ENVIRONMENT-RESPONSIVE PAPER FOR MEMBRANE CHROMATOGRAPHY

ENVIRONMENT-RESPONSIVE PAPER-SUPPORTED MEMBRANE FOR CHROMATOGRAPHIC BIO-SEPARATIONS: PREPARATION, CHARACTERIZATION AND APPLICATION

By

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ABSTRACT

Environment-responsive paper-supported composite membranes, consisting of interpenetrating network of poly(*N*-vinyl caprolactam) and cellulose fibres, suitable for hydrophobic interaction membrane chromatography of proteins were prepared. These membranes could be made to switch between hydrophobic and hydrophilic states in a reversible manner by addition or removal of anti-chaotropic salts. A series of systematic studies were conducted to examine the effects of polymer loading and salt concentration on hydraulic and antibody binding properties of these membranes. A membrane possessing a satisfactory balance of hydraulic permeability and antibody binding was further examined for use in an immunoglobulin G (IgG) purification process. The membrane demonstrated low fouling tendency, satisfactory IgG binding (c.a. 12 mg/ml bed volume), and was successfully used for IgG purification from simulated cell culture supernatant. The results obtained demonstrate that the poly(*N*-vinyl caprolactam)-paper composite and indeed similar material could be used as inexpensive membranes adsorbers for protein purification.

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CHAPTER 1

INTRODUCTION

Chromatography is indisputably the workhorse of downstream processing[1-2] as it plays a paramount role in most large-scale industrial bio-separations. It is conventionally performed by using packed bed column. This separation technique is capable of achieving remarkably high product purity. Hence, it is suitable for separations of therapeutic proteins[2], such as monoclonal antibodies and plasma proteins[3], with which high purity is of absolutely essential to meet the regulatory requirements. However, the attractiveness of chromatography is significantly hampered not only by its cost, but also by its intra-particle diffusion. Consequently, the diffusive mass transport limitation makes process time longer and scale-up of the process difficult[1-2, 4-12]. Also, high pressure drop issue[1-2, 8, 12] is often associated with the use of chromatography for its long diffusion time. As the development of pharmaceutical and bio-technological industries advances, the needs to reduce in dependency[2] on chromatography and to turn to other economic and efficient alternatives are inevitably rising.

One of the many alternative approaches to overcome the limitations of chromatography is the utilization of synthetic membrane. The main feature of membrane is its micro-porous structure which allows convective flow of solution through the pores and overcomes the diffusive mass transport limitation[1-2, 4-12]. By combining membrane technology and column chromatography, a membrane chromatography is emerged with

high throughput and high resolution features[4, 6-8]. Compared to gel-based column chromatography, membrane-based chromatography is relatively easy to scale-up and cost-effective[1-2, 4-12]. Also, membrane chromatography has low bed height to diameter ratio which creates low pressure drop across the membrane bed[6, 13]. Therefore, relatively high liquid flow rates can be exploited on membrane chromatography to reduce the process time without compromising the capture efficiency of the separation technique[1, 4, 6-9, 12].

As such, membrane chromatography has been long-established in research community as a promising large-scale separation process. However, owing to its novelty and limited commercial products[2, 8, 14], such technology is only well-received in selected industries. For instance, membrane chromatography is found suitable to perform purifications of large bio-molecules (> 250 kDa), such as plasmid DNA and virus[2, 8, 10]. Due to its low unit surface areas, such separation technique has also been restricted to certain purification processes, in which product or impurity of interest is not a primary constituent in the feed and appears in small amount[2, 5, 8, 10]. Examples of such applications are purification of therapeutic proteins and enzymes[2, 14], and removal of endotoxins[5, 12, 14-16].

Most separation chemistries employed in conventional chromatography have been adapted by membrane chromatography. Ion exchange (IEX) and affinity interactions are the most popular binding modalities in commercial and research applications,

respectively[2, 4, 8, 12]. Hydrophobic interaction, however, is far less reported in the literature, as compared to IEX and affinity interactions[8, 17-18]. Despite the unpopularity, such interaction has shown to be more discriminating and benign than other separation chemistries[8, 17-18]. It relies on anti-chaotropic salts to induce mildly hydrophobic interaction between proteins and adsorptive membranes, and separates proteins on the basis of hydrophobicity difference[3, 13]. As such, hydrophobic interaction membrane chromatography (HIMC) has been reported in the purifications of plasma proteins[18], heme proteins[19], therapeutic enzymes[3, 19] and recombinant proteins[4, 8, 17].

From the literature review, the typical membrane supports used to prepare hydrophobic interaction-based chromatographic membranes are polyethylene[20-21], styrene-divinylbenzene[19], glycidyl methacrylate-ethylene dimethacrylate (GMA-EDMA)[19] and poly(vinylidine difluoride)[6, 17-18, 22-24]. Most of the substrate membranes are characterized as either hydrophobic and/or relatively inert[6, 8]. As such, these composite membranes do not enjoy much satisfactory in solute separations due to their highly non-specific protein adsorption and low product recovery[25-26]. Hydrophilic gels[18, 24] are usually grafted on these membrane supports to facilitate dynamic change of hydrophobicity of the membranes when they are subjected to salt concentration change.

Alternatively, hydrophilic base matrices, such as cellulose, have been reported to overcome some of the hurdles in HIMC membranes[25-28]. Being an abundant bio-polymer in nature, cellulose is hydrophilic and compatible with bio-molecules. Therefore, this

material is perfect for constructing membrane support because it has low non-specific protein binding[25-27]. Hydrophobic ligands are covalently attached to the cellulose-based substrates to impart dynamic change of hydrophobicity on the membranes. An example of such composite membrane is the cellulose-based phenyl membrane adsorber which is recently introduced by Satorious Stedim Biotech[28] for commercial HIMC. Such membrane has hydrophobic phenyl ligands, covalently attached to the regenerated stabilized cellulose matrix (Hydrosart®), as adsorbents. The commercial membrane possesses low non-specific protein interaction and comparable binding efficiency to the conventional resins but with much shorter process time. In research applications, the use of cellulose filter paper as base matrix for HIMC membranes has also been reported recently. Such paper-based membrane relies on the grafted smart polymers to exhibit hydrophobic/hydrophilic property[27, 29].

Smart gels, also known as environment-responsive (ER) hydrogels, have been increasingly used in bio-separations and bio-processes[30-31]. Most of its membrane-based applications are found in size-exclusion-based ultra-filtration[24, 30, 32-35], but there are relatively few reports on using ER membranes in pervaporation[30] and membrane adsorption[23-24, 27, 29]. Such ER membranes can dynamically change its pore size and surface properties (hydrophobicity, ionic charge and affinity binding) in response to various environmental stimuli[24, 27, 29-30, 32-50]. Most studied ER membranes are made responsive to temperature and/or pH, as they are easy to manipulate[30, 33-35]. Recently, there is a handful of work on synthesis of salt-responsive membranes and its potential

application in HIMC[23, 27, 29]. These composite membranes are shown to yield substantial improvement in protein binding capacity and recovery without compromising the selectivity of the membranes.

Poly(*N*-isopropylacrylamide) (pNIPAAM) is the most extensively studied[42, 51-54] non-ionic smart polymer due to its sharp phase transition (near 32 °C) at physiological range. Other non-ionic smart polymers which also have been studied are poly(vinyl methyl ether) (pVME)[51-52] and poly(*N*-vinyl caprolactam) (pVCLM)[51-52]. These polymers can be cross-linked to form a three-dimensional hydrogel network that can change its physical and chemical properties when subjected to various environmental stimuli. Compared to pNIPAAM, pVCLM is a much bio-compatible and less toxic polymer because it does not produce small toxic amines when hydrolyzed[42, 51, 53-60]. Therefore, the study of pVCLM has become an interesting topic in recent years as an alternative smart polymer for bio-medical and bio-separation applications.

In light of the current research directions as described above, a novel composite membrane, comprising of cellulose filter paper, as membrane support, and cross-linked pVCLM hydrogels, as adsorbing media, is believed to resolve the current challenges confronted by HIMC membranes. This type of membrane has been reported to possess high permeability, due to the micro-porous structure of filter paper, and capability to demonstrate dynamic change in hydrophobicity, due to the smart polymeric hydrogels[27, 29]. In fact, cellulose filter paper is available at low cost and satisfies the requirements of

an ideal membrane support[25-26, 61-62], as outlined by the reviewers from both research and industrial field.

Therefore, the objectives of this project are formulated as follows:

1) To develop and characterize bio-compatible, ER paper-supported adsorptive membranes.
2) To systematically evaluate the effect of operating conditions on the protein binding performance of pVCLM-paper membrane as a hydrophobic interaction-based chromatographic membrane.

In Chapter 2, preparation of ER membranes by in-situ free radical polymerization to form an interpenetrating polymer network, comprising of cross-linked pVCLM hydrogels and filter paper, is discussed. The resulting composite membranes are first subjected to SEM characterization to compare the morphology difference between nascent and modified membranes. The net mass gain characterization is followed afterwards to study the effect of monomer and cross-linker content in reaction media on the mass gain of the membranes. Subsequently, the responsiveness of the membranes towards the change in salt concentration is examined, in terms of permeability change. Finally, the extent of permeability change and its consistency are investigated to confirm the repeatability of the membranes' behaviour.

Chapter 3 presents a feasibility study on the potential application of the ER membranes in purification of Immunoglobulin G by HIMC. Salt concentration effect on antibody solubility is first investigated to determine the antibody concentration to use, at which the antibody is fully soluble over the examined range of salt concentration. An ER membrane sample capable of demonstrating high permeability and significant permeability change at reasonably low operating pressure is then selected from previous characterization studies and used in the feasibility study. Important factors, such as polymer loading, salt concentration and feed conditions, were examined and optimized accordingly to determine the antibody binding capacity and selectivity of the composite membrane. The performance of ER membrane was compared against that of nascent membrane to demonstrate the attractiveness of using ER-paper membrane in HIMC.

