV grafting polymerization of a vinyl monomer on the surface of a polysulfone ultrafiltration membrane for preparation of a nanofiltration membrane. Results indicated that the membrane pore size was decreased as polyacrylic acid (PAA) was grafted onto the membrane through the UV-initiated polymerization.

Grafting is more sensitive to irradiation time than to the monomer concentration. Free radicals are generated, and polymerization is initiated in the presence of the smallest number of monomers during irradiation. Therefore, as long as the irradiation time is sufficient, the polymer chains can be formed on the membrane even if the monomer concentration is low [169]. In this study, the membrane roughness decreased after polymerization because acrylic acid (AA) was polymerized onto the pore surface and pore walls of the membrane.

Liu et al. showed that dye removal efficiency can be enhanced by diallyl dimethyl ammonium chloride (DADMAC) via UV-initiated polymerization on a polysulfone ultrafiltration membrane. With the effect of charge repulsion between the positively charge ligand and the salt, the dye removal can be readily obtained. Key kinetics parameters such as reaction time, monomer concentration, and irradiation intensity play a critical role in controlling the membrane water flux and salts rejection [170]. The main advantage of using a photosensitive membrane is that it involves a simple operation process without additional pre-treatment.
UV-initiated polymerization might require a photosensitizer to create free radicals on the membrane backbone. Two types of photosensitizers have been utilized for membrane surface modification. The type I UV-initiated initiator introduces the free radicals from the initiator self-cracking. The free radicals deposited on the membrane surface will abstract hydrogen atoms from this substrate, thereby transferring the free radicals to membrane substrate. Benzoin derivatives, peroxides and azo compounds are commonly used as type I photo initiators for hydrophilic modification of polyvinylidene fluoride (PVDF) and polyethylene terephthalate (PET) membranes [171, 172].

Type II initiators, such as benzophenone (BP) or BP derivatives, enhance polymerization by abstracting a hydrogen atom to generate free radicals from the polymer substrate, thus obtaining a higher grafting density. Type II initiators have often been applied to surface modification of polyethylene terephthalate (PET) [173], polyacrylonitrile (PAN) [174, 175], regenerated cellulose (RC) [176], polyethersulfone [177], and polypropylene (PP) [178]. In terms of UV-initiated polymerization, simultaneous polymerization and two-step polymerization are two common methods.

The simultaneous polymerization method includes the monomer and UV initiator in the operating solution. The obtained grafting density is low as polymerization occurs in the solution instead of on the membrane. However, BP initiator is insoluble in water, and thus limited solvents are available. Additionally, only certain solvents are effective for hydrogen abstraction reactions.

The two-step polymerization method involves pretreating the membrane prior to polymerization as follows: (i) the initiator is pre-coated to the membrane surface enhancing the BP concentration, and (ii) polymerization can be conducted in a variety of solvents resulting in a readily dissolvable monomer. Therefore, an improved grafting density is obtained compared to the
simultaneous polymerization. This solution works well for hydrophobic membranes and monomers with high solubility in water given that the BP deposited on the membrane is insoluble in water and this leads to reduced polymerization in the bulk solution.

Ahmad Rahimpour reported that the hydrophilic and lower fouling effect in the polyimide (PI) membranes modified by using ultraviolet light irritation [179]. That paper discussed the grafting performance in the absence and presence of BP during the polymerization. After grafting the monomer, such as, acrylic acid (AA), 2-hydroxyethylmethacrylate (HEMA), and 1,3-phenylenediamine (mPDA), onto the membrane surface, it was observed that the hydrophilicity of the PI membrane was improved in all cases. In the presence of a photo-initiator, the grafting degree was increased with increasing monomer concentration.

The essential factor is the hydrogen abstraction from the support polymer by the photo excited BP which generated starting radical for photo-initiated surface grafting promoting the polymerization progress [180]. Liu et al. reported that an AEX poly(butylene therephthalate) (PBT) nonwoven membrane was developed by UV-initiated poly(glycidyl methacrylate) (polyGMA) brushes grafted to the PBT fibers. The AEX membrane exhibited high protein separation efficiency by separating human immunoglobulin G (hIgG) from human serum albumin (HSA). The obtained purity and yield of hIgG flowing through the column were above 90% [181].

Himstedt et al. showed a commercially available nanofiltration membrane modified by UV-initiated polymerization growing polyacrylic acid nanobrushes from the surface of the membrane, which had minimal impact on the membrane resistance. The responsive polymer conformation was switched by feed pH; therefore, the water flux and sugar rejection could be tuned by the swollen and collapsed conformations of the grafted polymer [182].
Compared to other polymer grafting methods, UV grafting polymerization has several advantages such as grafting efficiency and cost [183]. UV grafting can be executed in air. Conversely, plasma polymerization requires the use of a vacuum, and atom transfer radical polymerization (ATRP) needs an oxygen-free environment. Moreover, the cost of UV grafting is much less than that of plasma because of the low energy required. In addition, the UV grafting polymerization method requires only the monomer, solvent, and UV light. The main challenge of ATRP comes from the catalyst removal [184]. In terms of grafting efficiency, the polymerization rate of UV grafting is higher than that of ATRP. However, uncontrolled grafting sites on the membrane are inevitable.

1.6.2 Atom Transfer Radical Polymerization (ATRP)

ATRP is a controllable radical polymerization technique via a transition metal catalyst. It was first reported by the group of Matyjaszewski in 1995 [185]. The narrow molecular weight of the grafted polymer chain and the architecture can be precisely tailored by ATRP. In terms of controllable chain growth technology, ATRP allows the design and manipulation of chain length and grafting density independently. In addition, ATRP has a more uniform surface than other free radical polymerization methods. ATRP reaction conditions are flexible. An appropriate solvent maintains membrane integrity. These characteristics are important for membrane modification for biological separation since membrane materials impose restrictions on the choice of solvent for the modification reaction. Further, the control of the molecular weight of the grafted polymer is important to avoid pore-filling [186].

The ATRP process is dependent on the equilibrium between activation and deactivation. A continuous growth reaction is initiated by utilizing an electron redox reaction between a metal
complex and an organic compound. Copper, iron, and nickel are generally involved in the reaction.

The scheme of ATRP is as follows:

\[
R-X + M_t^{n-Y/\text{ligand}} \quad \xrightarrow{k_{\text{act}}} \quad R^* + X-M_t^{n+1-Y/\text{Ligand}} \quad \xrightarrow{k_{\text{deact}}} \quad \text{termination}
\]

**Scheme 2** Transition metal complex of ATRP catalyzed

\[
R_p = k_p \times [M] \times [P^*] = k_p \times K_{eq} \times [M] \times [I_0] \times \left(\frac{Cu^{I}}{Cu^{II}}\right) \quad \text{Equation 1}
\]

where X, M, and Y represent the halogen atom, metal, and another halogen atom, respectively. \(k_{\text{act}}\), \(k_{\text{deact}}\), \(k_t\), \(R_p\), \(k_p\), \([P^*]\), \(K_{eq}\), \([I_0]\), \(Cu^I\), and \(Cu^{II}\) are the activation rate constant, deactivation rate constant, termination reaction rate constant, polymerization rate, propagation rate constant, radical concentration, equilibrium constant, initial initiator concentration, \(Cu^I\) concentration, and \(Cu^{II}\) concentration, respectively. The dormant species \(R-X\) represents halides, which react with activators and deactivators. The activators are ligand-stabilized transition metal complexes in their lower oxidation state (\(M_t^{n-Y/\text{ligand}}\)), and they react with dormant species to form active radicals (\(R^*\)). The deactivators are transition metal complexes in their higher oxidation state, and they cooperate with the transferred halide ligands (\(X-M_t^{n+1-Y/\text{ligand}}\)).

The reversible equilibrium reaction initially transfers the halogen atom on the initiator to the transition metal complex and oxidizes the metal complex to generate active free radicals. The deactivation rate of the active radical is higher than that of the dormant species (\(R-X\)), leading to the low concentration of free radicals in the system, which suppress chain transfer and chain termination reactions. The reaction is ideally continued until the monomer is completely consumed.
Nevertheless, in reality, the decreasing polymerization rate results from radical termination or catalyst oxidization.

For the ATRP reaction, selecting the appropriate monomer, initiator, catalyst, ligand, and solvent is important. The most important component of ATRP is the catalyst ratio (Cu$^\text{I}$ and Cu$^\text{II}$) as it is used to determine the equilibrium rate between active and dormant species. The monomer used in ATRP includes molecules with substituents that can stabilize the propagating radicals. The propagating rate is unique for every monomer. Therefore, optimizing the reaction condition with other species, such as catalysts, initiators, ligands, and solvents, is important.

The number of growing chains is determined by the initiator, and halide used. The reactivity of the halide follows the order tertiary > secondary > primary. In terms of catalytic activity, CuBr is better than CuCl. The ratio of Cu$^\text{I}$/Cu$^\text{II}$ is crucial for practical polymerization rate. Cu$^\text{II}$ is used to reduce the termination at the initial stage of polymerization.

One of the important aspects in an ATRP reaction is the selection of relevant ligands, which are used in combination with catalysts to form a catalyst complex. Ligand selection is based on the activity of monomers and type of catalyst metal. In addition, the appropriate solvent medium is critical. Solvent polarity strongly affects the ATRP polymerization rate.

**1.6.2.1 Surface-initiated Atom Transfer Radical Polymerization (SI-ATRP)**

SI-ATRP plays a key and distinct role in the surface modification that follows the same mechanism and is controlled by the same factors as a regular ATRP [184, 187, 188]. Polydispersity of the modified-polymer chain influences the transport, binding capability and anti-fouling properties of the membrane [189]. Nevertheless, SI-ATRP, with individually controlled chain length and chain density, offers a unique polymerization technique.
The surface-initiated modification process involves initiator immobilization and polymerization. Unlike the solution phase ATRP, SI-ATRP requires initiators to be immobilized on the media surface, such as membrane and resin prior to polymerization. The polymerization is conducted at the liquid/solid surface. The ability to control individually the grafting density and length are the main advantages of Si-ATRP.

Several types of commercial initiators have been developed for specific substrates. Additionally, the polymerization activity of ATRP makes block and gradient co-polymers possible, which is difficult to obtain through other free radical polymerization techniques. ATRP is also able to synthesize unique polymer shapes, such as comb and star configurations. Moreover, through nucleophilic substitution, the halide can introduce other functional groups. In Si-ATRP, the propagation and termination on the substrate may be distinguished from the reaction that occurred in solution. With higher grafting density, the end of the free radical polymer chain has a possibility to link and terminate. The quantitation of grafting density and chain length is another challenge. In the study of He et al., the weak cation-exchange membrane was acquired via a “post-polymerization modification” method. The weak cation-exchange membrane was modified by the SI-ATRP of glycidyl methacrylate (GMA) and a subsequent two-step derivation. By varying the grafting time of poly-GMA, a high adsorption capacity of 125.0 mg/mL was obtained using lysozyme as a model protein. Moreover, the effect of ligand density on the adsorption behavior of lysozyme was investigated.

With the prolongation of the grafting time, percentage utilization of carboxyl increases first and then decrease due to the mutual effects of the flexibility of the polymer chain, the steric hindrance, and the “adsorption-caused hindrance” effect [190]. Liu et al. showed comb-like salt-responsive copolymers grafted on regenerated cellulose membranes by ATRP. Polymer chain
length and chain density, along with the overall architecture, have a significant impact on protein binding and recovery. The results indicated that a high grafting density and short polymer chain can improve the binding capacity and recovery.

The pores could be blocked at a high grafting degree of the polymer leading to low recovery results at long primary polymer chains [47]. Wei et al. revealed that the grafting degree was controlled by varying the ATRP reaction time. A cation-exchange membrane with sulfonate as the exchange group was functionalized by ATRP on the regenerated cellulose membrane. The results demonstrated that with increasing ATRP reaction time, the lysozyme adsorption capacities increased. Static and dynamic binding capacity could reach a maximum capacity of ~140 mg/mL and ~90 mg/mL, respectively [191].

Scheme 3 Transition metal complex of SI-ATRP catalyzed [192].

1.6.3 Mixed-Matrix Membranes (MMMs)

Among the membrane modifications, the simplest modification method is that of MMMs, which is classified as an in-*situ* modification. Generally, the inorganic additives or other solid phases are incorporated in a continuous polymer matrix [193], which improves the membrane performance and demonstrates a unique feature of the new material by combining the easy processability of polymers and the distinguished selectivity of additives. Recently, simplifying the membrane fabrication process has become a topic of interest, and MMMs provide a promising
alternative for membrane manufacturing; consequently, both the academic and industry researches are focused on MMMs.

MMMs have been involved in a range of microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), even gas separation membrane, which directly evidences that MMMs have a large potential. In addition, in the other modifications discussed earlier, multi-step post-treatments are required to be integrated into their production process. In contrast, for MMMs, only one step is required. This not only is superior advantage but also effectively increases the performance of the membrane. In MMMs, several types of solid phase material have been used to fabricate the flat sheet membrane and hollow fibers via the conventional phase inversion method and the electrospinning technology.

The pioneering study on MMMs was conducted in the 1970s, where molecular sieve particles were imbedded in a rubbery polymer for the carbon dioxide capture [194]. The transport principle of this study combined the Knudsen diffusion of the molecular sieve and diffusion of the polymer. Later, a variety of different solid materials have been blended into the polymer for other specific applications.

In the phase inversion method, our group developed a zeolite MMMs for ammonium removal from aquaculture wastewater, and the total ammonia nitrogen TAN removal capacity can reach to 19.8 mg/g_{zeolite} when 7 ppm TAN was presented in the wastewater.

The zeolitic imidazolate framework (ZIF) was synthesized and mixed in poly(amide-b-ethylene oxide) (Pebax) to investigate its effect on the carbon dioxide capture behavior, and its showed higher CO$_2$/N$_2$ selectivity, above 50, which breakthrough the 2008 Robeson’s upper bound [158]. Additionally, due to the unique butanol selectivity of some ionic liquids (ILs), IL MMMs
for pervaporation were fabricated by Li, and the resulting membrane showed a 25% higher separation factor than the unmodified membrane when the temperature was maintained at 25°C [195]. To enhance the hydrophobicity of a membrane distillation (MD) membrane, graphene was used to tune the structure and surface property for improving the flux, and it also displayed strong robustness and stability in a 10 day long-term test [196].

The results are evidence that MMMs have excellent performance in different membrane applications, but the affinity and stability between the membrane and the additive will be a critical challenge for practical operation. For instance, the eluted of metal ions or particles may result in secondary pollution to the environment and loss of separation efficiency of the membrane with poor stability. The voids between the fillers and the polymer may also be a restriction to achieve efficiency and separation ability. Presently, MMMs cannot be considered the best method, but their simple formation will attract more attention in the future. Once these disadvantages are overcome, MMMs will be excellent membranes.

1.7 Research objective

Electrospinning technology is highly effective for the preparation of nanostructures from a variety of raw materials, from either natural or synthetic polymers to composites. Thus, in this decade, it has attracted the attention of researchers for the preparation of various nanostructures in extensive fields. This study aims at using the electrospun membranes to develop a highly efficient separation membrane and overcome the inherent limitations of flat sheet membrane. As electrospun nanofiber membranes have the advantage of high porosity due to their unique interconnected fiber structure, the separation performance is expected to improve substantially. Moreover, membrane modification methods, such as photoinduced graft polymerization, in-situ,
and atom transfer radical polymerization, have been investigated. Therefore, the main studies on this membrane technology will be described in this Ph.D. dissertation.

Research goals detailed in chapter 2 and chapter 3 are highly interdisciplinary involving materials fabrication, characterization, surface engineering and bioseparations. The overall goal is to develop topologically unique membranes with tunable hydrophobicity and pore size for protein purifications. In the chapter 2, grafting and optimizing polymeric anion exchange ligands on the electrospun membrane substrates is novel even though established surface chemistry has been adopted. In the chapter 3, the engineered responsive membranes will display higher capacity and higher recovery than current technology. The overall goal of chapter 4 is to develop an economically viable and continuous process for ammonium removal. In the chapter 5, the aim is to understand the factors that affect robustness of virus removal during the AEX polishing step. In particular, we aim to develop a fundamental understanding of the differences in robustness of AEX membrane adsorbers. The specific objectives of the proposed research are as follows:

**Objective 1** Evaluate the capacity and recovery of model proteins using in-house fabricated electrospun polysulfone and polyacrylonitrile nanofibrous membrane substrates. UV-initiated polymerization is conducted on electrospun membranes to form IEX membranes will be investigated. In particular, weak anion-exchange membranes will be fabricated by grafting poly(glycidyl methacrylate) on the electrospun membrane substrates via UV-initiated polymerization followed by the addition of diethylamine (DEA) via a ring-opening reaction. The effect of natural properties of the substrate on the performance of the IEX nanofibrous membranes will be studied. (Chapter 2)

**Objective 2** Evaluate the capacity and recovery of model proteins using in-house fabricated electrospun regenerated cellulose nanofibrous membrane substrates grafted with the responsive
PVCL ligands. ATRP would be used to graft the responsive ligand. The static and dynamic protein binding capacity and recovery will be determined using previously established protocols. Grafted polymer chain length and chain density will be optimized. (Chapter 3)

**Objective 3** Develop a continuous membrane process for ammonia removal from aqueous streams. Electrospun zeolite 13X containing nanofibers has been fabricated and investigated for ammonia removal from fishery water. The absorption kinetics, binding capacity, the effect of competing ions effect will be determined. Also, the performance of the membranes has been optimized. Regeneration and cleaning protocols have established. (Chapter 4)

**Objective 4** Investigate the key factors affecting the robustness of parvovirus removal during the AEX polishing step. Virus removal will be studied under different sets of conditions with varied pH, ionic strength, and impurity concentration. In addition, the comparative level of virus clearance under different operating conditions will be investigated. A mathematical model to demonstrate the interaction behavior between the variable parameter and log reduction of the virus will be developed. (Chapter 5)
References


[100] Z. Ma, M. Kotaki, T. Yong, W. He, S. Ramakrishna, Surface engineering of electrospun polyethylene terephthalate (PET) nanofibers towards development of a new material for blood vessel engineering, Biomaterials, 26 (2005) 2527-2536.


[190] M. He, C. Wang, Y. Wei, Protein adsorption by a high-capacity cation-exchange membrane prepared via surface-initiated atom transfer radical polymerization, RSC Advances, 6 (2016) 6415-6422.


Chapter 2. Electrospun Weak Anion-exchange Fibrous Membranes for Protein Purification

Shu-Ting Chen, S. Ranil Wickramasinghe, Xianghong Qian
(Reproduced with permission, copyright (2020) Membranes.)

Abstract

Membrane based ion-exchange (IEX) and hydrophobic interaction chromatography (HIC) for protein purification is often used to remove impurities and aggregates operated under the flow-through mode. IEX and HIC are also limited by capacity and recovery when operated under bind-and-elute mode for the fractionation of proteins. Electrospun nanofibrous membrane is characterized by its high surface area to volume ratio and high permeability. Here tertiary amine ligands are grafted onto the electrospun polysulfone (PSf) and polyacrylonitrile (PAN) membrane substrates using UV-initiated polymerization. Static and dynamic binding capacities for model protein bovine serum albumin (BSA) were determined under appropriate bind and elute buffer conditions. Static and dynamic binding capacities in the order of ~100 mg/mL were obtained for the functionalized electrospun PAN membranes whereas these values reached ~200 mg/mL for the functionalized electrospun PSf membranes. Protein recovery of over 96% was obtained for PAN-based membranes. However, it is only 56% for PSf-based membranes. Our work indicates that surface modification of electrospun membranes by grafting polymeric ligands can enhance protein adsorption due to increased surface area-to-volume ratio.
2.1 Introduction

The global market of biologics, particularly, protein therapeutics is growing rapidly [1, 2]. The production of protein-based human therapeutics such as monoclonal antibodies (mAbs) and Fc-fusion proteins involves cultivating mammalian cells such as Chinese hamster ovary cells (CHO) in complex cell culture suspension [3]. The desired protein therapeutics are typically secreted by the cells into the suspension media. The protein product must then be recovered and purified. The rapid advancement in upstream cell culture operations has led to a significant increase in product titers. However, this high-level of productivity is accompanied by establishing much higher cell density [4, 5] which places a much larger burden on the traditional downstream clarification and purification operations. Downstream processing becomes the bottleneck in the production of protein therapeutics and contributes significantly to the production cost [6]. High capacity and high recovery downstream purification unit operations are essential for the cost-effective purification of biologics.

