ENVIRONMENT-RESPONSIVE MEMBRANES FOR BIOSEPARATIONS

SYNTHESIS AND CHARACTERIZATION OF ENVIRONMENT-RESPONSIVE MEMBRANES FOR BIOSEPARATIONS

By

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ABSTRACT

Environment-responsive membranes were created by modification of a commerical polyvinylidene fluoride (PVDF) membrane support with a thermo-responsive hydrogel composed of poly N-vinyllactams cross-linked with bisacrylamide. The modified membranes were then characterized by their percentage mass gains as well as by their valve effect in response to changes in salt concentration. One set of membranes, with a large valve effect, was selected for highest retention of intermediately sized proteins was examined for ultrafiltration-based protein separation applications. A batch separation protocol featuring pulsed sample injection technique (PSIT) was then used to sieve single proteins and to fractionate a synthetic binary protein mixture and a synthetic ternary protein mixture with some success, demonstrating the potential of these environment-responsive membranes for use in multi-component separations. A second set of membranes, with a small valve effect, was selected for its ability to alter between hydrophobic and hydrophilic states under different environmental conditions and its potential in hydrophobic interaction membrane chromatography (HIMC) applications was successfully demonstrated by comparing against a benchmark membrane that is used successfully for HIMC applications in prior literature.

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Ruixiang Huang

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CHAPTER 1

INTRODUCTION

Environment-responsive (ER) membranes are mostly conventional filtration membranes that have been modified by grafting a layer of polymer chain grafts on the membrane surface or inside their pores or membranes that have been modified by the addition of an interpenetrating network of cross-linked hydrogel in their pore network structure [1-24].

These additions to the membrane general show a phase transition type behavior to two fundamental stimuli, temperature [11-13] and pH [1-10], resulting in a change in pore properties, charge or affinity to certain chemical moieties present in the substances that is being filtered. In addition, they can also be made responsive to other targeted stimulus (solute [24], solvent quality [15], electric fields [16-17], light [19-23], magnetic fields [19], and pressure [18]) by embedding the appropriate stimulus-responsive moieties (which produces the fundamental stimuli when the targeted stimulus is present) to the hydrogel network or the polymer chain grafts.

Practical applications for environment responsive membranes are that of in water and wastewater treatment, and that of multi-component separations in bio-process engineering.

Membranes used in water treatment applications have generally tend to foul very rapidly, due to the high particulate and organic content present in that of groundwater or wastewater. As a result, the trans-membrane pressure (TMP) in such applications (which are generally operated at constant flux mode) builds up very rapidly, greatly diminishing operating performance.

Measures to restore membrane performance proposed include periodic backflushing and chemical cleaning of the membranes. The former is very disruptive to operation, while the later measure often destroys the membrane integrity with the harsh nature of the chemicals used. Even with both measures, membrane performance is never fully restored.

ER membranes provide the possibility of reducing the frequency of back flushing and the usage of harsh chemicals during cleaning. By varying the pore size and membrane surface characteristic in response to environmental conditions, the foulants detach more readily, allowing back flushing to be completed far more quickly, and gentler agents to be used for membrane cleaning.

Multi-component separation in bioprocess engineering has been traditionally done by chromatography, which separate components dissolved in the mobile phase of the process on the basis of each component's relative mobility through the stationary phase. However, for a high purity separation and throughput, a very large, long chromatography column is required. This results in a very large process footprint ratio, making scale-up of chromatography processes very difficult.

Ultrafiltration has recently been utilized for large scale purification of macro-molecules. It utilizes a membrane barrier, and separates components based on differences in their size, charge and affinity towards the membrane. Because of its barrier-based nature, ultrafiltration has a smaller footprint ratio hence could be easily scaled up. However, its application has generally been limited to lone component purification, with multiple membranes required for a clean separation of multi-component mixtures.

ER membranes could enhance the potential of ultrafiltration by allowing sequential elution or permeation of different macromolecules by leveraging on pore size, charge and affinity binding changes caused by manipulation of environmental and operating conditions.

From the literature review, the substrate membranes are generally made from PVDF, polycarbonate, polyacrylonitrile (PAN), Telfon®, polyethylene and polypropylene [1-24]. The average pore size of the substrate seen in the literature review is around 0.2 microns (range: 0.09 -0.4 microns) or a MWCO ranging from 100,000 – 2,000,000 [1-24]. For pH-responsiveness, the polymer added is generally polyacrylic acid (PAA), polymethacrylic acid (PMAA), or poly(4-vinyl-pyrroridine) (P4VP) [1-10]. For solvent or temperature-responsive membranes, only poly-(N-isopropylacrylamide) gel grafting was reported [11-15]. The results from the studies show that permeability of the membrane is reduced significantly after modification in conditions which the chain or gel is swollen, but the permeability is often restored to levels close to the unmodified permeability in conditions in which the polymer is collapsed [11].

Filtration when the gel is in a collapsed state is between 4 – 1000 times higher than if the gel is in a swollen state. Filtration of model particles, like dextran, bovine serum albumin and others, were conducted in some of these studies, which show mixed results [1,3-8,11-12,15-17,19].

Given the significant interest in developing advanced separation processes for protein fractionation and the amount of research completed with ER membranes, it was surprising to find that none of the studies looked at how the dynamic properties of these membranes could be leveraged upon to facilitate multi-component separation. Furthermore, the literature

review above also found that most of the environment-responsive moieties studied previously are generally non-biocompatible, with some moieties being actually being toxic/irritant [1-6,10,15,17], carcinogenic [11,14,12] in their monomeric form, which are often present as residuals inside the membrane.

There are two objectives to this study project. The first was to synthesize and characterize environment-responsive membranes by in-situ polymerization of a bio-compatible, thermo-responsive hydrogel which was not studied by prior literature in the field of bioseparations using environment-responsive membranes. The second objective was to develop protocols leveraging on the dynamic properties of the membranes synthesized to facilitate multi-component separation, which involved using two sets of membrane synthesized with very different properties for ultrafiltration and membrane absorption applications respectively.

In chapter two, environment-responsive membranes were created by modification of a commerical PVDF membrane support with a thermo-responsive hydrogel composed of poly N-vinyllactams cross-linked with bisacrylamide. The modified membranes were then characterized by their percentage mass gains as well as by their valve effect in response to changes in salt concentration. One set of membranes, with a large valve effect, was selected for highest retention of intermediately sized proteins was examined for ultrafiltration-based protein separation applications. A batch separation protocol featuring pulsed sample injection technique (PSIT) was then used to sieve single proteins and to fractionate a synthetic binary protein mixture and a synthetic ternary protein mixture with some success, demonstrating the potential of these environment-responsive membranes for use in multi-component separations.

In chapter three, a second set of membranes, with a small valve effect, was selected for its ability to alter between hydrophobic and hydrophilic states under different environmental conditions and its potential in hydrophobic interaction membrane chromatography (HIMC) applications was successfully demonstrated by comparing against a benchmark membrane that is used successfully for HIMC applications in prior literature.

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CHAPTER 2

ENVIRONMENT-RESPONSIVE MEMBRANES FOR ULTRAFILTRATION-BASED BIOSEPARATIONS

Abstract

Environment-responsive ultrafiltration membranes were created by modification of a commerical PVDF membrane support with a thermo-responsive hydrogel composed of poly N-vinyllactams cross-linked with bisacrylamide. The modified membranes were then characterized by their percentage mass gains as well as by their valve effect in response to changes in salt concentration. A set of membranes selected for highest retention of intermediately sized proteins were examined for protein separation applications. A batch separation protocol featuring pulsed sample injection technique (PSIT) was then used to fractionate a synthetic binary protein mixture and a synthetic ternary protein mixture with some success, demonstrating the potential of these environment-responsive membranes for use in multi-component separations.

2.1 Introduction

Multi-component separation in bio-processes is traditionally done by chromatography, which separate components on the basis of relative mobility through a column. However, chromatographic equipment has large process footprint and scale-up of such processes is challenging. Membrane processes are being utilized for large scale purification of macro-molecules [1]. A membrane separates components based on differences in their size, charge and affinity towards the membrane. Due to its barrier-based nature, which results in a shorter path and smaller pressure drop, membrane filtration equipment has a smaller

footprint and is easily scaled up. However, application of membranes has generally been limited to two-component purification.

In the last two decades, novel environment-responsive membranes have been developed by various researchers [2-18]. Such membranes have stimuli-responsive moieties on the surface of the membrane or embedded in their membrane structure, which allow the membranes to dynamically change their structural, charge and affinity characteristics during filtration. Such membranes, when applied to membrane filtration could allow separation or elution by leveraging on pore size, charge and affinity binding changes caused by manipulation of environmental and operating conditions. The change in membrane pore and surface properties dynamically during filtration allow for the potential of novel multi-component separation protocols requiring just one membrane.

The earliest report of such membranes using in separation studies, to the best of the author's knowledge, was by Esienberg and Grozinsky [2], with membranes made from bovine hide corium collagen. The membrane permeability towards sucrose was controlled by electric fields applied across the solution and membrane, and large variation in flux by varying the electric field strength was reported. The rate of response to stimuli changes was also noted in that study. Subsequently, Iwata and Matsuda [3] studied the effects of environment-responsive polymer chains of poly-acrylic acid and polyacrylamide grafted on the surface of PVDF membranes and found by varying the length of the polymer chains grafted on the membrane surface, they were able to control the rejection of different macro-molecules under different conditions. The first

comprehensive determination of the change in molecular weight cut-off values of such membranes between different conditions, to the best of the authors' knowledge, was conducted by Masawaki et al [4]. The study utilized pH-responsive poly(4-VP-co-AN) membranes, filtering poly(ethylene-glycol) of a constant mass concentration at 1 g/L, but with different molecular weights, at pHs 4 and 10. It was found that the molecular weight cut-off of the membrane increased as the pH of the solution was increased. In addition to the above, there have been numerous other studies on the steady state behaviors of environment membranes at different conditions, utilizing different stimuli-responsive moieties, different membranes, different stimuli and different components to be separated [5-14].

It must be noted that none of the above studies have looked at how the dynamic properties of these membranes could be leveraged upon to facilitate multi-component separation. Furthermore, the literature review above also found that most of the environment-responsive moieties studied previously are generally non-biocompatible, with some moieties actually being toxic/irritant [3-7,9,15-17], carcinogenic [11,14,18] in their monomeric form, which are often present as residuals inside the membrane.

In this study, a novel biocompatible environment-responsive membrane is developed from commercially available PVDF membranes for the purpose of multi-component protein separation. The membranes are modified via in-situ thermal polymerization of two types of vinyllactam monomers with a N,N' methylene-bisacrylamide (hereby known as bisacrylamide) cross-linker and extracted repeatedly with water to remove the

unreacted reagents and unbound polymer and polymer gel. The modified membranes are then characterized by mass gain after moiety addition, response and extent of hydraulic permeability change while varying different solutions. A novel separation protocol is also presented, demonstrating the potential for the multi-component separation, using a synthetic mixture of two or more different proteins. The potential of the dynamic behaviour of such membranes could be leveraged on to achieve multi-component separation by single step filtration was assessed.

2.2. Experimental

2.2.1. Materials

Hydrophilized PVDF membrane discs (VVPP, 47 mm diameter) having a nominal pore size of 0.1 microns were purchased from Millipore and were used as the membrane base support and substrate on which the environment-responsive moieties were added upon to make the membrane environment-responsive. To ensure that there are no contaminants inside the membrane before moiety addition, acetone solution was used to extract the organics impurities within the membrane before the environment-responsive moieties were added.

The environment-responsive moiety that was added onto the membranes in this study is in form of a cross-linked hydrogel that is composed of varying compositions of N-vinyl-caprolactam, N-vinyl-pyrolidone monomers and bisacrylamide cross-linker. Iso-propanol was used as a liquid media in which the polymerization and cross-linking of the hydrogel within the membrane takes place. Azobisisobutyronitrile (AIBN) was used in this study as a thermal initiator in the polymerization reaction. All the above reagents, besides the membranes, were purchased from Sigma Aldrich. In addition cellophane sheets purchased from Arkwright were used to sandwich the membranes during polymerization as well.

20 mM phosphate buffer solutions of two different pHs (4.9, 7) at different NaCl concentrations (0 M, 1.5 M) were formulated using distilled de-ionized water obtained

from a Branstan Diamond Pure water purification unit. The phosphate and NaCl salts used to formulate the above buffers were all purchased from Sigma Aldrich.

To create the synthetic protein solutions, 5 commercially available proteins having slightly different molecular weights and hydrodynamic radiuses were purchased from Sigma Aldrich and mixed with the pH 7 low salt buffer solution formulated above. The proteins used are Bovine Serum Albumin from bovine serum (BSA) (MW=67kDa, minimum 98%, obtained via electrophoresis), Immunoglobulin from human serum (HIgG) (MW=155kDa, lyophilized powder, >= 95% purity, obtained via electrophoresis), Equine Ferritin from equine spleen (FER) (MW= 440 kDa, Type 1, saline solution, stock solution 111 mg/mL), Bovine Thyroglobulin from bovine thyroid (TG) (MW ~670kDa, powder form, >= 90%, agarose gel electrophoresis.) and Bovine Immunoglobulin M (BIgM) (MW ~950kDa, solution form (0.8 – 1.2 mg/mL), >=95%, obtained via HPLC).

2.2.2 Membrane Modification Procedure

The membranes discs are first soaked in acetone solution overnight to ensure that all extractable organic residuals within the membrane which might affect moiety addition are removed. The membranes are then dried in a constant humidity (50% relative) and temperature environment (25 ^{o}C) for at least 8 hours. The membrane is then weighed before moiety addition.

The monomers, cross-linker, initiator used to create the hydrogel are thoroughly dissolved in iso-propanol before the membranes are soaked in the said mixture for 10-15

minutes. The soaked membrane is then taken out of the mixture and sandwiched between two cellophane sheets on a glass plate. A roller was then applied across the surface of the sheets to ensure that no air bubbles were present in the reaction environment, which would inhibit the polymerization reaction. After rolling, scotch tape is applied on the edges of the cellophane sheet onto the glass tape to seal the membrane off from the atmosphere. The glass plate with the cellophane sandwiching the membrane is then placed into an oven at a constant temperature of 70 °C and left there for at least 8 hours in order for the polymerization process to be completed.

At the end of 8 hours, the glass plate is taken out of the oven and the membranes are then extracted in distilled de-ionized water at 70 °C for 3 days to remove the un-reacted monomers, other reaction byproducts and residuals in the membrane. At the end of 3 days, the membrane is washed again with distilled de-ionized water and left to dry in a constant temperature and humidity environment before it is characterized.

2.2.3 Membrane Characterization Apparatus

Following the drying of the membrane, the weight of the membrane after moiety addition is taken, and the mass gain percentage of the membrane under a particular modification condition is computed. The membrane is then mounted onto a custom-made membrane absorber module. The module is connected to a Masterflex peristaltic pump supplied by two buffer reservoirs with different buffer conditions, and the buffer supplied to the module is switched between the two buffers by the means of a flow switch. A pressure sensor is attached to the module to monitor trans-membrane pressure (TMP) changes

during the experiment, and the readings of the pressure sensor over time are automatically logged by a computer.

The system is operated at a constant flow mode, resulting in a constant flux through the membrane, while the buffer supplied is alternated between the different buffer solutions to determine both the extent of hydraulic permeability change at 2 different buffer conditions.