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CHAPTER 2

SYNTHESIS AND CHARACTERIZATION OF HYDROGEL COATED ENVIRONMENT-RESPONSIVE PAPER-BASED COMPOSITE MEMBRANE

Abstract

Bio-compatible pVCLM hydrogel had been successfully coated on cellulose filter papers in controllable manner to produce low-cost composite membranes with environment-responsive properties. The synthesis of poly(*N*-vinyl caprolactam)-paper membranes was investigated by studying the effect of monomer and cross-linker composition in reaction media on mass gain of composite membranes. The composite membranes were then characterized by their membrane morphology and dynamic change of hydraulic permeability in response to changes in salt concentration. A composite membrane, with an average net mass gain of 5.51 %, was capable of exhibiting high membrane permeability (c.a. 2.11 X 10^{-6} m.$(Pa.s)^{-1}$ per m of dried bed height). The environment-responsive membrane possessed micro-porous characteristic of filter paper and dynamic control of membrane permeability from pVCLM hydrogel, respectively.

2.1 Introduction

The growth of environment-responsive (ER) membrane technology has been sporadic in the last two decades[1]. Over the course of development, such membranes have been demonstrated as an attractive separation media because of their salient ability to

self-regulate their pore structure and surface properties. Smart polymers are incorporated in these membranes to impart responsiveness to a wide variety of environmental-stimuli, such as temperature[2-5], pH[2, 6-9], ionic strength[10-11], electric fields[12-13], solvent[14], light[15-16], pressure[17-18] and specific bio-molecules[19-20]. Most of the practical applications of ER membranes have been in water and wastewater treatment[21-22], gas separations[22] and bio-separations[6, 22-24]. In bio-separations, ER membranes are generally used for size-exclusion-based ultra-filtration[2-3, 5-6, 21-22]. However, there are relatively few reports on using ER membranes for pervaporation[22] and membrane-based adsorption[1, 21, 23-24].

These ER membranes are generally prepared either by co-casting of polymer blend, composed of responsive and membrane base materials, or by coating of responsive materials on polymer substrates[25]. The resulting membranes prepared from the former technique has functionality being embedded inside the structure of base polymer[7-8]. The latter method, however, can prepare two types of membranes. The first type of membrane possesses brush-like polymers attached to their inner pore surface with the loose-end extending towards the pore center[26-28] while the other type of membrane forms an interpenetrating polymer network (IPN) of cross-linked responsive hydrogel and polymer substrate[9, 23-24, 29-30]. While the preparation of IPN-based membranes is easy and straight-forward, the brush-based membranes have localized chain grafting with well-defined chain length. IPN-based ER membranes are usually prepared by soaking the substrates in a monomer solution, composing of pre-cursor monomer, cross-linker and

initiator, followed by free radical polymerization via UV, temperature or gamma irradiation.

Most smart polymers studied thus far are made responsive to either temperature, pH, or combination of temperature and pH. This is because these two variables are relatively easy to control [2-3, 6, 22] and, most importantly, they are essential to regulate the enzyme activities in human body. Hence, the development of controlled-release drug carrier based on smart polymers constitutes a significant part of smart-gel applications[31]. Poly(methacrylic acid) (PMAA) and poly(acrylic acid) (PAA) (shown in Figure 2.1) are the two most common polyelectrolytes[2, 6, 22, 31] used to make pH-responsive smart polymers. On the other hand, thermo-responsive polymers are based on non-ionic polymers[4, 22, 32], such as poly(*N*-isopropylacrylamide) (pNIPAAM), poly(vinyl methyl ether) (pVME) and poly(*N*-vinyl caprolactam) (pVCLM) (shown in Figure 2.1).

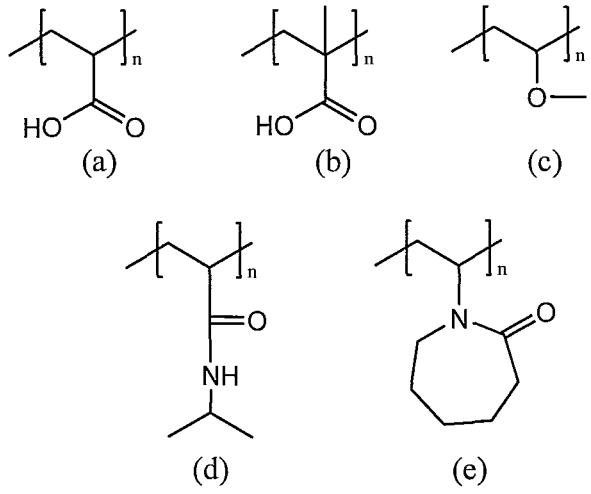

Figure 2.1 Chemical structure of some common environment-responsive polymers. (a) poly(acrylic acid); (b) poly(methacrylic acid); (c) poly(vinyl methyl ether); (d) poly(*N*-isopropylacrylamide); (e) poly(*N*-vinyl caprolactam).

Among the thermo-responsive polymer materials, pNIPAAM and its derivatives are the most studied smart polymers. These water soluble polymers can exhibit sharp phase transition at liquid critical solution temperature (LCST) (near 32 °C) in the physiological range. Such cross-linked polymer is easily prepared by free-radical polymerization[4]. Despite its distinct characteristics, pNIPAAM and other acrylamide-based polymers are considered non-biocompatible as they can produce small toxic amines and acrylic acid when subjected to hydrolysis[4, 29, 33-40]. Hence, they are not suitable for biomedical and biopharmaceutical applications.

On the other hand, pVCLM is another well-studied thermo-responsive polymer which is relatively biocompatible as it does not form toxic molecules when hydrolyzed.

pVCLM has seven-membered lactam rings and a LCST in physiological range (32 to 34 °C)[4]. Compared to pNIPAAM, pVCLM has a more well-defined thermo-responsive property[21, 36]. The homo-pVCLM gel can exhibit mass swelling ratio as high as 110 (ratio of gel mass at equilibrated state to gel mass at dried state)[41]. Smart-polymers based on pVCLM are reported in biomedical applications, such as drug delivery and enzyme carrier[35-36, 38-40]. More recently, ER membranes based on pVCLM have successfully demonstrated its potential application in membrane filtration[5, 21] and chromatography[1] by making use of its dynamic change of pore size and surface properties.

In the present study, a biocompatible and highly permeable ER membrane was developed by the formation of interpenetrating polymer network (IPN) between cross-linked pVCLM polymers and cellulose-derived filter paper. The modified membranes were subsequently characterized by net mass gain and membrane morphology. This was followed by studying membrane responsiveness and permeability change towards the change of salt concentration in aqueous solution. Composite membrane with optimal characteristics was selected for further investigation on protein binding and chromatographic separation studies in Chapter 3.

2.2 Materials and Methods

2.2.1 Materials

WHATMAN cellulose filter paper (Grade No.5) was purchased from VWR and served as polymer substrate on which hydrogel was coated. Lamination pocket,

purchased from Grand and Toy, was used to sandwich and seal the filter paper during polymer reaction.

All chemicals were used as received unless otherwise specified. Monomer solution containing *N*-vinylcaprolactam (*N*VCLM) monomers, methylenebisacrylamide (MBA) cross-linkers, 50/50 blend of diphenyl(2,4,6-trimethylbenzoyl)-phosphine oxide and 2-hydroxy-2-methylpropiophenone UV-initiators and iso-propanol solvent were used to synthesize hydrogel. Sodium phosphate (both mono- and dibasic) and ammonium sulphate were used to prepare buffer solutions. All the above chemicals were purchased from Sigma-Aldrich, except iso-propanol which was obtained from Fisher Scientific.

Two types of buffer solutions were used for the experiments, namely salt-containing and salt-free buffer solutions. Henceforth, these buffers are referred to as Buffer A and Buffer B, respectively. Both buffer solutions were prepared using 20 mM sodium phosphate (pH 7.0) with the former containing an additional 1.1M ammonium sulphate in it. All buffer solutions were prepared using de-ionized water provided by a Diamond Nanopure water purification unit (Barnstead, Dubuque, IA). The solutions were subsequently micro-filtered and degassed using a micro-filtration unit fitted with a 0.45 μm cellulose acetate membrane before use.

2.2.2 Preparation of PVCLM-paper-based Membrane

pVCLM-paper composite membranes were prepared as described by Huang et al.[21] with minor alterations. Five monomer solutions of different monomer and cross-linker compositions, in mass percentage, were prepared in Petri dishes according to the mixing compositions shown in Figure 2.2. In each monomer solutions, 3 g of solvent and 0.2 g of UV-initiator were added.

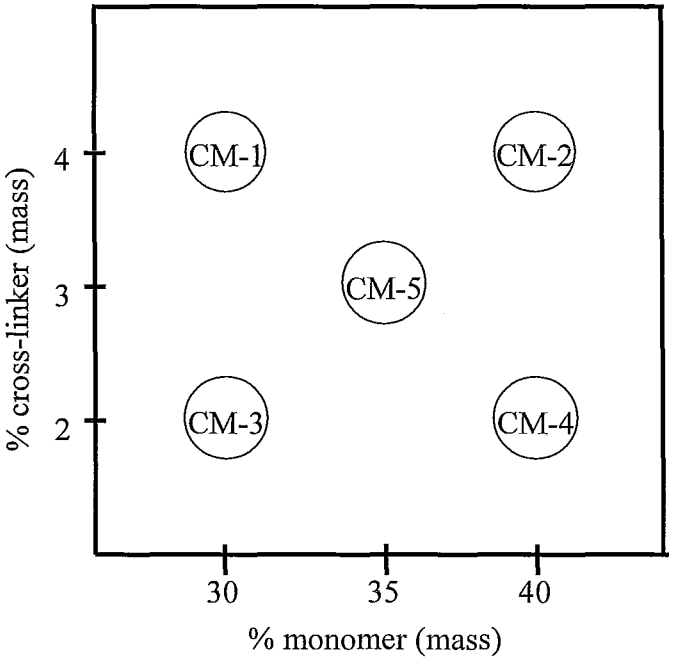

Figure 2.2 Design of experiment with cross-linker and monomer content in reaction media, expressed in % mass, as tuning parameters.