Ion-exchange (IEX) and hydrophobic interaction (HIC) chromatography are routinely used during the downstream purification of protein therapeutics. After the initial capturing step using protein A chromatography, the feed stream typically is further processed by additional polishing steps such as IEX and HIC chromatography operated under flow-through mode to further reduce the host cell proteins (HCPs), DNA, aggregates and other impurities present [7]. However, resin-based packed-bed chromatography suffers from high pressure drop and slow pore diffusion which leads to longer processing time and potentially denaturation of the product. Membrane adsorbers are promising technologies to replace resin-based chromatography [8-14]. Membrane-based IEX and HIC adsorbers can overcome afore-mentioned limitations [15, 16]. Moreover, the performance of membrane adsorbers is largely independent from the feed flow rate. However, membrane
capacity is typically lower compared to that of resin. Significant efforts have been dedicated to develop high binding capacity and/or high recovery membrane adsorbers by grafting ligands on membrane substrates using UV-initiated polymerization or atom-transfer radical polymerization (ATRP) [8-14, 17-19].

Electrospun membranes provide a 3-D scaffold which enhances surface area to volume ratio for protein adsorption. Electrospinning has attracted attention as a versatile and robust method for fabricating nanofibrous membranes [20-22]. Compared with membranes produced through temperature or non-solvent induced phase inversion processes [23], electrospun nanofibrous membranes have a much higher porosity due to their unique interconnected fiber structures. Using the electrospun membrane as a substrate to attach ligands can increase the available grafting area for protein binding. Previous study [24] evaluated the performance of electrospun polyethersulfone (PES) affinity membranes. These PES affinity membranes demonstrated a high specific binding selectivity for IgG molecules and low non-specific protein adsorption as well as low flow-through pressure drop due to their large pore sizes. Another study [25] reported the fabrication of electrospun carbon nanofibrous mats, a promising alternative to the packed-bed media for bioseparation applications. The binding capacity for lysozyme of the mats reached over 200 mg/g of adsorption media. In addition, these mats showed high feed flow rate and low pressure drop due to their large pore sizes. Earlier work [26] also tested the effects of compression and the number of bed layers for the dynamic binding capacity of regenerated cellulose based IEX electrospun membranes. The highest dynamic binding capacity for lysozyme reached ~21 mg/mL for carboxylate adsorbents at a compressive pressure of 1 MPa. An increase in the compressive pressure decreased dynamic binding capacity. Moreover, a decrease in dynamic binding capacity was also observed as the number of bed layers increased from 4 to 12 due to the reduction in
surface area resulting from membrane compression. High static binding capacity reaching 284 mg/g of adsorbents for lysozyme obtained from grafting critic acid on electrospun ethylene-vinyl alcohol (EVOH) nanofibrous membranes [27]. Previous work focused largely on grafting monomeric cation-exchange ligands on electrospun membrane substrates. No systematic investigation has been conducted on the performance of grafted polymeric anion-exchange electrospun membranes. Here the effects of ligand chain length, chain density as well as the substrate material and fiber diameter on protein binding capacity are studied.

There are several approaches to graft ligands on electrospun membrane substrates. Previous studies [24-27] conjugated a monomeric acid, base or affinity ligand directly to the functional group on the substrate. However, there has been significant work [8-14, 17-19] on surface modification by grafting polymers on the flat sheet membrane substrates using ATRP and UV-initiated polymerizations. These results show that there exists an optimal ligand chain density and chain length for maximizing protein binding capacities for IEX, affinity and HIC membrane chromatography [19]. Surface modification with UV initiated reaction or polymerization is fast and robust with extensive application for IEX membranes [28, 29]. Previous review [16] showed that the UV irradiation time, initiator concentration and the overall grafting degree affect protein binding capacity and membrane throughput significantly. Too high a grafting density can result in low binding capacity due to ligand aggregation and reduced protein accessibility. At relatively low grafting degree, ~100 mg/mL protein binding capacity of BSA can be obtained. Besides ligand, substrate morphology and porosity also play an important role [11]. Previous work [30] investigated the effects of degree of GMA grafting of non-woven substrate on the kinetics of non-specific protein adsorption. Their results indicate that higher surface area nonwoven substrate promotes faster protein adsorption.
As earlier work [24-27] focused on grafting monomeric cation exchange ligands, our work of grafting and optimizing polymeric anion exchange ligands on the electrospun membrane substrates is novel even though established surface chemistry has been adopted. Since ATRP requires inert condition and is difficult to implement at a large scale, UV-initiated polymerization on electrospun membranes to form IEX membranes was investigated here. In particular, the weak anion-exchange membranes were fabricated by grafting poly(glycidyl methacrylate) to the electrospun membrane substrates via UV-initiated polymerization followed by the addition of diethylamine (DEA) via a ring-opening reaction. Both polyacrylonitrile (PAN) and polysulfone (PSf) substrates were investigated. The properties of functionalized membranes were characterized using Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and zeta potential measurement. Furthermore, adsorption experiments were performed to determine the static and dynamic protein binding capacities of the functionalized membranes.

2.2 Materials and Method

2.2.1 Materials

Polysulfone (PSf, MW 60 kg/mol) in powder form as membrane material was purchased from BASF (Ludwigshafen, Germany). Polyacrylonitrile (PAN) in powder form as membrane material was kindly provided by R&D membrane center from Chung Yuan Christian university, Taiwan [31]. N, N-dimethylformamide (DMF), methanol (ACS grade), Tris (hydroxymethyl) aminomethane (Tris, biotechnology grade), and sulfuric acid (ACS grade) were purchased from VWR (Radnor, PA). Glycidyl methacrylate (GMA), Sodium phosphate dibasic (Reagent Plus, >=99.0%), sodium phosphate monobasic monohydrate and benzophenone (BP) were purchased from Sigma-Aldrich (St. Louis, MO). Inhibitors in GMA were removed through a pre-packed
inhibitor remover column (Sigma-Aldrich, St. Louis, MO) prior to use. 1-Butanol (>99% ACS grade) and diethylamine (DEA, reagent grade 99%) were purchased from Alfa Aesar (Ward Hill, MA). Bovine serum albumin (BSA) were acquired from Lee BioSolution (Maryland Heights, MO). All chemicals were used without further purifications except GMA. Deionized water (DI) was obtained from Milli-Q ultrapure water purification system (Millipore, Billerica, MA).

2.2.2 Methods

2.2.2.1. Fabrication of fibrous membranes

Electrospinning was used to fabricate polysulfone (PSf) nanofibrous membranes. PSf powders were dissolved in DMF (20 w/v %) under continuous stirring at 70 °C for about 24 h until a homogeneous solution was obtained following our earlier work [32]. The mixed solution was subsequently used for the fabrication of electrospun membranes using a syringe pump. Here the pump flow rate, applied voltage, needle gauge, and the distance between the needle tip and collector were fixed at 0.5 mL h⁻¹, 25 kV, 22-gauge needle, and 25 cm, respectively for the fabrication of PSf membranes. The reaction temperature was maintained between 21-25 °C and the relative humidity at 23% during the electrospinning of PSf. The nanofiber mat was prepared by collecting nanofibers over a period of 4 h.

To fabricate PAN nanofibrous membranes, PAN powder was dissolved in DMF (8 w/v%) with stirring to form a homogenous solution. The casting solution was then placed into a syringe pump and electrospun onto the aluminum foil following the similar procedure to the fabrication of PSf membrane. The flow rate, applied voltage and the distance between the needle tip and the collector are 0.6 mL h⁻¹, 20 kV and 15-20 cm respectively. The nanofiber mat was prepared by collecting nanofibers for 4 h.
2.2.2.2. UV-initiated polymerization of GMA

The PSf and PAN nanofibrous membranes were further surface modified with tertiary amine groups. Two step modification procedure is illustrated below in Scheme 1 following the previous chemistry [33]. The PSf fibrous and PAN fibrous membranes were first washed with methanol and dried at 40 °C under reduced pressure using a vacuum oven. PAN membranes were hydrolyzed prior to the use in 2 M NaOH solution at 50 °C for 2 h. The hydrolyzed membranes were then rinsed with DI water until the pH of the water reached 7.0. The membranes were subsequently dried in a vacuum oven before modification. Butanol was used as a solvent for UV initiated grafting of GMA with benzophenone (BP) as the UV initiator. A grafting solution contained 20% (v/v) GMA and 1% (w/v) BP. A pre-weighed membrane (6.2 cm², ~13 mg) was dispersed evenly with GMA grafting solution until it is completely soaked. The membrane was then sandwiched between two petri dishes. One side of the membrane was exposed to UV lamp (model UVAPRINT 100, Honle UV technology, Germany) with intensity of 44.5 mW/cm². The distance between the membrane and the lamp was maintained to be around 40 cm. After completion of the predetermined reaction time, the membrane was washed in methanol to remove any unreacted GMA and BP. Thereafter, the membrane was finally dried in a vacuum oven for 4 h at 40 °C. Thereafter, the membrane was grafted with GMA on the other side of the membrane following the same procedure.
Scheme 1 Surface modification of hydrolyzed electrospun PAN membrane (A); and electrospun PSf membrane (B).
GMA-grafted membranes (PSf-GMA and PAN-GMA) were then immersed in a 50% (v/v) diethylamine (DEA) aqueous solution at 30°C with gentle shaking overnight. Thereafter the membranes were washed repeatedly with a DI water and methanol mixture to remove any unreacted species. To reduce nonspecific protein binding, these modified membranes were then hydrolyzed in a 0.1 M sulfuric acid solution at 50 °C overnight to convert any remaining epoxy group to hydroxyl groups. The membranes were again washed with DI water after hydrolysis [33]. After that, the membranes were dried at 40 °C under reduced pressure using vacuum oven. The grafting degree was calculated based on the amount of GMA grafted as shown in Equation (1) below where \( W_0 \) is the original membrane weight (mg) and \( W_1 \) is the weight after GMA and DEA grafting (mg). Triplicate measurements were conducted to obtain the accuracy of the standard deviation.

\[
\text{Degree of grafting (\%)} = \frac{W_1 - W_0}{W_0}
\]  

(1)

Similarly, nanofibrous PAN membranes were modified. However, PAN membranes were hydrolyzed before use in 2 M NaOH solution at 50°C for 2 h. The hydrolyzed membranes were then rinsed in DI water until the pH of the water reached 7.0. The membranes were subsequently dried in a vacuum oven before modification with GMA and DEA.

2.2.2.3. Membrane morphology

Surface and cross-sectional morphology of these fabricated nanofibrous membranes were imaged using a scanning electron microscopy (SEM). Cross-sectional images of the membranes were obtained by fracturing the membranes in liquid nitrogen.
2.2.2.4. Zeta potential

The surface charge in terms of Zeta potential on the membrane was measured using Beckman Coulter Delsa NanoHC (Brea, CA). A flat cell was used for measuring the zeta potential on the membrane surface. Dry membranes were immersed in the buffer solution with predefined pH value. NaCl solution was used for calibration and triplicate measurements were carried out for each membrane [34].

2.2.3. Membrane Binding Capacity Determination

2.2.3.1. Static protein binding capacity measurement

The static binding capacity were measured for these modified electrospun membranes using bovine serum albumin (BSA) as a model protein. BSA has an isoelectric point (pI) of 4.7 and is negatively charged at neutral pH. Static protein adsorption isotherms were measured on unmodified and GMA-DEA modified membranes. Six different initial BSA concentrations at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL in 20 mM Tris-HCl buffer at pH 7.0 were used to measure the amount of protein adsorbed by the membranes. The membranes were cut into 4.9 cm² disks. Each membrane was placed in a 60 mL glass bottle from VWR (Radnor, PA). All membranes were first equilibrated with a pH 7 tris buffer for 1 h. Thereafter, all membranes were challenged with a specified protein solution and equilibrated for 20 h at room temperature under gentle shaking. The equilibrium concentrations of BSA solutions were first measured using UV absorbance at 280 nm with a UV–VIS spectrophotometer (Thermo Scientific™ GENESYS 10S UV-Vis). BSA binding capacity of the membrane at each protein concentration was calculated below:

\[
\text{Binding capacity} = \frac{\text{Amount of protein bound to a membrane}}{\text{membrane volume}}
\]  

(2)
2.2.3.2. Dynamic binding capacity measurement

Protein stock solutions were prepared by dissolving 30 mg of BSA protein in 10 mL of 20 mM Tris-HCl buffer (pH 7.0, Buffer A), which contained no other salt. Then this stock solution of BSA was added to the 20 mM Tris-HCl buffer containing 1 M of NaCl (Buffer B) to obtain a solution of 3 mg/mL protein solution. All the buffers and protein solutions were then filtered with Whatman 0.2 μm PES membrane before the dynamic protein binding test. Membranes were soaked in buffer A for 10 minutes before use. Each run used four membranes packed in a stainless-steel flow cell (Mustang Coin® module, Pall Corporation). A flow distributor was placed on each side of the membrane stack to obtain a uniform flow across the membranes. All runs were conducted with ÄKTA FPLC (GE Healthcare Bio-Sciences Corp). The runs were automated using the Unicorn software 7.3 for binding and elution. The system was initially equilibrated with buffer A (adsorption buffer) at a flow rate of 1 mL/min for 10 minutes. Feed protein solution containing 3 mg/mL BSA was then loaded onto the membrane at a flow rate of 1 mL/min for 10 minutes for a total of 10 mL feed. Unbound proteins were then washed away from the membrane using buffer A (adsorption buffer) for 10 minutes at 1 mL/min, followed by elution with buffer B (elution buffer) at a flow rate of 1 mL/min for 10 min. The runs ended when the UV absorbance at 280 nm reached a constant. Three fractions were collected including loading, washing as well as elution. Protein concentrations in these fractions were determined by UV at 280 nm. Protein binding capacity was calculated using Equation (2) whereas protein recovery was determined using the following formula:

\[
\text{Recovery} = \frac{\text{Amount of eluted protein}}{\text{Amount of protein bound to the membrane}} \quad (3)
\]
2.3. Results and Discussion

2.3.1. Membrane characterization

Chemical composition, surface morphology and membrane properties of the PSf and PAN based electrospun nanofibrous membranes were characterized using FTIR, scanning electron microscopy (SEM) and zeta potential measurements.

2.3.1.1. Chemical composition of membrane surface

FTIR spectra for the fabricated nanofibrous PSf membranes are shown in Figure 1. The FTIR spectrum of the base PSf membrane (black line, bottom) exhibits a number of characteristic peaks. After grafting GMA on the membrane (red line, middle), two distinctive new peaks can be observed at 905 cm\(^{-1}\) and 1730 cm\(^{-1}\) respectively [35]. The 905 cm\(^{-1}\) peak comes from the epoxy group whereas the 1730 cm\(^{-1}\) peak arises from the ester carbonyl group after modification with GMA [36]. At the same time, the 1235 cm\(^{-1}\) peak from the tertiary amine from the base membrane becomes less prominent. The FTIR spectra indicate that GMA was successfully grafted onto the PSf membrane. After further modification with DEA followed by converting the remaining epoxy to OH group, a prominent tertiary amine peak at 1235 cm\(^{-1}\) was again observed. The epoxy peak at 905 cm\(^{-1}\) almost disappears indicating successful conversion and modification to form PSf-GMA-DEA membranes [37].
Figure 1 FTIR spectra of PSf base membrane (black), after modification with GMA (red), and further modification with DEA to form PSf-GMA-DEA membrane.

FTIR spectra of PAN membranes before and after modification are shown in Figure 2. The base PAN membrane (black) before hydrolysis and PAN-GMA-DEA membrane (blue) all exhibit a broad peak at around 3400 cm\(^{-1}\) due to the presence of surface -OH groups. After GMA modification (red), the broad -OH peak almost disappears. The carbonyl group from the ester group at 1730 cm\(^{-1}\) becomes significantly enhanced after the modification. The sharp peak at 2243 cm\(^{-1}\) arises from the stretching of the residue -CN group in the base membrane (black). This peak becomes much weaker after the membrane surface is modified with GMA (red). The broad peak in the range of 1065 cm\(^{-1}\) was attributed to the stretching of C-N bonds [33]. The bending peaks from the epoxy group after GMA modification were observed in the range of 904 and 847 cm\(^{-1}\) (red) [38]. The amine peak at 1230 cm\(^{-1}\) becomes much weaker upon surface modification with GMA, but with much enhanced intensity after the DEA modification. The FTIR spectra again suggest successful modification of the PAN membrane with GMA and DEA.
Figure 2 FTIR spectra of hydrolyzed PAN membrane (black), PAN membrane modified with GMA (red) and membrane modified with GMA-DEA (blue).
2.3.1.2. Membrane Structure and Morphology

Figure 3 SEM morphology of unmodified PSf (a), PSf-GMA with 7 min UV (b), PSF-GMA-DEA (c), unmodified mPAN (d), mPAN-GMA with 15 min UV (e), and mPAN-GMA-DEA (f).
Scanning electron microscopy (SEM) images of electrospun nanofibrous PSf and PAN membranes before and after surface modifications were shown in Figure 3. It can be seen that PSf based membranes exhibited uniform nanofibrous structures. For the base PSf membrane, the average diameter of PSf fibers is in the range of 1.25 µm ± 0.23 µm. However, the fiber diameter increased significantly as more modifications were performed on the base membrane as can be seen from Figures 3(a-c). The fiber diameter for PSf-GMA-DEA membrane is almost twice as much as that of the base PSf membrane. Moreover, the fiber surface also becomes visibly rougher after GMA and DEA modification. This dramatic increase in fiber diameter can be explained by the polymerization process adopted here. When grafting the polymer, the UV initiator BP and monomer GMA were mixed together in the prepared solution, in which the membranes were soaked to uptake the mixtures. In addition to grafting GMA polymers chemically bonded to the membrane substrate, this UV-initiated polymerization can also lead to the polymerization of the free GMA polymers adsorbed physically on the surface of the membrane substrate [39]. The physically adsorbed free GMA polymers lead to the significant increase in fiber diameter and increase roughness since the polymer chains were not tightly packed. A two-step process involving the initial initiator adsorption followed by the subsequent polymerization can reduce the probability of forming free polymer chains.

The mPAN electrospun membranes exhibit clearly a thinner fibrous structure than PSf membranes as shown in Figure 3(d-f). The average diameter of mPAN fibers was estimated to be in the range of 150 to 340 nm before surface modification. Upon 15 min of UV initiated polymerization of grafting GMA, average fiber diameter only increased slightly. The significantly reduced grafting degree and the less hydrophobic nature of the mPAN membrane compared to that of PSf membrane likely lead to the much smaller fiber diameter. Further modification with DEA,
fiber size has visibly increased. This is probably due to fact that reaction of DEA to the epoxy group resides in each GMA monomer unit leading to a substantial increase in the grafted polymer molecular weight.

2.3.1.3. Zeta potential

![Graph](image1.png)

**Figure 4** (a) Zeta potential of mPAN base membrane, modified mPAN-GMA-DEA membranes with 3, 6, and 15 min UV polymerization, (b) Zeta potential of PSf base membrane, modified PSf-GMA-DEA membranes with 3, 6, and 7 min UV polymerization.
Zeta potential of the electrospun PAN base membrane and modified membranes at 3, 6, and 15 min UV polymerization times were measured and shown in Figure 4. It can be seen that the base membrane (black) at the pH range between 3 and 11 has a negative surface charge. This is due to the fact that after NaOH hydrolysis, some of the -CN groups on PAN membrane are converted to the negatively charged -COO\(^-\) groups. After the GMA-DEA surface modification, the zeta potential increases at each pH value increases. This is due to the incorporation of the tertiary amines in the membrane surface leading to an increase in the surface charge. Moreover, the longer the UV polymerization or the GMA chain is, the higher the DEA number incorporated into the surface layer, thereby the higher the number of tertiary amines resides on membrane surface, which in turn leads to a higher zeta potential. After 15 min of UV polymerization and the subsequent DEA addition, the zeta potential becomes largely positive except at pH 11 indicating higher density of the positive charges on the membrane surface and subsequently higher anion-exchange capacity. The zeta potential trend of modified PSf membranes as a function of pH is similar to that of PAN electrospun membranes. However, the former is slightly higher overall than the latter at the same pH value and the same grafting condition.

2.3.1.4. Grafting degree.

Grafting degrees of modified PSf and PAN nanofibrous membranes with respect to UV polymerization times are shown in Figure 5. As mentioned previously, the UV polymerization was conducted on both sides of the membrane in order to functionalize more tertiary amines on the membrane surface. PSf electrospun membrane was found to sustain damages when the UV exposure was longer than 7 min. This is due to the fact that membrane base materials are sensitive to UV light [40]. Longer UV exposure leads to a decreased mechanical strength and reduced chemical stability. As a result, UV polymerization of grafting GMA was conducted for 1, 3, and 7
min only for the PSf membranes. The grafting degree was determined after both GMA and DEA modifications. It can be seen from Figure 5 that when the UV polymerization time increased from 1 to 7 min, the grafting degree increased dramatically from about 25% to over 100% indicating the efficiency of the polymerization. However, as mentioned previously, it is likely that polymers that are not chemically bonded to the membrane surface are formed as well during the polymerization reaction since the initiator and monomer were mixed together.