The membrane permeability, k_{mem} (m^2), is then determined from the above flux experiments and calculated using equation (2.1) [19, 20]:

$$k_{mem} = \frac{Q \cdot d \cdot \eta}{A \cdot \Delta P} \qquad (2.1)$$

where Q is the volumetric flow rate through the membrane (m^3/s), d the membrane thickness (m), η the solvent viscosity (Pa·s), A is the active membrane surface area (m2), and ΔP the trans-membrane pressure (TMP) (Pa). The permeate flux, J_v, is calculated from Q/A. The membrane permeability is calculated from the slope of the straight line obtained by plotting $Q \cdot \eta / A$ as a function of $\Delta P/d$.

After screening and characterizing various membranes modified via the above characterization tests, the membranes that seemed to show the possibility of retaining certain large proteins while allowing relatively unhindered permeation of other smaller proteins were selected for further experimentation.

2.2.4 Single Protein Ultrafiltration Sieving Experiments

The method and system used in the batch protocol runs used is adapted from that of Ghosh and Cui [21]. A 5 mL stirred cell with the membrane mounted inside the module is integrated with the AKTA Prime-plus chromatography system. The system is supplied with two buffer reservoirs containing two different 20 mM phosphate buffers at pH 7 or 4.7 with significantly different buffer conditions, one having a 0 M NaCl concentration (Buffer A), the other having either a 1.25 M NaCl or 1.5 M NaCl concentration (Buffer B). A lower salt concentration (1.25 M NaCl) is used for Buffer B when the particular protein (HIgG and FER) has a propensity to aggregate at high salt concentrations. The system is monitored for its permeate UV absorbance, conductivity, pH and system TMP changes, which the readings are automatically logged onto the computer via the Labview software interface supported by the AKTA prime plus system.

The low salt buffer is first fed into the module at a known constant flux until a steady baseline is reached for all the monitored parameters. A 0.5-2.0 mL protein solution containing a known concentration of either BSA, HIgG, FER or TG is then injected into the system and the changes in the permeate's UV absorbance, pH, conductivity and system TMP changes are monitored for the time length it takes to pass 2-3 full cell volumes of buffer solution through the module.

After 2-3 full cell volumes have been filtered, the buffer supplied to the module is then switched to the high salt buffer and the changes in the permeate's UV absorbance, pH, conductivity and system TMP changes are monitored for the time length it takes to pass

2-3 full cell volumes of buffer solution through the module, after which the buffer supplied to the module is switched back to the low salt buffer to pass another 3 full cell volumes of buffer solution through the module.

A 2mL sample of buffer is collected at the following sections of such a run:

1.) The permeate immediately following the injection of the protein solution. (Known as 'initial permeate'.)

2.) The permeate immediately before the buffer change to a high salt buffer. (Known as 'permeate just before first buffer change'.)

3.) The permeate immediately following the buffer change to a high salt buffer. (Known as 'permeate just after first buffer change'.)

4.) The permeate immediately following the buffer change back to the low salt buffer. (Known as 'permeate after second buffer change'.)

5.) The retentate inside the stirred cell at the end of the run. (Known as 'retentate'.)

The samples collected are then analyzed along with the feed sample through a size exclusion chromatography column via a Varian Star high pressure liquid chromatography system to determine the retentive properties of the membranes for the single protein solution at two different buffer conditions.

2.2.5 Batch Separation Protocol featuring Pulsed Sample Injection Technique (PSIT)

A 5 mL stirred cell with the membrane mounted inside the module is integrated with the AKTA Prime-plus chromatography system. The system is supplied with two buffer reservoirs containing two different 20 mM phosphate buffers at pH 7 or 4.7 with significantly different buffer conditions, one having a 0 M NaCl concentration (Buffer

A), the other having 1-2 M NaCl concentration (Buffer B). The system is monitored for its permeate UV absorbance, conductivity, pH and system TMP changes, which the readings are automatically logged onto the computer via the Labview software interface supported by the AKTA prime plus system.

The low salt buffer is first fed into the module at a known constant flux until a steady baseline is reached for all the monitored parameters. A 0.5 mL protein mixture containing a known quantity of HIgG and FER (Sample A) is then injected into the system and the changes in the permeate's UV absorbance, pH, conductivity and system TMP changes are monitored for the time length it takes to pass 2-3 full cell volumes of buffer solution through the module.

After 2-3 full cell volumes have been filtered, the buffer supplied to the module is then switched to the high salt buffer and the changes in the permeate's UV absorbance, pH, conductivity and system TMP changes are monitored for the time length it takes to pass 2-3 full cell volumes of buffer solution through the module, after which the buffer supplied to the module is switched back to the low salt buffer to pass another 2-3 full cell volumes of buffer solution through the module.

A 2mL sample of sample is collected at the following sections of such a run:

1.) The permeate immediately following the injection of the protein solution. (Known as 'initial permeate' – Sample B.)
2.) The permeate immediately before the buffer change to a high salt buffer. (Known as 'permeate just before first buffer change'.)

3.) The permeate immediately following the buffer change to a high salt buffer. (Known as 'permeate just after first buffer change' – Sample C.)

4.) The permeate immediately following the buffer change back to the low salt buffer. (Known as 'permeate after second buffer change' – Sample D.)

5.) The retentate inside the stirred cell at the end of the run. (Known as 'retentate' – Sample E.)

The samples are then analyzed along with the feed sample via protein A affinity chromatography on an AKTA Prime-plus system to determine the retentive properties of the membranes for the binary protein solution at two different buffer conditions, and also through a size exclusion chromatography column via a Varian Star high pressure liquid chromatography system.

2.3. Results and Discussion

2.3.1 Membrane Modification

It was found that the composition of the co-polymers had no effect on the resultant percentage mass gain of the modified membranes. It is also observed that the percentage mass gain of the membrane is determined positively proportional to both the weight percentage of cross-linker added to the reaction media as well as the weight percentage of the hydrogel polymer pre-cursors in the reaction media.

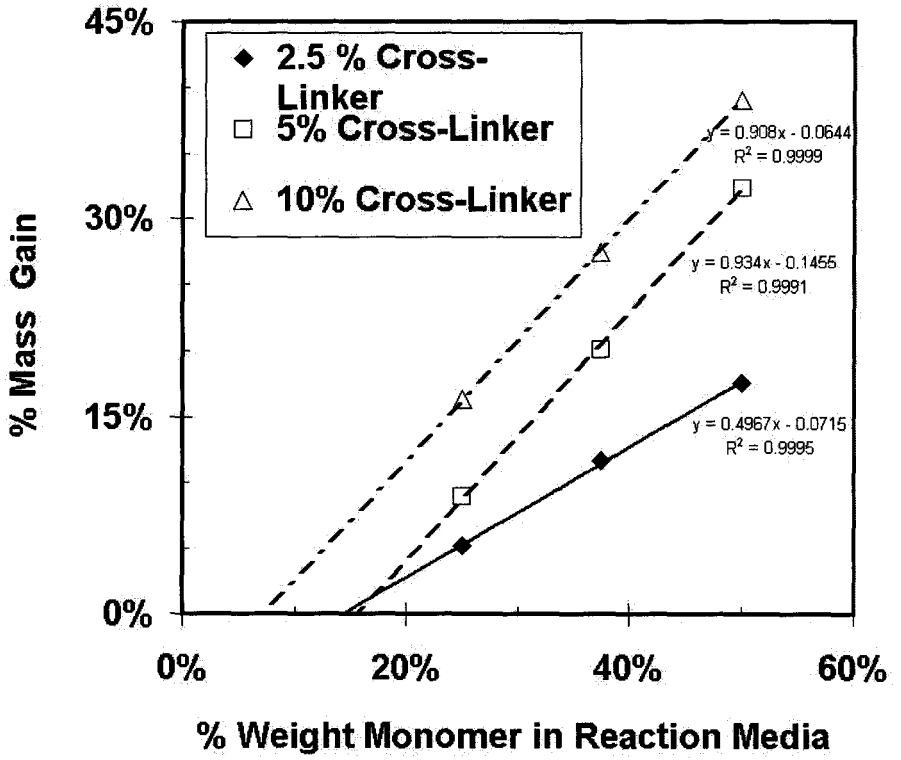

Figure 2.1 Membrane characterization by mass gain and composition

As can be seen from Figure 2.1, the percentage mass gain of the membrane is a function of the weight percentage of the monomer pre-cursor present in the reaction media and

that of the weight percentage of the bisacrylamide cross-linker present in the reaction media. The relationship appears to be generally linear in nature, where it is shown that at a constant percentage weight of bisacrylamide cross-linker added, the percentage mass gain by the membrane, at least observed from the data available, is approximately a linear function of the percentage weight of the monomer pre-cursor present in the reaction media. The regression lines in Figure 2.1 are drawn to guide the eyes.

Table 2.1 shows the characteristics of the modified PVDF membranes when subjected to constant flux filtration, under different buffer conditions, along with results from an unmodified hydrophilic PVDF membrane for comparison. From Table 1, it is seen that the effect of the above two factors on the valve range also appear to be multiplicative, as can be seen by the similar low valve range valves obtained for membranes modified under conditions 1, 2, and 3 due to either the weight percentage of bisacrylamide cross-linker added being low, or the weight percentage of monomer pre-cursor added being low, or both. Conversely, the higher valve range values for membranes modified under conditions 4 and 5 is due to the modification conditions possessing at least a moderate amount weight percentage of both bisacrylamide cross-linker and monomer pre-cursor added to the reaction media.

Table 2.1 Membrane permeability characteristics at different salt conditions

Membrane Condition	k_{mem} $x10^{16}$ (m^2) Low Salt	k_{mem} $x10^{16}$ (m^2) High Salt	Valve Range	$\sqrt{k_{mem}}$ $x10^9$ (m) Low Salt	$\sqrt{k_{mem}}$ $x10^9$ (m) High Salt
Unmodified hydrophilic 0.1 micron PVDF membrane (<10% change in $\sqrt{k_{mem}}$ value)	4.51	5.51	1.22	21.2	23.5
1 (~35% change in $\sqrt{k_{mem}}$ value)	1.08	2.90	2.68	10.4	17.0
2 (~60% change in $\sqrt{k_{mem}}$ value)	1.93	5.22	2.71	13.9	22.8
3 (~60% change in $\sqrt{k_{mem}}$ value)	0.25	0.67	2.73	4.96	8.20
4 (~100% change in $\sqrt{k_{mem}}$ value)	0.07	0.38	5.30	2.66	6.13
5 (>200% change in $\sqrt{k_{mem}}$ value)	0.038	0.38	9.85	1.95	6.13

The membrane permeability, k_{mem}, was calculated using equation (2.1) with the results displayed in Table 2.1. The membrane permeability, k_{mem}, provides insight into the microstructure of the modified membrane, where the value of $\sqrt{k_{mem}}$ is a measure of the spacing within the membrane and hydrogel network [19, 20]. In the absence of methods yielding accurate parameters for the membrane pore size after modification, the value of $\sqrt{k_{mem}}$ can be used as a crude estimate for the average spacing between the networks of the hydrogel chain structure at different conditions [19, 20]. This could be crudely interpreted as some measure of the average effective pore radius of the membrane. However, it must be noted that the nature of the PVDF support, being of a network nature, has a very large pore size distribution. Therefore, the reported values of $\sqrt{k_{mem}}$ will significantly underreport the effective pore radii of the membrane, as evidenced by the $\sqrt{k_{mem}}$ of the unmodified membrane being only one-quarter to one-fifth of the nominal pore diameter of the unmodified PVDF membrane shown in Table 2.1.

For an effective environment-responsive membrane to be used in multi-component protein separation, the said membrane must have a $\sqrt{k_{mem}}$ valve in closed valve mode that is significantly smaller than the larger proteins that is to be retained, while the value of $\sqrt{k_{mem}}$ in open valve configuration must be sufficiently large enough to allow permeation of the intermediate sized proteins. Therefore, the approximate size of the proteins to be separated must also be known. Table 2.2 shows the typically reported Stokes radius (or hydrodynamic radius) of some commercially available proteins. By comparing the values of $\sqrt{k_{mem}}$ in closed valve position from Table 2.1 and the hydrodynamic radius of commercially available proteins from Table 2.2, we find that condition 1, 2 and 3 yield values of $\sqrt{k_{mem}}$ in closed valve positions that are between 5-14 nm, when multiplied by a factor of 4 or 5, yield a nominal pore size between 25 – 70 nm, which is very much larger than the intermediate and larger proteins shown in Table 2. Hence they are deemed to be unsuitable as candidates for further study in multi-component protein separation; given the pore size estimate is underreported by the value of $\sqrt{k_{mem}}$.

Table 2.2 Typically reported Stokes radius (hydrodynamic radius) of proteins

Protein name	**MW (kDa)**	**Hydrodynamic radius (R_s) ($x10^9$m)**	**Hydrodynamic diameter ($x10^9$m)**
Bovine Immunoglobulin M (BIgM)	950	12.65[25]	25.3
Bovine Thyroglobulin (TG)	669	8.5 [25]	17
Equine Ferritin (FER)	440	6.1 [26]	12.2
Human Immunoglobulin G (HIgG)	155	5.29 [25]	10.6
Bovine Serum Albumin (BSA)	67	3.5 [25]	7

The membranes made from condition 4 and 5, using the above calculation method, yield nominal closed valve position pore diameters of 10 and13.3 nm respectively. By taking a more conservative approach due to the large distribution in pore size inherent to the membranes, it was determined that membranes prepared at condition 5 would have the highest possibility of retaining certain large marco-molecules while allowing other smaller marco-molecules to permeate while in its closed valve configuration at the low salt buffer concentration, and would allow intermediate sized marco-molecules to permeate through while in its open valve configuration at high salt buffer concentrations.

2.3.2 Protein Ultrafiltration

2.3.2.1 Single Protein Sieving Protocol featuring PSIT

All the results from this point onwards pertain to a particular set of membranes prepared at condition 5. Table 2.3 shows the summary of the average sieving coefficient parameters determined by a large number of single protein sieving runs using the PSIT method as outlined above. Figures 2.2-2.5 show the filtration operation curve of these experiments at a constant pH of 7. The legend for Figures 2.2-2.5, 2.6, 2.8 is shown in Figure 2.10 at the end of this chapter (pg 2-35), before the chapter references.