Fresh filter papers, with respective diameter and average weight of 9 cm and 0.5 g, were conditioned for 5 hrs at 23°C and 50 % relative humidity in a constant temperature and humidity (CTH) room prior to weight measurement. The measured papers were then dipped in the individual monomer solutions for 15 mins, allowing papers to adsorb

reaction media. Immediately after the liquid sorption, the wet papers were removed and then sealed in laminating pouches using the Eagle35 laminating machine (General Binding Corporation) to reduce air bubbles in the reaction environment and minimize the inhibition of subsequent polymer reaction. The pouches were then subjected to UV-curing process for 20 mins, operating at 350-400 nm wavelength and 3.30 mW/cm^2 intensity.

When the reaction was completed, the membranes were removed from the pouches and washed excessively with running de-ionized water for 10 mins to extract un-reacted monomers and leachable polymer chains in the membranes. Following the washing step, the membranes were submerged in fresh water in Petri dishes overnight. At the end of the procedure, the extracted membranes were cleaned and dried in the CTH room at the same temperature and relative humidity as before until constant paper weight was attained in order to measure the mass gain.

2.2.3 Membrane Chromatography Setup

The setup of membrane chromatography system as described by Ghosh[42] is summarized below. The system was assembled by two integral parts, namely membrane adsorber module and AKTAprime liquid chromatography system (GE Healthcare Biosciences, NAIE D'urfe, QC, Canada). The membrane module, with an effective diameter of 1.8 cm, was custom-made[43] to provide an excellent flow distribution. Stacks of membrane discs, obtained from membrane samples, were fitted within the module.

The membrane module was integrated with the AKTAprime system which provided continuous reading on UV-absorbance (at 280 nm), conductivity, and pH of the effluent stream from the module as well as transmembrane pressure across the module. All the above operating parameters were recorded and logged into a computer using Prime View software.

2.2.4 Mass Gain Characterization

One of the most straight-forward ways to validate the success of polymer coating on filter papers was to determine the mass gain percentage of the modified membranes. As the term suggested, the mass gain percentage was computed by comparing the net mass increase, caused by the polymer coating, to the mass of the nascent filter paper. The equation for mass gain percentage (MG) is

$$MG = \frac{m - m_o}{m_o} X\ 100\% \tag{2.1}$$

where m and m_o denote the dried paper mass before and after polymer coating.

2.2.5 Scanning Electron Microscopy (SEM) Characterization

Alternatively, the polymer grafting on filter paper could also be validated via the morphological images of the modified paper surfaces using Philips 515 scanning electron microscopy (SEM). Additionally, SEM was also used to provide visual insights on the pore diameter and porosity of the membranes, before and after modification, for qualitative comparison. Small samples with approximately 2 mm long and 2 mm wide

were cut from the membranes. The samples were then subjected to a thin layer of gold coating in a sputter coated machine, prior to SEM imaging.

2.2.6 Hydraulic Characterization of Membranes

As shown in Figure 2.3, membrane chromatography system is used throughout the experiments to evaluate the hydraulic properties of the membranes. The system was supplied with two separate buffer reservoirs; one contained Buffer A while the other filled with Buffer B. The buffer solution was pumped into the membrane module, fitted with membrane discs. The effluent coming out from the membrane module was then channelled to the online detector to monitor the operating parameters before going to waste. The liquid flow rate was adjusted to either constant flux or step mode, depending on the experimental goals.

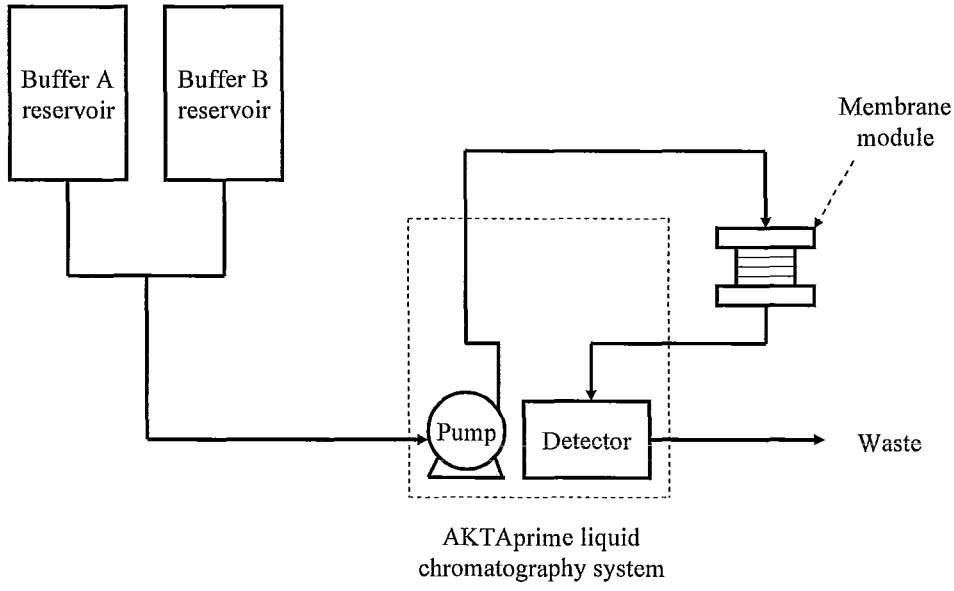

Figure 2.3 Membrane chromatography system for hydraulic characterization.

The study of hydraulic properties of the membranes was consisted of three parts. In the first part, the transmembrane pressure across the membrane bed was collected for each membrane samples at a constant liquid flow rate of 1ml/min. Three membrane discs were installed in the membrane module. Salt-free buffer solution was used to feed the membrane module because the membrane was at its smallest pore size at this condition. Therefore, the maximum operating pressure of each membrane, resulting from pore shrinkage, was used as the design criteria in selecting an optimal membrane for subsequent hydraulic characterization studies.

The second part examined the dynamic change of the membranes in hydraulic properties by observing the change in pressure system as the salt content in the mobile liquid phase varied. The membrane stack, loaded in the membrane module, was subjected to sequential buffer switch from Buffer A to Buffer B in cyclic fashion. The resulting pressure profile with respect to the change in salt content was recorded. Based on the profile, dynamic change of the membranes in hydraulic permeability was evaluated for its consistency and reliability.

In the final part, the hydraulic permeability of the optimal membranes was determined over a range of operating flow rates (0.5 - 3.0 ml/min) under salt-present and salt-free conditions by passing Buffer A and B, respectively, across the membrane bed. For each case of buffer solution, the system pressure corresponded to the operating flow rate was recorded and subsequently plotted against the permeate flux (J_v) to generate a

linear plot. The J_v was determined by computing the ratio of liquid flow rate (Q) to cross-sectional area (A) of the membrane discs. The hydraulic permeabilities of the membrane, in salt-present and salt-free conditions, were calculated from the slopes of respective linear plots.

2.3 Results and Discussion

2.3.1 Polymer Reaction and Membrane Synthesis

The pVCLM-paper composite membranes were successfully prepared by UV-initiated free radical polymerization. In this polymerization, isopropanol (IPA) was used as the solvent because VCLM is not water-miscible in monomer form. Moreover, IPA could also be used as a perfect medium for heat dissipation[29] during the polymerization since it has relatively low toxicity and volatility (MP at 82.3 °C). The use of UV light as the radical initiator was justified by the fact that UV-initiated polymerization is, in general, clean, rapid, and not temperature dependent (ie. can react at room temperature). Furthermore, the phosphine oxide initiator possesses photo-beaching characteristic which does not only serve as an indicator of the reaction but also promote uniform polymer grafting via layer-by-layer fashion[29]. The overall chemical reaction for the polymerization is illustrated below.

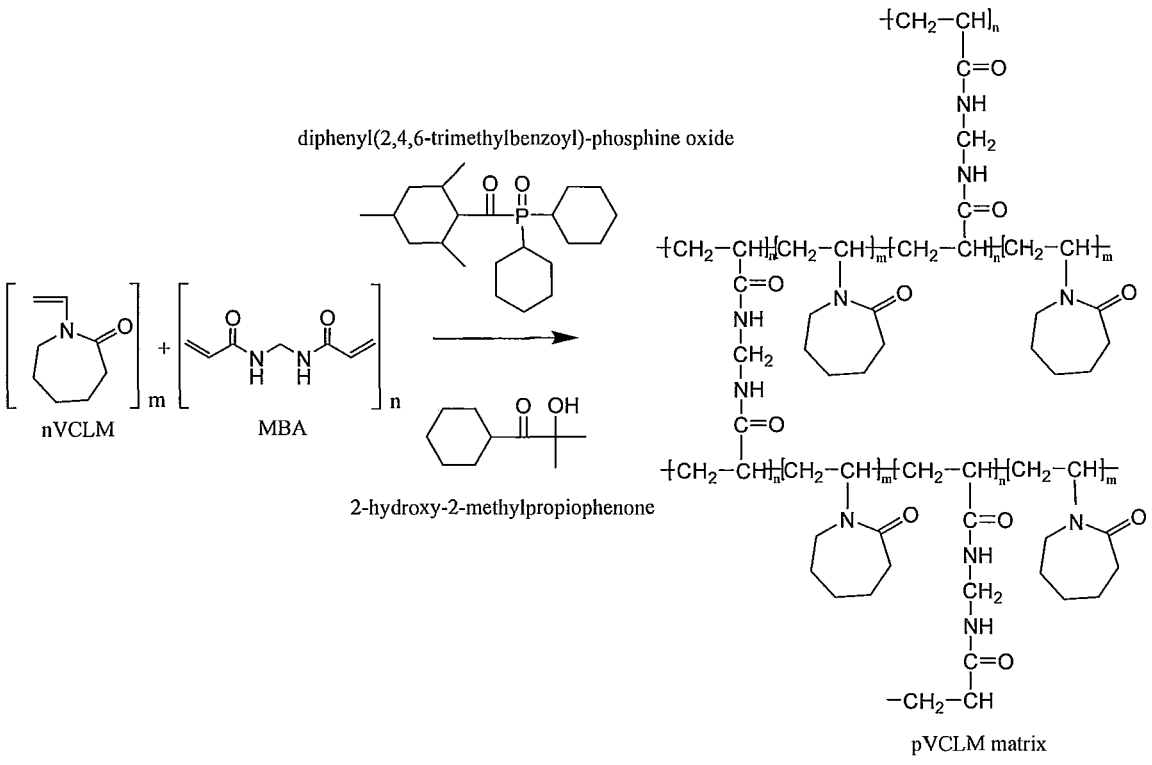

Figure 2.4 Chemical reaction of cross-linked pVCLM hydrogel.