![Figure 5](image)

**Figure 5** Grafting degree as a function of UV polymerization time for both electrospun PSf and PAN membranes.

On the other hand, for PAN electrospun membranes, grafting degree also increases with the increase of UV polymerization time. After 15 min of UV polymerization and subsequent DEA addition, ~55% grafting degree was achieved, significantly less than those grafted on PSf membranes. This difference probably lies in the fact that UV initiated polymerization can occur even without the initiator BP for the PSf membrane. The contact angle for unmodified electrospun PSf membrane was estimated to be 128.0 ± 4.8° whereas it was measured to be 73.1 ± 3.5° for unmodified hydrolyzed PAN membranes. Since multiple factors including surface roughness
contribute to water contact angle, contact angles for flat sheet membranes were also measured. Contact angles for flat sheet PSf and PAN membranes are measured to be 70.0°± 1.7° and 45.0°± 4.6°, respectively. These observations confirm that the PSf base membrane is more hydrophobic than the PAN membrane in agreement with previous studies [41, 42]. As a result, more initiator BP and monomer GMA molecules can be adsorbed on the membrane surface leading to an increased polymer chain density thereby increased grafting degree. Moreover, increased initiator and monomer uptake by the PSf membrane leads to the increased number of free UV-initiator polymer chains.

2.3.2. Static and dynamic protein binding capacity tests

![Figure 6](image)

**Figure 6** Static binding capacities of unmodified (virgin) and modified PSf and PAN membranes at different UV polymerization times. Error bars represent the standard deviation from 3 measurements. For PAN, the virgin membrane is after NaOH hydrolysis.

Static protein binding tests were conducted for the electrospun base and modified PSf and PAN membranes as a function UV polymerization time as shown in **Figure 6**. The membranes were equilibrated with the protein solutions for 20 h. The time dependent binding capacities of the
two membranes are shown in Figure S1 in the supplementary document. The BSA binding capacity increases as a function of time and reaches a stable value after about 12 h. The unmodified PSf electrospun membrane exhibits non-specific protein binding capacity of close to 100 mg/mL whereas the PAN membrane only has about 20 mg/mL. As indicated previously, PSf base membrane is more hydrophobic than the hydrolyzed electrospun PAN base membrane since PAN possesses negatively charged -COO\(^-\) groups. After 3, 6 and 7 min of UV initiated polymerization and the final functionalization of DEA, the BSA binding capacity of PSf membrane increased dramatically to about ~260 mg/mL. This significant increase in protein binding capacity is correlated with the more than 100% of the grafting degree observed for the PSf membranes. The overall increase in the protein binding capacity is correlated with the increase in grafting degree. Even though PAN membranes exhibit relatively low protein binding capacity, the overall trend in its binding capacity with regard to grafting degree is still valid. The static binding capacity for modified electrospun PAN membranes increased from ~40 mg/mL after 3 min UV polymerization to about 100 mg/mL after 15 min UV polymerization and the addition of DEA. The high protein binding capacity of PSf-GMA-DEA membrane partly comes from its more hydrophobic base membrane and partly from its higher grafting degree.

The high protein binding capacities of the electrospun and surface grafted PSf-GMA-DEA and PAN-GMA-DEA membranes come partly from their high surface-volume ratio compared to flat sheet membranes. In addition, surface modification which increased the density of tertiary amine is also partly responsible for the enhanced protein binding. This indicates that electrospun nanofibrous membranes with appropriate functionalization are promising for improved performance during downstream protein purification. The high non-specific binding of proteins on PSf-GMA-DEA membrane remains to be a challenge for industrial applications. Moreover,
additional investigation is necessary for the separation of protein mixtures with different pIs at appropriate feed conditions.

Dynamic binding capacities of the fabricated membranes were determined using FPLC Chromatograms for FPLC runs with both surface-modified electrospun membranes are shown in Figure 7. The PSf-GMA-DEA membrane was surface modified with 7 min of UV polymerization whereas the PAN-GMA-DEA membrane was modified with 15 min of UV polymerization. The PSf-GMA-DEA membrane exhibited a BSA protein binding capacity of 201.3 mg/mL membrane volume as compared to the value of 87.2 mg/mL for the corresponding PAN-GMA-DEA membrane. However, in the case of PSf-GMA-DEA membrane, only ~56% of the BSA was recovered upon elution which was significantly lower than the value of ~96% for the corresponding PAN-GMA-DEA membrane. The protein binding and recovery results are tabulated in Table 1. The fact that PSf-GMA-DEA has a much higher protein binding capacity and relatively lower protein recovery and that PAN-GMA-DEA has a lower protein binding capacity and a much higher recovery agrees with the properties of the membranes and our previous static protein binding tests. The former is more hydrophobic and thus more non-specific protein binding leading to a higher binding capacity but lower recovery. The latter is more hydrophilic with much less non-specific protein binding leading to a lower binding capacity but increased recovery.
**Figure 7** Chromatograms of dynamic protein binding runs using PSf-GMA-DEA with 7 min UV polymerization (a), and mPAN-GMA-DEA with 15 min UV polymerization (b).

**Table 1** Productivity comparison for different substrate membranes. Error bars represent the standard deviation of calculated values using data from 2 measurements.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Dynamic Binding Capacity (BSA, mg/mL membrane volume)</th>
<th>BSA yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSf</td>
<td>201.3 ± 4.8</td>
<td>55.9 ± 5.1</td>
</tr>
<tr>
<td>PAN</td>
<td>87.2 ± 2.6</td>
<td>96.4 ± 3.2</td>
</tr>
</tbody>
</table>
2.4. Conclusions

Here PSf and PAN surface modified weak anion-exchange electrospun nanofibrous membranes were fabricated by UV-initiated polymerization. The physiochemical properties of the surface modified membranes were characterized using SEM and FTIR. The changes in surface charge and charge density as a function of UV polymerization time were measured by zeta potential and correlated with the polymer grafting degree. Due to the high substrate porosity resulting from the high surface-to-volume ratio of the nanofibrous materials, the surface modified PSf-GMA-DEA and mPAN-GMA-DEA membranes exhibit high static and dynamics protein binding capacities. BSA protein binding capacity increases as the grafting degree increases in agreement with surface charge measurements. However, after 15 min of UV exposure, the mechanical integrity of PSf membrane is compromised. The PSf-GMA-DEA membrane is more hydrophobic than the mPAN-GMA-DEA membrane leading to its increased non-specific binding of the protein. The BSA recovery of mPAN-GMA-DEA membrane reached over 96% whereas it is only ~56% for the PSf-GMA-DEA. Our study indicates that electrospun nanofibrous mPAN-GMA-DEA membrane is promising as a high capacity high recovery ion-exchange membrane for protein purifications.

Acknowledgments

This work was supported by the Center for Advanced Surface Engineering (CASE), under the National Science Foundation (NSF) Grant No. OIA-1457888 and the Arkansas EPSCoR Program.
References


Supplementary

**Figure S1** BSA static binding capacities at different equilibrium times for both surface modified electrospun PSf-GMA-DEA (7 min UV polymerization time) membrane and mPAN-GMA-DEA (15 min UV polymerization time) membrane. For both membranes, four pieces of membranes were soaked in 4 beakers separately containing 3 mg/mL BSA in 20 mM Tris buffer at pH 7.0. The equilibration process was conducted at room temperature under gentle shaking. Protein binding capacities were measured after 1, 5, 12, 20 h of equilibration from 4 beakers respectively.
Chapter 3. High efficiency hydrophobic interaction chromatographic (HIC) membrane utilizing electrospinning technique

Abstract

Phenyl-based membrane hydrophobic interaction chromatography (HIC) is a purification process based on hydrophobicity differences between impurities and products and commonly used in biopharmaceutical production and. Existing technologies suffer with the drawbacks like low product yields and protein denaturation issues. Herein, responsive HIC membranes have been investigated for the purification of proteins in a bind-and-elute mode to overcome the aforementioned drawbacks. Poly N-vinylcaprolactam (PVCL) is a prototype polymer that is salt- and thermo-responsive which can be triggered by maintaining ideal external conditions. In the scope of this study, the responsive polymer, PVCL, was functionalized on the electrospun regenerated cellulose membrane by atom transfer radical polymerization (ATRP). Large surface-to-volume ratios and high porosity leads electrospun membrane offer more room for grafting ligand resulting in the higher ligand density. An optimal chain density was established based on the performance of the fabricated HIC membranes in terms of protein binding and recovery. Langmuir-Freundlich isotherm pattern was obtained from the results of the static adsorption studies with BSA as the model protein. Both high dynamic binding capacity (~12 mg/mL) and high recovery (~96%) could be achieved at BSA concentration of 0.1 g/L using the fabricated responsive HIC membrane. At high feed concentration of 1 g/L, the dynamic binding capacity of ~30 mg/mL was obtained. Our study demonstrated that substantial improvements in recovery and binding capacity were observed using the electrospun HIC membranes relative to the flat-surface membranes.
3.1 Introduction

The rapid development of biopharmaceuticals in recent decades has led to an increased demand in research field of the purification methods as higher product purity is demanded by regulatory agencies [1]. The efficacy of the purification steps must be considerably increased to lower the excessive cost in downstream processing. In related scenarios, downstream processing costs account for 50%–80% of the entire production cost [2]. Accordingly, the biopharmaceutical industry is facing a bottleneck in an attempt to enhancing the efficacy of purification processes to reduce costs while simultaneously sustaining product quality. Significant efforts have been made in recent years to overcome the existing challenges to improve the specificity and efficacy of the purification methods involved in the downstream processing.

Hydrophobic interaction chromatography (HIC) is a chromatographic technique commonly used in many bio-separation applications such as those for enzymes, monoclonal antibodies, hormones, vaccines, growth factors, interferons, and truncated forms of recombinant proteins [3, 4]. A related theory to this technique first appeared in 1949, but the term HIC was proposed by Hjerten in 1973 [5]; subsequently, the technique became substantially developed. HIC is quite different from several other purification processes because the interaction between water molecules constitutes the driving force for binding [3]. The applied chromatographic medium comprised of different extent of salts, has a major role to play in the reversible interaction, which acts as a controller between the hydrophobic surface on the protein and the ligand. Therefore, various parameters like applied ligand, salt type, temperature, and ionic strength considerably affect overall chromatographic performance [2, 6-8]. In particular, the performance related to protein binding and recovery in chromatography is dependent on protein hydrophobicity, ligand
types, salt type, and salt concentration of the buffer [9]. Different interactions between various proteins and ligands lead to different types of selectivity for different feed streams [10, 11].

Resin-packed chromatography has been rigorously used in industrial product processing; however, several drawbacks have gradually emerged following an exponential increase in the overall demand for products. Slow pore diffusion and high hydraulic pressure requirements cause lower productivity and higher operational costs, respectively; nonetheless, functionalized membrane absorbers provide a practical solution to overcoming those drawbacks because of the open microporous structure of membranes [12]. Although low ligand capacity levels engendered by a relatively low surface-to-volume ratio constitute a difficulty that must be resolved, membrane chromatography is still a promising technique for future use. Till date, many researchers have been paying increasing attention to the binding and elution of HIC membranes for protein purification [13]. Most of these studies have focused on utilizing membrane adsorbers to bind and elute targeted proteins [7, 14, 15]. In such methods, the targeted proteins bind to hydrophobic ligands in high-ionic-strength buffers and elute upon application of low-ionic-strength buffers [7]. This distinct high salt binding and low salt elution enable HIC to be used in the purification step. However, application of HIC has been limited due to the overall lower efficiency of the ligands used to bind the proteins and also, due to difficulty in elution of the bound proteins [16]. Hence, several attempts have been made in recent years to improve the selective responsive nature of the ligands to improve the HIC performance in bind and elute mode [14].

In this context, the thermoresponsive polymer, poly(N-vinylcaprolactam) (PVCL), possesses a relatively low critical solution temperature (LCST) in between 30 °C to 50 °C [17, 18]. This temperature range is directly related to molecular weight of polymer and polymer concentration. When the temperature exceeds the LCST, the polymer conformation would collapse
and become hydrophobic, thus promoting protein binding. In contrast, when the temperature is below the LCST, the polymer would become swollen, thus promoting protein desorption [11, 19]. However, the LCST of PVCL is still higher than the operating temperature which is normally room temperature during manufacturing step. Common practice is to vary the ionic strength during the LCST transition instead of increasing the temperature for protein purification. With an increase in ionic strength, the LCST of the polymer decreases. Different salt types lead to different LCSTs under the same ionic strength condition [20, 21]. In addition, the advantages of using PVCL are its biocompatibility and low toxicity. Herein, another thermo-responsive polymer, namely poly(N-iso-propylacrylamide) (PNIPAM), generates a low-molecular-weight amine during hydrolysis, which is toxic to biological systems; whereas, PVCL does not generate any low-molecular-weight amine during the hydrolysis process as the amide bond is located within the enclosed cyclic moiety [2, 22, 23].

Over the past decade, Qian et al. have contributed significantly towards development of high-efficiency membrane chromatography and used the peculiar thermo-responsive polymer, namely PVCL, to enhance the overall protein binding capacity and understand the underlying binding mechanism [2, 7, 14]. However, most of these studies have used flat-surface membranes as substrates. Further, the previous works have demonstrated the bovine serum albumin (BSA) adsorption performance of a commercial regenerated cellulose (RC) membrane grafted with PVCL. A high grafting density was found to be highly unfavorable for BSA recovery due to the intrinsic steric hindrance. Moreover, the usage of longer polymer chain was found to be restricted due to conformation swelling. When BSA was loaded in 1.8 M (NH₄)₂SO₄ solution, the dynamic binding capacity estimated at 90% breakthrough for the protein was 4.8 mg/mL, with the corresponding recovery reaching as high as 96% [17]. The dynamic binding capacity was successfully enhanced
by introducing poly(hydroxyethyl methacrylate) (HEMA) to act as the primary backbone. Subsequently, PVCL brushes were developed as a secondary layer to form a comb-like ligand. The corresponding results indicated that a long primary chain inhibited protein recovery and binding capacity. When BSA was loaded in 1.8 M (NH₄)₂SO₄ solution, at a suitable density of ligands, the dynamic binding capacity as high as 12.6 mg/mL could be reached, with the corresponding recovery being 78% [7]. Further, Liu et al. indicated that the LCST of PVCL could be substantially dependent on salt concentration as well as salt type. In their study, PVCL was modified on a commercial RC membrane. A comparative analysis of the dynamic as well as static binding capacities of IgG and BSA was reported when loaded in Na₂SO₄, Al₂(SO₄)₃, (NH₄)₂SO₄, and ZnSO₄ solutions under the same ionic strength condition. The results indicated that the protein binding capacities associated with the aforementioned solutions followed the order: Na₂SO₄ > (NH₄)₂SO₄ > Al₂(SO₄)₃ > ZnSO₄. Moreover, IgG was determined to have a higher binding capacity than BSA in the same feed stream, which could be attributed to the higher hydrophobicity of IgG. The dynamic binding capacity of BSA was noted to be 6 mg/mL when BSA was loaded in 1.2 M Na₂SO₄ solution, and the corresponding recovery was approximately 85% [2]. Overall, the previous studies have demonstrated that grafting hydrophobic ligands on flat-surface membranes would limit binding capacity due to the low surface area. Hence, a significant scope was yet to be explored in this context to improve the limited binding capacity by increasing the surface area of the membrane by implementing advanced fabrication methods.

Electrospinning is a practical solution for overcoming the limitation of a low surface-to-volume ratio. The peculiar technique involves multiple controllable parameters and can be applied to fabricate membranes exhibiting high porosity along with an interconnected structure [24]. Thus, this attractive technique has been explored in many applications in recent years, including water
treatment, tissue engineering, and oil–water separation applications. In last decade, researchers have typically focused on developing electrospinning procedures for preparing ion-exchange membranes [25]. In some cases, a considerable breakthrough was achieved in terms of binding capacity enhancement which could be attributed to the large specific surface area to enhance ligand density obtained using electrospinning method exhibiting a 3D structure with high porosity, and avoid protein fouling by large pore size [26-31]. In last few years, few studies have been reported the fabrication of HIC membranes using electrospinning method for various applications. Therefore, there is significant scope in terms of development of electrospun HIC membranes for their prospective application to address the existing issues commonly faced in bio-separation industry.

In our study, ATRP surface modification technique was used to develop optimal HIC membranes. In addition, RC membrane was fabricated through electrospinning method and applied as a substrate membrane with an aim to achieve high surface area to volume ratio. Although the binding capacity was higher when sodium sulfate was employed in the buffer solution in our previous study, herein, ammonium sulfate have been used since high sodium sulfate concentration tends to denature the target proteins. The fabricated membranes were characterized using Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS) and scanning electron microscopy (SEM). Furthermore, adsorption experiments were conducted to determine the static and dynamic protein binding capacities of the functionalized membranes. The electrospun HIC membranes were ultimately challenged with mixture proteins like BSA, IgG, and Lysozyme solutions to provide insights about the binding mechanism and future practical applications.
3.2 Experimental

3.2.1 Materials

N, N, N, N, N-pentamethyl diethylenetriamine (PMDETA, 99%), 2-bromo-2-methylpropionyl bromide (BIB, 98%), 2-hydroxyethyl methacrylate (98%), N-vinylcaprolactam (98%), 4-(dimethylamino) pyridine (DMAP, >99%), copper(I) chloride (>99.99%), copper (II) chloride (>99.99%), and cellulose acetate (CA, Mn ~30 k) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Triethylamine (TEA, >99%) and N, N dimethylacetamide (DMAc, 99%) were sourced from Alfa Aesar (Ward Hill, MA, USA). Acetonitrile (>99.8%), methanol (99.8%), acetone (>99.5%), and ammonium sulfate (proteomics grade) were purchased from VWR (Radnor, PA, USA). Boric anhydride and BSA were acquired from Avantor Performance Materials (Center Valley, PA, United States). Purified human IgG4 monoclonal antibody was obtained from Eli Lilly (Indianapolis, IN, United States). Deionized (DI) water was generated using Thermo Fisher Scientific (Waltham, MA) DI water system.

3.2.2 Preparation of HIC electrospinning membrane

In a typical electrospinning method, CA (13.8 wt%) was dissolved in Acetone/DMAc (2:1, w/w) mixture as the medium. The resultant mixture was stirred in room temperature to obtain a homogenous solution and used as the casting solution. The aforementioned casting solution was filled into a syringe pump and electrospun onto aluminum foil using an electrospinning system (laboratory grade). For this system, the syringe needle tip was connected to the high-voltage power supply anode, while the grounded collector plate was connected to the cathode. The applied voltage level was set to be 12.5 kV maintaining the distance between the collector–needle tip at 15–20 cm and controlled the flow rate of the syringe pump at 0.3 mL/h. A nanofiber mat was prepared by collecting nanofibers for 8 h. After the membranes were collected from the screen, a CA membrane
was set in a fume food for 1 day to evaporate the residual solvent. Following the annealing process, the CA membrane was peeled from aluminum foil and hydrolyzed in aqueous sodium hydroxide (0.1 M) solution for overnight without stirring, resulting in the complete removal of the acetyl groups in the CA molecules through a hydrolysis reaction. Thereafter, the membrane samples were stored in DI water bath until further use.

3.2.3 Membrane surface modification using surface initiated ATRP

The reaction scheme for modifying HIC membranes through ATRP is presented in Figure 1. An HIC membrane was modified using surface initiated ATRP following the method reported in our previous study [2]. First, a regenerated cellulose (RC) obtained after hydrolysis of CA membrane was dried in a vacuum oven for 8 h to remove residual moisture content before immobilizing with an initiator in 40, 80, and 200 mM BIB/acetonitrile solutions for a constant period. Subsequently, a mixture solution was prepared by adding a monomer PVCL, copper (II) chloride, copper (I) chloride, and ligand PMDETA into the methanol/water solution (50 v/v%). Argon was injected to degas the mixture solution for 15–20 min. The monomer ratio of CuCl, CuCl₂, and PMDETA was fixed at 200:1:0.2:2. Furthermore, the immobilized membrane was placed into a clean round-bottom three-necked flask, and the flask was subjected to vacuum drying and an argon back-filling process thrice to ensure that it was completely filled with argon. Finally, the polymerization process was initiated by injecting the derived mixture solution into the flask. After reaching a constant period of ATRP, the modified membranes were rinsed three times using 50 v/v% methanol/water solution and subsequently, three times using DI water. The resultant membrane was kept in DI water bath on the shaker to remove any residual solvent.
3.2.4 Characterization of the HIC membranes

All membrane samples were cleaned with DI water and dried in a vacuum oven (12.5 L, VWR International, Radnor, PA, USA) overnight prior to characterization. A static contact angle analysis was used to determine the influence of changes in ionic strength of solution on the membrane surface. Images were observed by an optical angle meter (FDS Corp., Garden City, NY, United States). First, a membrane sample was attached onto a clean microslide with double-sided tape (3M, Maplewood, MN, USA) for the measurement. The membrane faced down to the certain buffer solution comprised of different salt concentration with varying ionic strength for a while followed by injection of air. Quintuplicate measurements were conducted to obtain the accuracy of the standard deviation.