Table 2.3 Summary of sieving coefficient parameters

pH	Salt	Average S_{app} (BSA)	Average S_{app} (HIgG)	Average S_{app} (FER)	Average S_{app} (TG)
4.7	High	Not tested	0.37	0.25 ± 0.13	Not tested
4.7	Low	Not tested	0.22 ± 0.05	0.16 ± 0.08	Not tested
7	High	Approx. 1	0.27	0.04	0.03
7	Low	Approx. 1	0.39 ± 0.22	0.11	<0.005

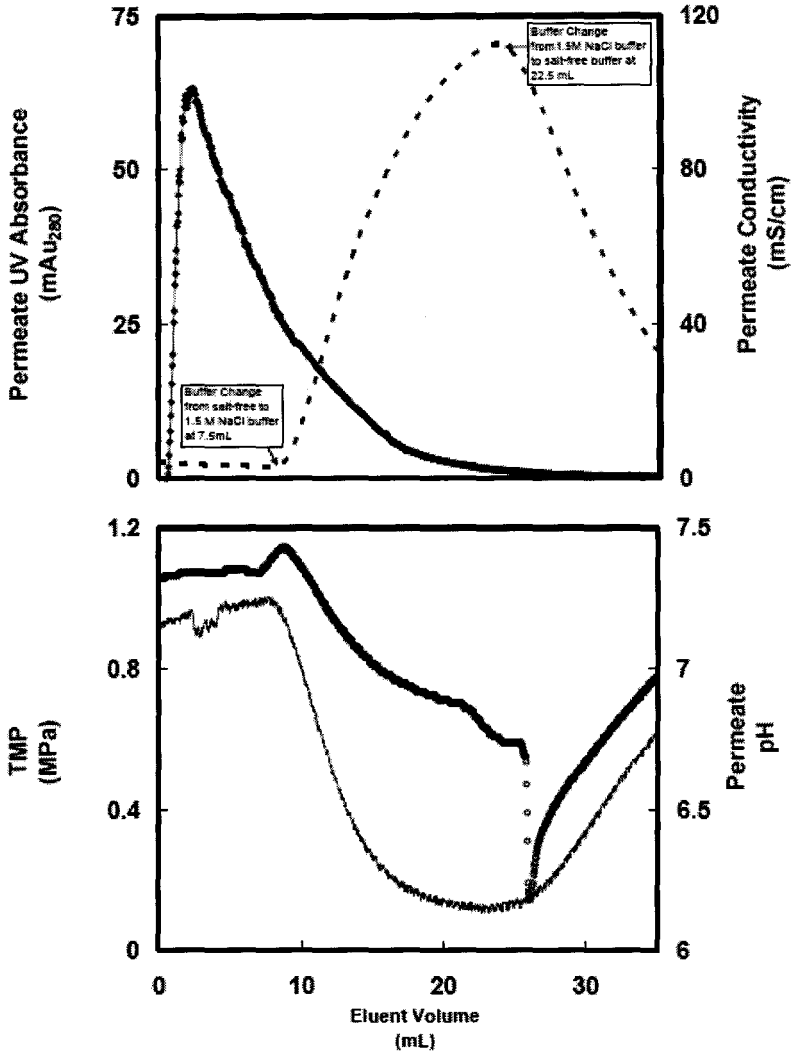

Figure 2.2 UV, conductivity, TMP and pH Profile of PSIT protocol of BSA filtration, initial cell concentration 1.2 mg/mL, constant pH 7.

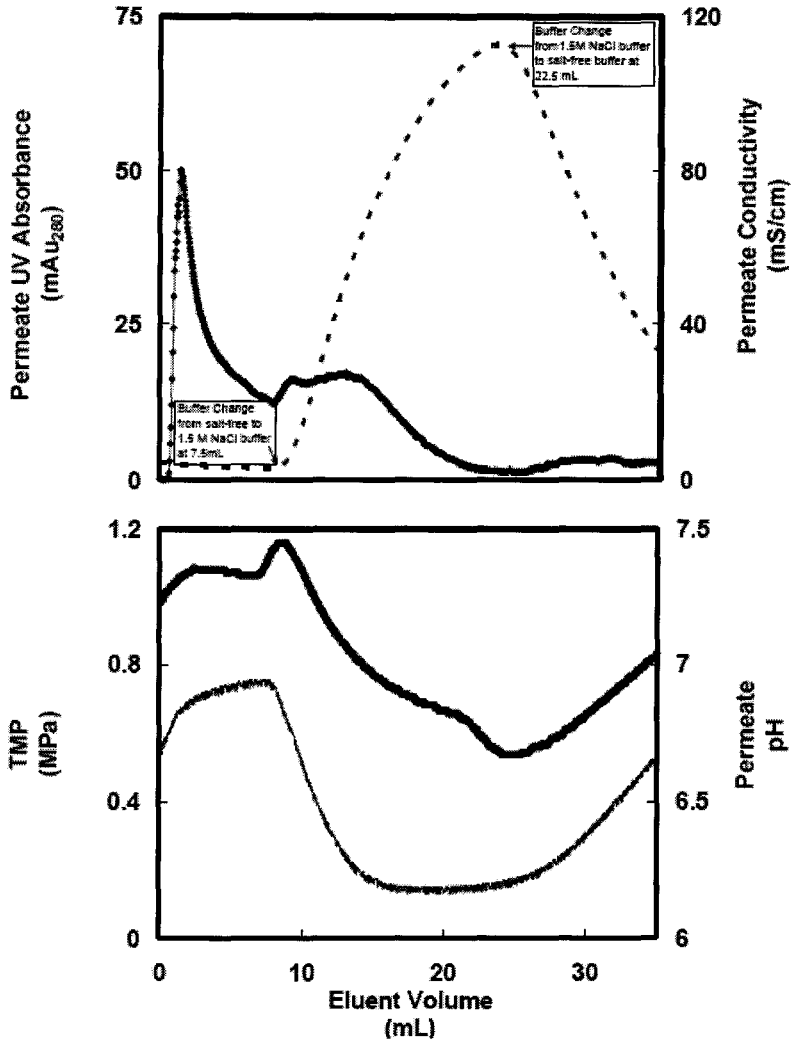

Figure 2.3 UV, conductivity, TMP and pH Profile of PSIT protocol of HIgG filtration, initial cell concentration 1.2 mg/mL, constant pH 7.

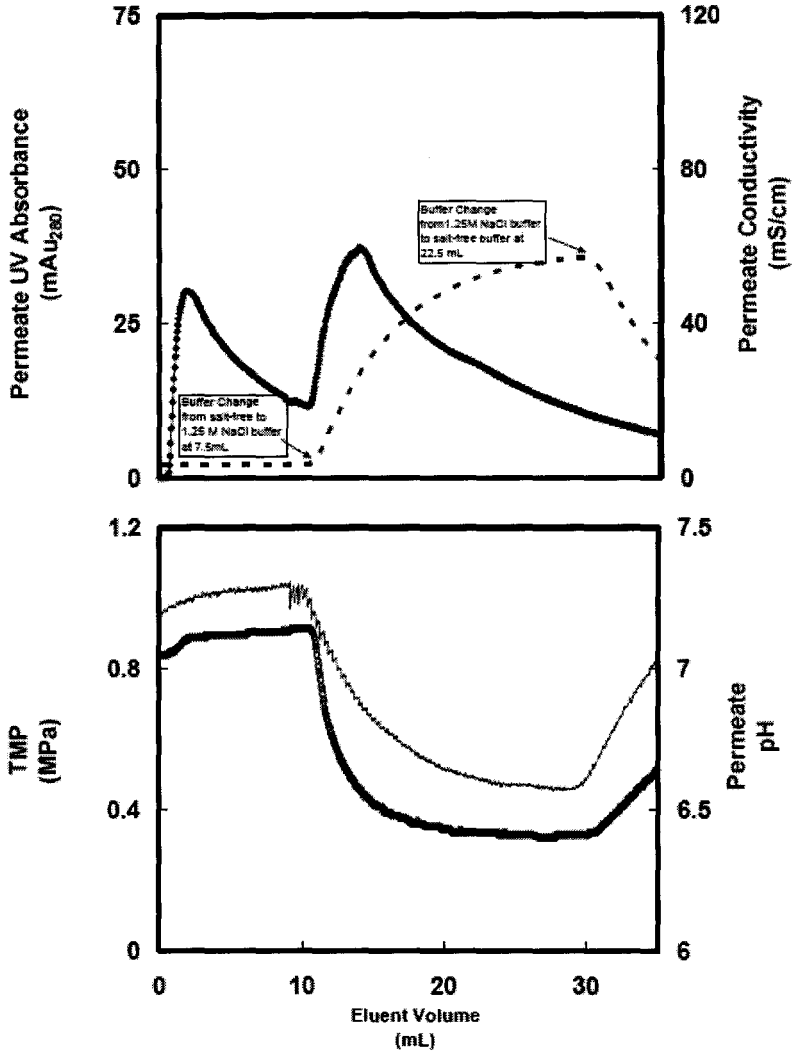

Figure 2.4 **UV, conductivity, TMP and pH Profile of PSIT protocol of FER filtration, initial cell concentration 2 mg/mL, constant pH 7.**

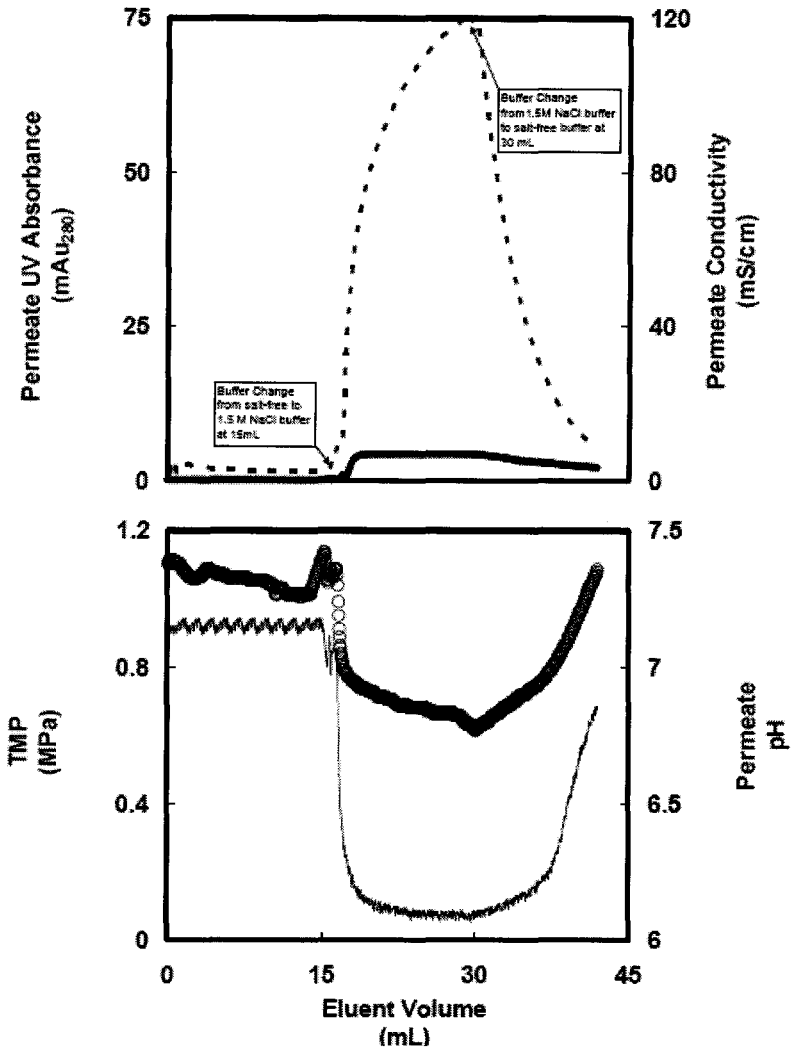

Figure 2.5 UV, conductivity, TMP and pH Profile of PSIT protocol of TG filtration, initial cell concentration 1.2 mg/mL, constant pH 7.

It is concluded that BSA, in its both monomeric and dimeric forms, permeates the membrane unhindered, while TG is almost completely retained by the membrane at the same pH. Hence the modified membrane is inferred to have a closed valve pore size which is at least larger than 7 nm (hydrodynamic diameter of BSA), and an open valve pore size will retain most proteins at around 17 nm (hydrodynamic diameter of TG).

For HIgG and FER, the approximate sieving coefficients vary significantly depending on buffer condition in which the proteins were filtered by the membrane. HIgG had its highest sieving coefficient when filtered at its approximate isoelectic point, pH 7, in low salt buffer (0.39), and had its lowest sieving coefficient when filtered in low salt pH 4.7 buffer (0.22), where it is positively charged. FER had its highest sieving coefficient when filtered at its approximate isoelectric point, pH 4.7, in high salt buffer (0.25), and its lowest sieving coefficient when filtered in high salt pH 7 buffer (0.04), when it is negatively charged.

Prior literature has suggested that a protein would have its smallest apparent size at isoelectric point, and hence would have its highest sieving coefficient at that point when subjected to ultrafiltration [23]. HIgG has been frequently reported in prior literature to aggregate into micron-sized precipitates at high salt concentrations near its isoelectric point [22], which allows for easy retention on the membrane. Given HIgG has a relatively high sieving coefficient (0.39) when filtered at low salt pH 7 buffer, HIgG must be permeating relatively unhindered through the membrane, even in its closed valve condition. Any minute aggregation of HIgG on the retentate side would reduce the effective concentration of soluble HIgG on the retentate side for membrane permeation, lowering the value of the apparent sieving coefficient of the membrane for HIgG.

FER also tends to aggregate at high salt concentrations, this property being used in the FER purification from cell lysate from equine spleen. However, the aggregation phenomenon of FER is not well studied, although it is claimed in the same paper that

proteins with iron have a marked tendency to precipitate at physiological pH [24]. Given that the permeation of FER through the membrane is significantly more hindered at pH 7 than pH 4.7, as evidenced by the higher sieving coefficients at pH 4.7, any minute reduction in the soluble FER due to protein aggregation during filtration at pH 7 into micron-sized precipitates will result in a significant reduction in the concentration of FER in permeate.

2.3.2.2 Novel Two Component Batch Fractionation Protocol featuring PSIT

Figure 2.6 shows the outcome of the proposed novel batch separation protocol featuring PSIT when applied to filtration of the binary protein solution buffer, while Figure 2.7 the HPLC-SEC analysis of some of the permeate samples collected at different time intervals of the separation protocol, and Table 2.4 shows the results of the protein A analysis on the same permeate samples collected.