The polymer reaction was initiated by irradiation of UV-initiator, followed by propagation of co-polymer chains which were made up of bi-functional cross-linker and *N*VCLM monomer. The resulting non-ionic polymer was a 3-dimensional polymeric hydrogel network which interpenetrated cellulose paper web and formed a micro-porous composite membrane with environment-responsive property.

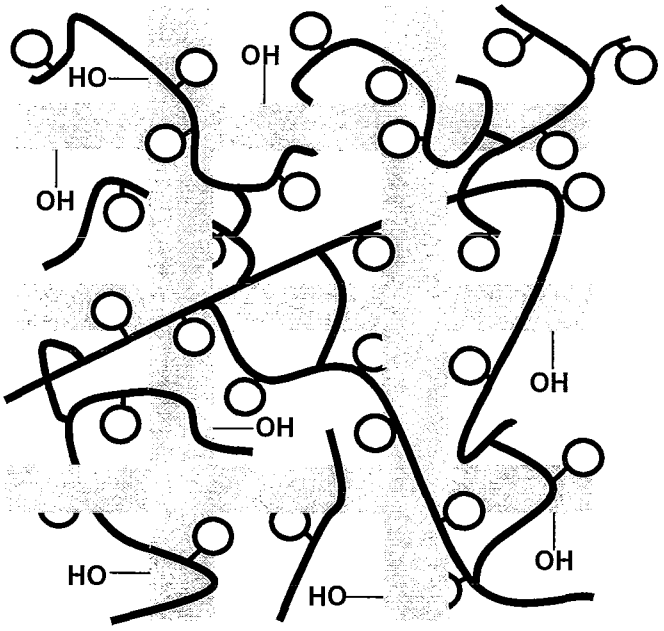

Figure 2.5 Cartoon image of IPN of pVCLM and filter paper.

Figure 2.5 proposed a cartoon image of the interpenetrating polymer network of pVCLM and cellulose fibre. The network of fibre mesh is largely spaced in the drawing, indicating the high permeability of filter paper. The circular objects denote the 7-membered lactam rings extended from C-C backbones. As shown in the figure, it is speculated without experimental evidence that no polymer chain is covalently bonded to the cellulose and the coating of pVCLM polymer on filter paper is entirely based on inter-network entanglement, as purported in literature[44].

2.3.2 SEM Images of Membranes

Figure 2.6 compares a set of SEM images of filter papers, before (A) and after (B and C) the polymer treatment. As vividly shown in this figure, the coating of hydrogel on filter paper is successful as parts of the paper fibres are covered by a layer of polymeric

gel. The extent of polymer coverage was governed by the amount of polymer loaded on the membrane. In general, the higher the mass gain in composite membrane, the larger the area of filter paper was covered by hydrogel. In comparison to nascent filter paper, the composite membranes had relatively low porosity, small pore size and occluded pores due to the polymer coating. Such occurrence could adversely affect the performance of the membrane as would be discussed in the later section.

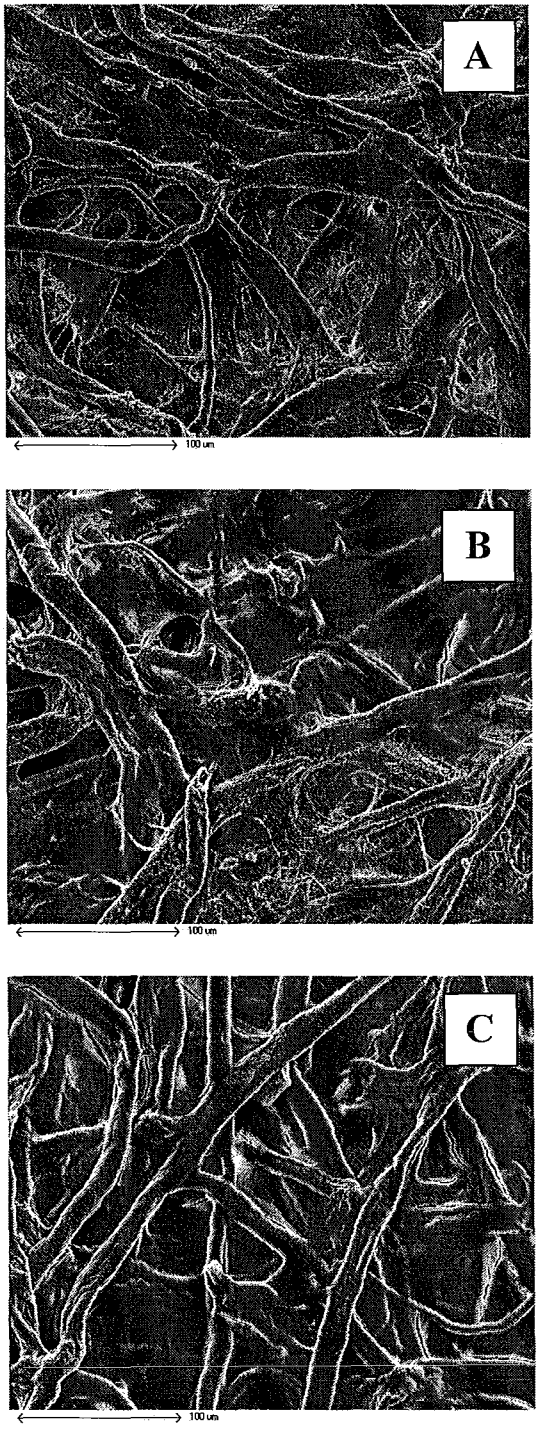

Figure 2.6 Comparison of surface morphology of nascent filter paper (A), and pVCLM-paper with 5.51 % (B), and 8.96 % (C) mass gain.

2.3.3 Effect of Monomer and Cross-linker Content on Mass Gain of Membrane

A systematic study to examine the concurrent effect of monomer and cross-linker content, in reaction media, on mass gain in composite membrane is summarized in Figure 2.1. Five different composite membranes were prepared from monomer solutions, containing different monomer and cross-linker concentration, but fixed initiator and solvent amount. Table 2.1 summarizes the mass gain of composite membranes for all five treated paper samples. An apparent trend was observed in mass gain of membrane such that it increased with monomer and cross-linker content in reaction media. The observation was found to be in good agreement with those reported in literature[21, 29].

Table 2.1 Summary of percentage mass gain and transmembrane pressure of composite membranes prepared from different monomer and cross-linker contents in monomer mixture.

Paper Sample	% Mass Gain	Pressure Range (MPa)
CM-0	0.00	0.04
CM-1	8.96 ± 0.65	0.11
CM-2	13.91 ± 0.98	> 1
CM-3	2.77 ± 0.58	0.04
CM-4	5.51 ± 0.91	0.06
CM-5	10.34 ± 0.73	0.18

Based on Table 2.1, CM-2 membrane achieves the highest mass gain while CM-3 membrane scores the lowest mass gain. The change in mass gain was attributed to several factors. Firstly, the amount of cross-linker and monomer present in reaction media could restrict the growth of pVCLM gel network across the paper web. Since the cross-linker, in CM-3 membrane case, was the limiting reactant, all cross-linkers were consumed

completely to form clumps of gel across the paper web while the free polymer chains were remained in the reaction media. Consequently, the free polymer chains were extracted in the subsequent washing stage and, therefore, a low mass gain membrane was obtained.

Secondly, IPA is a chain transfer agent[29] which impedes the growth of polymer chains and yields low molecular weight polymers. As a result, the formation of interpenetrating polymers network between pVCLM gel and paper web was suppressed by the short polymer chains. Hence, for the solvent volume used in this study, the percentage mass of cross-linker in reaction media must be greater than 2 % in order to form gel on filter paper.

2.3.4 Effect of Mass Gain on Transmembrane Pressure

Also from Table 2.1, another relationship is identified between the mass gain in composite membrane and the transmembrane pressure, measured across a stack of three membrane discs, in the presence of 20mM sodium phosphate buffer solution. The system pressure was found to be directly related to the mass gain of the membrane. As the polymer loading increased, the porosity and the pore size of filter paper were greatly reduced. Additionally, the likelihood of pore obstruction was also increasing with polymer loading. Such effects are evident as manifested in the SEM images located in Figure 2.6. As a consequence, all the above-mentioned effects could lead to severe liquid flow resistance, thereby creating a high pressure drop across the membrane stack. This

was demonstrated in the case of CM-2 membrane such that the transmembrane pressure value was not available in Table 1 as it exceeded the operating pressure limit (1 MPa) of AKTA system. On the other hand, CM-3 membrane had the lowest operating pressure and was found comparable to original filter paper.

2.3.5 Effect of Salt Concentration on Hydraulic Permeability of Membrane

Figure 2.7 presents the transmembrane pressure profile of membrane module, fitted with three CM-4 membrane discs, as a function of salt concentration. Similar pressure-conductivity profiles were also obtained for other prepared pVCLM-paper membrane samples.