A Fourier-transform infrared spectroscopy (FTIR) instrument (IRAffinity, Shimadzu, MD, USA) was applied to determine chemical functional groups on the membrane surface based on a specific emission wavelength. An X-ray photoelectron spectroscopy (XPS) instrument from Thermo Fisher Scientific Inc. (Waltham, MA, USA) was also used in order to determine the chemical composition of the membranes. Scanning electron microscopy (SEM) instrument (FESEM S-4800) purchased from Hitachi Co. (Tokyo, Japan) was used to characterize the surface morphology of the modified as well as unmodified membranes.
3.2.5 HIC membrane performance

Grafting degree is an essential factor for determination of the extent of successful ATRP grafting. The entirety of the membranes derived in this study was dried in a vacuum oven at 40 °C temperature; in addition, variations in the weight of the sample before and after ATRP were noted. The grafting degree was calculated based on the amount of PVCL grafted as shown in Equation 1.

\[
\text{Grafting degree (\%)} = \frac{\text{membrane weight after modified (mg)} - \text{unmodified membrane weight (mg)}}{\text{unmodified membrane weight (mg)}}
\]  

(2)

For static binding capacity tests, first, the membranes were cut into 4.9 cm² disks. Each membrane was placed in a 60 mL glass bottle from VWR (Radnor, PA). The entirety of the membranes was equilibrated for 1 h with adsorption buffer (20 mM Phosphate + 1.8 M Ammonium Sulfate, pH 7.0, buffer A). Subsequently, all the equilibrated membranes were challenged for 5 h with BSA as model protein at room temperature under gentle shaking. Additionally, five protein solutions of different concentrations were shaken simultaneously. The equilibrium concentrations of protein solution were first measured by recording the UV absorbance at 280 nm with a UV–VIS spectrophotometer (Thermo Scientific™ GENESYS 10S UV-Vis); these equilibrium concentrations of protein solution could be further determined by their standard curves. Binding capacity and protein recovery could be calculated as follows:

\[
\text{Binding capacity} = \frac{\sum \text{Amount of protein bound to a membrane (mg)}}{\text{membrane volume (mL)}}
\]  

(3)

\[
\text{Recovery} = \frac{\sum \text{Amount of protein eluted (mg)}}{\sum \text{Amount of protein bound to a membrane (mg)}}
\]  

(4)
For dynamic binding capacity, ÄKTA Pure (GE Healthcare Bio-Sciences Corp, Boston, MA, United States) was employed to conduct the fast protein liquid chromatography (FPLC) experiments. For developing the test method, Unicorn software version 7.3 was used to automate experiments on BSA binding and elution. BSA solutions were prepared by dissolving 10 mg of BSA into 10 mL of buffer A, which contained salt. IgG₄ stock and lysozyme solutions were prepared by dissolving 1 mg of IgG₄ or lysozyme into 10 mL of buffer A. Prior to the execution of binding tests, the entirety of the derived protein and buffer solutions were filtered using a Whatman 0.2-μm Polyethersulfone (PES) membrane filters. Specifically, four membranes (0.04 mL) were loaded into a stainless-steel flow cell (Mustang Coin module, Pall Corporation, Port Washington, New York, United States) equipped with two flow distributors to establish a uniform flow across all membranes. The membranes were equilibrated in the forward flow configuration in buffer A (adsorption buffer) for 10 min at 1 mL/min. A protein solution (1 mg/mL) was loaded onto the membrane for 10 min at a flow rate of 1 mL/min. Unbound proteins were then washed from the membrane surface using buffer A (adsorption buffer) for 5 min at 1 mL/min, followed by a step change to running buffer B (elution buffer; no salt) to the membrane at the same flow rate. The run was stopped until the UV absorbance at 280 nm was constant. The elution fraction and washing fraction (includes loading fraction) were collected, and the volumes were determined accordingly. Washing fraction, protein concentration in the sample solution, and elution fraction was calculated through UV absorbance at 280 nm.

3.2.6 Correlations from Statistical Analysis

Statistical design of experiments (DOE) studies provide an effective way for optimization and selection of experimental condition based on a model generating phenomenological model [32, 33]. The model was developed to optimize and predict the experimental flow conditions to achieve
best membrane performance. Further, the model was statistically validated using the regression coefficient, predicted responses, and statistical tests combined with the experimental data points performed as recommended in the design table. Two-level three-factorial was applied to investigate and validate process parameters influencing the protein binding capacity. The process parameters like ammonium sulfate concentration (0.6 to 1.8 M) in loading buffer and protein concentration (0 to 1 g/L) was considered in the model. The output parameter, protein binding capacity, was considered as the response. Coded symbols like $X_1$ and $X_2$, represents the salt concentration (M) and protein concentration (g/L), respectively (Table 1). In this study, the response and the independent variables were fitted with two factor interactions as below.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{12}X_1X_2$$

where $Y$ is the predicted value, $b_0$ is the constant, $X_1$ is the salt concentration, $X_2$ is the protein concentration, $b_1$ and $b_2$ are linear coefficients, $b_{12}$ is cross-product coefficients, $Y$ is response of protein binding capacity. All the calculations were performed using software package Design-Expert (Version 11; Stat-Ease, Minneapolis, MN). The statistical $R^2$-value as shown in eqn. (4) was used to be evaluate the accuracy of the regression equation.

$$R^2 = \sum (X_{exp} - X_{pred})^2 \quad (5)$$

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coded symbol</th>
<th>Actual values of coded levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt concentration (M)</td>
<td>$X_1$</td>
<td>0.6 1.2 1.8</td>
</tr>
<tr>
<td>Protein concentration (g/L)</td>
<td>$X_2$</td>
<td>0.1 0.5 1.0</td>
</tr>
</tbody>
</table>

Table 1 Coded Values of the Variables
3.3 Results and discussion

3.3.1 Physicochemical properties of HIC membranes

An RC fibrous membrane was obtained through hydrolysis when the acetyl group was converted into a hydroxyl group; subsequently, PVCL was grafted onto the fiber surface after immobilization of the BIB initiator. Several characterization methods were utilized to investigate the successful surface modification and functional groups present on HIC membrane in detail. FTIR characterization method was used to determine the stretching and bending frequencies of functional groups under IR light [32]. The FTIR spectra of the CA, RC, and surface modified membranes are presented in Figure 2. The homemade CA membrane exhibited a significant characteristic peak at around 1630 cm$^{-1}$, which was ascribed to the carbonyl (C=O) group [7]. As the CA membrane was modified to RC membrane, a new broad peak was observed at around 3400 cm$^{-1}$ and was attributed to the hydroxyl group. Moreover, the observed carbonyl group peak in case of CA membrane, vanished for the electrospun RC membrane obtained after hydrolysis. These observations confirmed the successful completion of the hydrolysis reaction. Each PVCL monomer contained a carbonyl group and upon polymerization the resultant polymer covered the membrane surface. Hence, a new carbonyl group peak and weaker hydroxy group peak were observed for the RC PVCL membrane upon successful grafting of the PVCL monomer on the membrane surface through ATRP [7]. The stretching vibrations of the N–H and O–H groups in RC PVCL membrane is corresponded to the broad bands at 3000–3700 cm$^{-1}$ [33]. The surface elemental composition was determined from the XPS analysis, and the corresponding spectra are presented in Figure 3. Elemental N on the RC PVCL membrane was contributed by the cyclic amide group on the PVCL monomer, and the signal of elemental O was weaker because the
polymerized PVCL overlapped with the surface. Combining the derived XPS and FTIR findings, it was confirmed that the PVCL was grafted successfully on the RC membrane through ATRP.

Figure 2 FTIR spectra of the CA, RC, and surface modified RC PVCL membranes.
Figure 3 XPS analysis spectra for unmodified RC membrane and RC-PVCL modified membrane.

Surface SEM images recorded for the CA, RC, and RC PVCL membranes are depicted in Figure 4, respectively to analyze any change in morphology during the surface modification reactions. The average fiber diameter of cellulose acetate ranged from 330 to 440 µm, similar to earlier reported study [25]. After the hydrolysis reaction, no significant alteration on the morphology RC membrane with uniformly smooth roughness was observed. However, significant amount of roughness was observed on the surface of the RC PVCL membrane, which could be attributed to the polymerization reaction. Moreover, the fiber arrangement changed significantly. During ATRP, the PVCL monomer generated free radicals and grafts on the surface through the BIB initiator. Additionally, PVCL created a crosslinking effect between fibers, which might have caused some of the fibers to be tied together resulting in a significant change in the morphology.
of the membrane. However, the main factor influencing efficiency was determined to be the adsorption ability of the surface functional groups rather than the membrane morphology.

Figure 4 SEM images of cellulose acetate nanofibers (a) regenerated cellulose nanofibers (b), and modified RC PVCL membrane (c).

Contact angle analysis is a practical approach for observing the hydrophobicity of a membrane [34]. An electrospun membrane typically exhibits a textile- and scaffold-like structure, conferring the membrane surface with hydrophobicity because of the existence of air gaps between air droplets and fibers. The contact angle analysis results derived in this study are illustrated in Figure 5. The electrospun CA membrane exhibited a contact angle of approximately 130°, whereas a flat-surface CA membrane had a contact angle of nearly 80° [35], which can be attributed to its scaffold-like structure. Therefore, the electrospun CA membrane was more hydrophobic than the flat-surface CA membrane. However, following hydrolysis treatment, the contact angle of the RC membrane was considerably reduced to approximately 18° mainly due to the increased attractive forces between the water molecules with the surface hydroxyl groups. The contact angle of the RC PVCL membrane was increased to approximately 63° after PVCL grafting was conducted. According to the results of our previous study, applying droplets of solutions with different salt concentrations to membranes revealed hydrophobic properties in the membranes. Upon increasing the salt concentration, significant reduction in LCST of the polymer was obtained. Furthermore, hydration of ions results in decrease in the LCST. A direct interaction between
cation-amide O cause first-shell of PVCL dehydration [36, 37]. In other words, an enhanced hydration free energy by increasing the salt concentration results in a decrease in the LCST. Additionally, distinct salt types exhibit a corresponding hydration free energy, which leads to differentiation in the capability of stabilizing the hydrophobic conformation of the polymer. This finding can be explained by the Hofmeister series [11, 38-40]. In this study, after an increase in the ammonium sulfate concentration (0 to 1.8 M), the contact angle gradually raised from approximately 63° to 120°, which is in agreement with the aforementioned principle.

![Figure 5](image)

**Figure 5** Under water air bubble contact angle measurement results as function of ammonium sulfate concentration. The contact angle in the results were converted to the water contact angle.

Grafting degree was calculated to determine the extent of grafting of a membrane compared with an unmodified membrane (Equation 1), which is widely used in grafting from modification,
including ATRP, UV photo grafting, and plasma grafting methods. In this study, the ATRP time was controlled to investigate changes in grafting degree. The grafting degree of the membranes increased rapidly during the first 3 h because of the less steric obstacle while the highest grafting degree (~10%) was observed at 12 h (Figure 6). However, the grafting degree of the membranes in the other condition still increased gradually, except for that at 5 h. Subsequently, static binding ability was investigated.

![Figure 6](image)

**Figure 6** Degree of grafting as function of polymerization time for PVCL grown on membrane.

### 3.3.2 Static binding and mechanism

Static binding experiments were performed using BSA as the model protein to evaluate the performance of the fabricated HIC membranes. In this context, the static binding capacity for BSA was evaluated as a function of ATRP time for RC-PVCL membranes as shown in **Figure 7**.
Ammonium sulfate (1.8 M) with 20mM phosphate was employed as the buffer, and the static binding time period was set to 5 h. The results revealed that the static binding capacity increased as the ATRP time increased due to the longer polymeric chain length. Beyond the ATRP time of 6 h, there was no significant increment in the protein binding capacity, and it became almost constant after 8 h. Generally, a shorter ATRP time is preferred by membrane manufacturers which results in lower producing cost. Furthermore, in order to understand the binding energies and their underlying mechanisms, the 1.8 M ammonium sulfate solution was employed to determine BSA static binding isotherms, as presented in Figure 8. The adsorption behavior of the electrospun HIC membranes could be described using the Langmuir–Freundlich isotherm model. The Langmuir–Freundlich model combines the advantages of the Langmuir and Freundlich models. The isotherm tends to favor the Langmuir model when $n_{LF}$ is 1, which is the equation for determining adsorption affinity (Equation \( q = \frac{Q_{\text{max}} (a_{LF} C_{eq})^{n_{LF}}}{1 + (a_{LF} C_{eq})^{n_{LF}}} \) (5)). Interestingly, the isotherm favors the Freundlich model when $n_{LF}$ approaches 0. Therefore, a homogenous or heterogenous binding system can be modeled using a Langmuir–Freundlich isotherm [41, 42]. Results of static binding capacity experiments was successfully fitted in the Langmuir–Freundlich model according to the following equation:

\[
q = \frac{Q_{\text{max}} (a_{LF} C_{eq})^{n_{LF}}}{1 + (a_{LF} C_{eq})^{n_{LF}}} \quad (5)
\]

where $q$ represents the amount of proteins adsorbed at equilibrium (mg/mL membrane volume), $Q_{\text{max}}$ represents the maximum adsorption capacity of a membrane (mg/mL membrane volume), $C_{eq}$ represents the aqueous-phase concentration at equilibrium (mg/mL), $a_{LF}$ represents the adsorption affinity constant (mL/mg), and $n_{LF}$ represents the heterogeneity index. In this study, the $R^2$ value corresponding to the static binding capacity of the electrospun HIC membrane was
0.9988. In addition, the binding mechanism indicated towards a Langmuir isotherm pattern as evident from the heterogeneity index approaching towards a value of to 1. The fact indicated that the binding mechanism is favorable to monolayer adsorption. Each binding site can be occupied by at most one BSA molecule. The maximum binding capacity regressed from the Langmuir–Freundlich model matched well with the experimental data as depicted in Figure 8.

![Figure 7](image-url) **Figure 7** Static binding capacity as function of polymerization time for PVCL grown on membrane.
Figure 8  Langmuir-Freundlich curve for ATRP 6 h PVCL modified membrane.

3.3.3 The statistical analysis of HIC membrane

A detailed analysis was performed to evaluate the effects of the feed conditions on binding capacity as prescribed from DOE studies in Table 2. Table 3 summarizes obtained binding capacity for the experiments. Response surface methodology (RSM) was employed to optimize the feed conditions to obtain an empirical relationship between binding capacity and the two independent variables which can be expressed as per the following regression eqn. (6):

\[ Y = 13.33 + 5.13X_1 + 8.79X_2 + 3.0X_1X_2 \]  

The statistical significance of the above equation was verified using the standard analysis of variance (ANOVA) method for the phenomenological model as presented in Table 4. The model $F$-value of 47.52 and the values of probability (P) > F (0.0001) implied that the interaction
terms considered were statistically significant. The coefficient of determination \((R^2)\) was determined to be 0.96 for binding capacity, indicating that the statistical model could be used to explain 96% of the variability in the response and only about 4% of total variation cannot be attributed to the independent variables. These results further confirm that the model is strong and can be used to predict the responses in the range of the process parameters considered in this study.

**Figure** depicts the observed binding capacity versus the predicted responses from the empirical model. As evident from **Figure**, the predicted data points based on the responses from empirical model agree well with the measured protein binding capacity results in the range of the operating variables. Furthermore, the F- and p-values reveal the significance of the independent parameters in the order: protein concentration > salt concentration. The response surface curves were plotted to examine the interaction of the variables and to determine the optimum level of each variable for maximum response. The effects of salt concentration and protein concentration on the binding capacity are presented in

**Figure**. It is evident from Figure 10 that at low salt concentration range, protein binding capacity would be significantly low. Significant improvement in the binding capacity could be obtained by increasing the protein concentration and salt concentration. This could be attributed to the promoted aggregation and precipitations of protein in presence of high salt concentrations. As a result of disruption of the hydration barriers between protein molecules, higher amount of salt causes water molecules surrounding the protein to move into the bulk solution. The hydrophobic zones of the protein surface thus become exposed, providing sites of attraction between neighboring protein molecules. Therefore, with increase in protein concentration, a high attractive binding between protein molecules often leads to a significant increase in binding capacity. The predicted relatively low binding capacity in low salt concentration could be explained using low
level of LCST transition of PVCL. This observation is in good agreement with contact angle results. The membrane surface is relatively hydrophilic at 0.6 M salt concentration buffer (Figure 5). Furthermore, the coefficient of empirical relation indicated the increment in the salt concentration, protein concentration as well as interaction between protein and salt concentration demonstrated positive impact on the binding capacity. However, the irreversible protein aggregation under high salt concentration is predicted to attribute to the limited protein recovery.

Table 2 The binding capacity under different feed conditions.

<table>
<thead>
<tr>
<th>Run No.</th>
<th>Salt conc. (M)</th>
<th>Protein conc. (g/L)</th>
<th>Binding capacity (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>1</td>
<td>12.65</td>
</tr>
<tr>
<td>2</td>
<td>1.2</td>
<td>0.5</td>
<td>11.61</td>
</tr>
<tr>
<td>3</td>
<td>1.2</td>
<td>0.1</td>
<td>5.79</td>
</tr>
<tr>
<td>4</td>
<td>1.8</td>
<td>0.1</td>
<td>7.95</td>
</tr>
<tr>
<td>5</td>
<td>0.6</td>
<td>0.1</td>
<td>1.89</td>
</tr>
<tr>
<td>6</td>
<td>1.8</td>
<td>1</td>
<td>30.69</td>
</tr>
<tr>
<td>7</td>
<td>1.8</td>
<td>0.5</td>
<td>16.55</td>
</tr>
<tr>
<td>8</td>
<td>1.2</td>
<td>1</td>
<td>25.05</td>
</tr>
<tr>
<td>9</td>
<td>1.2</td>
<td>0.5</td>
<td>11.28</td>
</tr>
<tr>
<td>10</td>
<td>0.6</td>
<td>0.5</td>
<td>9.88</td>
</tr>
</tbody>
</table>
Table 3 Estimated regression co-efficient obtained from Analysis of variance (ANOVA) for the model study for the model.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F ratio</th>
<th>Prob &gt; F</th>
<th>R²</th>
<th>Adjusted R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>657.62</td>
<td>3</td>
<td>219.21</td>
<td>47.52</td>
<td>0.0001</td>
<td>0.9596</td>
<td>0.9394</td>
</tr>
<tr>
<td>Error</td>
<td>0.0545</td>
<td>1</td>
<td>0.067</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>658.29</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4 Significance of the regression coefficient in terms of F-values obtained from ANOVA study.

<table>
<thead>
<tr>
<th>Variable Terms</th>
<th>F Ratio</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁-Salt conc.</td>
<td>34.21</td>
<td>0.0011*</td>
</tr>
<tr>
<td>X₂-Protein conc.</td>
<td>100.58</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

*p-value less than 0.05 indicates model terms are significant.
**Figure 9** Correlation between the predicted binding capacity and experimentally determined actual binding capacity for DOE studies.
3.3.4 Dynamic binding and mechanism

Further, dynamic binding tests were conducted using BSA and IgG to evaluate the performance of the fabricated HIC membranes. All these experiments were conducted using a variety of feed streams at 0.1 and 1 g/L BSA. As mentioned earlier, the polymer chain length was found to be the key factor influencing binding capacity. In Table 5, any increment in the grafting degree has a positive impact on the BSA binding, however, a significant reduction in the recovery was observed. The results are in good agreement with the fact that the higher grafting degree was inferior to the protein recovery [7]. A longer polymer chain is unfavorable for polymeric conformation changes during LCST switching leading to limited recovery. Also, the longer
polymer chain length may entrap the protein during polymer transition resulting in inefficient protein desorption, thus, a significantly lower recovery was obtained. Therefore, even with increasing binding capacity beyond ATRP time of 6 h, the corresponding membrane was chosen for the subsequent experiments for its optimal performance considering both the factors.

Table 5 Recovery and dynamic protein binding capacity based on grafting degree and protein loading concentrations.