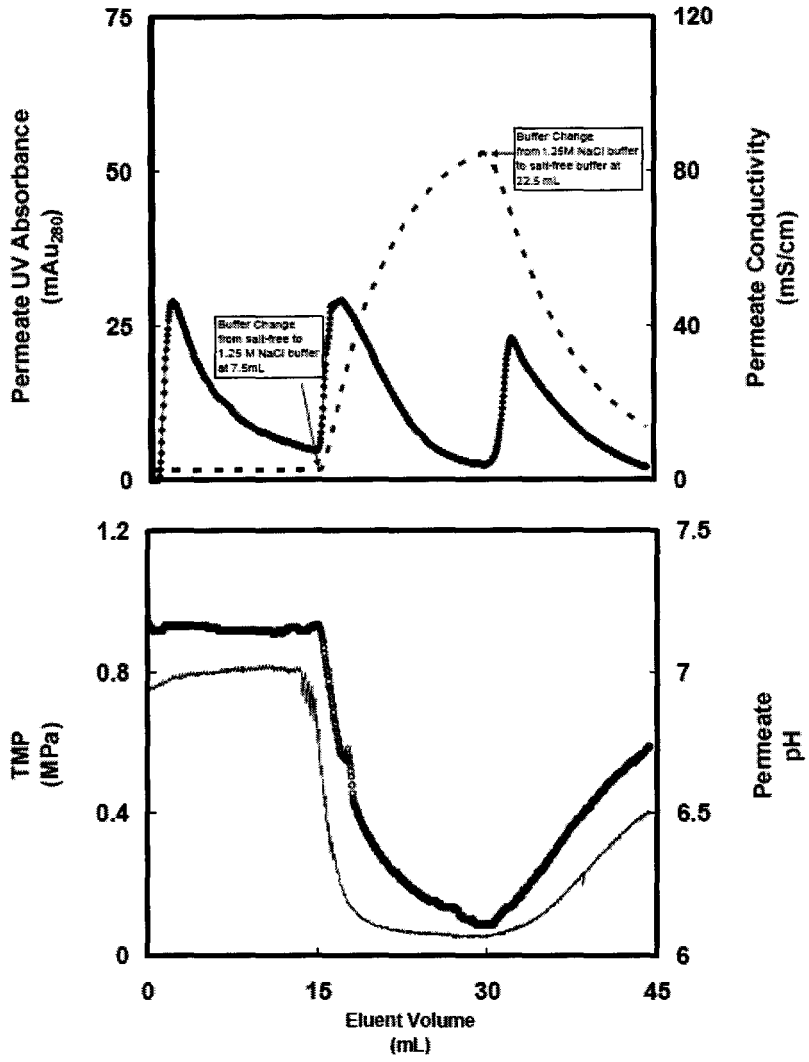

Figure 2.6 **UV, conductivity, TMP and pH Profile of two protein PSIT protocol, initial cell concentration – 0.39 mg/mL HIgG, 0.1 mg/mL FER.**

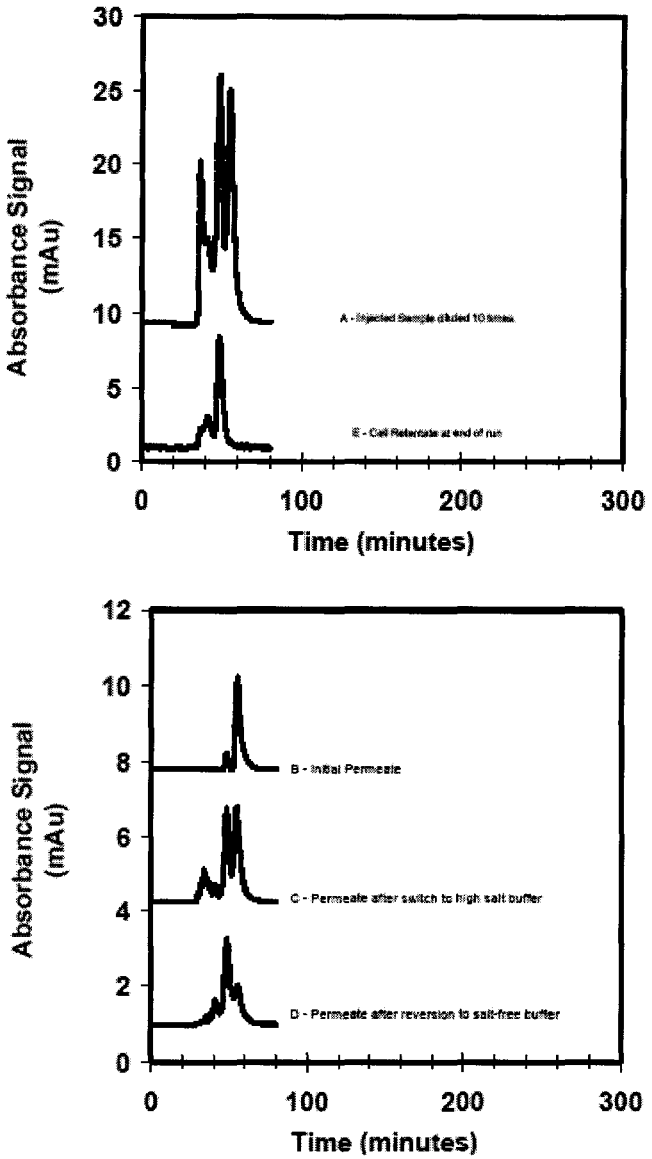

Figure 2.7 **HPLC size exclusion column (SEC) results of feed, permeate and retentate samples from two protein PSIT protocol. (A – injected sample, B – initial permeate, C – high salt permeate, D – reversion to low salt permeate, E – cell retentate at end of run).**

Table 2.4 Protein-A column analysis of 2 protein PSIT protocol permeate samples

Species (mg/mL)	Initial Cell Concentration (0mL)	Initial Permeate (1-3mL)	Permeate just before first buffer change (13-15mL)	Permeate just after first buffer change (16-18mL)	Permeate just after second buffer change (31-33mL)	Retentate Concentration (45 mL)
HIgG	0.39	0.11	0.007	0.034	0.007	0.002
FER	0.10	0.022	0.013	0.029	0.012	0.037

The following observations are made by a comparison of the results of the proposed novel separation protocol shown in and Table 2.4, Figure 2.6-2.7.

The initial permeate absorbance peak, according to the HPLC-SEC and Protein A column analysis of the initial permeate in Table 2.4, shows a proportionally larger percentage of HIgG than was present in the injected protein solution, indicating that the membrane is selective towards HIgG over FER. Towards the end of the first low salt phase of the protocol, after approximately 3 time constants, the HIgG content in the permeate has dwindled to a value that is significantly lower than that of the FER present in the permeate just before the first buffer change, indicating depletion of HIgG on the retentate side.

After the start of the high salt phase of the protocol, HPLC-SEC and Protein A analysis of the permeate sample collected around this section shown that both the HIgG and FER content in the permeate has increased over that of the permeate just before the first buffer

change, and that FER has now become a more significant part of the permeate composition than in the permeate just before the first buffer change.

At the start of the second low salt phase, a third permeate absorbance peak was observed, and Protein A analysis of the permeate sample collected around this section has showed that the overall concentration of the permeate has declined back to the levels of the permeate just before the first buffer change, and the composition of the permeate is also highly similar to that of the permeate just before the first buffer change, while HPLC-SEC results show that the FER content of the permeate at this time is significantly larger than during the first low salt phase. This peak could be explained by PVDF binding of protein during the high salt phase as well as formation of retainable protein aggregates during the high salt phase, and the elution of PVDF bound protein and dissolution of the above mentioned protein aggregates.

HPLC-SEC and Protein A analysis of the retentate remaining in the stirred cell at the end of the run shows that essentially all of the HIgG injected into the stirred cell at the start of the protocol has permeated through the membrane, while 63% of the FER has permeated through the membrane during the execution of the protocol, mostly during high salt buffer filtration.

From results obtained, the membranes fabricated appear to be a suitable candidate for further study for multi-component protein separation in batch operation mode, having clearly demonstrated its potential for usage in multi-component separation.

2.3.2.2 Novel Three Component Batch Fractionation Protocol featuring PSIT

To provide more concrete proof that the membrane is able to perform multi-component separation, a three-component batch fractionation is attempted.

Figure 2.8 shows the outcome of the proposed novel batch fractionation protocol featuring PSIT when applied to filtration of a synthetic three protein solution buffer, while Figure 2.9 the HPLC-SEC analysis of some of the permeate samples collected at different time intervals of the separation protocol.

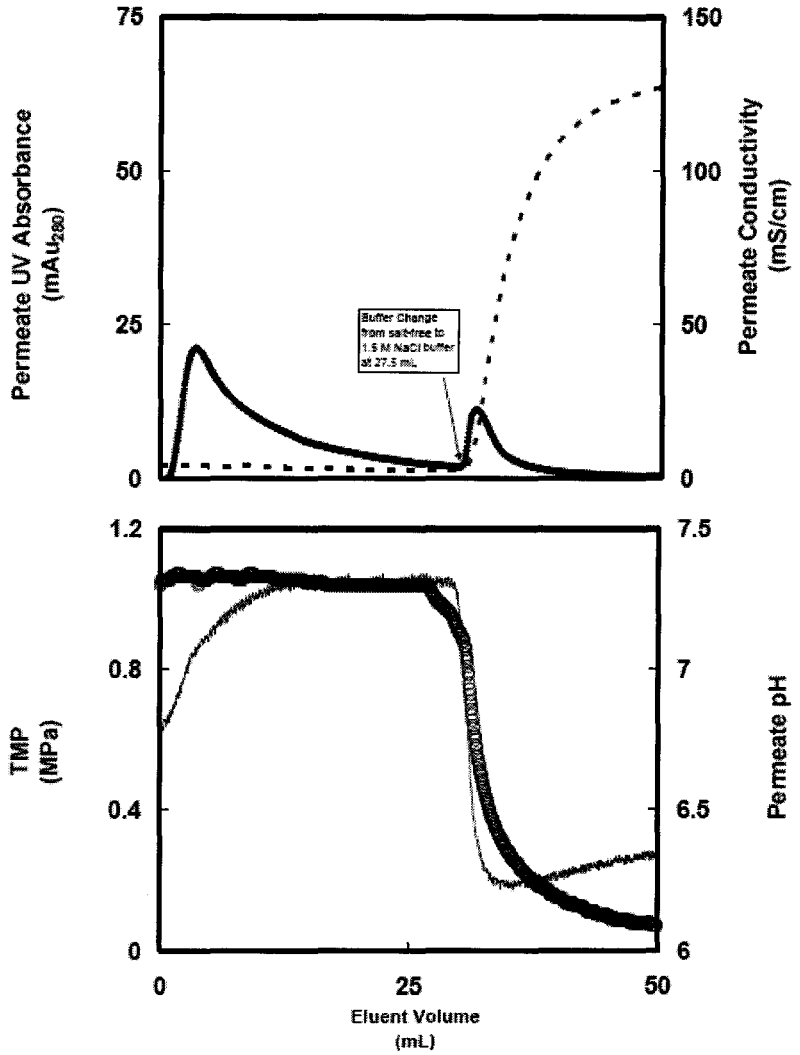

Figure 2.8 **UV, conductivity, TMP and pH Profile of three protein PSIT protocol, initial cell concentration: 0.24mg/mL HIgG, 0.12 mg/mL FER, 0.12 mg/mL BIgM.**

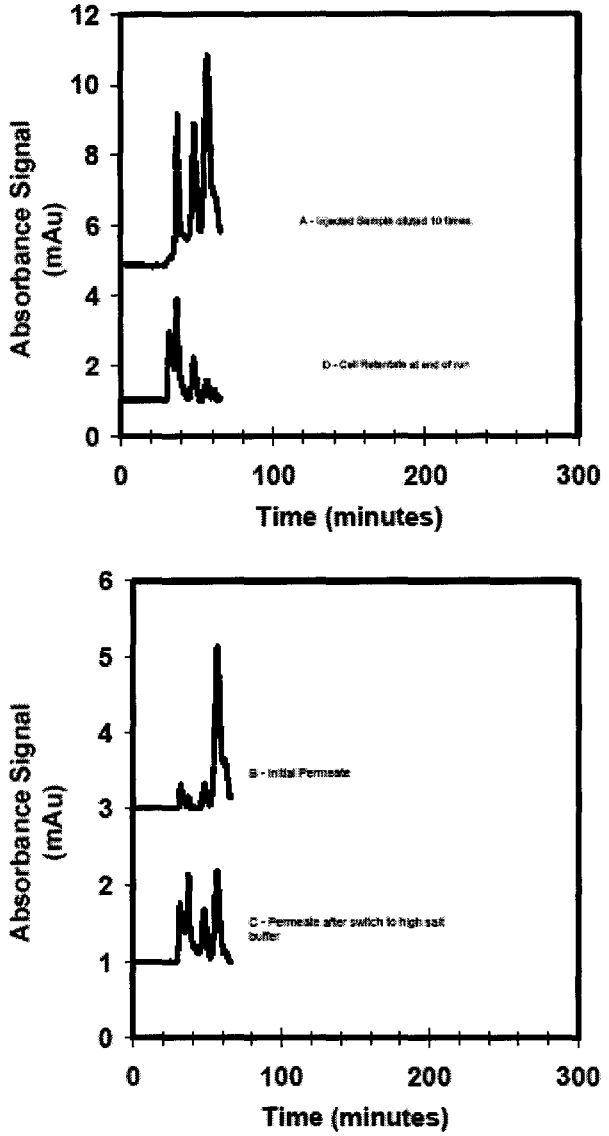

Figure 2.9 HPLC-SEC results of feed, permeate and retentate samples from three protein PSIT protocol. (A – injected sample, B – initial permeate, C – high salt permeate, D – cell retentate at end of run).

The following observations are made by a comparison of the results of the proposed novel fractionation protocol shown in Figure 2.8 and Figure 2.9.

The initial permeate absorbance peak, according to the HPLC-SEC and Protein A column analysis of the initial permeate in Table 2.4, shows a proportionally larger percentage of HIgG than was present in the injected protein solution, indicating that the membrane is selective towards HIgG over FER and BIgM.

After the start of the high salt phase of the protocol, HPLC-SEC collected around this section shown that both the FER and BIgM content in the permeate has increased over the initial permeate, and that FER and BIgM has now become a more significant part of the permeate composition.

HPLC-SEC analysis of the retentate remaining in the stirred cell at the end of the run shows that essentially all of the HIgG injected into the stirred cell at the start of the protocol has permeated through the membrane, while about half of the FER has permeated through the membrane during the execution of the protocol, mostly during high salt buffer filtration, and most of the BIgM still remains on the retentate side of the membrane.

The results shown in this section presents a stronger proof that the above membrane is a suitable candidate for further study for multi-component protein separation in batch operation mode. However, optimization of the process conditions would be required for a cleaner separation.

4. Conclusions

Based on the above results and explanations advanced to explain the results obtained, the following points are advanced as the conclusions for this study:

1.) Environment-responsive membranes have been successfully created through modification of a commerical PVDF membrane support with a thermo-responsive hydrogel composed of poly N-vinyl-lactams and bisacylamide cross-linker.

2.) The modified membranes are characterized by their percentage mass gain and membrane permeability, and it was found that the percentage mass gain of the modified membrane is found to be linearly correlated with the percentage weight of the monomer pre-cursor in the reaction media as well as the percentage weight of the bisacrylamide cross-linker added to the reaction media. The membrane permeability is found to be a multiplicative function of both the percentage weight of the monomer pre-cursor in the reaction media as well as the percentage weight of the bisacrylamide cross-linker added to the reaction media. The most appropriate modification condition for protein separation purposes in this study appear to be condition 5, based on the valve range and $\sqrt{k_{mem}}$ values calculated. Membranes made from condition 5, were therefore selected for further experimentation in protein filtration studies.

3.) The single protein sieving experiments utilizing a batch protocol featuring PSIT shown that the membranes selected were suitable for use in multi-component separation. The sieving coefficients obtained from single protein sieving experiments indicate that

the sieving coefficients of different proteins against the membrane can be changed by changing both the salt and pH of the buffers.

4.) The potential of the novel batch fractionation protocol was demonstrated through the filtration of a synthetic binary protein mixture composed of Equine Ferritin and Human Immunoglobulin G. The results show that smaller proteins could be fractionated from larger proteins using the membrane, through a protocol leveraging on the environment-responsive nature of the membrane's permeability, as well as the phenomena of the smallest apparent size of protein being at isoelectric point, to maximize the separation resolution of the proteins.

5.) Further proof of the potential of the novel batch fractionation protocol was demonstrated through the filtration of a synthetic three protein mixture composed of Bovine Immunoglobulin M, Equine Ferritin and Human Immunoglobulin G. The results show that using the above membranes, three different proteins having different sizes could be fractionated to a certain extent, through a protocol leveraging on the environment-responsive nature of the membrane's permeability, as well as the phenomena of the smallest apparent size of protein being at isoelectric point, to maximize the separation resolution of the proteins. However, optimization of the process conditions would be required for a cleaner separation than what is shown here.

Legend

For Figures:

2(a,b),3(a,b),4(a,b),
5(a,b),6(a,b),8(a,b).