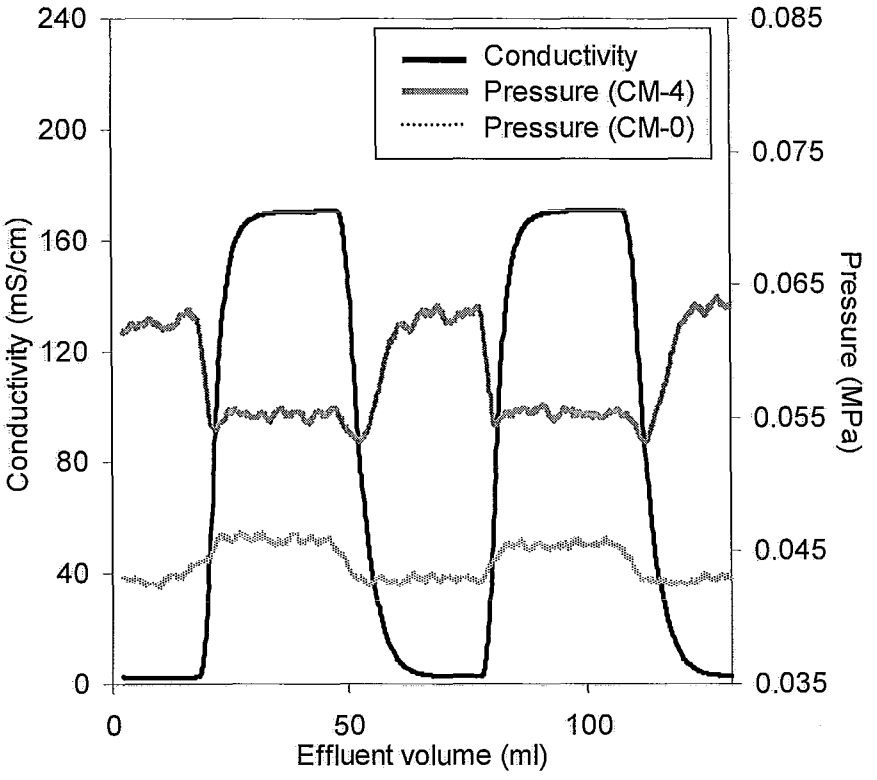

Figure 2.7 Comparison of pressure profile as a function of salt concentration for pVCLM-paper (CM-4) and nascent filter paper (flow rate: 1.0 ml/min; binding buffer: 1.1 M ammonium sulphate in 20 mM phosphate buffer at pH 7.0; eluting buffer: 20 mM phosphate buffer.

It was clearly shown that the composite membrane was responsive to salt concentration in liquid phase. The pressure across the module was inversely related to the salt concentration in liquid phase. When the concentration of ammonium sulphate increased, as reflected by the rise in conductivity value, the pressure in the system decreased rapidly. This was because the hydrogel network underwent a phase transition and collapsed as the salts brought down the gel's LCST below the ambient temperature[11]. As a result, the pore size of the membrane enlarged immediately after the phase transition

which, in turn, reduced the resistance of liquid permeating through the membranes. On the other hand, the pressure increased to its original value when the salt concentration in the liquid phase decreased. During the phase transition, the collapsed gel network reverted to its hydrated state, thereby contracting the pore size and increasing the flow resistance. From the repeated attempts of sequential change in salt concentration, the pressure pattern was found to be similar. This result clearly demonstrated the reversible nature of the property change in the composite membrane.

Also from Figure 2.7, the most striking difference between original and environment-responsive filter papers is the pressure-conductivity profile. The original filter paper exhibited an opposite pressure-conductivity profile when compared to modified filter paper. The negligible pressure increase was partly driven by the increase in viscosity of flowing liquid containing ammonium sulphate[24]. Additionally, the salt presence could also induce weak hydrophobic interaction[24] between the adjacent paper fibres via the non-polar surface regions of cellulose chains. As a result, paper formed a tighter network and increased the flow resistance. Compared to modified filter paper, the overall transmembrane pressure profile of original filter paper was lower due to the absence of polymer coating on paper fibres. Additionally, the magnitude of pressure change in original filter paper, caused by the change in salt concentration, was also found to be less significant than that of modified filter paper.

Based on the results of this study, CM-4 membrane was found to possess reasonably high hydraulic permeability and substantial pressure change during the buffer switch. Henceforth, it was chosen to be evaluated for the rest of the work in this chapter. Despite its excellent hydraulic permeability, CM-3 membrane was not selected because of its operating pressure being similar to that of original filter paper. As such, CM-3 membrane could not exhibit the distinctive change in hydraulic permeability which was an important factor to verify the functionality of the membrane. The rest of the membranes were eliminated because they yielded high system pressure, which could adversely affect the performance of membrane chromatography.

2.3.6 Hydraulic Permeability of Composite Membrane

Figure 2.8 presents the flux-pressure profiles of three CM-4 membrane discs evaluated in Buffer A and B solutions. Based on the figure, the permeate flux ($J_v = Q/A$) is found to be a function of transmembrane pressure in both buffer solutions. The relationship appeared to be linear such that the transmembrane pressure increased with permeate flux. The hydraulic permeability of CM-4 membrane was found to be lower in salt-free condition, 1.68 X 10^{-6} m.$(Pa.s)^{-1}$ per m of dried bed height, compared to that obtained in the presence of salt, 2.55 x 10^{-6} m.$(Pa.s)^{-1}$ per m of dried bed height. The difference in permeability was due to the dynamic behaviour of the gel as discussed previously. The permeability values of the composite membrane were found to be an order of magnitude higher than the reported permeability range of commercial adsorptive membranes (1.6 X 10^{-7} m.$(Pa.s)^{-1}$ to 3.3 X 10^{-7} m.$(Pa.s)^{-1}$ per m of dried bed height)[24].

Therefore, these novel composite membranes could operate at higher liquid flow rate to reduce the process time without compromising the system pressure. The high permeability was inherently due to the micro-porous characteristic of filter paper.

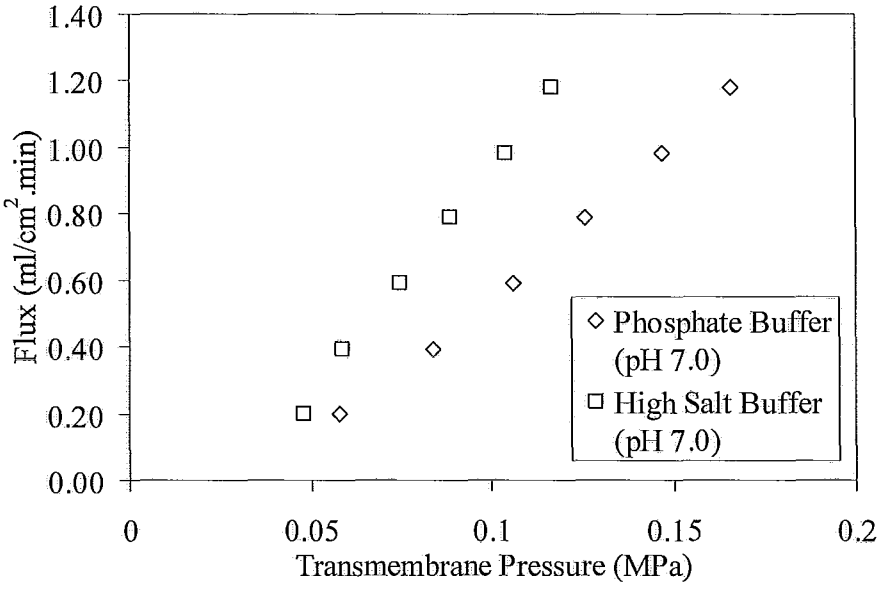

Figure 2.8 The difference in flux and pressure relationship for CM-4 composite membrane evaluated in (1) 20 mM phosphate buffer containing 1.1 M ammonium sulphate and (2) 20 mM phosphate buffer, both at pH 7.0 (flow rate: 1.0 ml/min; number of disc: 5)

2.4 Conclusions

Non-ionic pVCLM hydrogels had been successfully coated on cellulose filter papers in controllable manner to produce low-cost composite membranes, which was responsive to salt concentration. The composite membranes were characterized by their pore structure, net mass gain and membrane permeability. From the results of the characterization studies, the following can be concluded.

(1) The mass gain of composite membrane was found to be strongly dependent on the composition of monomer and cross-linker in reaction media, such that the net mass gain increased with the percentage mass of monomer and cross-linker.

(2) SEM study showed that the coating of polymeric gel on filter paper could change the pore size and the porosity of membrane support, depending on the coating extent. Excess of polymer loading could lead to poor permeability and high pressure drop across the membrane.

(3) The modified membranes were found to be responsive to salt concentration in aqueous solution as indicated by the change in permeability when the salt concentration changed. The membrane permeability was high in salt-containing environment but was low in salt-free condition.

(4) The permeability change in composite membrane was found to be both reversible and consistent over a series of repeated cyclic change in salt concentration. The permeability values of CM-4 composite membrane were found to be significantly higher than those of commercial adsorptive membranes.

(5) CM-4 membrane, with an average net mass gain of 5.51 %, demonstrated high permeability and significant pressure change during the salt concentration change.

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CHAPTER 3

APPLICATION OF ENVIRONMENT-RESPONSIVE PAPER-BASED COMPOSITE MEMBRANE IN CHROMATOGRAPHIC SEPARATION OF PROTEINS

Abstract

Environment-responsive paper-supported composite membranes, consisting of interpenetrating networks of poly(*N*-vinyl caprolactam) and cellulose fibres, were prepared for potential application in protein separations by hydrophobic interaction membrane chromatography. These membranes could be made to switch between hydrophobic and hydrophilic states, in a reversible manner, by addition or removal of anti-chaotropic salts. The effect of polymer loading and salt concentration on antibody binding capacity of composite membranes with different polymer loadings was systematically studied. A set of membrane possessing high hydraulic permeability and antibody binding was further examined for use in an immunoglobulin G (IgG) purification process. The membrane demonstrated low fouling tendency and high IgG binding (c.a. 12 mg/ml bed volume). Also, high product purity and recovery were attained in the purification of IgG from simulated mammalian cell culture supernatant, demonstrating the potential of environment-responsive paper-based membrane in performing rapid and inexpensive membrane chromatographic separations.