<table>
<thead>
<tr>
<th>Grafting degree (%)</th>
<th>BSA loading concentration (g/L)</th>
<th>Protein binding capacity (mg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>0.1</td>
<td>7.95 ± 0.75</td>
<td>98.17 ± 0.02</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>30.69 ± 0.34</td>
<td>51.54 ± 1.96</td>
</tr>
<tr>
<td>9</td>
<td>0.1</td>
<td>14.29</td>
<td>80.64</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>43.43</td>
<td>21.09</td>
</tr>
</tbody>
</table>

In the scope of the present study, the effect of polymer density was investigated in detail. Various initiator concentrations of 40, 80, and 200 mM was used at a fixed immobilization time of 3 h. Each condition was performed duplicate, and the derived results were averaged and are reported herein. The membrane treated with the initiator concentration of 80 mM, exhibited the highest dynamic binding performance under various feed streams (Table 6). Membranes with a low polymer density (40 and 80 mM) exhibited high recovery (>95%) when lower amount of BSA was present in feed stream. A membrane with high polymer density (200 mM) was associated with a high protein binding capacity but a low recovery rate in presence of lower concentration of BSA concentration in feed. Also, the protein entrapment into the structure of the fiber is likely to be enhanced at high ligand density, which explains the low recovery yield. Increased amounts of steric hindrance lead to a conformation change in the polymer. A high initiator concentration was
associated with a high polymer density, and a high polymer density engendered higher steric hindrance, which could inhibit swelling and extend the hydration chains in low-salt-concentration buffer. A high chain density is likely to restrict PVCL conformational transition [7]. This could lead to a lower recovery rate due to unfavorable BSA desorption. Furthermore, the improvement in the binding capacity associated with the increase in loading concentration could be attributed to the dispersion BSA molecule leading to a strong attractive irreversible protein binding with the membrane and, hence, low recovery (Table 7). Mono-layer adsorption in the presence of less amount of bound protein leads to high recovery. There is likely a trade-off between the dynamic capacity and recovery. Thus, further experiments were performed with membranes treated using an initiator concentration of 80 mM.

Earlier studies [7] have indicated that binding capacity is affected by the relationship between polymer chain length and density. Herein, a related study was performed investigating the effect of polymer density on binding capacity. PVCL was directly deposited on a 0.45-µm flat surface RC membrane. The optimal polymer density determined within 5 h of immobilization could result in the most favorable binding capacity and recovery. The 90% dynamic binding capacity was obtained to be 4.8 mg/mL, and the corresponding recovery was 96% [17]. At a low feed concentration, a high protein binding capacity and high recovery were observed. Moreover, a comb-like PVCL HIC membrane, which was modified on 0.45- and 1-µm RC membranes, was developed [7]. A high density and short chain could enhance the binding capacity but reduce the overall recovery. A dynamic binding capacity of 12.6 mg/mL and recovery of 78% could be obtained when 1 g/L BSA was used under the improved modification condition. Therefore, the use of an electrospun membrane as a substrate plays a crucial role in improving the binding capacity of an HIC membrane. The improved dynamic binding capacity of 30.69 mg/mL could be achieved
through the use of an electrospun HIC membrane. A high protein binding capacity at a high feed concentration and a high recovery rate at a low feed concentration were also observed in this study. This fact could be explained by a weak attraction between BSA could improve irreversible protein binding in case of feed with lower concentration of BSA.

Table 6 Recovery and dynamic protein binding capacity based on various initiator and protein loading concentrations.

<table>
<thead>
<tr>
<th>Initiator conc.(mM)</th>
<th>BSA loading concentration (g/L)</th>
<th>Protein binding capacity (mg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40mM</td>
<td>0.1</td>
<td>4.79 ± 0.11</td>
<td>97.54 ± 2.27</td>
</tr>
<tr>
<td>40mM</td>
<td>1</td>
<td>24.95 ± 0.56</td>
<td>57.24 ± 2.33</td>
</tr>
<tr>
<td>80mM</td>
<td>0.1</td>
<td>7.95 ± 0.75</td>
<td>98.17 ± 0.02</td>
</tr>
<tr>
<td>80mM</td>
<td>1</td>
<td>30.69 ± 0.34</td>
<td>51.54 ± 1.96</td>
</tr>
<tr>
<td>200mM</td>
<td>0.1</td>
<td>6.22 ± 0.11</td>
<td>83.39 ± 0.22</td>
</tr>
<tr>
<td>200mM</td>
<td>1</td>
<td>28.38 ± 1.75</td>
<td>41.44 ± 0.01</td>
</tr>
</tbody>
</table>

Table 7 Recovery and dynamic protein binding capacity based on protein loading concentrations under 80mM initiator concentration ATRP 6h.

<table>
<thead>
<tr>
<th>BSA loading concentration (g/L)</th>
<th>Protein binding capacity (mg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>7.95 ± 0.75</td>
<td>98.17 ± 0.02</td>
</tr>
<tr>
<td>0.3</td>
<td>9.93</td>
<td>91.56</td>
</tr>
<tr>
<td>0.5</td>
<td>16.55 ± 2.09</td>
<td>75.67 ± 3.32</td>
</tr>
<tr>
<td>1</td>
<td>30.69 ± 0.34</td>
<td>51.54 ± 1.96</td>
</tr>
</tbody>
</table>

The effect of flow rate on the dynamic protein binding capacity and recovery is presented in Table 8. Such an effect exerted on the binding capacity of the electrospun HIC membrane was
limited as expected characteristics of such membranes. The observed dynamic binding capacities were within the error bar range as the flow rate was increased from 0.5 mL/min to 2 mL/min. However, the recovery rate reduced as the flow rate was increased. It could be attributed to the fact that the lower flow rate might be favorable for swelling of the polymer swelling leading to more protein desorption. The effect of fiber diameter on the protein binding capacity and recovery was investigated. Table 9 listed the natural properties of two different fabrication condition membranes. Because of the higher voltage applied, the diameter of fiber was along with reduced. The modification condition of these two membranes remained at 80mM initiator concentration and ATRP 6h. The membrane with thinner fibers exhibits smaller pore sizes, which could be due to the tightly packed of fibers leading to smaller pore sizes. The relatively high protein binding capacity and low recovery were observed with more delicate fibers membrane. Such observation suggests that this can be due to an increase in the grafting density leading to improved binding capacity and a higher number across points of fibers resulting in a limited recovery. The across points of fibers would entrap the protein cause finite protein recovery.

Table 8 Flowrate-dependent protein binding and recovery under 80mM initiator concentration and ATRP 6h.

<table>
<thead>
<tr>
<th>BSA loading concentration (g/L)</th>
<th>Flowrate (mL/min)</th>
<th>Protein binding capacity (mg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>28.87 ± 0.79</td>
<td>57.51 ± 1.23</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>30.69 ± 0.34</td>
<td>51.54 ± 1.96</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>27.36 ± 0.93</td>
<td>41.72 ± 1.80</td>
</tr>
</tbody>
</table>
Table 9 The effect of fiber diameter on protein binding capacity and recovery.

<table>
<thead>
<tr>
<th>Polymer conc. (%)</th>
<th>Voltage (kV)</th>
<th>Fiber diameter (nm)</th>
<th>Pore size (um)</th>
<th>BSA loading conc. (g/L)</th>
<th>Protein binding capacity (mg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>1</td>
<td>330-440</td>
<td>3.24±0.27</td>
<td>0.1</td>
<td>7.95 ± 0.75</td>
<td>98.17 ± 0.02</td>
</tr>
<tr>
<td>13.8</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>30.69 ± 0.34</td>
<td>51.54 ± 1.96</td>
</tr>
<tr>
<td>20</td>
<td>0.1</td>
<td>220-350</td>
<td>2.88±0.39</td>
<td>1</td>
<td>33.58</td>
<td>79.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Additional experiments were used to determine the binding capacity of IgG in the feed containing 1.8M ammonium sulfate (Table 10). An average binding capacity of 16 mg/mL and recovery of 85% was obtained under the operating condition of 0.1 g/L IgG. In addition, an average binding capacity of 47 mg/mL was achieved when 1 g/L IgG was loaded. Himstedt et al. [17] observed a IgG capacity of 21 mg/mL, whereas Liu et al. [2] reported a IgG capacity of 12 mg/mL. Therefore, the higher dynamic binding capacity of the electrospun HIC membrane reported in this study is a considerable breakthrough. These results highlight the potential benefits derived from designing a responsive electrospun HIC membrane. The observed dynamic binding capacity of IgG was obtained to be higher than that of BSA which could be attributed to the considerably higher hydrophobicity of IgG as compared to BSA. Protein hydrophobicity is essential in protein separation for obtaining HIC membranes.
Table 10 Dynamic protein binding capacity and recovery results for IgG.

<table>
<thead>
<tr>
<th>IgG loading concentration (g/L)</th>
<th>Protein binding capacity (mg/mL)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>16.31 ± 0.23</td>
<td>85.32 ± 1.38</td>
</tr>
<tr>
<td>0.5</td>
<td>28.78 ± 0.18</td>
<td>23.95 ± 1.17</td>
</tr>
<tr>
<td>1</td>
<td>47.68 ± 1.29</td>
<td>14.22 ± 1.93</td>
</tr>
</tbody>
</table>

A high binding capacity at a high feed protein concentration is beneficial to biopharmaceutical protein purification. The development of a tailored responsive ligand cooperating with an electrospun membrane enables the efficient fractionation of proteins and overcomes binding limitations. An electrospun membrane with high surface area to volume ratio provides a favorable platform which is not hampered by the inherent restrictions in flat-surface membranes. Specific proteins can be fractionated at distinct ionic strengths due to their different degrees of hydrophobicity.

3.4 Conclusions

A responsive HIC membrane was fabricated using electrospinning method which exhibited high protein binding performance. Further, thermoresponsive polymer, PVCL, was grafted on electrospun RC membranes which served as tentacles. PVCL conformation could be switched by changing the ionic strength, resulting in a polymer with higher hydrophobicity. A high protein binding capacity of over 30 mg/mL could be achieved. However, the recovery rate was reduced at a high protein loading. Easy dispersion of BSA leads to stronger interaction between the protein molecules. The electrospun HIC membrane was noted to efficiently purify proteins at a low feed concentration. Unlike flat-surface membranes, electrospun membranes offer high dynamic
capacities because of their high surface-to-volume ratio, which provides more room for grafting polymer chains. A highly efficient, electrospun, responsive HIC membrane has promising application for downstream purification processes in the field of biopharmaceuticals.

Acknowledgements

The authors gratefully acknowledge the financial support for this research provided by University of Arkansas, USA.
References


Chapter 4. High Performance Mixed-Matrix Electrospun Membranes for Ammonium Removal from Wastewaters

Abstract

Mixed-matrix electrospun membranes were developed to investigate ammonium removal from aquaculture wastewaters for the first time from wastewaters. Particles derived from the inexpensive zeolite 13X were successfully incorporated into polyethersulfone (PES) matrices. The fabricated mixed-matrix electrospun membranes demonstrate high ammonium removal capacity reaching over 90 mg/g_{zeolite}, more than 4 times higher than the previously fabricated mixed-matrix membranes via phase inversion. Moreover, the membranes fabricated exhibit high permeability and ease of regeneration. Over 90% of total ammonium nitrogen (TAN) can be removed from low TAN wastewaters such as aquaculture wastewaters. In addition to zeolite 13X, other zeolite particles including zeolite Y, zeolite 3A and 4A were incorporated into the membrane matrix. The inexpensive zeolite 13X show the highest ammonium exchange capacity.
4.1 Introduction

The biological production of ammonia (NH$_3$) is part of the natural metabolic process across all the fauna from humans to fish. Ammonia is a major protein metabolite released to the environment. It is also an essential nutrient for plants and a key component of the nitrogen cycle. Depending on the acidity or pH of the aquatic system, the equilibrium between the molecular form (NH$_3$) or protonated form (NH$_4^+$) can shift dramatically with one or the other being the dominant species. Environmental protection agency (EPA) regulates the discharging limit of total ammonia nitrogen (TAN) including both NH$_4^+$ and NH$_3$ from the wastewater treatment plants. Municipal wastewater treatment typically employs aerobic process to convert TAN to nitrite (NO$_2^-$) and further to nitrate (NO$_3^-$). Nitrate is ultimately converted to N$_2$ and released to air via the anaerobic process. The biological conversion process is generally effective once established but slow. However, it does take a long time to establish the bacterial culture during the initial start-up phase. In addition, it may be ineffective when there is a sudden surge of TAN in the wastewater as biological conversion is slow.

Ammonia is released to the aquaculture water during protein metabolism by fish and via bacterial digestion of organic matter. Ammonia is rather toxic to fish and has to be maintained below 0.05 mg/L or ppm. This puts the maximum TAN in aquaculture water to be ~1.5 ppm at neutral pH. In order to maintain this level of TAN, the aquaculture water has to be often partially replaced with fresh water. Zeolite materials have been employed for the removal of ammonium ion from wastewaters [1-6]. Previous studies indicate that the inexpensive natural zeolite 13X can perform ion exchange with ammonium ion with good capacity [6]. However, zeolite 13X is not stable in water and cannot be applied effectively as an ammonium absorbent. Our previous work [7] has shown that a mixed-matrix membrane incorporating cheap natural zeolite 13X particles
can effectively remove NH$_4^+$ from aquaculture wastewater via ion-exchange to reach TAN concentration below 1 ppm. No leach of zeolite materials has been observed. Three different methods were used to fabricate the mixed-matrix membranes and fibers. One involves phase inversion by mixing the zeolite particles with the polysulfone (PSU) in 1-methyl-2-pyrrolidinone (NMP). The maximum amount of zeolite loading incorporated without compromising the mechanical property of the membrane is 50% of the PSU. Our results indicate that the higher the amount of zeolite particles incorporated, the higher the capacity of the mixed-matrix membrane for removing ammonium ion via ion-exchange. The maximum capacity reached is 19.8 mg of TAN per g of zeolite (mg/g$_{\text{zeolite}}$) for the membrane with 15% PSU and zeolite loading of 50% PSU. The second approach is the formation of zeolite-containing fibers by mixing the zeolite with PSU in NMP similar to the preparation of mixed matrix membrane. The prepared solution was then dispensed into water via a syringe. These fibers exhibit a maximum ammonium exchange capacity of 5.4 mg/g$_{\text{zeolite}}$ at 15% PSU and zeolite loading of 50% PSU. The third approach involves the pore-filling of zeolite through the supporting structure from the back of an existing 30 kD polyethersulfone (PES) membrane. The maximum capacity reached is 10.4 mg/g$_{\text{zeolite}}$. In all these approaches, it seems that the capacity is limited by the amount of zeolite particles incorporated.

In order to incorporate more zeolite particles into the membrane matrix, here electrospinning technique has been used to fabricate mixed-matrix membranes. Electrospun membranes demonstrate a high surface-volume ratio and high porosity. As a result, the membranes fabricated typically demonstrate high capacity and high permeability. Previously we fabricated weak anion exchange electrospun membranes for protein purifications [8]. Here mixed matrix
electrospun membranes are fabricated for ammonium removal from synthetic wastewater containing both low and high TAN concentration levels.

4.2 Material and methods

4.2.1 Materials

Polyethersulfone (PES; MW ~60,000) in powder form as membrane material was purchased from BASF (Ludwigshafen, Germany). N, N-dimethylformamide (DMF) (≥99.8% ACS grade), sodium hypochlorite (12.5% in aqueous solution) and sodium chloride (NaCl) (ACS grade) were purchased from VWR (Radnor, PA). Ammonium chloride (≥99%) was purchased from Alfa Aesar (Haverhill, MA). Liquid phenol (> 89%) was purchased from Sigma-Aldrich (St. Louis, MO). Sodium nitroprusside dihydrate (≥98%) was purchased from MP biomedicals (FKA ICN BIOMED) (Santa Ana, CA). Sodium hydroxide (≥98%, ACS grade) was purchased from Amresco (Solon, OH). Sodium citrate dehydrate (≥99%) was purchased from J.T. Baker (Phillipsburg, NJ). All chemicals were used without further purifications. Zeolite 13X molecular sieve (Stream Chemicals, 1/16” pellets (Linde 13X)). Zeolite Y, sodium, molecular sieves 4A powder, and molecular sieves 3A powder were purchased from Alfa Aesar (Haverhill, MA). Deionized water (DI) was obtained from Milli-Q ultrapure water purification system (Millipore, Billerica, MA). A commercial PAN membrane, (MWCO 400,000, Ultura) which was used as a backing layer for spinning nanofibers, provided by Ultura (Oceanside, CA).

4.2.2 Fabrication of Mixed-Matrix Nanofibrous Membranes

Zeolite 13X particles were grinded mechanically to obtain fine a powder. After grinding, particle size of 13X was measured using laser diffraction and shown in Figure 1. The majority of the particles are between 9-12 μm. An electrospinning process was used to fabricate PES
nanofibers. Zeolite 13X and PES powders with a fixed weight ratio were dissolved in DMF under stirring at 70°C. Thereafter a homogeneous casting solution was obtained after 24 h of stirring and mixing. The solution was then injected into a syringe pump.

Nanofibers were deposited onto the backside of the PAN membrane during the electrospinning process. Here the syringe pump flow rate is fixed at 0.5 mL h$^{-1}$. The applied voltage was kept at 25 kV and the distance between the needle tip and fiber collector is 25 cm. These are critical parameters to control the fiber diameter and the properties of the fibrous membranes formed. The x%PES–y%zeolite was used to denote the membranes with different compositions, where x represents the wt% of PES to the polymer-solvent mixed casting solution, y represents the wt% of 13X with respect to PES in the mixture.

![Figure 1 Typical zeolite 13X particle size distribution measured by laser diffraction.](image)

**4.2.3 Characterization**

**4.2.3.1 Particle Size Analysis**

The particle size distribution of typical mechanically grinded zeolite 13X particle suspension solution was characterized using a laser diffraction particle size analyzer (LS 13 320, Beckman...
Coulter, Inc., USA). The suspension solution was placed into sonication bath for 1 hour before analysis.

### 4.2.3.2 Membrane Morphology

The membrane surface and cross-section morphology were investigated by field-emission scanning electron microscopy FESEM Model S-4800 (Hitachi Co., Tokyo, Japan) with 1 kV electron beam. The membrane samples were dried in vacuum oven at 40°C at least 24 h to remove residue solvent and moisty before sample preparation. Also, the membrane dipped in liquid nitrogen for few seconds to obtain uniform cross-section. Further, the membranes were cut into appropriate size and mounting on the SEM sample holder by carbon conductive tabs (Ted Pella, Redding, CA).

### 4.2.4 Performance of Mixed Matrix Membranes

Filtration experiments were performed using a stirred normal filtration cell (Sterlitech corporation, Kent, WA) connected to a pressurized feed vessel from a nitrogen tank. During filtration, the feed solution was stirred to minimize concentration polarization. Prior to each test, the membrane coupons were compacted by filtering DI water until a steady permeate flux was obtained. The permeate flux was calculated based on the time-derivative of the permeate mass, which was measured by collecting the filtered water on a digital balance (Mettler Toledo, PL602-s) connected to a computer.

To evaluate membrane performance, 7 to 60 ppm TAN feed solutions containing ammonium chloride (NH4Cl) were filtered through the compacted membranes using the dead-end filtration cell. Filtration pressure was adjusted for testing different membranes in order to control the permeate flux and subsequently the residence time for ammonium ion exchange. Multiple permeate samples were collected for each membrane to determine its ammonium removal capacity.
TAN concentration in the sample was determined via phenol hypochlorite method [9] using a UV–VIS spectrophotometer (Thermo Scientific™ GENESYS 10S UV-Vis). TAN removal rate was calculated by the concentration of TAN in the filtrate collected divided by the concentration of TAN in the feed using the following equation:

\[
TAN\,\text{removal}\,\% = \frac{C_{\text{permeate}}}{C_{\text{feed}}} \times 100
\]  

(1)

where \(C_{\text{filtrate}}\) and \(C_{\text{feed}}\) represent the concentration of TAN in the permeate and feed solutions respectively. TAN removal capacity was determined by calculating the total amount of TAN removed over the course of filtration experiment divided by the amount of zeolite incorporated in the membrane in mg/g using the following equation:

\[
\text{TAN\,Removal\,Capacity} = \frac{\sum C_{\text{permeate}} \times V_{\text{permeate}}}{W_{\text{zeolite\,13X}}}
\]  

(2)

4.3 Results and discussion

4.3.1 The Effects of Zeolite Loading on TAN Removal

In order to investigate the effects of zeolite 13X loading on ammonium removal, mixed matrix membranes were fabricated incorporating zeolite 13X equivalent to 0, 50 and 100 wt% with respect to PES in the casting solution. The filtration experiments were conducted using 45 mm in diameter cut membrane coupons from the fabricated electrospun nanofiber membranes. Prior to ammonium exchange experiments, membranes were compacted using DI water until a stable flux was obtained. Since the mechanism for ammonium removal from the feed solution is via ion-exchange process, residence time of the feed solution with the membrane is an important parameter. Based on the DI water fluxes of the fabricated mixed matrix membranes and our earlier studies [7], a flux of about 70 LMH can ensure sufficient time for the ion-exchange process while
maintaining the efficiency of the filtration process. More details on the effects of flux on ammonium removal will be discussed in the later section.