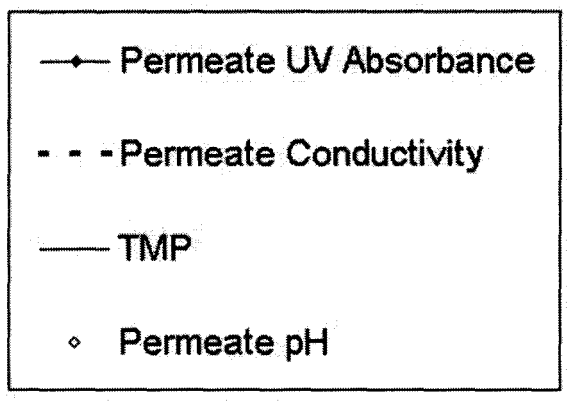

Figure 2.10 Legend.

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CHAPTER 3

HYDROGEL COATED MEMBRANES FOR MEMBRANE CHROMATOGRAPHY

Abstract

Environment-responsive absorptive membranes were created by modification of a commercial hydrophobic PVDF membrane support with a thermo-responsive hydrogel composed of poly N-vinyllactams cross-linked with bisacrylamide. The modified membranes were then characterized by their percentage mass gains as well as by their contact angle under salt-free and high salt buffer conditions. A set of membranes having properties which translate to probable high protein binding capacity were selected and examined for membrane chromatography applications. The membranes were compared against commercial 0.22 micron hydrophilized PVDF membranes from Millipore, which were shown previously to have potential to be used for applications involving membrane chromatography. It was found that the new membranes had around 33-50% higher h1gG1-CD4 saturation binding capacity, depending on conditions, and were able to bind 3 times more hIgG1-CD4 from Chinese Hamster Ovary(CHO) cell culture media.

3.1. Introduction

Membrane chromatography is a bioseparations technique that is fast gaining popularity in the purification of antibodies and other expensive biologics [1-5]. The advantages of porous membranes over chromatographic packed bed media include faster separation and ease of scale up, which results in better process economics [6]. The mode of operation for membrane chromatography is carried out as "Bind, Wash, Elute" and has been successfully performed for a wide range of protein purification applications [4, 7, 8], an

extremely attractive technique to employ when the target molecule has a very low concentration in the feed solution. Considering monolconal antibodies (mAb) produced by mammalian cell culture rarely exceeds 0.5 mg/mL, the use of membrane chromatography for the purification of mAb from cell culture media is particularly attractive [9-11].

Humanized IgG are preferred as biopharmaceutials and tremendous opportunities in the area of protein based therapeutics has been realized with due to research and development efforts in this area. Known for its biological and therapeutic properties in providing symptomatic relief for patients with either refractory psoriasis or rheumatoid arthritis, hIgG1-CD4 is a humanized IgG1 type anti-CD4 mAb [12].

IgG1 type mAbs are usually purified by affinity separation, through Protein-A or Protein-G based chromatographic media, which are expensive, difficult to scale up, and known to leach out from the affinity media. Being immunotoxic in nature, they are not the ligand of choice for large scale antibody purification for human therapeutic use [13]. In addition, protein denaturation and dimerization often occur due to the use of acidic buffers to elute the bound mAbs from Protein-A or Protein-G based media, which are highly undesirable for biopharmaceutical applications [14].

Hydrophobic interaction chromatography (HIMC) yields a good separation of IgG from most cell culture media constituents, given that antibody binding on the chromatographic media is highly selective in nature at high anti-chaotropic salt concentrations, and is

relatively gentle as compared to other types of chromatographic separations [15]. While purification of mAbs using column based HIMC chromatography has been widely reported [16, 17] HIMC using membrane chromatographic media has not [3, 18 -20].

In recent years, novel hydrogels have been incorporated into membranes used in membrane chromatography to further increase the respective membranes' binding capacity [21]. One class of such hydrogels used is made through the cross-linking of vinyl-lactam monomers. It is known that the polymers formed from a certain monomers in this class can exhibit a lower critical solution temperature (LCST) at around physiological temperature, as a result of a shift from a general hydrophilic state below the LCST to that of a hydrophobic state above LCST, resulting in a general chain collapse as water molecules surrounding the chains are expelled from the polymer chains. The change in hydrophobicity of the hydrogel can be quantitatively determined through contact angle experiments under different conditions, in addition to the determination of phase transition behaviour through discrete scanning calorimetry. The switching behavior of the hydrogel between hydrophobic and hydrophilic states can be exploited in membrane chromatography applications by coating the membrane with a layer of the above-mentioned hydrogel. Figure 3.1 shows the relevant functional group of such a hydrogel when coated on a PVDF membrane.

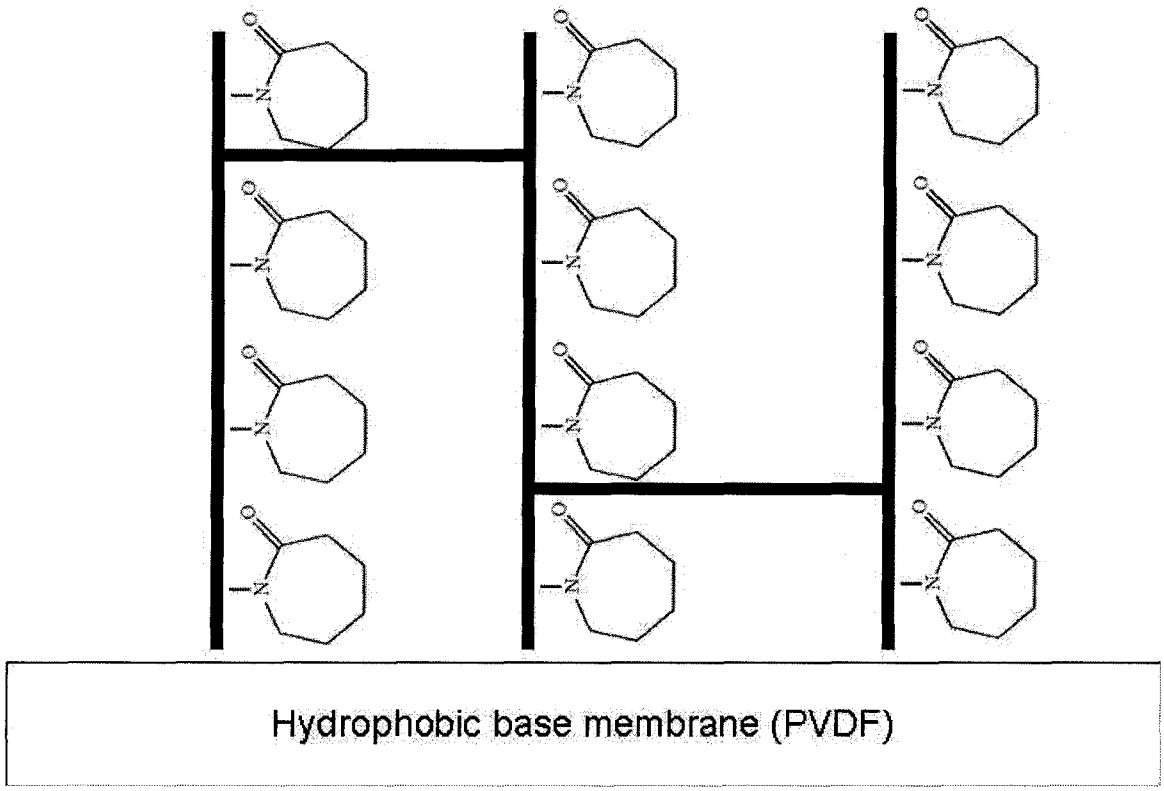

Figure 3.1 Chemical structure of the relevant functional group, caprolactam, attached to the backbone of the hydrogel coated on the PVDF membrane pores.

Figure 3.2 shows the rough mechanics of how the above mentioned behavior can be utilized in membrane chromatography. When in a thermodynamic state corresponding to a condition above the LCST, the hydrogel coated on the membrane exists in a collapsed state, where the hydrophobic sites of the membrane are exposed, allowing for more hydrophobic molecules, like IgG, to bind readily to the membrane. When the conditions are changed, resulting in a thermodynamic state corresponding to that which is below the LCST, the hydrogel coated on the membrane will begin to swell with water molecules, resulting in the shielding of the hydrophobic sites of the membrane by water molecules as

well, which displaces the previously bound hydrophobic molecules, resulting in an elution of the bound substances.

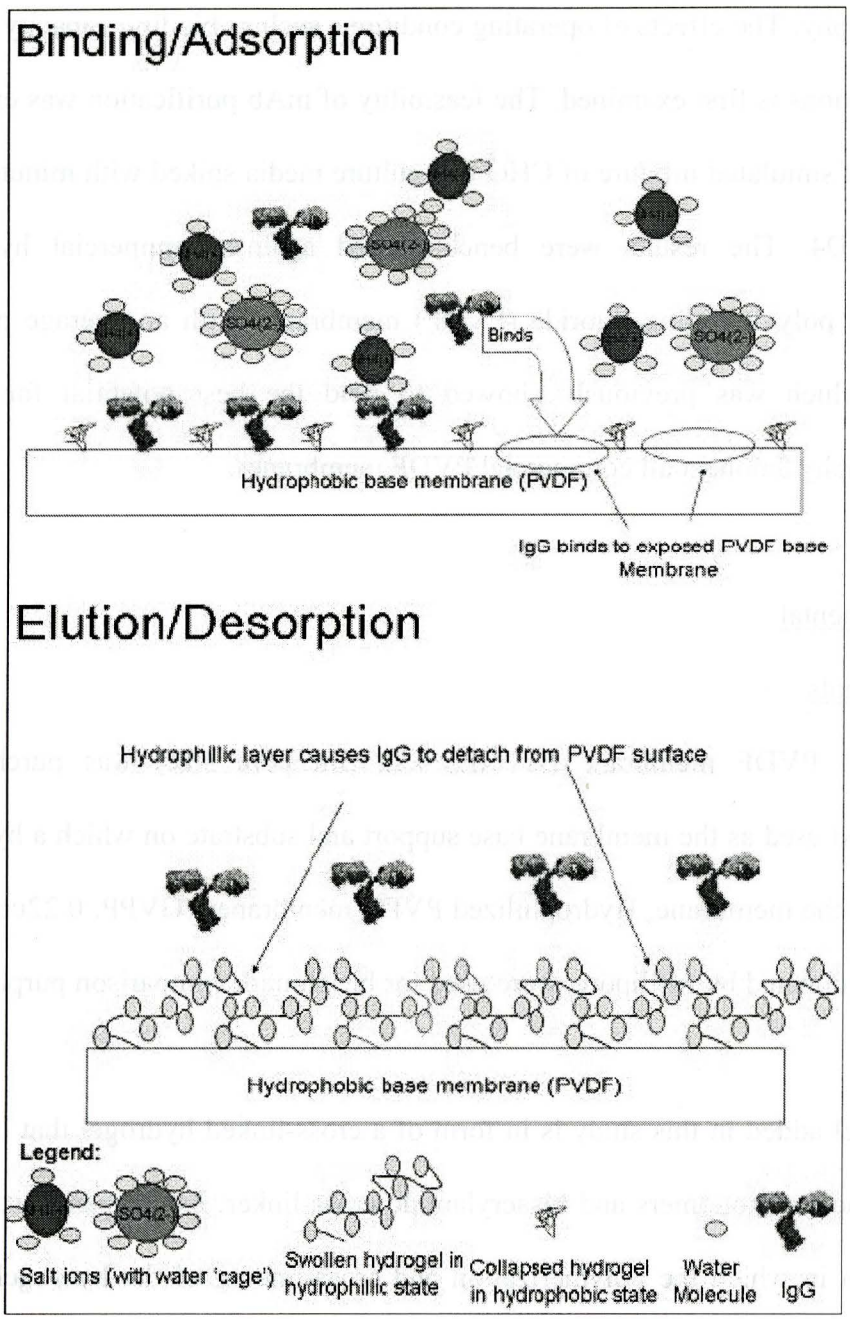

Figure 3.2 Mechanics of binding of the hydrogel coated PVDF membrane.

This paper discusses the use of a hydrogel-coated absorptive microporous polyvinylidine fluoride (PVDF) membranes having an average pore size of 0.22 μm for the separation of hIgG1-CD4 from mammalian cell culture media by hydrophobic interaction membrane chromatography. The effects of operating conditions such as binding capacity at different buffer conditions is first examined. The feasibility of mAb purification was examined by separation of simulated mixture of CHO cell culture media spiked with minute quantities of hIgG1-CD4. The results were bench-marked against commercial hydrophilized microporous polyvinylidine fluoride (PVDF) membranes with an average pore size of 0.22 μm, which was previously showed to hold the best potential for membrane chromatography amongst all commercial PVDF membranes.

3.2. Experimental

3.2.1. Materials

Hydrophobic PVDF membrane (GVHP, 0.22 μm pore size) was purchased from Millipore and used as the membrane base support and substrate on which a hydrogel was grafted onto the membrane. Hydrophilized PVDF membranes (GVPP, 0.22um pore size) were kindly donated by Millipore, were used for benchmark comparison purposes.

The hydrogel added in this study is in form of a cross-linked hydrogel that is composed of N-vinyllactam monomers and bisacrylamide cross-linker. Iso-propanol was used as a liquid media in which the polymerization and cross-linking of the hydrogel within the membrane takes place. A 50/50 blend of diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide/2-hydroxy-2-methylpropiophenone was used in this study as a photo initiator in the

polymerization reaction. All the above reagents, besides the membranes, were purchased from Sigma Aldrich. In addition laminating pockets were purchased from Grand and Toy was used to sandwich the membranes during polymerization as well.

hIgG1-CD4 (batch no. 10) was kindly donated by the Therapeutic Antibody Centre, University of Oxford, UK and was used as obtained. Serum-free CHO cell culture media (catalog no. C1707) was purchased from Sigma Adlrich.

20 mM phosphate buffer solutions (pH 7) at ammonium sulfate ($(NH_4)_2S0_4$) concentrations (0 M, 1.25 M, 1.5 M) were formulated using distilled deionized water obtained from a Branstan Diamond Pure water purification unit. Acetone solution purchased from Sigma Aldrich was used to extract organic impurities from the membranes before hydrogel addition. The phosphate, ammonium sulfate, citrate and sodium chloride salts used to formulate the above buffers were all purchased from Sigma Aldrich.

3.2.2 Membrane Modification Procedure

The membranes discs are first soaked in acetone solution overnight to ensure that all extractable organic residuals within the membrane which might affect moiety addition are removed. The membranes are then dried in a constant humidity (50% relative) and temperature environment (25 ^{o}C) for at least 8 hours. The membrane is then weighed before moiety addition.

The monomers, cross-linker, initiator used to create the hydrogel are thoroughly dissolved in iso-propanol before the membranes are soaked in the said mixture for 10-15 minutes. The soaked membrane is then taken out of the mixture and placed inside a laminating pouch and put through a laminating machine to ensure that no air bubbles were present in the reaction environment, which would inhibit the polymerization reaction. After lamination, the closed laminating pouch with the membrane sealed inside is placed in a UV radiation chamber and irradiated with 360 W/m^2 of UV irradiation for 1 hour to initiate UV polymerization.

After UV polymerization is completed, the membrane is then taken out of the laminating pouch and then extracted in distilled de-ionized water at 70 ^{o}C for 3 days to remove the un-reacted monomers, other reaction byproducts and residuals in the membrane. At the end of 3 days, the membrane is washed again with distilled de-ionized water and left to dry in a constant temperature and humidity environment before it is characterized.