3.1 Introduction

The advent of membrane and its fusion with chromatography technology has created a new class of separation process, known as membrane chromatography, with high throughput and high selectivity characteristics. Unlike the traditional gel-based chromatography, the membrane-based chromatography also offers the convenience of easy scale-up, maintenance, and process integration[1-4]. Together, these advantages make membrane chromatography an attractive separation process from economic standpoint. As such, membrane chromatography has been found useful in pharmaceutical and bio-technological industries, particularly in fast protein purification in which the target protein has very low concentration in the feed solution[4-7]. Such membrane-based bio-process can also decrease the likelihood of inactivation of bio-molecules[2, 8] by means of short process time and low pressure drop across the membrane bed.

Hydrophobic interaction is a relatively less explored separation chemistry utilized in membrane chromatography[4, 9-10]. It relies on the reversible hydrophobic interaction between proteins and adsorptive membranes to separate proteins based on their hydrophobicity difference. Therefore, such interaction is regarded as selective[10-12]. Anti-chaotropic salts are exploited to induce the hydrophobic interaction between proteins and adsorbent, and to preserve the biological activity of the protein molecules[12]. Most of the applications of hydrophobic interaction membrane chromatography (HIMC) have been found in the purifications of plasma proteins[10], heme proteins[13], therapeutic enzymes[12-13] and recombinant proteins[1, 4, 9].

The typical membranes used in HIMC are of two types, namely hydrophobic membranes coated with hydrophilic gels and hydrophilic membranes grafted with hydrophobic ligands[2, 4, 10, 14-15]. These membranes can vary their hydrophobicity level based on the surrounding salt content in the mobile phase liquid. However, some of these membranes are reported to have either low protein binding capacity or high non-specific protein adsorption[16-17]. As such, they often suffer fouling and loss in product recovery. Hydrophilized PVDF membranes, grafted with a variety of hydrophilic ligands[2, 10, 16], have successfully minimized the non-specific binding issue but the cost of the membranes is relatively expensive due to complicated and energy-consuming nature of the production methods[2].

In order to tackle the challenges in HIMC's membranes and achieve the desired chromatographic separation, a proper selection of membrane support is essential. Cellulose filter paper is a cheap membrane support which possesses low non-specific protein binding, high permeability, and good mechanical strength[16, 18]. Being a natural polymer, cellulose is also hydrophilic and compatible with bio-molecules. Most reported paper-based membranes are affinity-type[16-20] and few other reports on ion-exchange variant[21-22]. A DEAE-cellulose composite membrane, developed by Sato et al.[21], can directly fractionate proteins from a particle-containing feed solution based on ion-exchange interaction. Yang and Chen[16-17] and Hou et al.[19] have demonstrated the use of cellulose composite as affinity-type membranes for IgG and biopolymer separations,

respectively. All of these membranes demonstrate high water permeability and reinforced mechanical strength. These membranes are prepared by either covalent coupling of ligands via reactive hydroxyl groups or grafting of cross-linked polymers[4, 14, 16, 18].

In more recent development, environment-responsive membranes have been exploited in HIMC[23-25]. Cross-linked hydrogels are coated on membrane surface or embedded within membrane structure to impart stimuli-responsive property on the membrane and, thus, enhance the capture efficiency of membranes. These intelligent polymers can undergo a reversible phase separation and change the membrane surface property from hydrophilic to hydrophobic state in the presence of anti-chaotropic salt. Additionally, the change in surface property is also accompanied by a dynamic change in pore properties when subject to external stimuli, as illustrated in Figure 3.1. The pore size shrinks (inducing hydrophobic surface) and expands (creating hydrophilic surface) in salt-present and salt-free environments, respectively. Such membranes are prepared by modifying the commercial membranes via polymer coating method[2, 23-27].

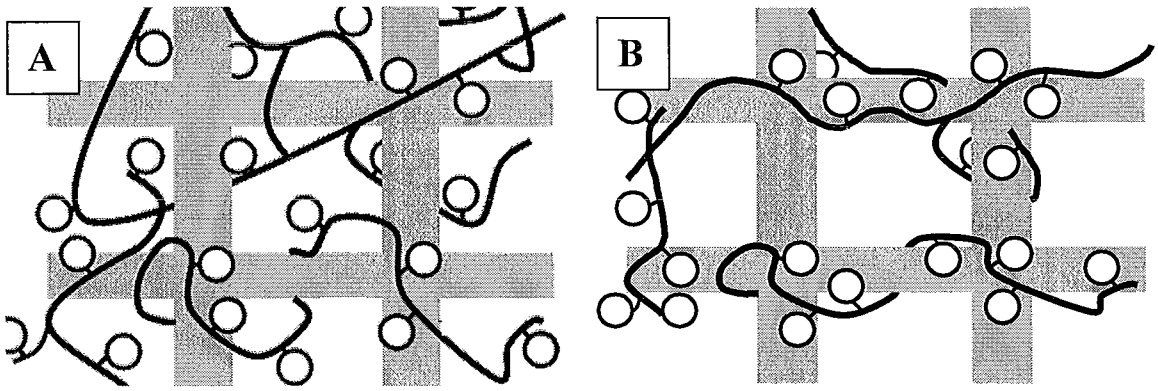

Figure 3.1 Morphology and permeability change in ER membrane when subjected to salt concentration change. (A) in salt-free environment; (B) in salt-present environment.

Among the materials used to compose the smart polymers, poly(*N*-isopropylacrylamide) (pNIPAAM) and its derivatives are the most studied ones[27-31]. Yu et. al[25] and Chen et. al.[23] have used homo-pNIPAAM and its derivative, with which hydrophilic PEG chains were incorporated, to form salt-responsive paper-based membranes and use in purification of monoclonal antibody via hydrophobic interaction. UV-polymerization of pNIPAAM on polyethylene and polypropylene membranes has also been reported in literature[28, 32]. These acrylamide-type polymers, however, are not biocompatible because they are known to impose high cellular cytotoxicity[31] and release toxic by-products upon hydrolysis[27-28, 30-31, 33-38].

On the other hand, a recently well-studied thermo-responsive polymer that is safe and compatible with bio-molecules is poly(*N*-vinylcaprolactam) (pVCLM). Apart from drug release and enzyme carrier studies[30, 35-38], the application of pVCLM has also been reported in membrane modification. The use of pVCLM with Cibacron Blue ligand in

dye-affinity chromatography[39] was reported successful in purifying lactate dehydrogenase, a crucial enzyme for catalyzing the inter-conversion of pyruvate and lactate. Lequieu et. al.[40] has synthesized a thermo-responsive pVCLM-PET composite membrane for size-exclusion-based separation of macromolecular mixture. In most recent publication, the salt- responsive pVCLM-PVDF membranes are being used in multi-component protein fractionation and chromatographic antibody separation using ultrafiltration and HIMC, respectively[24].

The aim of present work is to synthesize salt-responsive pVCLM-paper-based membranes for potential application in HIMC. These membranes were prepared by coating polymerization of cross-linked polymer network onto the surface of filter paper. The modification method described in this work was fast and cheap compared to the industrial production methods. Polymer loading and salt concentration effects on membrane performance were investigated independently and tuned accordingly for the feasibility studies of hydrophobic-based protein binding and separation. As an application, IgG and Chinese Hamster Ovarian (CHO) cell culture medium were used in the work.

3.2 Materials and Methods

3.2.1 Materials

WHATMAN Grade No. 5 filter papers with an average pore size of 2.5 μm were purchased from VWR and used as membrane support for hydrogel coating. Lamination

pockets were conveniently purchased from Grand and Toy and used to seal the paper samples during polymerization.

All chemicals were used as received unless otherwise specified. Protein products, such as IgG (≥ 95%) and serum-free CHO cell culture media, were purchased from Sigma-Aldrich. Chemicals required for paper modification, including *N*-vinyl caprolactam (*N*VCLM) monomers, methylenebisacrylamide (MBA) cross-linkers, and 50/50 blend of diphenyl(2,4,6-trimethylbenzoyl)-phosphine oxide and 2-hydroxy-2-methylpropiophenone UV-initiators, were also purchased from Sigma-Aldrich. Other chemicals, such as sodium phosphate (both mono- and dibasic), sodium citrate, sodium chloride, citric acid and ammonium sulphate were also purchased from Sigma-Aldrich. HPLC-graded iso-propanol was purchased from Fisher Scientific.

All buffer and protein solutions were prepared using ultra-pure water obtained from a Diamond Nanopure water purification unit (Barnstead, Dubuque, IA). Two types of primary buffer solutions were used for the experiments, namely salt-containing and salt-free buffer solutions. Henceforth, these buffers are referred to as Buffer A and Buffer B, respectively. Both buffer solutions were prepared using 20 mM sodium phosphate (pH 7.0) with the former contained ammonium sulphate in it. Other buffer solutions, used for sample analysis, were 100 mM sodium citrate (pH 3.0) and 150 mM sodium chloride in 20 mM sodium phosphate solution. All solutions were micro-filtered and degassed using a micro-filtration unit fitted with a 0.45 um cellulose acetate membrane prior to use.

3.2.2 Preparation of PVCLM-paper-based Membrane

The novel IPN technique used to synthesize pVCLM-paper composite membranes was mostly adapted from Huang et al.[15] and is summarized below. Five monomer solutions of different monomer and cross-linker compositions, in mass percentage, were prepared in Petri dishes according to the mixing compositions shown in Figure 3.2. 3 g of solvent and 0.2 g of UV-initiator were added in each monomer solutions.