**Figure 2** Percentage of TAN removed as a function of filtrate volume collected for three mixed matrix membranes incorporating 0, 50 and 100 wt% zeolite 13X with respect to PES polymer in the performance of membrane with various zeolite content.

Here a flux of 70 LMH was maintained by adjusting the pressure for all three membranes with different zeolite 13X loadings. Figure 2 shows the percentage of TAN removed as a function of the filtrate volume collected for three membranes. All feed solutions contain 15 ppm TAN. Permeate samples were analyzed for each 50 mL collected. The 20%PES-0%Zeolite13X membrane without incorporating zeolite13X showed less than 20% TAN removal during the first 200 mL of filtrate collected. Thereafter TAN removal quickly decreases to almost 0 for the remaining 300 mL of filtrate collected. This indicates that PES membranes possess some negative charge sites and could perform limited ion-exchange with the feed solution with TAN. However, its TAN removal capacity is very limited. When 50 wt% of zeolite 13X was incorporated into the membrane (20%PES-50%Zeolite13X), the membrane showed over 97% TAN removal during the
first 100 mL of filtrate. However, TAN removal rate quickly decreased during the next 200 mL of filtrate to just over 20%. The TAN removal rate continued to decrease during the remaining 200 mL of filtrate. However, when 100 wt% of zeolite 13X was incorporated into the membrane (20%PES-100%Zeolite13X), TAN removal rate consistently reached over 95% for the first 250 mL permeate collected. Thereafter, TAB removal rate decreased to about 93% at 300 mL filtrate and ~52% at 500 mL reaching ~12% at 800 mL filtrate. It can be seen that the amount of TAN removed is directly proportional to the amount of zeolite 13X incorporated into the membrane in agreement with our previous findings [7].

**Figure 3** SEM images of cross-sectional surfaces (top panel) and top surfaces (bottom panel) of fabricated mixed matrix membrane (20%PES-100%Zeolite13X) with increased left to right magnifications.

Figure 3 shows the SEM images of the cross-sectional surfaces (top panel) and membrane top surfaces (bottom panel) for one of the fabricated MMMs at 20%PES-100%Zeolite13X
composition. The magnification increases from left to right for the images taken. The zeolite particles can be visibly seen. Fiber diameters have also been measured based on these images as shown in Figure 4. Electrospun nanofiber diameters varied from 200 to 900 nm with the highest percentage appeared between 400–500 nm. The diameters were measured without taking zeolite particles into consideration since these particles are much larger in the μm scale.

![Figure 4](image)

**Figure 4** The nanofiber diameter distribution of electrospun 20%PES-100%Zeolite13X membranes based on the images from SEM.
4.3.2 The Effects of Filtration Flux on Ammonium Exchange and Removal

Since the mechanism for ammonium ion removal from the wastewaters is ion-exchange, both the kinetics and thermodynamics of the ion-exchange process are important and need to be studied. Here electrospun MMMs with the same composition of 20%PES-100%Zeolite13X were fabricated with approximately the same weight as shown in Table 1. These membranes were tested with 6 ppm TAN synthetic wastewaters at a flux of 70, 140, 210 and 280 LMH respectively. The percentage of TAN removed in every 50 mL filtrate was measured and plotted as a function of the feed volume as shown in Figure 5. Different flux values represent different kinetics for the ammonium ion exchange process. A total of 2000 mL feed volume was tested. It can be seen that for ammonium removal rate reached over 90% during the initial 650-850 mL of the feed depending on the flux. Thereafter, the flux declined rapidly over the next 1000 mL of the feed and eventually
reduced to less than 10% at the last 50 mL of the filtrate collected. However, there are some differences between experiments with different fluxes. The total amount of TAN removed for each experiment was calculated and shown in Table 1. Since the actual membrane weight thereby its zeolite 13X content is slightly different, the total TAN amount removed does not necessarily reflect the effect of flux on the TAN removal capacity. The TAN removal capacity in mg over the total amount of zeolite 13X incorporated in the membrane in g was shown in the last column in Table 1. It can be seen that the lowest flux at 70 LMH exhibited the highest TAN removal capacity reaching 53.7 mg/g. The removal capacity reduced slightly to 52.2 and 52.4 mg/g for the 140 and 210 LMH experiments respectively. The TAN removal capacity reduced much more to 48.8 mg/g for filtration experiment at 280 LMH flux. For the remaining experiments, flux of 70 LMH was kept for all the remaining experiments.

**Table 1** The TAN Removal Capacity as a Function of Filtration Flux

<table>
<thead>
<tr>
<th>Membrane Composition</th>
<th>Membrane Weight (g)</th>
<th>Flux (LMH)</th>
<th>Total TAN Removed (mg)</th>
<th>TAN Removal Capacity (mg/g zeolite)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% PES - 100% Zeolite13X</td>
<td>1.18 ± 0.06</td>
<td>70</td>
<td>31.7 ± 2.26</td>
<td>53.7 ± 3.83</td>
</tr>
<tr>
<td>20% PES - 100% Zeolite13X</td>
<td>1.18 ± 0.09</td>
<td>140</td>
<td>30.8 ± 1.02</td>
<td>52.2 ± 1.73</td>
</tr>
<tr>
<td>20% PES - 100% Zeolite13X</td>
<td>1.10 ± 0.09</td>
<td>210</td>
<td>28.8 ± 1.89</td>
<td>52.4 ± 3.44</td>
</tr>
<tr>
<td>20% PES - 100% Zeolite13X</td>
<td>1.05 ± 0.10</td>
<td>280</td>
<td>25.6 ± 2.53</td>
<td>48.8 ± 4.87</td>
</tr>
</tbody>
</table>
4.3.3 The Effects of TAN Concentration on Its Removal Capacity

The effects of feed concentration on the dynamic exchange capacity were also investigated. The feed solutions containing 7, 15, 30 and 60 ppm TAN concentrations were filtered through freshly fabricated membranes with 20%PES-100%Zeolite13X with similar weights. The TAN removal rate was plotted as a function of filtration feed volume passed through the membrane. The flux was kept at 70 LMH based on previous studies. It can be seen that TAN removal rate reduced substantially as the TAN concentration in the feed multiplies. Table 2 lists the weights of the membranes fabricated, the corresponding feed concentrations, the total amount of TAN removed and TAN removal capacity in mg of TAN per gram of zeolite 13X incorporated in the membrane. It can be seen that the dynamic exchange capacity does not change much when the TAN concentration increases. The ammonium ion exchange seems to be rather rapid and is not affected by the high amount of TAN present in the feed. This is consistent with the previous flux data that TAN removal is a rather rapid process and that only a slight decrease in the capacity was observed even at the highest filtration flux of 280 LMH. This also indicate the potential application of these MMMs for the removal of TAN at low concentration feed streams such as aquaculture wastewaters, but also for other industrial processes producing high TAN feed streams.
Figure 6 The effects of TAN concentration in the feed on its removal rate at LMH 70 for the fabricated membranes with the same composition.

Table 2 TAN removal capacities of fabricated MMMs with the composition 20%PES-100%Zeolite13X at feed concentrations of 7, 15, 30 and 60 ppm respectively.

<table>
<thead>
<tr>
<th>Membrane Composition</th>
<th>Weight (g)</th>
<th>Flux (LMH)</th>
<th>Feed Conc. (ppm)</th>
<th>Total TAN Removed (mg)</th>
<th>TAN removal capacity (mg/g_{zeolite})</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%PES-100%Zeolite13X</td>
<td>1.15 ± 0.09</td>
<td>70</td>
<td>7</td>
<td>31.7 ± 1.38</td>
<td>55.1 ± 2.38</td>
</tr>
<tr>
<td>20%PES-100%Zeolite13X</td>
<td>1.16 ± 0.07</td>
<td>70</td>
<td>15</td>
<td>31.5 ± 1.52</td>
<td>54.4 ± 2.62</td>
</tr>
<tr>
<td>20%PES-100%Zeolite13X</td>
<td>1.20 ± 0.13</td>
<td>70</td>
<td>30</td>
<td>31.1 ± 2.17</td>
<td>51.8 ± 3.62</td>
</tr>
<tr>
<td>20%PES-100%Zeolite13X</td>
<td>1.17 ± 0.11</td>
<td>70</td>
<td>60</td>
<td>31.4 ± 2.01</td>
<td>53.7 ± 3.44</td>
</tr>
</tbody>
</table>
4.3.4 The Effects of Membrane Regeneration on Its Performance

The previous studies on the effects of filtration flux and feed concentration on the TAN removal rate and TAN removal capacity for the fabricated mixed matrix membranes used freshly made membranes with the same composition and approximately the same weight. The reuse and regeneration of the membranes were also investigated. Here after the membrane was filtered with a 2000 mL 6 ppm feed solution, the regeneration of the membranes was conducted by filtering the membrane using 2 M NaCl solution at 70 LMH until no TAN in the filtrate was detected. This process takes about 12 hours and 2000 mL of the salt solution. Since NH$_4^+$ ion and Na$^+$ have the same charge and are very similar to each other in terms of their ionic radii, it is expected that the exchange between the two ions are rather efficient. Indeed, it appears that after the first regeneration of the membrane, the TAN removal rate remains more or less the same as shown in Figure 7. The filtrate volumes with 90% TAN removal for both the virgin membrane and the regenerated membrane reached 850 mL. However, there is some slightly loss of TAN removal capacity. During the subsequent 2nd and 3rd regenerations, only 750 mL and 700 mL respectively of the filtrates reached 90% TAN removal rate. The loss of membrane capacity for TAN removal is more apparent as also can be seen clearly in Figure 7. This loss of capacity is likely due to the diffusion of the NH$_4^+$ ions into the inner regions of the zeolite 13X particles leading to the unavailability of those sites for further adsorption of the ammonium ions. However, it can be seen for this study that these MMMs can be potentially used multiple times after regeneration using a 2 M NaCl salt solution. Moreover, if NaNO$_3$ salt solution is used instead of NaCl, NH$_4^+$ can be recovered in the format of NH$_4$NO$_3$ solution, which can be directly used as a fertilizer. Not only these MMMs can be employed to remove TAN, but also N nutrient can be easily recovered by another ion-exchange process. Further studies are underway to investigate nutrient recovery.
4.3.5 The Effects of Zeolite Type on the Performance of Fabricated Membranes

In mentioned previously, zeolite 13X is a rather inexpensive and abundant natural zeolite from volcanic ashes, it will not contribute much to the cost of fabricating these mixed-matrix membranes. On the other hand, there are many other zeolite types available including zeolite 3A, 4A and Y. These zeolite particles possess specific properties such as high purity and with specific pore sizes. They also have a different Al/Si ratio which is an important indicator for the ion-exchange capacity of the zeolites. Figure 8 shows the percentage of TAN removal as a function of the filtrate volume collected for the mixed-matrix membranes incorporating zeolite 13X, 3A, 4A and Y. All the membranes contain 20%PES and the same weight percentage of the zeolite particles. It can be seen that zeolite 13X demonstrates the highest TAN removal efficiency, followed by zeolite 4A, zeolite 3A with zeolite Y possessing the lowest TAN removal efficiency.
Figure 8 The percentage of TAN removal as a function of the filtrate collected for mixed matrix membranes incorporating different zeolite particles of 13X, 3A, 4A and Y. The membranes have the same mass composition of 20%PES-100% Zeolite.

Since these membranes incorporating different zeolite particle types have slightly different weights, the TAN removal capacity was calculated as shown in Table 3. The weight of the membrane, the flux value for the filtration experiments, the permeate volume reached 90% TAN removal and the total amount of TAN removed are also listed in Table 3. It can be seen that zeolite 13X has the highest TAN removal capacity at 52.5 mg/g. Zeolite 4A has the second highest TAN removal capacity at 48.1 mg/g. The other two zeolite particles 3A and Y have substantially reduced capacity at 43.1 and 43.6 mg/g respectively.
Table 3 TAN removal capacities for MMMs incorporating different zeolite types with the same mass composition of 20%PES-100% Zeolite at feed concentration of 6 ppm. The weight of the membrane, and the filtrate volume with more than 90% TAN removal, the total amount of TAN removed are also shown.

<table>
<thead>
<tr>
<th>Membrane Composition</th>
<th>Weight (g)</th>
<th>Flux (LMH)</th>
<th>Permeate volume with &gt; 90% TAN removal (mL)</th>
<th>Total TAN Removed (mg)</th>
<th>TAN Removal Capacity (mg/g zeolite)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20%PES-100% Zeolite 13X</td>
<td>1.15 ± 0.07</td>
<td>70</td>
<td>800</td>
<td>30.2 ± 0.75</td>
<td>52.5 ± 1.30</td>
</tr>
<tr>
<td>20%PES-100% Zeolite Y</td>
<td>1.08 ± 0.11</td>
<td>70</td>
<td>400</td>
<td>23.6 ± 1.87</td>
<td>43.6 ± 3.46</td>
</tr>
<tr>
<td>20%PES-100% Zeolite 3A</td>
<td>1.12 ± 0.09</td>
<td>70</td>
<td>400</td>
<td>24.2 ± 1.79</td>
<td>43.1 ± 3.19</td>
</tr>
<tr>
<td>20%PES-100% Zeolite 4A</td>
<td>1.13 ± 0.08</td>
<td>70</td>
<td>650</td>
<td>27.2 ± 1.59</td>
<td>48.1 ± 2.81</td>
</tr>
</tbody>
</table>

The differences between these different zeolite types can be due to several reasons. One of the major differences between these zeolite types is their ion-exchange capacity. Ion-exchange capacity is strongly dependent on the Si/Al ratio for the zeolite material. The higher the Si/Al ratio is, the lower the ion-exchange capacity will be. The higher Al content increases the negative charge density of the zeolite. For zeolite 4A and 3A, the Si/Al ratio is 1 for both. For zeolite Y, the ratio is 2.46. For zeolite 13X is 1.23. Clearly zeolite 4A and 3A should have the highest ion-exchange capacity. However, pore size of the zeolite cages also plays an important role, zeolite 3A, 4A and 13X has pore size of 3, 4 and 10 Å respectively. Smaller pore size tends to limit the passage of the hydrated ions and their diffusion to the binding sites. As a result, zeolite 13X has the highest ammonium ion exchange capacity, zeolite Y and 3A have similarly low ammonium ion exchange capacity. Moreover, the filtrate volume with larger than 90% TAN removal for each membrane is also shown in Table 3. The highest volume is zeolite 13X with 850 mL, followed by zeolite 4A
at 650 mL with zeolite 3A and Y at 400 mL consistent with the capacity values. In addition to the ammonium exchange capacity, cost of these zeolites is also a critical consideration of these mixed matrix membrane applications for ammonium removal. Zeolite 4A, 3A and Y are significantly more expensive than zeolite 13X. MMMs incorporating with zeolite 13X appear to be the best performing membranes for industrial applications.

4.3.6 Comparison of TAN removal capacity between composite membranes and fibers

The performances of electrospun mixed-matrix membranes and previously fabricated membranes via phase inversion or pore filling on TAN removal are compared and listed in Table 4. The 13X pore-filled PES membrane (MWCO=30 kDa, EMD Millipore) has the lowest TAN removal capacity at 10.4 mg/g_zeolite_. The phase inversion mixed-matrix membrane exhibits an intermediate TAN removal capacity at 19.8 mg/g_zeolite_. The electrospun membrane has the highest capacity of 91.3 mg/g_zeolite_. The low capacity for pore-filled membrane is clear since zeolite particles are aggregated together when filled in the pores of the PES ultrafiltration membrane. For the membrane fabricated via phase inversion, zeolite particles are more dispersed in the polymer matrix compared to pore-filled membrane leading to an increase in the ion exchange capacity. For the electrospun membrane, it is more porous compared to the corresponding phase inversion one leading to a significant increase in the ion-exchange capacity by more than 4 times.

Table 4 show shows the capacity in terms of the amount of water can be treated per membrane area in L/m^2 if 90% of the TAN is removed in the filtrate assuming that the concentration of TAN in the wastewater is 7 ppm. It can be seen that phase inversion fabricated membrane has the lowest volume TAN removal capacity. This is due to the limited loading of zeolite 13X particles possible for the mixed-matrix membrane without compromising its mechanical integrity. The pore-filled membrane has a higher capacity due to the fact that more zeolite particles can be filled and
incorporated into the membrane pores. The electrospun membrane has the highest volume capacity at 750 L/m². This is due to the fact that electrospun membrane is able to incorporate significantly more zeolite particles and that the membrane is more porous compared to the corresponding phase inversion membrane. Electrospun mixed-matrix membrane can treat over 21 times more wastewater than phase inversion counterpart.

Table 4 Difference in TAN removal capacity levels (L/m²) between composite membranes.

<table>
<thead>
<tr>
<th>Composite Membrane</th>
<th>TAN removal capacity (mg/g&lt;sub&gt;zeolite&lt;/sub&gt;)</th>
<th>TAN removal capacity (L/m²)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase inversion Mixed-matrix Membrane (15%PSU-50%Zeolite)</td>
<td>19.8</td>
<td>35</td>
</tr>
<tr>
<td>Pore-filled</td>
<td>10.4</td>
<td>140</td>
</tr>
<tr>
<td>Electrospun Mixed-matrix Membrane (20%PES-100%Zeolite)</td>
<td>91.3</td>
<td>750</td>
</tr>
</tbody>
</table>

*The volume of the wastewater treated is based on the water contains 7 ppm TAN only without any other competitive ions present.

4.4 Conclusion

Electrospun mixed-matrix membranes incorporating natural zeolite particles 13X were fabricated for the removal of ammonium ions from the wastewaters. These fabricated membranes demonstrate a high ion exchange capacity and high permeability. The capacity for removal of TAN reaches 91.3 mg/g<sub>zeolite</sub> incorporated, more than 4 times higher than the previously fabricated mixed-matrix membranes via phase inversion. The fabricated electrospun membrane has a throughput of 750 L/m² to reduce the synthetic 7 ppm TAN feed to less than 1 ppm. The membranes are stable and can be regenerated by filtering 2 M NaCl through the membrane. No leaching of zeolite particles has been observed. Compared to zeolite Y, 4A and 3A particles that
are incorporated into the mixed-matrix electrospun membranes, the naturally occurring inexpensive zeolite 13X exhibits the highest ammonium exchange capacity.

Acknowledgments

Funding for this work was provided by Arkansas Bait and Ornamental Fish Growers Association through the National Science Foundation Industry/University Cooperative Research Center for Membrane Science, Engineering and Technology, the National Science Foundation (IIP 1361809) and the University of Arkansas.
References


Chapter 5. Factors Affecting Robustness of Anion Exchange Chromatography: Selective Retention of Minute Virus of Mice Using Membrane Media

Abstract

To identify conditions for effective capture of minute virus of mice (MVM) using anion exchange chromatography (AEX), mobile and stationary phase factors affecting MVM binding with membrane AEX media were investigated. The initial study was conducted for Membrane A for a range of feed conditions using bovine serum albumin (BSA), which was used as a model molecule mimicking the most general set of host cell proteins. The effects of pH, conductivity and concentration of BSA on MVM binding were systematically investigated. It was found that BSA concentration has the most significant impact on MVM binding followed by the conductivity of the feed solution. The effect of pH on MVM binding is also detected but has a less impact compared to other two factors in the range of feed conditions investigated. In addition to Membrane A, three other AEX membranes (Membrane B, Membrane C, Membrane D) were investigated for MVM binding at a selected feed condition. It appears that the charge density of the ligand has the most significant impact on MVM binding performance of AEX membranes from stationary phase perspective.
5.1 Introduction

The purification of monoclonal antibodies (mAbs) and other protein-based therapeutics routinely begins with an affinity chromatography capturing step [1, 2]. Subsequently, additional chromatographic polishing steps including anion exchange (AEX), cation exchange (CEX), or hydrophobic interaction chromatography (HIC) are performed to remove the remaining impurities including host cell proteins (HCPs), DNA, leached Protein A, putative viruses and product aggregates to acceptable levels [3-5]. In addition, at least two viral clearance steps with orthogonal mechanisms are commonly included by European Medicines Agency [4, 6-10]. Virus clearance steps generally include low pH or detergent virus inactivation, anion exchange chromatography (AEX) or size-exclusion based viral filtration. Manufacturers must demonstrate an adequate overall viral safety profile prior to obtaining regulatory approval for product licensure.