3.2.3 Membrane Characterization Apparatus

Following the drying of the membrane, the weight of the membrane after moiety addition is taken, and the mass gain percentage of the membrane under a particular modification condition is computed.

The contact angles of the membranes at different buffer conditions were measured by the sessile drop method with a Ram-Hart Model 100-00 contact angle goniometer. Membrane samples were positioned on the measuring stage of the apparatus, and a 0.5 pL

sessile drop of water was placed on the membrane and the contact angle of the membrane under that particular buffer condition was measured. Angles on both sides of each drop were measured to assure symmetry.

After screening and characterizing various membranes modified via the above characterization tests, the membranes that had contact angle differences between different buffer conditions larger than the commercial 0.22 μm hydrophilic PVDF membranes, while still exhibiting acceptable TMP (<0.5 MPa) at a flux of 0.372 cm/min were selected for further experiments in membrane chromatography.

In addition to the above characterization tests, confocal laser scanning microscopy and SEM images of the selected set of membranes were taken compared against the same images taken for the commercial GVPP 0.22 μm hydrophilized PVDF membranes.

To obtain the confocal laser scanning images for the membrane at binding conditions, 1 mL of 2 μg/mL of fluorescently labeled IgG at 1.5 M ammonium sulfate in 20mM sodium phosphate at pH 7 solution was filtered through the one piece of both VCL-2 and the commercial GVPP 0.22 μm hydrophilized PVDF membranes at a flux of 0.372 cm/min.

To obtain the confocal laser scanning images for the membrane at eluting conditions, 1 mL of 2 μg/mL of fluorescently labeled IgG at 0M ammonium sulfate in 20mM sodium

phosphate at pH 7 solution was filtered through the one piece of both VCL-2 and the commercial GVPP 0.22 μm hydrophilized PVDF membranes at a flux of 0.372 cm/min.

The confocal laser scanning images were taken using a Carl Zeiss: LSM 510. The membrane was magnified using a 63 × objective and the scan speed was set at 8. Image size was set at 512 × 512.

SEM images of both VCL-2 and the commercial GVPP 0.22 μm hydrophilized PVDF membranes were taken using a Philips SEM 515.

3.2.4 Experimental Protocols

The membranes discs are all housed within a membrane module with an effective diameter of 18.5 mm for all experiments [22].

All experiments are carried out at 1 mL/min at ambient temperature, ie, 24 °C. This was essential in determining the appropriate feed conditions to be used in the membrane chromatographic experiments. All solutions used were prepared using a 20 mM sodium phosphate base buffer at pH 7. The saturation binding capacity of the membranes at different feed conditions (mAb concentrations and ammonium sulfate concentrations) was determined by breakthrough and elution experiments using pure mAb samples. The mAb which was bound was eluted from the membrane using an ammonium sulfate free buffer (Buffer B). Protein separation experiments were carried out by injecting large

volumes of simulated feed solutions (mAb spiked mammalian cell culture media) prepared in appropriate buffers into the membrane module.

To determine the composition of the feed, flowthrough, flowthrough and eluent, a Varian high pressure liquid chromatography (HPLC) system integrated with a size exclusion column (SEC) was used. The mobile phase for the HPLC-SEC system was an aqueous solution of 0.25M sodium chloride solution (NaCl). The % purity and composition of the hIgG1-CD4 is computed as the positive area under the curve for the peak centered around 58 minutes of the chromatogram divided by the total positive area under the chromatogram. Such a computation was possible due to the fact that the hIgG1-CD4 peak was well defined and did not overlap with the peaks of the media proteins.

Finally, for qualitative analysis, a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) analysis of the feed, flowthrough and eluent was obtained using an 10% non-reducing gel [23]. The feed, flowthrough and eluent samples were first pre-concentrated and desalted by using 10kDa MWCO disposable centrifuge filter tubes from Millipore.

3.3. Results and Discussion

3.3.1 Contact Angle at Different Buffer Conditions

Table 3.1 shows the contact angle of the different membranes at different buffer conditions. From Table 3.1, the membrane VCL-2 was selected on the basis of a lower pressure drop and with a sufficiently large change in hydrophobicity between salt-free and high salt buffer (more than 2 times the contact angle difference as compared to GVPP 0.22 μm PVDF membrane).

Table 3.1 Comparison of contact angle between different membranes

Membrane Type	GVPP (Durapore 0.22 hydrophillic PVDF membrane)	GVHP (Durapore 0.22 hydrophobic PVDF membrane)	'VCL-1' (Durapore 0.22 hydrophobic PVDF membrane, GVHP, modified with hydrogel, 22% mass gain)	'VCL-2' (Durapore 0.22 hydrophobic PVDF membrane, GVHP, modified with hydrogel, 6% mass gain)
20mM Phosphate Buffer, pH 7	75.7	122.1	51.3	41.0
20mM Phosphate Buffer, pH 7 with 5 M NaCl	103.6	134.6	124.5	98.8
Difference in Contact Angle between membrane in salt-free and high salt buffer	27.9	12.5	73.2	57.8
Remarks	Commercial hydrophilized PVDF media, membrane chromatography benchmark	Original unmodified hydrophobic membrane	TMP too high for membrane chromatography	TMP appropriate for membrane chromatography, contact angle about twice of GVPP benchmark

3.3.2 Confocal and SEM Images of Membranes

Figure 3.3 shows the comparison of the difference between VCL-2 and the commercial GVPP 0.22 μm hydrophilized PVDF membranes at both binding and eluting conditions, with florescent IgG bound to the membrane. By a visual comparison of the two membranes at binding conditions, it is immediately clear that at the same buffer conditions of 1.5 M ammonium sulfate, VCL-2 can easily bind more than twice as much IgG as the commercial GVPP 0.22 μm hydrophilized PVDF membranes. Furthermore, by comparison of the two membranes at eluting conditions, it is also clear than although VCL-2 does exhibit some irreversible binding of IgG on its surface, as compared to the amount of florescent IgG bound to the VCL-2 membrane at binding conditions, it is not very significant.

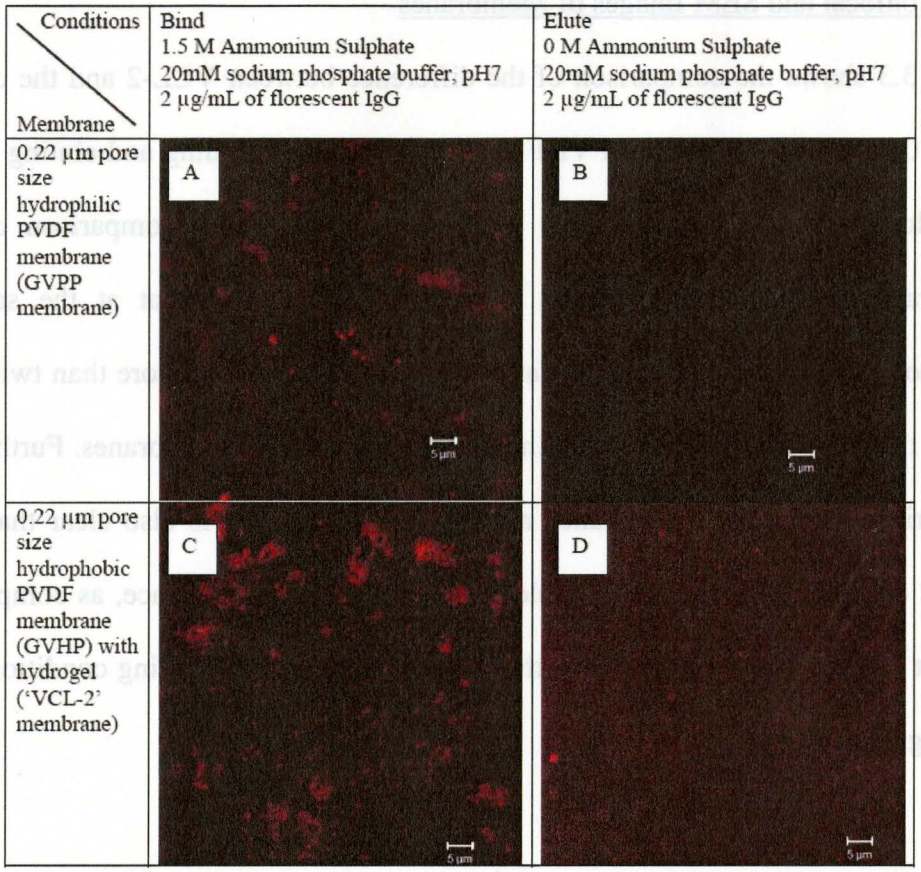

Figure 3.3 Differences between 'VCL-2' and the commercial GVPP 0.22 µm hydrophilized PVDF membranes at both binding and eluting conditions, with florescent IgG bound to the membrane. A – GVPP membrane under binding conditions, B – GVPP membrane under eluting conditions, C – 'VCL-2' membrane under binding conditions, D – 'VCL-2' membrane under eluting conditions.

Figure 3.4 shows the distribution of bound IgG throughout the thickness of the membrane at binding conditions. It appears than most of the IgG bound is located at the centre of the membrane. This is due to the fact that at binding conditions, the coated hydrogel is in its collapsed hydrophobic state, and has retreated into the centre of the membrane to minimize total contact area with the aqueous buffer. As such, most of the IgG bound at

binding conditions is bound to an environment-responsive hydrogel which is able to exhibit a hydrophobic/hydrophilic transition under certain conditions.

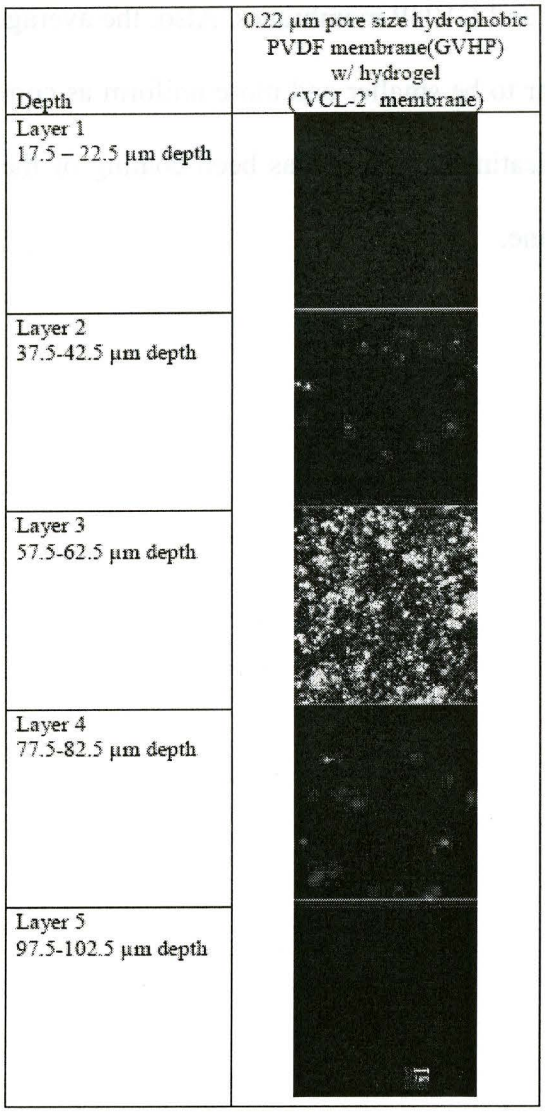

Figure 3.4 Confocal layer scan through thickness of 'VCL-2' membranes under bind conditions. Each layer imaged is approximately 20 µm apart from the next, and has a scan depth of 5 µm.

Figure 3.5 shows a comparison of the SEM images between the nascent GVHP hydrophobic membrane, hydrophilic GVPP 0.22 µm membranes and VCL-2 membranes. From the images, there appears to be a successful coating of polymeric material onto the

VCL-2 membrane. When compared to the GVPP membrane, the VCL-2 membrane appears to have a porous structure where the polymer network appears to look less 'jagged' than the GVPP and GVHP membranes. Also, the average pore size of the VCL-2 membranes also appear to be smaller and more uniform as compared to the GVPP and GVHP membranes, indicating that there has been coating of the pores by the hydrogel grafted onto the membrane.

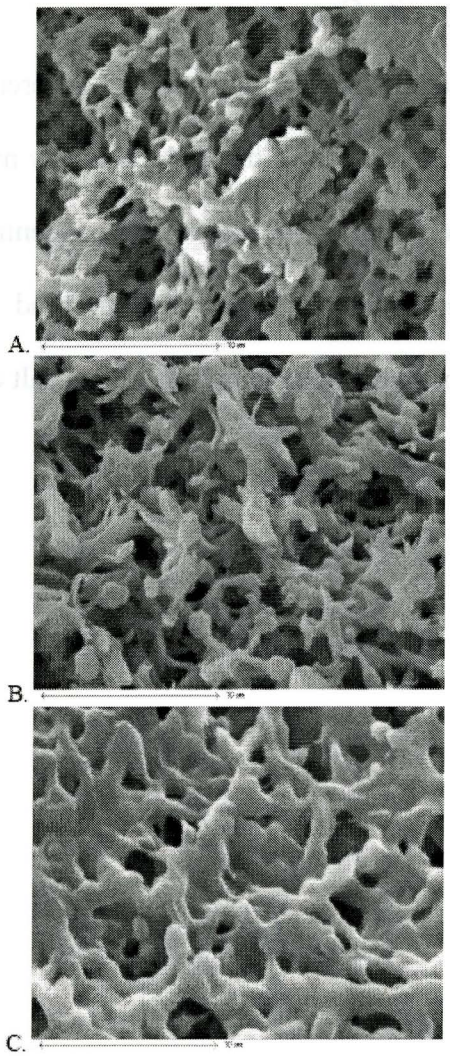

Figure 3.5 **SEM comparison between the nascent hydrophobic GVHP 0.22 μm membranes (A), hydrophilic GVPP 0.22 μm membranes (B), and 'VCL-2' membranes (C). Samples have been spluttered with gold prior to imaging.**

3.3.3 Saturation Binding Capacity Comparison

Table 3.2 shows the saturation binding capacity of different membranes at different binding conditions. As can be seen from the table, the new membranes are able to bind hIgG1-CD4 at a much lower ammonium sulfate salt concentration than the GVPP 0.22 μm membranes, while simultaneously being able to bind approximately 60% more hIgG1-CD4 at conditions above 1.5 M ammonium sulfate salt concentration.