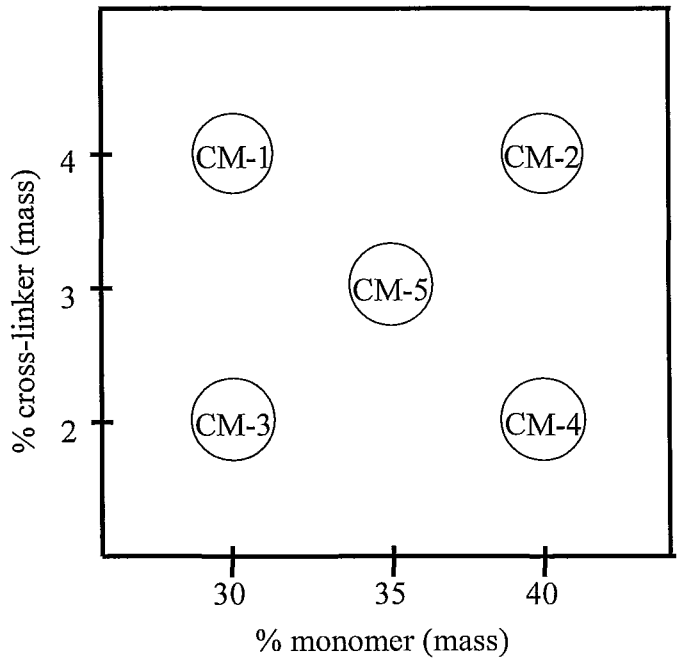

Figure 3.2 Design of experiment with cross-linker and monomer content in reaction media, expressed in % mass, as tuning parameters.

Fresh filter papers with an average weight of 0.5 g were conditioned for 5 hrs at 23°C and 50 % relative humidity in a constant temperature and humidity (CTH) room before taking the weight measurement. The measured papers were then submerged in the monomer solutions for 15 mins to allow papers to adsorb reaction media, after which the

wet papers were removed and then sealed in laminating pouches using the Eagle35 laminating machine (General Binding Corporation). The sealing was an important step to minimize the inhibition of polymer reaction by reducing the air bubbles trapped in the reaction environment. The pouches were subsequently subjected to UV-irradiation, operating at 350-400 nm wavelength and 3.30 mW/cm^2 intensity, for 20 mins.

When the reaction was completed, the resulting membranes were removed from the pouches and washed excessively with ultra-pure water for 10 mins to remove un-reacted monomers and leachable polymer chains from the membranes. The membranes were then soaked in fresh ultra-pure water in Petri dishes overnight before they were dried, at the same temperature and relative humidity as before, in CTH room. The mass gain in composite membranes was determined after the paper weight achieved a steady reading.

3.2.3 Membrane Chromatography Setup

The membrane chromatography system was composed of two main components; a custom-built membrane adsorber module and an AKTAprime liquid chromatography system (GE Healthcare Biosciences, NAIE D'urfe, QC, Canada). The membrane module[41] had an effective diameter of 1.8 cm. Paper samples were cut into small discs and then fitted in the module. The membrane module was integrated with the AKTAprime system. UV-absorbance (at 280 nm), conductivity, and pH of the effluent

stream leaving the module as well as transmembrane pressure across the module were continuously monitored and logged into a computer using Prime View software.

3.2.4 Antibody Solubity in Salt Solution Experiments

Prior to antibody binding study in membrane chromatography, the influence of anti-chaotropic salt on the solubility of antibody must first be investigated. This step was absolutely crucial as it defined the operating window of the appropriate feed condition to be used in the following membrane chromatographic experiments.

All experiments were performed at ambient temperature i.e. 24 ^{o}C. The examined concentration range of salt and IgG was 0 to 1.5 M and 0.1 to 0.9 mg/ml, respectively. A variety of protein solutions, containing IgG and ammonium sulphate at different concentrations, were prepared using 20 mM sodium phosphate buffer solution. In addition to protein samples, protein-free buffer solutions of different salt concentrations were also prepared for system calibration. After the preparation, all samples were centrifuged at 4250 rpm for 20 mins to segregate precipitated antibody from aqueous solution. The concentration of antibody in the supernatants was then measured at 280 nm wavelength using a UV-vis spectrophotometer (Cary 100 Bio, Varian).

For each prepared antibody concentration, the percentage of antibody precipitated (P) were computed by using equation 3.1.

$$P = \frac{A_o - A_i}{A_o} X\ 100 \tag{3.1}$$

where A_o and A_i denote the absorbance of salt-free and salt-containing antibody solutions, respectively.

3.2.5 Antibody Binding Experiments

The IgG binding capacities of the membrane was determined by running bind-wash-elute experiments through the membrane chromatography system, as illustrated in Figure 3.3.

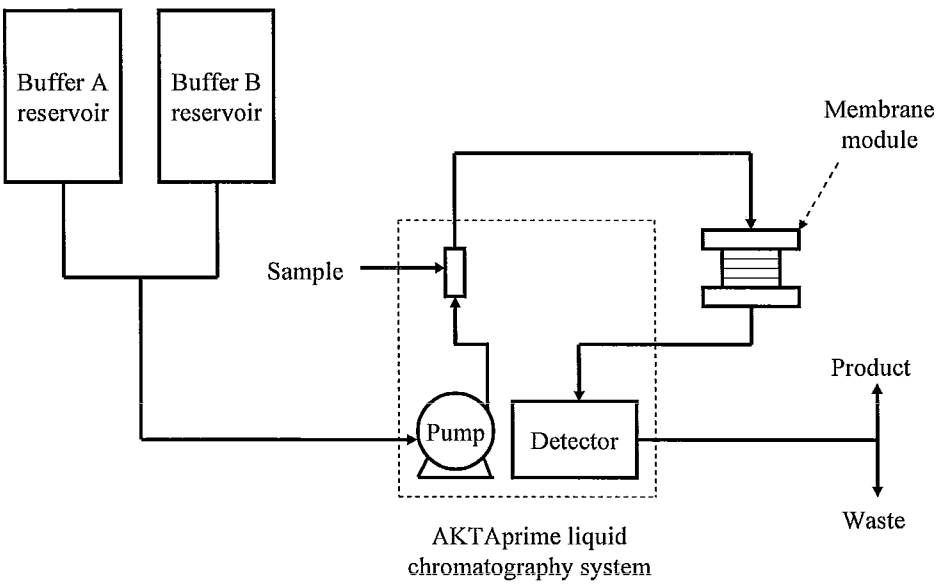

Figure 3.3 Membrane chromatography system for antibody binding experiments.

The experimental methodology began with equilibrating the system with salt-containing buffer solution, followed by pulsing feed sample into the module. In all experiments, the salt concentration in feed sample must keep the same as that in binding buffer solution. After sample injection, the binding buffer solution was continuously feed into the module to wash out any loosely bound antibody until the UV absorbance returned to the baseline. The bound antibody was then recovered using salt-free buffer solution. All binding experiments were carried out at 1.0 ml/min. The amount of antibody recovered was quantified from the eluted peak area, based on appropriate calibration.

Since the salt concentration in binding buffer and the mass gain in composite membrane were the two important factors affecting the antibody adsorption on the membranes, the antibody binding study was split accordingly into two parts. Readers should be aware that there are also other operating parameters (liquid flow-rate, feed volume, temperature, pH and so forth) affecting the binding performance of the membrane, which will not be discussed in this chapter.

The effect of salt concentration on antibody binding was initially studied. This was performed by preparing a collection of feed samples, in which fixed amounts of IgG were added to 20 mM sodium phosphate buffer solutions containing different concentrations of ammonium sulphate (0.9, 1.1, 1.3, 1.5 M). As for the second part of the antibody binding experiments, the influence of polymer loading on the membrane was

investigated such that a variety of prepared membranes with different polymer mass gains were evaluated for their antibody binding capacity.

3.2.6 Antibody Separation Experiments

The experimental methodology for antibody separation experiment was similar to that for antibody binding experiment. The binding buffer consisted of ammonium sulphate in 20 mM sodium phosphate while 20 mM sodium phosphate buffer, itself, was used as the eluting buffer. The feed samples were prepared by spiking CHO cell culture medium with appropriate amounts of IgG in binding buffer. The salt concentration in feed sample must keep the same as that in binding buffer solution. Sample loops were used to inject the feed samples into the membrane module. The feed, flow-through, and eluted peak samples were collected throughout the experiment and then analyzed for IgG content and purity.

The collected samples were desalted and concentrated in 10 kDa centrifugal filter tubes through batch centrifugation, prior to sample analysis. After sample treatment, the samples were analyzed using HiTrap Protein-A FF affinity column and Superdex size exclusion column (SEC). The Protein-A column experiments were conducted by injecting the samples to the Protein-A column via AKTA liquid prime chromatography system. All experiments were performed at 1.0 ml/min flow rate with binding and eluting buffers being 20 mM sodium phosphate at pH 7.0 and 100mM sodium citrate buffer at pH 3.0, respectively. As for SEC experiments, the SEC was integrated with a high

pressure liquid chromatography (Varian ProStar) and the running buffer solution used was 150 mM sodium chloride in 20mM sodium phosphate. All SEC experiments were conducted at a constant flow rate of 0.2 ml/min and the duration of each run was set to 150 minutes. In each run, 250 μl of sample was injected into the column. Using an appropriate calibration, IgG purity and recovery were determined from the area under the peaks in chromatograms.

3.3 Results and Discussion

3.3.1 Solubility Study of Antibody in Anti-chaotropic Salt Solution.

Figure 3.4 shows the influence of ammonium sulphate concentration and protein concentration on the solubility of IgG. Based on this figure, salt concentration is found to have a paramount role in affecting the solubility of IgG. The anti-chaotropic salts could increase or reduce the solubility of IgG, depending on the IgG concentration in the protein solution. The solubility profile of IgG as a function of salt concentration can be distinguished into two types, salting-in and salting-out profiles.