Minute virus of mice (MVM) belongs to Paroviridae and is an adventitious virus that historically contaminated biomanufacturing processes [11]. Therefore, MVM removal capability of a purification process is often evaluated as a representative case for other adventitious viruses. Evaluation of viral clearance during anion exchange chromatography typically adopts a scale down model by spiking the feed solution with ~7-8 logs of virus particles. AEX is operated under flow-through mode where virus particles and impurities (HCPs and DNA) are bound to substrate whereas the product of interest flow-through.

AEX resins or membranes make use of the fact that the isoelectric points (pIs) of many mAbs (pI, ~7-9) is notably higher than those of HCPs and DNA (~4-6) as well as model viruses such as MVM (pI ~6.0) and xMuLV (pI ~5.8) [3, 4, 7-9, 12]. Consequently, by choosing an appropriate pH (e.g., pH 7) of the solution condition, these contaminants will be negatively charged and are removed by their electrostatic interaction with the positively charged ligands (typically
quaternary amine (\(-\text{NR}_4^+\) where R represents the same or different alkyl groups) and primary amine (\(-\text{NH}_2\) groups) functionalized on the resins or membranes. The mAb product is subsequently recovered in the flow-through. Since electrostatic interaction is non-specific in nature, competitive binding of positively charged ligands with different negatively charged species (HCPs, DNA and virus particles) will occur [3, 4]. Depending on the level of impurity in the feed solution, competitive binding of the impurity to the AEX ligands may lead to a reduction of the viral clearance [13]. As a result, virus clearance is anticipated to be largely modulated by the presence and the typical level of impurities in feedstreams, and the pI of the mAb.

The strength of electrostatic interaction depends on the magnitude of the charges on the positively charged ligands and negatively charged impurities. For the ligands, the charge on the quaternary amine will remain more or less the same at different pH conditions. However, for the primary amine as a weak base, its charge will be affected by the pK\(_b\) value of the amine group and subsequently the pH of the solution. On the other hand, primary amine (\(-\text{NH}_2\)) is both a hydrogen-bond donor and hydrogen-bond acceptor which enables it to form hydrogen bonding interactions with the afore-mentioned impurities in addition to the electrostatic interaction. On the contrary, quaternary amine (\(-\text{NR}_4^+\)) cannot form hydrogen bonds with other functional groups. Besides ligand type, the charges on the specific impurities such as HCPs and virus particles as well as on the protein therapeutics are dependent on the pH and conductivity of the feed solution. This arises from the fact that the pK\(_a\) or pK\(_b\) values of the acid or base groups on the side chains or end groups of the exposed amino acids are dependent on their local physio-chemical environment [14-16].

In addition, the conductivity of the feed solution also affects electrostatic interaction significantly as high conductivity (or ionic strength) reduces electrostatic interaction due to the
screening effect. As a result, the ligand-impurity interaction based on electrostatic interaction can be significantly affected by the pH and conductivity of the feed solution.

On the other hand, if virus particles, mAb product or impurities carry opposite charges, there is a strong driving force for them to attract each other and form complexes [5, 17-19]. The formation of complexes between mAb, virus particle and/or HCP will depend on the structure, physiochemical property, solution condition as well as the solubility of the individual species at the specific solution condition. Binding capacity could be affected by the ligand type and its charge density as well as substrate properties such as porosity and pore-size distribution. The pore structures and the volume fraction of the substrate matrix will also affect the partitioning of the contaminant species including virus particles as well as mAbs. Besides charge, this partitioning also depends on the ratio of the size of the solute to that of the pore or polymer matrix characteristics. Due to the different geometry between different adsorptive membranes as well as resins, there could have intrinsic differences in the partitioning of the solute particles between different substrates. Given this interplay between many variables, it is expected that the binding robustness of AEX resins versus membranes will be different. Moreover, different membranes with different pore sizes and pore-size distributions as well as different ligand type and ligand density will demonstrate differences in virus binding.

Anion-exchange chromatography is often used as a polishing step during the downstream purification of mAbs and other protein therapeutics [20]. Resin-based AEX remains to be commonly used by biopharmaceutical industry even though it often suffers from high pressure drop and difficulty to scale up [21]. Its separation performance is also affected by the flow-rate due to the mass-transfer limitations. Use of membranes as chromatographic support materials overcome the mass transfer limitations associated with resin-based chromatography. Pressure
drops are lower and process flow rates are much higher, essential to overcome bottlenecks in the purification train. The use of membrane-based anion and cation exchange (anion and cation exchange adsorbers) is gaining wider acceptance. They are used in flow through mode for removal of contaminants such as virus particles, HCPs and DNA [22, 23]. Dominated by fast convective flow [24], the dynamic binding capacity of the membrane is typically independent on flow rate.

In this study, we focused on the commercially available anion exchange membranes to remove MVM. The key factors affecting the robustness of virus removal during the AEX polishing step were investigated in detail. Robustness was hypothesized to be affected by two groups of variables: feed/mobile phase properties and stationary phase properties (ligand, ligand density, and other membrane properties). Virus removal performance was investigated under different set of conditions by varying the pH, ionic strength, and impurity concentration. The purpose of this study was to identify the comparative level of virus binding under different operating conditions for four commercial anion exchange membranes. Based on the observed data, a phenomenological model was developed to demonstrate the interaction behavior between the variable parameters and binding of the virus. During the first part of the study, Bovine Serum Albumin (BSA, pI 5.4) was used as a model HCP to investigate the effect of HCP level on MVM binding using Membrane A membrane. One feed condition that exhibited the highest sensitivity to MVM binding was selected for investigation of different commercial AEX membranes including Membrane B, Membrane C, and Membrane D. The second part of the study focused on the effects containments in the actual mAb feedstream from on MVM binding, where mAb A was purified after both Protein A affinity chromatography and AEX steps and mAb B was purified only by Protein A capture step.
5.2 Experimental Methods and Materials

5.2.1 Experimental Design

Chromatography method. The effects of feed conditions including pH, ionic strength and the level of competition in terms of the BSA concentration on MVM binding were investigated initially with flow-through anion-exchange membrane chromatography with Membrane A. The buffer was chosen as the 50 mM tris buffer throughout the study. The feed streams were spiked with 7.5 logs/mL MVM based on qPCR titer. The design of experiments (DOE) for the chromatography run is shown in Table 1. The conductivity of the feedstream is controlled by varying the NaCl salt concentration. Three pH conditions (6, 7 and 8) were investigated. BSA was used to represent as a model HCP to compete for MVM virus binding. Its concentration varied from 0, 1 to 10 g/L. Protein stock solutions were prepared by dissolving certain amount of BSA protein in specific buffer condition following DOE condition. All the buffers and protein solutions were then filtered with Whatman 0.2 μm PES membrane before test. Membranes were preconditions following by manual before used. All runs were conducted with ÄKTA FPLC (GE Healthcare Bio-Sciences Corp). The runs were automated using the Unicorn software 5.3 for binding and elution. The system was initially equilibrated with buffer A (adsorption buffer) at a constant flow rate for 10 minutes. Feed solution was then loaded onto the membrane at a constant flow rate. To remain the same loading density, the proportional loading volume will be used to challenge the different membranes. The loading density is defined as amounts of protein (mg) per membrane volume (mL). Unbound proteins were then washed away from the membrane using buffer A (adsorption buffer), followed by elution with buffer B (elution buffer) at a constant flow rate. The runs ended when the UV absorbance at 280 nm reached a constant. Three fractions were collected including product, washing as well as strip. Protein concentrations and virus titer in these
fractions were determined by UV at 280 nm and TCID\textsubscript{50} (tissue culture infectious dose 50) assay. All runs were conducted in duplicate. The flowrate was fixed at 2 mL/min for all runs.

**Table 1** Design of Experiments (DOE) for investigating the effect of feed condition on MVM binding under 50 mM Tris buffer with Membrane A.

<table>
<thead>
<tr>
<th>Exp. No</th>
<th>pH</th>
<th>NaCl (mM)</th>
<th>BSA (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>150</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>150</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>7</td>
<td>150</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>8</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>7</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>13</td>
<td>8</td>
<td>150</td>
<td>1</td>
</tr>
</tbody>
</table>

Once the effects of pH, conductivity and BSA concentration on MVM binding with Membrane A membrane were established, other membranes with different manufacturers were used at one selected feed condition where MVM binding is found to be most sensitive. Finally, viral binding with two mAb feedstream (mAb A and mAb B) containing different levels of impurities were investigated at the selected condition.

**5.2.2 Materials**

*Membranes*. Commercially available membranes were purchased from manufactures. Table 2 shows the properties of the membranes including membrane volume (MV), ligand type, charge density, pore size and surface area.
Table 2 Properties of Membranes investigated.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Membrane A</th>
<th>Membrane B</th>
<th>Membrane C</th>
<th>Membrane D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane Volume (mL)</td>
<td>0.86</td>
<td>0.08</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>Base Membrane Material</td>
<td>Polyethersulfone</td>
<td>Stabilized Reinforced Cellulose</td>
<td>Stabilized Reinforced Cellulose</td>
<td>Polyacrylamide Composite</td>
</tr>
<tr>
<td>Ligand Type</td>
<td>Quaternary Amine</td>
<td>Primary Amine</td>
<td>Quaternary Amine</td>
<td>Quaternary Amine</td>
</tr>
<tr>
<td>Surface area (cm²)</td>
<td>36</td>
<td>78.5</td>
<td>2.9</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Raw and feed materials. In addition, 100 kDa amicon ultracentrifuge filters were purchased from MilliporeSigma (Burlington, MA). Tris and sodium chloride (biotechnology grade) were purchased from G-Biosciences (Saint Louis, MO). Sodium hydroxide (ACS grade, ≧98%), hydrochloric acid (6 mol/L), and Spectra/Por® 1-5 Standard RC Dry Dialysis Trial Kits (Spectrum® Laboratories) were purchased from VWR (Radnor, PA). Sodium phosphate dibasic (ReagentPlus®, ≧99.0%) and sodium phosphate monobasic monohydrate (ACS reagent, ≧98%) were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) were purchased from Lee BioSolution (Maryland Heights, MO). DNase for quantitative PCR was purchased from Promega (Madison, WI). iTaq universal SYBR green supermix was purchased from Bio-Rad (Hercules, CA), QIAquick PCR purification kit was purchased from Qiagen (Germantown, MD), TOPO® TA Cloning® Kit for Sequencing was purchased from Life Technologies (Carlsbad, CA), and One Shot™ TOP10 Chemically Competent E. coli was purchased from Thermo Scientific (Waltham, MA). The mAb A purified after Protein A capturing and AEX polishing steps, and mAb B purified after Protein A capturing step were provided by AstraZeneca (Gaithersburg, Maryland, U.S.). Both mAb A and B have an isoelectric point (pI) of ~8.0. An ultrafiltration/diafiltration (UF/DF) step was used to prepare the mAb feedstream to 50
mM tris + 50mM NaCl at pH 7. The targeted mAb concentration after UF/DF was ~10 g/L. After UF/DF, the mAb stock stored in -80°C before used. The mAb feed concentration was adjusted according to the membrane loading density investigated.

Buffer preparation. Buffers containing 50 mM Tris and 0, 50 or 150 mM NaCl were prepared initially. The pH of the buffers was then adjusted by titrating 2 M HCl into the above prepared buffers to reach targeted value of 6, 7 and 8.

5.2.3 Charge Density Measurement

Surface charge densities and volume-based charge densities were obtained from manufacturers if available. The volume-based charge densities of Membrane A and B membranes were determined by titration method described below. Membranes were initially soaked by DI water for 30 min. These membranes were then soaked in 0.1 M NaOH solution overnight. After that, membrane samples were washed again by the DI water until the pH of the solution reached 7. Thereafter, membranes were immersed in 2 M NaCl overnight to exchange the OH⁻ ions with Cl⁻ ions. Both are counter ions to the positively charged amine groups in the membranes. The solutions after the ion exchange were then titrated using 0.01 M HCl. The charge density is defined as the number of OH⁻ ions per unit membrane volume [3].

5.2.4 Membrane Porosity Determination

To determine the porosity of membranes investigated, lysozyme was used as a tracer following the previous studies [25]. Experiments were conducted using 50 mM Tris containing 250 mM NaCl at pH 8.0 [25]. Lysozyme was dissolved in the buffer to reach 2 g/L concentration. Membranes were initially equilibrated with the buffer solution without the protein for 30 min. A total of 10 mL of prepared lysozyme solution was then injected and effluent was monitored by UV
absorbance at 280 nm. The injection experiment performed at 5 different flow rates in the presence and absence of membranes. Lysozyme retention volume was calculated as the average of from five flow rates. The five flow rates are all within the recommended operation range by manufacturers. The porosity of the membrane was determined based on the following equations:

\[ V_{voids} = V_{sys\ with\ MA} - V_{sys\ w/o\ MA} \]  (1)

where \( V_{voids} \) is the total voids including both membrane pores and module dead volumes. \( V_{sys\ with\ MA} \) is the retention volume in the presence of a the membrane module. \( V_{sys\ w/o\ MA} \) is the retention volume in the absence of the membrane module. The pore volume was then calculated:

\[ V_{pore} = V_{voids} - V_{module} \]  (2)

where \( V_{pore} \) is the pore volume and \( V_{module} \) is the dead volume of the membrane module. The dead volume of the module was measured by gauge after breaking the module. The porosity was determined by the ratio of the void volume of the pore to the reported membrane volume.

\[ Porosity \ (\%) = \frac{V_{pore}}{MV} \times 100\% \]  (3)

### 5.2.5 MVM Production and Purification

Minute virus of mice (MVM) is a representative parvovirus that can infect Chinese Hamster Ovary (CHO) cell culture. The initial stock of the MVM (ATCC® VR1346™) was purchased from American Type Culture Collection (Manassas, VA). A further production and purification of MVM were performed based on an adapted protocol from literature [26]. In-house produced MVM stocks typically have a titer of \(-10.5\text{-}11.5\) logs/mL measured by qPCR. The overall HCP concentration in the virus stock was determined to be less than 0.4 g/L. During MVM spiking studies, the targeted virus titer is \(7.5\) logs/mL using \(~100\) μL or less of the virus stock for
20-60 mL feedstream. As a result, the HCP concentration from the virus stock in the feed is low, on the order of $10^{-5}$ g/L. More details on the production and purification of our in-house produced MVM virus stock is described in a previous publication [6].

5.2.6 MVM Titer Assays

5.2.6.1 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) method was used to quantify the number of copies of viral genomes in virus stocks and feedstream by a Bio-Rad CFX ConnectTM Real Time System (Hercules, CA) with Bio-Rad CFX Manager software. The standard curve was made by qPCR amplification of serial diluted recombinant plasmid ranging from $10^1$-$10^9$ copies/mL. Three repeats were done to plot the standard curve, with 95% confidence limit and a mean ± 0.5 log considered as an acceptable criterion. More details on the qPCR protocol can be found in an earlier publication [6].

5.2.6.2 TCID$_{50}$ assay

Viral titer of the feed and the filtrate was determined by tissue culture infectious dose 50 (TCID$_{50}$) assay based on the method developed previously. Briefly, the indicator cell line NB324K was donated from Peter Tattersall at Yale University. The cells were seeded into 96-well plate to reach a desired confluency of 20-50%. The samples were diluted in serial 10-fold dilutions with the seeding medium. Each dilution was inoculated onto one column (6-wells) at 100 µL/well. The negative control wells were inoculated with the same seeding medium. Plates were incubated in 37°C, 5% CO$_2$ incubator. After ten days, all the wells were inspected under microscope for cytopathic effect (CPE). Two replicates were done for each sample. Spearman-Kärber method (your reference or Dougherty, R., The Spearman-Karber Method. in R.J.C. Harris (Ed.),
Techniques in Experimental Virology, Academic Press, New York, 1964; p. 169–223) was used for calculation of TCID\textsubscript{50} titer.

**5.2.6.3 Large volume plating assay**

Large volume plating (LVP) assay was also performed for the flow-through samples when no CPE was observed with the TCID\textsubscript{50} assay. When no CPE was observed in the flow-through fractions, LVP assay was also performed for those fractions to reduce the detection limit. The indicator cells were cultured in a 96 well plate following the same procedure as for TCID\textsubscript{50} assay. Each diluted sample was transferred into 50 mL disposable polystyrene reservoir and mixed well. Finally, each sample was inoculated onto 12 columns, all 96 wells, at 200 µL/well. Plates were returned to 37 °C, 5% CO\textsubscript{2} incubator. After ten days, all the wells were inspected under microscope for cytopathic effect [27, 28].

**5.2.7 Anion Exchange Membrane Chromatography Runs**

Membrane A, Membrane C, and Membrane B, Membrane D were placed into the FPLC system, wetted, and equilibrated as described earlier [4]. Feedstream were prepared by spiking the BSA solutions with 7.5 logs/mL (qPCR) MVM particles. BSA concentrations at 0, 1 and 10 g/L were investigated. The feed volume in the range of 2-20 mL was determined by the membrane volume (MV) of the commercial membranes to reach the same loading density. The flow-through, washing and strip fractions were collected separately. Each membrane was first equilibrated in buffer A (50 mM Tris with 0, 50, or 150 mM NaCl depending on the feed buffer) at 2 mL/min for 10 minutes before use. After loading, the membrane was then washed with the same volume of the adsorption buffer, followed by a step change to buffer B (50 mM Tris with 1 M NaCl) for the elution. Protein concentrations in different fractions were determined using UV at 280 nm, which used adsorption buffer to be blank for flowthrough and washing fraction and elution buffer used
to be blank for strip fraction. MVM titers were determined by TCID$_{50}$ assay. The log reduction value (LRV) of the virus particles was calculated for TCID50 assays using the following formula:

$$LVR = \log \frac{c_{MVM, feed}}{c_{MVM, flow-through}}$$  \hspace{1cm} (7)

where $c_{MVM, feed}$ and $c_{MVM, flow-through}$ are the titers in the feed and flow-through fractions. Higher LRV correspond to higher binding strength of MVM.

### 5.2.8 Correlations from Statistical Analysis

A phenomenological model using JMP software from SAS Institute (Cary, NC) was developed to understand the virus binding (LRV) with regard to conductivity, pH and the HCP concentration (represented by BSA) in the feed. DOE provides an effective way for selecting experimental conditions and generating prediction model [29, 30]. The coded symbol $X_1$, $X_2$, and $X_3$ represents pH, salt concentration (mM), and protein concentration (g/L) respectively. The DOE condition listed in Table 1. Center points of the parameter are experimental runs where between the low and high levels of the parameter. In this study, the center point is 7, 75mM, and 5 g/L, which respectively represent pH, salt concentration, and BSA concentration. A second-order polynomial equation was used to fit the data.

### 5.3 Results and Discussion

#### 5.3.1 The Effects of Feed Condition and BSA Concentration on MVM Clearance

The AEX chromatographic runs with Membrane A based on the DOE as shown in Table 1 were performed for a total of 14 feed conditions with one repeat, which reduced random errors. The targeted feed titer for all the conditions was 7.5 logs/mL measured by qPCR. The LRVs were calculated based on the TCID$_{50}$ assay. When no CPE was observed in the flow-through fractions,
LVP assay was also performed for those fractions. Table 3 shows the feed conditions including pH, NaCl concentration in mM and BSA concentration in g/L, and the impact on MVM binding (LRVs). When the BSA concentration is 10 g/L or when the salt concentration is 150 mM, significant virus breakthrough was observed with LRV < 1 except for one condition with an LRV of 1.75. BSA is a model HCP that binds competitively to prevent MVM binding as both are negatively charged in a solution above pH 6. A 10 g/L of BSA concentration could saturate the binding sites on the Q membranes leading to a dramatic reduction in MVM LRV to less than 0.5. On the other hand, the effect of 150 mM NaCl concentration in the feed solution on virus binding is not as dramatic since the conductivity of the feed solution is still relatively low at ~19 mS/cm. Higher conductivity leads to a stronger charge screening effect and a weaker electrostatic interaction. In 6 of the 14 conditions, complete virus retention (pH 8, 50 mM NaCl and 1 g/L BSA) were observed with TCID$_{50}$ assay, where conditions are in favor of MVM binding (low conductivity and low competition).
Table 3 LRV values for the AEX runs with Mustang Q at different feed conditions

<table>
<thead>
<tr>
<th>Variables</th>
<th>Response (TCID&lt;sub&gt;50&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH (X&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>NaCl (mM) (X&lt;sub&gt;2&lt;/sub&gt;)</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
</tr>
<tr>
<td>6</td>
<td>150</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>150</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td>7</td>
<td>150</td>
</tr>
<tr>
<td>8</td>
<td>150</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
</tr>
</tbody>
</table>
The correlation between the predicted LRV and measured LRV with TCID$_{50}$ assay for DOE experiments with Mustang Q.