Table 3.2 Binding capacity hIgG1-CD4 on GVPP and 'VCL-2' membranes

Experimental Conditions	Membrane	Incipient breakthrough binding capacity (mg/mL)	10% breakthrough binding capacity (mg/mL)	Saturation binding capacity (mg/mL)
1.0 M Ammonium Sulphate, mAb concentration 0.220 mg/mL Flow rate 1 mL/min	GVPP (Durapore 0.22 hydrophillic PVDF membrane)	N/A	N/A	Negligible (<1 mg/mL)
	'VCL-2'(Durapore 0.22 hydrophobic PVDF membrane, GVHP, modified with hydrogel)	2.058	2.389	3.407
1.25 M Ammonium Sulphate, mAb concentration 0.220 mg/mL Flow rate 1 mL/min	GVPP	N/A	N/A	Negligible (<2 mg/mL)
	'VCL-2'	5.400	5.974	8.331
1.5 M Ammonium Sulphate, mAb concentration 0.220 mg/mL Flow rate 1 mL/min	GVPP	5.476	5.734	5.886
	'VCL-2'	6.199	7.749	8.856
1.7 M Ammonium Sulphate, mAb concentration 0.220 mg/mL Flow rate 1 mL/min	GVPP	8.015	9.387	21.598
	'VCL-2'	8.589	10.977	28.294

3.3.4 Spiked CHO Cell Culture Media Membrane Chromatography Separation and Purification Comparison

Figures 3.6 and 3.7 shows the results of the separation and purification of hIgG1-CD4 from a 5 mL spiked CHO cell culture media using membrane chromatography for the two membranes at 1.5 M and 1.7 M ammonium sulfate concentrations while Table 3 shows the purity and recovery of the hIgG1-CD4 of the experiments, comparing the 'VCL-2' membrane against the commercial GVPP 0.22 μm hydrophilized PVDF membrane.

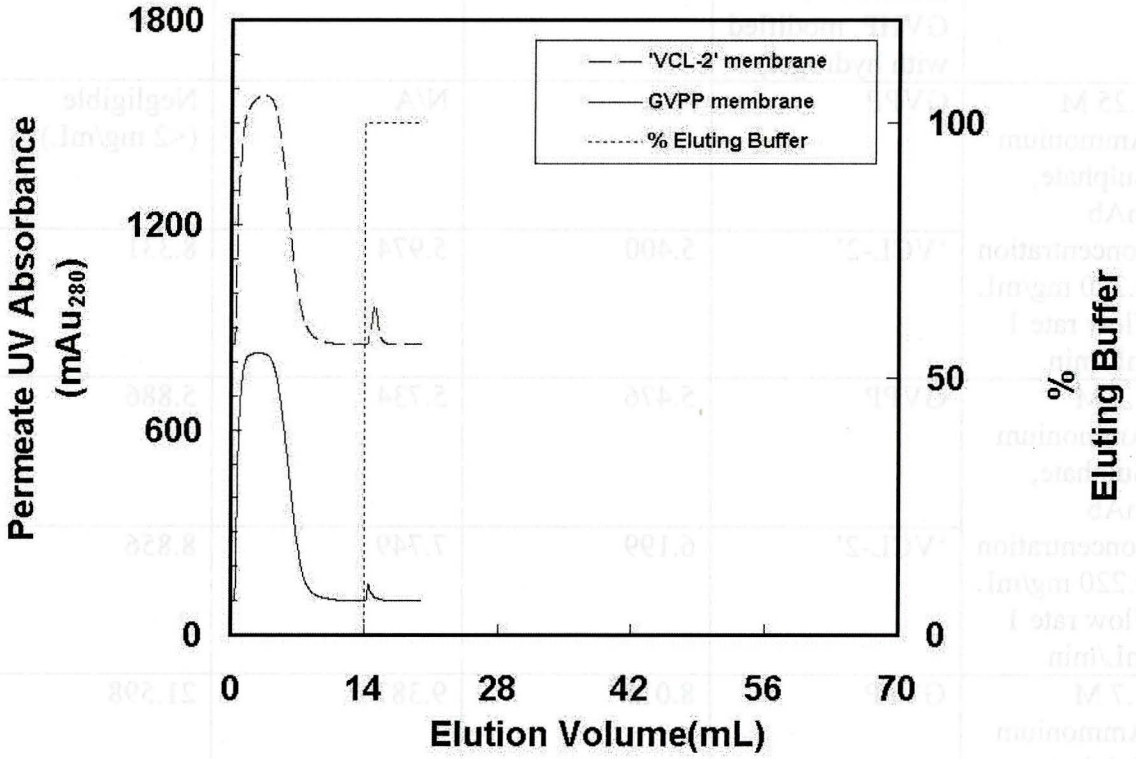

Figure 3.6 Purification of hIgG1-CD4 from CHO-cell culture media. membrane diameter 18mm, number of discs 2, flowrate 1 mL/min, mAb concentration in feed 0.1 mg/mL, ammonium sulfate concentration in feed 1.5 M.

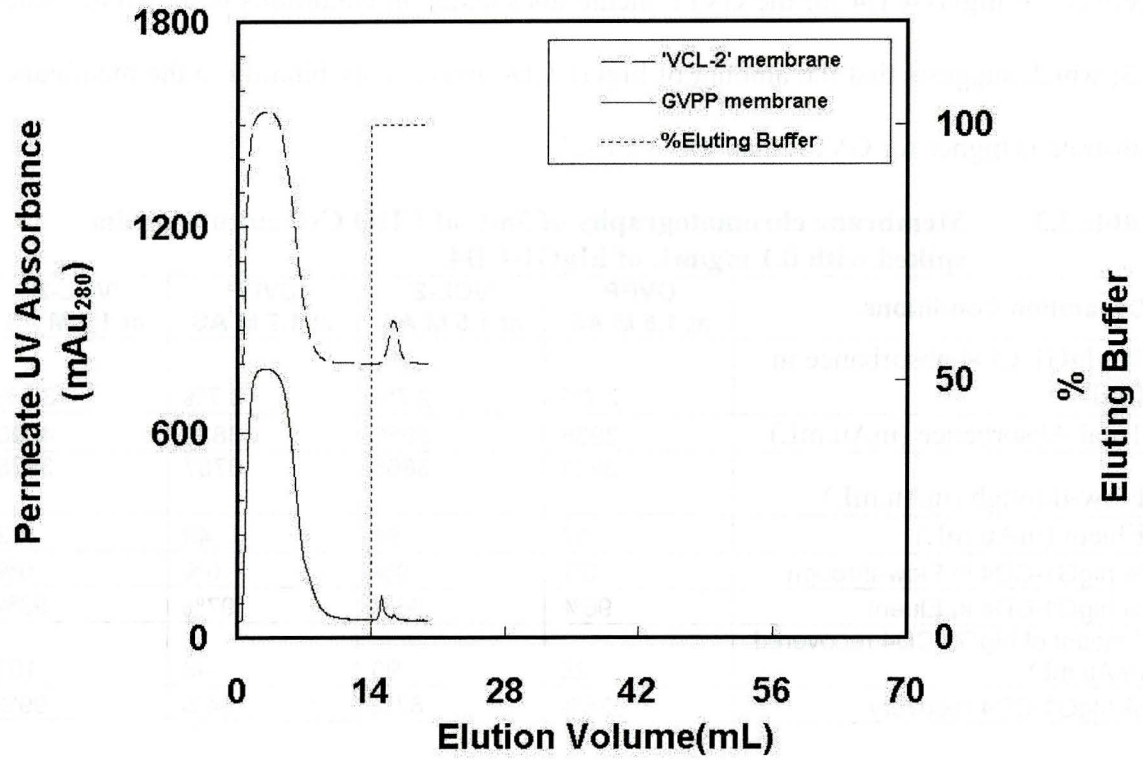

Figure 3.7 Purification of hIgG1-CD4 from CHO-cell culture media. membrane diameter 18mm, number of discs 2, flowrate 1 mL/min, mAb concentration in feed 0.1 mg/mL, ammonium sulfate concentration in feed 1.7 M.

As can be seen, the absolute recovery of hIgG1-CD4 is approximately three times higher at both 1.5 M and 1.7 M ammonium sulfate with 'VCL-2' than the commercial GVPP 0.22 μm hydrophilized PVDF membrane. The % purity of the hIgG1-CD4 eluent when the 'VCL-2' membrane is used is comparable to that of GVPP membrane under all conditions examined.

Analysis of the feed and eluent by the HPLC is shown in Figures 3.8, 3.9 and Table 3.3. As can be seen from Table 3, for both membranes, there is essentially no hIgG1-CD4 present in the flowthrough for both membranes under all conditions. However, a lower %

recovery of hIgG1-CD4 for the GVPP membranes under all conditions is shown in Table 3.3; which suggests that the amount of hIgG1-CD4 irreversibly binding to the membrane substrate is higher for GVPP than for 'VCL-2'.

Table 3.3 Membrane chromatography of 5mL of CHO Cell culture media spiked with 0.1 mg/mL of hIgG1-CD4.

Separation Conditions	GVPP at 1.5 M AS	'VCL-2' at 1.5 M AS	GVPP at 1.7 M AS	'VCL-2' at 1.7 M AS
% hIgG1-CD4 absorbance in feed	2.7%	2.7%	3.7%	3.7%
Total Absorbance (mAu.mL)	3938	3959	3836	4090
Flow-through (mAu.mL)	3911	3865	3787	3928
Eluent (mAu.mL)	27	94	49	162
% hIgG1-CD4 in Flow-through	0%	0%	0%	0%
% hIgG1-CD4 in Eluent	96%	96%	97%	93%
Amount of hIgG1-CD4 recovered (mAu.mL)	26	90	48	151
% hIgG1-CD4 recovery	25%	87%	34%	99%

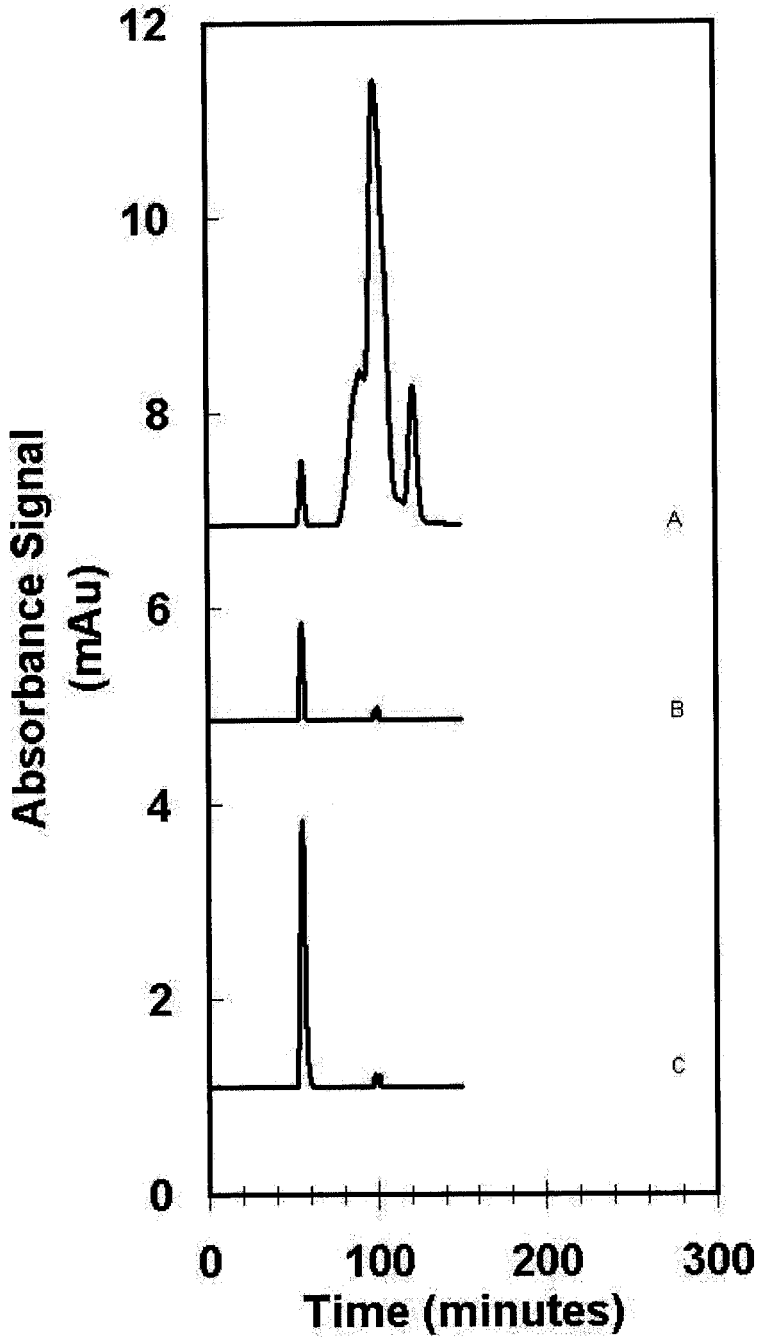

Figure 3.8 **SEC Analysis and comparison of separation experiment shown in Figure 6: A - CHO Cell Culture with 0.1 mg/mL hIgG1-CD4 Feed at 1.5 M Ammonium Sulphate, B - Eluent from commercial GVPP membrane, C- Eluent from membrane 'VCL-2'.**

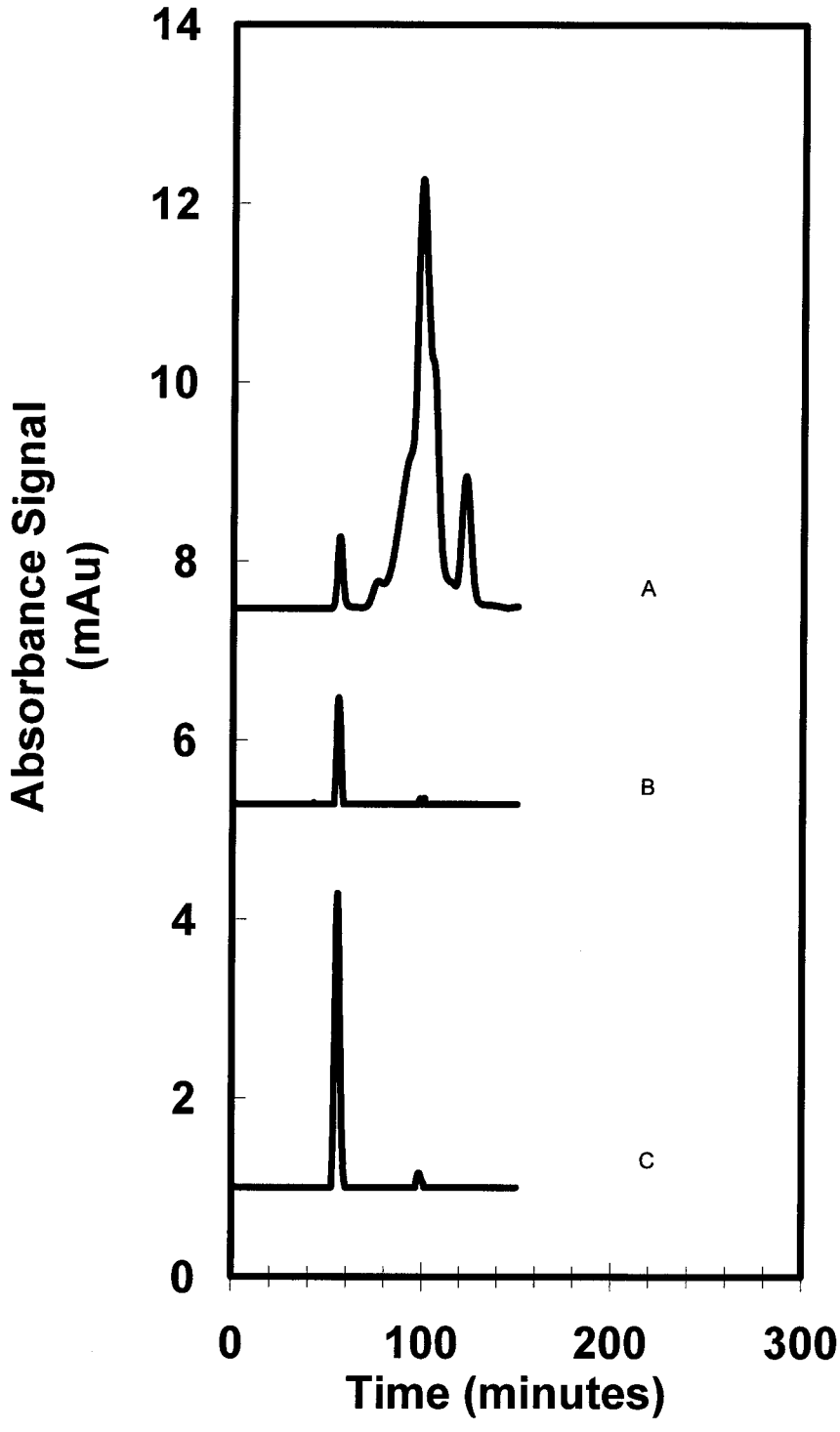

Figure 3.9 **SEC Analysis and comparison of separation experiment shown in Figure 7: A - CHO Cell Culture with 0.1 mg/mL hIgG1-CD4 Feed at 1.7 M Ammonium Sulphate, B - Eluent from commercial GVPP membrane, C- Eluent from membrane 'VCL-2'.**

Furthermore, from Figure 3.8 and 3.9, it would appear that membrane 'VCL-2' also releases the hIgG1-CD4 in a more concentration fashion than the GVPP membrane, given that the eluent hIgG1-CD4 peak is higher for membrane 'VCL-2' than the GVPP membrane under all conditions examined, despite of 'VCL-2' having a phase transition which is considered to be gradual.

The non-reducing SDS PAGE of feed, flow-through and eluent for all the experiments depicted in Figure 3.6-3.9 is shown in Figure 3.10. As can be seen, the hIgG1-CD4 bands are noticeably darker for the eluent samples collected from the 'VCL-2' membranes than the GVPP membranes, with no media proteins presence, suggesting that the overall recovery of the 'VCL-2' membranes is higher than that of the GVPP membranes, while allowing for a comparable level of purification.

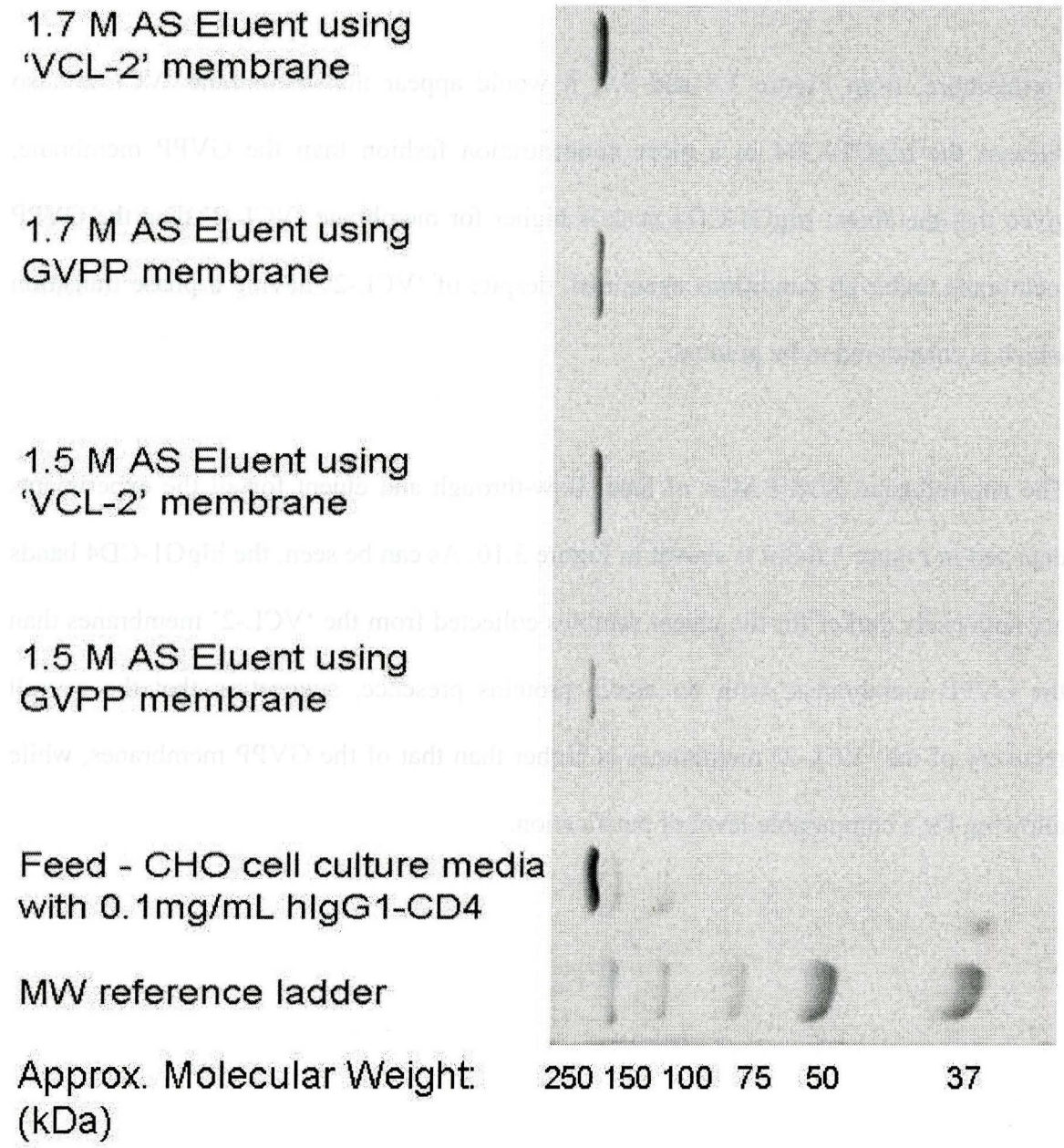

Figure 3.10 **SDS-PAGE obtained with feed and purified mAb samples from experiment carried out to purify hIgG1-CD4 from CHO cell culture media.**

4. Conclusions

Based on the above results and explanations advanced to explain the results obtained, the following points are advanced as the conclusions for this study:

1.) Environment-responsive absorptive membranes have been created through modification of a commercial hydrophobic PVDF membrane support with a thermo-responsive hydrogel composed of poly N-vinyl-lactams and bisacylamide cross-linker.

2.) The hydrogel grafted membranes are characterized by their % mass gain and contact angle differences. It is found that the hydrophobic membranes with the least amount of mass gain shown the greatest difference in contact angle at different buffer conditions.

3.) The saturation binding capacity of these hydrogel grafted membranes is higher than commercial GVPP 0.22 micron pore size hydrophillized PVDF membranes, which were previously shown to hold potential in membrane chromatography applications.

4.) The hydrogel grafted membranes are also able to bind three times more hIgG1-CD4 from CHO cell culture media as compared to commercial 0.22 micron pore size hydrophillized PVDF membranes, and at a purity level which is comparable to that of the commercial 0.22 micron pore size hydrophillized PVDF membranes.

5.) The hydrogel grafted membranes exhibit less irreversible hIgG1-CD4 binding than the GVPP membranes, and are also able to elute the bound hIgG1-CD4 in a more concentrated form.

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CHAPTER 4
CONCLUSIONS

The performance of the novel membranes synthesized in this study project is highly dependent on the amount of gel coating on the membrane, as well as the nature of the base membrane material.

In Chapter 2, environment-responsive ultra-filtration membranes have been successfully created through filling of a commercial hydrophilized PVDF membrane support with a thermo-responsive hydrogel composed of poly N-vinyllactams and bisacylamide cross-linker.

The modified membranes are characterized by their percentage mass gain and membrane permeability, and it was found that the percentage mass gain of the modified membrane is found to be linearly correlated with the percentage weight of the monomer pre-cursor in the reaction media as well as the percentage weight of the bisacrylamide cross-linker added to the reaction media. The membrane permeability is found to be a multiplicative function of both the percentage weight of the monomer pre-cursor in the reaction media as well as the percentage weight of the bisacrylamide cross-linker added to the reaction media.

For ultrafiltration applications, the most appropriate modification condition for protein separation purposes in this study appear to be condition 5, based on the valve

range & $\sqrt{k_{mem}}$ values calculated. Membranes made from condition 5, were therefore selected for further experimentation in protein filtration studies.

The single protein sieving experiments utilizing a batch protocol featuring PSIT shown that the membranes selected were suitable for use in multi-component separation. The sieving coefficients obtained from single protein sieving experiments indicate that the sieving coefficients of different proteins against the membrane can be changed by changing both the salt and pH of the buffers.

The potential of the novel batch fractionation protocol was demonstrated through the filtration of a synthetic binary protein mixture composed of Equine Ferritin and Human Immunoglobulin G. The results show that smaller proteins could be fractionated from larger proteins using the membrane, through a protocol leveraging on the environment-responsive nature of the membrane's permeability, as well as the phenomena of the smallest apparent size of protein being at isoelectric point (pI), to maximize the separation resolution of the proteins.

Further proof of the potential of the novel batch fractionation protocol was demonstrated through the filtration of a synthetic three protein mixture composed of Bovine Immunoglobulin M, Equine Ferritin and Human Immunoglobulin G. The results show that using the above membranes, three different proteins having different sizes could be fractionated to a certain extent, through a protocol leveraging on the environment-responsive nature of the membrane's permeability, as well as the

phenomena of the smallest apparent size of protein being at pI, to maximize the separation resolution of the proteins. However, optimization of the process conditions would be required for a cleaner separation than what is shown here.

The results presented in Chapter 2 are novel in that the a previously unreported type of environment-responsive membrane is produced, based on the in-situ polymerization of a hydrogel that has not been studied for the modification of PVDF membranes, composed of polyvinyllactams with a bisacrylamide crosslinker. Furthermore, novel protocols leveraging on the environment-responsive nature of the membrane's permeability, as well as the phenomena of the smallest apparent size of protein being at pI, to maximize the separation resolution of the proteins were proposed in this study, which was absent in the prior literature, which focused mostly on the steady state behaviour of environment-responsive membranes.

In Chapter 3, environment-responsive adsorptive membranes have been successfully created through coating of a commercial hydrophobic PVDF membrane support's internal pore structure with a thermo-responsive hydrogel composed of poly N-vinyllactams and bisacylamide cross-linker.

The difference between these membranes synthesized in Chapter 3 and the membranes synthesized in Chapter 2 is that those ultrafiltration membranes were created using a hydrophilized PVDF membrane support, while these adsorptive membranes were created through the use of a hydrophobic PVDF membrane support.

Furthermore, the percentage mass gain of the ultrafiltration membranes synthesized in Chapter 2 are of a much higher magnitude than those adsorptive membranes synthesized in Chapter 3, for they require extensive coating & filling of the internal pore structure to reach the appropriate pore size necessary to be able to carry out size-exclusion of the model proteins examined, while the membranes synthesized in Chapter 3 are characterized by low percentage mass gain, as the main function of the hydrogel in this case is to act as an expanding hydrophilic barrier to displace the adsorbed proteins from the hydrophobic PVDF membrane surface during the elution stage of membrane chromatography.

The hydrogel coated membranes synthesized in Chapter 3 are characterized by their percentage mass gain and contact angle differences, and it is found that the hydrophobic membranes with the least amount of mass gain shown the greatest difference in contact angle at different buffer conditions.

It was determined that the saturation binding capacity of these hydrogel coated membranes is higher than commercial GVPP 0.22 micron pore size hydrophillized PVDF membranes, which were previously shown to hold potential in membrane chromatography applications.

The hydrogel grafted membranes are also able to bind three times more hIgG1-CD4 from CHO cell culture media as compared to commercial 0.22 micron pore size

hydrophillized PVDF membranes, and at a purity level which is comparable to that of the commercial 0.22 micron pore size hydrophillized PVDF membranes. The hydrogel coated membranes also exhibit less irreversible hIgG1-CD4 binding than the GCPP membranes, and are also able to elute the bound hIgG1-CD4 in a more concentrated form.

The results presented in Chapter 3 are novel in that no prior reports of environment-responsive adsorptive membranes synthesized from a hydrophobic base PVDF membrane coated with that of polyvinylcaprolactm hydrogel. Furthermore, these novel membranes also possess a superior saturation and dynamic binding capacity towards hIgG1-CD4, with comparable low non-specific binding, when put to a direct comparison against a known benchmark, Millipore's Durapore® GVPP 0.22 μm hydrophilized PVDF membrane, under identical operating conditions.

With regards to possible future direction for the work presented in Chapter 2; while the potential of multi-component separation using only one single filtration stage is demonstrated, the results of the three component separation shown is far from ideal, as the largest protein, BIgM, which should be retained appears to permeate readily through the membrane during open valve operation, possibly due to the opening of the pores while the pH of the retentate buffer is still relatively close to the pI of BIgM.

This is due the fact that the PVDF base membrane has a large pore size distribution, and although pore filling of the base membrane with hydrogel would to some extent minimized the final pore size distribution of the modified membrane product

when the membrane is operated in closed pore mode, it does not remove the fact that there remains the fact that the pore filling cannot be entirely uniform throughout the membrane, even under the most tightly controlled conditions. As such, the opening of the internal pore structure of the membrane when conditions favour the collapse of the hydrogel will also result in an increase in the pore size distribution of the membrane, unintentionally allowing the largest proteins to permeate through the membrane readily.

Furthermore, given that the size differences of different proteins are generally in the range of 1-5 nanometres, and that the buffer conditions can sometimes result in a smaller protein not at its pI having a bigger apparent hydrodynamic size than a larger protein at its iso-electric point, which may explain why while TG (pI 4-5), seems to be easily retained by the membrane even in open valve conditions at pH 7, BIgM (pI 7-9), which is larger, permeates through under the same experimental conditions, which adds a further complication to the use of such membranes to separate proteins exclusively through size-exclusion mechanisms. [1]

Finally, it must be noted that this study has not looked in any detail with regards to the phenomena of protein-protein interactions (which is why TG was only used in single protein sieving studies) and protein-membrane interaction, clearly evident in the results shown in Chapter 2, which will further hinder research in this area.

Based on the results so far, a recommendation to get around the issue of uneven pore filling would be to utilize a low protein binding, ultrathin base membrane with a

narrow pore size distribution and having a thickness similar to that of the proteins being filtered, and coating that membrane with an appropriate environment-responsive hydrogel that also exhibits low protein binding. While it does not address the issue of protein-protein interaction, which might still hinder practical application of environment-responsive membranes for multi-component separation in ultra-filtration, it would at least solve the other issues appear to be hindering progress in this area of research.

With regards to possible future direction for the work presented in Chapter 3; it is observed that the 'VCL-2' membranes appear to release bound hIgG1-CD4 in a more gradual manner than the GVPP membranes, as evident from a broader elution peak, and it can be inferred that these membranes transit between hydrophobic and hydrophilic conditions in a more gradual manner.

As such, there exists a possibility of using such adsorptive membranes for multi-component separation of antibody monomer, dimer, trimer and associated aggregates; as it is reported that the monomers, dimmers, trimers and associated aggregates of antibodies have slight differences in hydrophobicities; currently, multi-component membrane chromatographic separation of such mixtures using GVPP membranes have been attempted and met with some success [2], although it is apparent that a membrane exhibiting a more gradual transition from a hydrophobic to hydrophilic state would be highly desired for such applications.

Chapter References

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