The Response surface methodology (RSM) fit of a second order polynomial function to LRV as a function of pH, NaCl and BSA concentrations using JMP resulted an empirical equation (5) describing the relationship between LRV and the three independent variables (pH, salt content and BSA concentration):

\[
LRV = -0.77 + 1.37 \times \Delta pH - 2.56 \times \%salt - 3.27 \times \%BSA - 0.12 \times \Delta pH \times \%salt + 1.02 \times \Delta pH \times \%BSA - 0.227 \times \%salt \times \%BSA - 0.61 \times (\%salt)^2 + 0.29 \times (\%BSA)^2
\]

where \( \Delta pH = pH - 7 \), \( \%salt = \frac{C_{NaCl}}{75} \) and \( \%BSA = \frac{C_{BSA}}{5} \) with \( C_{NaCl} \) and \( C_{BSA} \) representing the concentrations of NaCl in mM and BSA in g/L in the feed solutions respectively.

Figure 1 exhibits the correlation between predicted LRV and actual measurements with TCID$_{50}$ assay with $R^2$ reached 0.98. The coefficients of the empirical relation (8) indicates that increasing pH has a positive effect on LRV, increasing BSA and NaCl concentrations has a negative effect on LRV at the first order. The second order coefficients are more complex. Variations of LRV as
a function of three individual variables are plotted in Figures 2-4, which will be described in more detail below. Additional two confirmation experiments were performed: (1) pH=7, C_{NaCl}=50 mM, C_{BSA}= 1 g/L, (2) pH=7, C_{NaCl}=150 mM, C_{BSA}= 0.5 g/L. The predicted LRVs are both ~2.2 logs with the actual measured value of 2.8 and 1.0 logs respectively.
Figure 2 The effects of pH on MVM clearance at three different salt concentrations (left) and BSA concentrations (right) in the feed streams for Mustang Q.

Figure 3 The effects of salt concentration on MVM clearance at three different pH values and levels of competition measured by BSA concentrations in the feed streams for Mustang Q.
Figure 4 The effects of level of competition measured by BSA concentrations on MVM clearance at three different pH values and salt concentrations in the feed streams for Mustang Q.

Figure 2 plots the effects of pH on LRV for MVM clearance at three different pH levels for three NaCl concentrations (left) and three BSA concentrations (right). It can be seen that LRV increases with the increase of pH for all three salt concentrations. This is due to the fact that the charge on MVM particle becomes more negative as the pH increases resulting a stronger electrostatic interaction with the positively charged quaternary ammonium ion ligand which does not vary the charge with pH. Moreover, the figure also shows that the increase of LRV with pH is more sensitive to NaCl concentration at 50 mM compared to other two salt conditions. This is due to the fact that at higher salt concentration of 150 mM, the LRVs at all pH conditions are dominated by the weakened electrostatic interaction. At salt concentration of 50 mM, the charge increase at higher pH also plays a role leading to an increase in the LRV value. When the feed solution contains no added salt, the LRV as a function of pH does not change as significantly as in the case of feed with moderate salt concentration since the LRV at pH 6 with 0 mM NaCl is already relatively higher than at the corresponding 50 mM salt concentration feed. The variation of LRV as a function of pH at three different BSA levels exhibits similar trend as shown on the right-hand
panel of Figure 2. The increase of BSA concentration in the feed dramatically reduced the LRV values. At 10 g/L BSA, the LRV is virtually reduced to almost 0. At 1 g/L BSA feed solution, LRV has the most apparent increases with the increase of pH. In the absence of BSA in the feed solutions, no virus breakthrough was observed for all three pH conditions.

Figure 3 shows the effect of salt concentration on LRV at three pH conditions (left panel) and at three BSA concentrations (right panel). It is apparent that the increase of salt concentration in the feed reduces LRV for all the conditions. It seems that solution conductivity has a stronger effect than for the effect of pH on LRV. This can be inferred from equation (9) where the first-order coefficient for the salt concentration is larger than the corresponding value for pH. Figure 4 shows the effect of BSA concentration on LRV at different pH conditions (left-panel) and at three different salt concentrations (right-panel). It can be seen at both panels, the reduction in LRV is more dramatic as BSA concentration in the feed increases indicating that HCPs in the feed has a significant effect on viral clearance. It can also be inferred from Equation (9) that the first-order coefficient for BSA is the largest among the other two first-order coefficients. These coefficients are 1.37, -2.56 and -3.27 for pH, salt and BSA concentrations respectively. Therefore, during AEX viral clearance, the HCP level in the feed has the strongest effect on the LRV, followed by the conductivity of the feed solution and finally the feed pH in the range of conditions investigated here. From above study for Mustang Q membrane, it seems that LRV is sensitive most to the condition at pH 7, 50 mM NaCl and 1 g/L BSA. This is the condition for the subsequent studies to investigate the effects of membrane type on MVM clearance.

5.3.2 Comparison The Performances of AEX Membranes for Viral Clearance

Four anion exchange membranes (Membranes A, B, C, and D) were investigated side-by-side for MVM binding at the selected feed condition with pH 7, 50 mM NaCl and 1 g/L BSA. All
the feedstream were spiked with 7.5 logs/mL (qPCR) MVM virus. The flowrate for all the runs was fixed at 2 mL/min, which is within the recommended range by the manufacturers. Virus loading density was kept to be similar for all the membranes at ~7.5 logs (qPCR)/mL. The feed volume for each membrane was different depending on the individual MV. It was ~20 mL for Membrane A and Membrane C, 2 mL for Membrane B and 5 mL for Membrane D. The TCID\textsubscript{50} assay was used to determine the virus titers.

**Table 7** Membrane surface and volume charge densities, pore sizes and porosity for the four membranes investigated.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Membrane A</th>
<th>Membrane B</th>
<th>Membrane C</th>
<th>Membrane D</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Membrane Volume (mL)</strong></td>
<td>0.86</td>
<td>0.08</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Surface Charge Density (μeq/cm\textsuperscript{2})</strong></td>
<td>0.61</td>
<td>18-22</td>
<td>2-5</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Volume Charge Density (mmol/mL)</strong></td>
<td>0.056</td>
<td>0.65-0.80</td>
<td>0.072-0.18</td>
<td>1.0</td>
</tr>
<tr>
<td><strong>Pore Size (μm)</strong></td>
<td>0.8</td>
<td>3-5</td>
<td>3-5</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Porosity (%)</strong></td>
<td>64</td>
<td>N/A</td>
<td>62</td>
<td>85</td>
</tr>
</tbody>
</table>
Figure 5 Comparison of MVM binding for 4 commercially available anion exchange membranes under pH 7, 50 mM Tris, 50 mM NaCl buffer condition and 1 g/L BSA feed concentration spiked with 7.5 logs/mL.

Figure 5 shows the LRV for the 4 different AEX membranes investigated. No virus breakthrough was observed in the flow-through fractions from the Membranes B, C, and D. As a result, the LRVs shown in Figure 5 are from LVP assay in the flow-through fractions from these membranes. Virus breakthrough was detected in the flow-through fractions in the Membrane A experiment resulting in a relatively low LRV. To better understand the performance differences in MVM binding between these AEX membranes, the pore structure and ligand charge densities were compared. Volume ligand charge densities calculated based on membrane volume, for Membranes B is obtained from the manufacturers directly. Volume charge densities for Membrane A, Membrane C and Membrane D were measured in the current study. The corresponding surface charge densities were derived from the volume charge densities except for Membrane D which has a 3-D matrix structure. The porosities of the membranes except for Membrane B were measured during the current study. Table 5 lists surface and volume charge densities, pore sizes and
porosities of these membranes. It can be seen that charge density plays a dominant role in the performance of MVM binding. The pore size of Membrane A at 0.8 μm lies between those of Natrix (0.4 μm) and Sartobind membranes (3-5 μm). Its porosity at 64% is similar to that of Membrane C at 62%. However, the charge density of Membrane A at 0.056 mmol/mL is significantly lower than Membrane B (0.65 – 0.80 mmol/mL), Membrane D (1.0 mmol/mL) and also lower than Membrane C (0.072-0.18 mmol/mL). It is clear that charge density is more critical for MVM binding at the same virus loading for the membranes investigated. This is particularly true for MVM binding since MVM particle sizes are in the range of 18-24 nm, much smaller than membrane pore sizes. For large virus particles, pore size and porosity will probably become more important.

5.3.3 Effect of different mAb types on MVM clearance

MVM binding for two monoclonal antibodies (mAb A and mAb B) from Astrazeneca were investigated using two AEX membranes, Membrane A and Membrane D. Membrane A and Membrane D were chosen because these two membrane consist of the same ligand type and exhibit respectively the lowest LRV and the highest LRV when challenged with a critical buffer condition shown in Figure 5. mAb A is purified after Protein A chromatography followed by AEX polishing step whereas mAb B is less purified after only a Protein A capturing step. The actual HCP concentrations in both mAbs are in the order of ~100-500 ppm. Both mAbs have been buffer exchanged to the buffer investigated previously with 50 mM Tris at pH 7 with 50 mM NaCl. Two mAb concentrations were used at 1 g/mL and 10 g/mL to achieve different loading densities. The feedstream were spiked with 7.5 logs/mL MVM particles (qPCR). The flowrate was kept the same as before for both Membrane A and Membrane D runs at 2 mL/min. Feed volume was kept the same as before for Membrane A at 20 mL for a loading density of 23 and 230 mg/mL MV
respectively for the 1 and 10 g/L mAb feedstream. For Membrane D, the first set of runs were conducted with a feed volume of 5 mL with a corresponding loading density of 25 and 250 mg/mL MV. The second set of experiments were conducted with a feed volume of 20 mL with a corresponding loading density of 100 and 1000 mg/mL MV.

![Figure 6](image.png)

**Figure 6** LRV for MVM binding during AEX run with Membrane A for both mAb A and mAb B feedstream at two loading densities of 23 and 230 mg/mL MV.

Figure 6 shows the LRV for MVM binding during AEX run with Membrane A for mAb A and mAb B at two different protein loading densities of 23 and 230 mg/mL (MV). TCID$_{50}$ assay was used to determine the feed and flow-through MVM titers. When no CPE was observed, LVP assay was used to determine the titer in the flow-through fractions as was done previously. For mAb A after both Protein A and AEX steps, the LRV reached over 5 at both loading densities. No
virus breakthrough was observed in the flow-through fractions. For mAb B, the LRV also reached over 5 for the 23 mg/mL loading density with high level of virus binding. However, at loading density of 230 mg/mL, breakthrough was observed and the LRV was reduced to 2.83. Clearly, HCPs in the feed solution affect MVM binding for the AEX process with Membrane A. For more purified mAb A, the amount of HCPs in the feed is relatively low and it does not reduce the LRV for MVM binding. On the other hand, with less purified mAb B, the relative higher concentration of HCPs affects MVM binding with Membrane A, particularly at the high loading density of 230 mg/mL (MV).

![Graph showing LRV for MVM binding during AEX run with Membrane D for both mAb A and mAb B feedstream at two loading densities of 23 and 230 mg/mL MV.](image)

**Figure 7** LRV for MVM binding during AEX run with Membrane D for both mAb A and mAb B feedstream at two loading densities of 23 and 230 mg/mL MV.
Figure 7 shows the LRV for MVM binding for Membrane D membrane for both mAbs at two different loading densities of 23 mg/mL and 230 mg/mL MV. For all the runs, LRV reached over 4 with complete viral binding. It indicates that Membrane D is more robust in MVM binding due to its high ligand density. This agrees with our results described previously with BSA as a model HCP. Pore size does not seem to play any important role in MVM binding.

![Graph showing LRV for MVM binding](image)

**Figure 8** LRV for MVM binding during AEX run with Membrane D for both mAb A and mAb B feedstream at two loading densities of 100 mg/mL and 1000 mg/mL MV.

To further challenge the capacity of Membrane D membrane for MVM binding, a much higher loading density of 1000 mg/mL MV was investigated for both mAb A and mAb B as shown in Figure 8. LRVs for 100 mg/mL MV loading density were also included. For both mAb A and
mAb B, significant virus breakthrough was observed at 1000 mg/mL MV. For more purified mAb A, an LRV of 2.67 was obtained whereas for a less purified mAb B, an LRV of 1.17 was obtained indicating the influence of HCP impurity on MVM binding.

### 5.4 Conclusions

The factors affecting MVM binding during AEX runs were investigated. The initial study was conducted for a range of feed conditions using BSA a model HCP with Membrane A membrane. The effects of pH, conductivity varied by using different NaCl concentrations and the concentration of BSA on MVM binding was systematically investigated. From mobile phase perspective, it was found that BSA concentration (amount of HCP in the feed) has the most significant impact on MVM binding followed by the conductivity of the feed solution. The effect of pH on MVM binding remains to be important but has a less impact compared to the level of impurities and feed conductivity in the range of feed conditions investigated. In addition to Membrane A, three other AEX membranes were investigated for MVM binding at the same selected feed condition. Data suggests that the charge density of the ligand is most significant on the MVM binding performance of AEX membranes from stationary phase perspective.

### Acknowledgements

Funding for this work was provided by AstraZeneca through the National Science Foundation Industry/University Cooperative Research Center for Membrane Science, Engineering and Technology, the National Science Foundation (IIP 1361809) and the University of Arkansas. We also acknowledge MilliporeSigma for donating some of the Membrane D membranes for the study.
References


Chapter 6: Conclusions and Future Direction

6.1 Conclusions

In the scope of this study, electrospun membranes were developed using different types of substrate with multiple polymerization methods for various applications from ammonium ion removal to protein purification. Moreover, we also investigated the impact of crucial factors on MVM clearance during anion exchange membrane chromatography. Our major conclusions are summarized below. Our work indicates how the electrospun membrane with high surface-to-volume ratio provide many advantages over functionalized flat sheet membrane.

In the case of the weak anion exchange electrospun membranes, experimental results show that the surface modified PSf-GMA-DEA and mPAN-GMA-DEA membranes exhibit high static and dynamics protein binding capacities due to high substrate porosity resulting from the high surface-to-volume ratio of the nanofibrous materials. The PSf-GMA-DEA membrane is found to be more hydrophobic than the mPAN-GMA-DEA membrane leading to increased non-specific binding of the protein. Our study indicates that electrospun nanofibrous mPAN-GMA-DEA membranes are promising as ion-exchange membrane for application in protein purification.

A new set of HIC membranes were also developed and applied for protein purification. Parameters like polymer chain length and polymer density were explored in detail and found to play important role in maximizing binding capacity and recovery of electrospun membranes. High protein binding capacity is achieved with the fabricated responsive HIC electrospun membrane. However, recovery is found to be reduced at a high protein loading. The mono-layer adsorption at low protein concentration leads to high recovery. Furthermore, the responsive HIC electrospun membrane is found to exhibit high separation performance in terms of purifying IgG and lysozyme.
Our work also shows how electrospun membranes provide a novel solution for ammonia removal. The laboratory fabricated mixed matrix electrospun membranes were found to remove 90% of TAN with a volume of 800 mL at 7 ppm (feed concentration). The mixed matrix electrospun membrane further demonstrates high TAN removal at various feed concentrations. With increasing zeolite particle loading, TAN removal is successfully increased. The derived mixed-matrix membranes exhibit high levels of water flux and can be regenerated easily using 2 M NaCl solutions. The TAN removal capacity of the mixed matrix electrospun membrane derived in the present study is compared with the removal capacity of mixed-matrix phase inversion and pore-filled composite membranes. Overall, the mixed matrix electrospun membrane demonstrates significantly higher removal capacity relative to the other membranes.

In addition to the studies on electrospun membranes, a systematic investigation was also performed to explore the impact of feed condition and membrane properties on MVM clearance during membrane anion exchange chromatography. The selected pH range of the feed plays an important role in guaranteeing containments have a negative charge. In terms of feed conditions, BSA concentration and salt concentration were found to be the two key factors for virus removal. High conductivity is found to be unfavorable for the Coulombic interaction between the virus particle and positively charged ligand, leading to disruption of the MVM adsorption. Limited LRV comes from a great number of BSA competing for the binding sites with MVM particles. The membrane media with higher charge density provides more binding sites for containment adsorption, while higher porosity offers an easily accessible structure for binding impurities which led to a better viral clearance. This study elucidates the correlation of feed condition by the DOE analysis and provides an understanding of the mechanism of adsorption between different products through charge density and porosity.
6.2 Future direction

In this study, HIC and IEX membranes were fabricated using electrospinning methods. Moreover, the effect of IEX membrane properties on the binding capacity has been investigated. Further, the effect of the polymer density and polymer chain length on the binding capacity and recovery of responsive membranes has also been studied in detail.

The current technology suffers from protein denaturation, low product yields, the possibility of high pressure and lengthy processing time. The large usage of salt is one of the major drawbacks in processes involving HIC. The proposed responsive membrane based HIC could overcome all these limitations with tunable selectivity, enhanced product recovery, and milder processing conditions. Therefore, increasing the hydrophobicity of ligand would lower the required salt concentration for binding. Further, it could be worthwhile to incorporate hydrophobic monomers for lowering the LCST.

On the other hand, the stability of the electrospun membrane is non-uniform for different batch membranes leading to different efficiency of functionalized electrospun membrane fabricated in different batches. Again, a comparative separation performance of electrospun membrane could be performed with the 3D printed membrane which has intrinsic structure with consistent high surface-to-volume ratio. Insights obtained from these experimental studies would help design better responsive HIC membranes and weak anion exchange membrane.

In the case of the weak ion exchange membranes fabricated during this study, the non-specific binding was observed. Choosing an appropriate substrate to reduce the non-specific binding with shorter functionalization time could help improve the overall performance of the weak IEX membrane.
Besides the improvement of membrane structure, fundamental ligand characterization also is critical to increase recovery. As our results show, the different ATRP or UV-exposure reaction time affect membrane performance because of the molecular weight and grafting density of the grafted ligands. However, there is no precise method that can detail the analysis of the polymer ligands on the membrane directly. Up to now, the researchers still use the conventional characterization grafting the ligand on the standard surface such as silica wafer or gold or performing solution ATRP in the same condition.

It will be important to develop a method to analyze the resulting polymer chain length accurate on the membrane surface. For the membrane modifications, the reversible addition-fragmentation chain transfer (RAFT) with xanthates as chain transfer agents showed well-controlled polymerization of non-conjugated N-vinyl monomers in the previous study. It may be a potential modification method to develop a highly efficient functionalized membrane chromatography.

Furthermore, electrospinning technology is explored to fabricate a novel zeolite incorporated membrane for ammonium removal. Herein, a good ammonium removal efficiency is obtained in this study as compared to the prior art. However, an exclusive and fundamental study would be required in the future to deeply investigate the mechanism of ammonium absorption. According to the real lake feed stream result, the other matters like ions or electrolytes may affect the ammonium removal. Additionally, to adapt different feed streams comprised of ammonium ion-based feed streams, a detailed analysis of the environmental parameters like temperature, pH value, and viscosity would be required. Currently, several framework materials, for example, metal-organic frameworks (MOF) and covalent organic frameworks (COF), show excellent
selection regardless of gas or ions separation. Incorporation of such materials into the electrospun membrane could be performed in the future.

In this study, we functionalized several nanofiber membranes via a traditional needle electrospinning setup. Although the membranes displayed excellent performance in various applications, the production was slow and limited to a batch process. It may cause the development of electrospun scale-up is limit due to the high cost of production. To overcome these disadvantages, multi-needles electrospun technique has been developed to carry out continuous production of a large area and uniform fiber. However, the main issue of multi-needles electrospun technique comes from electrical field interfere between needles. The asymmetric electric field may lead to a significant deviation of the single-needle jet. Applying an electrical field auxiliary device and changing the arrangement of the needles could be reduced the mutual influence of the electric field between the needles, but it hard to be completely overcome this effect. Recently, the needleless method has been developed to overcome the inherent drawbacks of the needle spinning method; the relevant researcher began to create the needleless electrospinning device. The brushing device realizes the reciprocating motion and applies the polymer solution to the linear metal electrode through the small hole. The polymer solution forms uniformly distributed small liquid beads on the elongated metal electrode by surface tension. Under the traction of the electrostatic field, a Taylor cone is created, and the spinning phenomenon occurs. Needleless method can produce uniform electrospun fibers on a large scale, thereby improving productivity and reducing costs. Along with the development of the needleless method, the electrospun membrane may be more competitive in the market.

Ion exchange chromatography is also explored in the polishing step used in downstream processing. The effect of the mobile phase on viral clearance is also explored in detail. On the
other hand, the effect of stationary phase properties on viral clearance still requires some investigates. Strengthening the understanding of an experimental framework such as confocal microscopy to assess species migration depth will help improve the objectivity of the conclusions. Additionally, establishing a theoretical framework for understanding differences in the robustness of different membrane media as well as resins will facilitate mechanism insights.