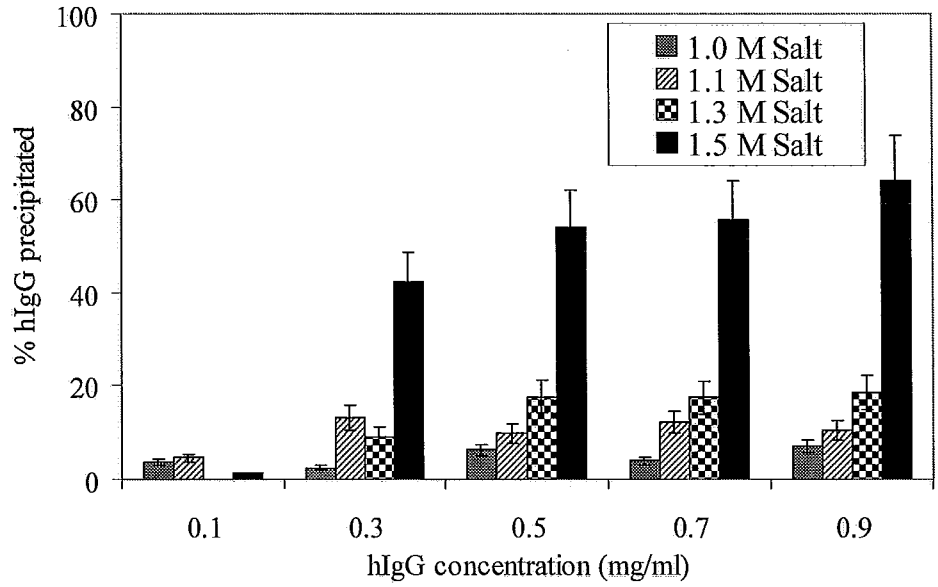

Figure 3.4 Solubility of IgG in different ammonium sulphate solutions.

When 0.1 mg/ml of IgG solution was used, the solubility behaviour of the protein was found to conform to the salting-in theory. This was due to the formation of an electrostatic double-layer[11], surrounding the IgG, which retains the protein molecules in solution and, therefore, increases their solubility. On the other hand, protein solutions with IgG concentration greater than 0.1 mg/ml obeyed the salting-out theory (i.e. protein solubility decreases with salt concentration). This reverse profile was attributed to the electrostatic shielding[11], on the charged groups of IgG, which facilitated protein interaction and induced protein precipitation.

Also, more IgG was found precipitated when the protein concentration increased while the salt concentrations were kept constant. The increase in IgG precipitation was because of the limited amount of salt available in the system to solubilise the increasing

amount of excess IgG in the solution. Therefore, more proteins experienced salt-out effect and formed precipitates.

The standard error range reported in the figure was based on a series of sample measurements, which were performed immediately after sample preparation. The experimental variations, such as UV sensors, biological properties, temperature, relative humidity, and time duration, could account for the reported error range. Thus, the information conveyed in Figure 3.1 must be served only as a general reference and readers are advised to use this information at their own discretion.

From the results of the solubility study, 0.1 mg/ml of IgG solution was selected for performing the remaining experiments in this chapter. This was because at least 95 % of the proteins in 0.1mg/ml of IgG solution were completely dissolved in the salt concentration range examined. This allowed the salt concentration in feed solution to be adjusted over a large operating window. Compared to 0.1 mg/ml of IgG solution, the operating window was severely constrained by the protein solubility at higher protein concentration. This large operating window could be explored subsequently to investigate the effect of salt concentration on protein binding capacity of the adsorptive membranes.

3.3.2 Effect of Salt Concentration on Antibody Binding Capacity of pVCLM-paper Membrane

Prior study performed in Chapter 2 had shown that CM-4 membranes were characterized to have relatively high permeability and fairly significant pressure change, during the buffer switch, to indicate the phase transition of gel. Hence, CM-4 membrane discs were selected in this study to examine the effect of salt concentration on protein binding capacity of membranes.

The general result of antibody binding studies showed that the hydrogel-coated membrane could reversibly bind IgG via hydrophobic interaction. This was because pVCLM hydrogel was a non-ionic water soluble material which could undergo reversible phase separation and exhibit hydrophobic behaviour in the presence of salts. Hence, the IgG could adsorb on the polymer in salt-present environment and be recovered by elution in salt-free aqueous solution.

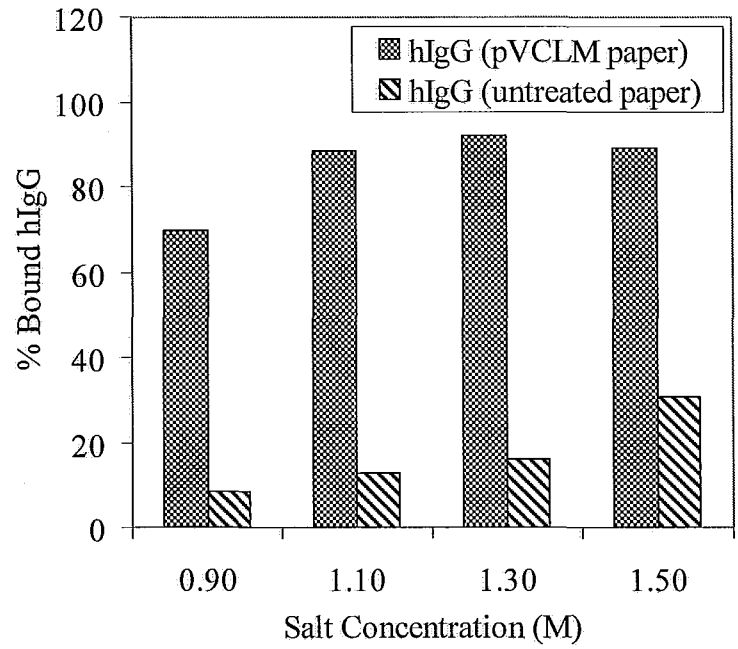

Figure 3.5 IgG binding on CM-4 membrane and original filter paper at different salt concentration levels (IgG concentration in feed: 0.1 mg/ml; feed volume: 2 ml; flow rate: 1 ml/min; membrane bed volume: 0.140 ml, number of disc: 3).

Figure 3.5 presents a comparison study of protein binding on pVCLM-paper membrane and original filter paper over an examining range of salt concentration. As can be seen from the graph, the coating of pVCLM hydrogel on filter paper significantly improve the IgG binding capacity, by approximately 8 to 11 folds. The dramatic enhancement in IgG binding capacity was attributed to the relatively hydrophobic lactam rings of pVCLM, which induced much greater hydrophobic interaction between polymer and antibody than that of untreated filter paper. Additionally, similar protein binding capacity was obtained in the salt concentration range of 1.1 M to 1.5 M. Such discovery is very attractive from the operating point of view as the binding condition could be

alleviated by using a lower salt concentration than those reported in the literature[9-10, 42] without compromising the binding capacity of the protein. In the subsequent desalting stage, the salt-free buffer volume can also be reduced to remove salts from the recovered product.

The study of salt concentration effect on CHO cell culture binding capacity was also investigated to compare the binding preference of the composite membrane on both types of protein. The CHO cell culture medium used in these experiments was composed of recombinant human insulin, insulin-like protein (LONG R3 IGF-1), and enzymatically hydrolyzed soy protein, which are all relatively hydrophilic proteins[43]. Therefore, the binding of media proteins on the composite membrane was expected to be much lower than that of IgG, as corroborated in Figure 3.6. Nevertheless, the binding of media proteins on the composite membrane slowly increased with salt concentration due to salting-out effect.

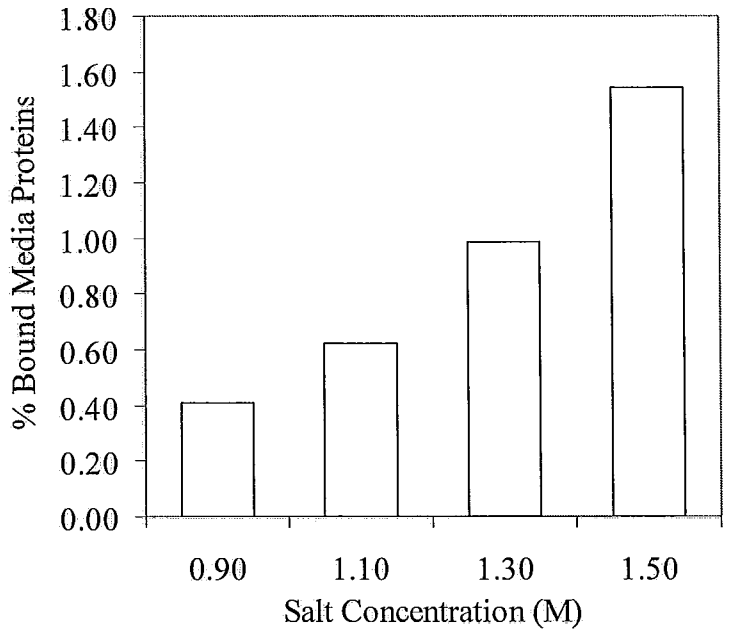

Figure 3.6 CHO media proteins binding on CM-4 membrane at different salt concentration levels (CHO media proteins concentration in feed: 0.5 μg/ml; feed volume: 2 ml; flow rate: 1 ml/min; membrane bed volume: 0.140 ml, number of disc: 3).

Based on these results, the protein binding of composite membrane was found to be quite selective at low salt concentration, such that the membrane had stronger affinity for IgG than CHO cell culture media proteins. However, the selectivity of the membrane slowly diminished at high salt concentrations. To preserve high selectivity of the membrane without compromising the IgG binding on the composite membrane, 1.1 M ammonium sulphate in binding buffer was chosen for performing the remaining studies in this chapter.

3.3.3 Effect of Polymer Loading on Protein Binding Capacity of pVCLM-paper Membrane

Since polymer coating can alter the morphology of filter paper, the influence of pVCLM loading on antibody binding capacity of composite membranes was examined. Figure 3.7 compares the antibody binding capacity of paper samples treated with different levels of polymer loading. As expected, the IgG binding capacity was boosted substantially for all pVCLM-paper membranes. The increase of IgG binding on composite membrane diminished with the polymer loading until 8.96 % mass gain, after which the binding capacity dropped as the polymer loading increased. Such binding trend suggested that there was an optimal range of polymer loading on filter paper which could maximize the binding capacity of IgG on the composite membrane.

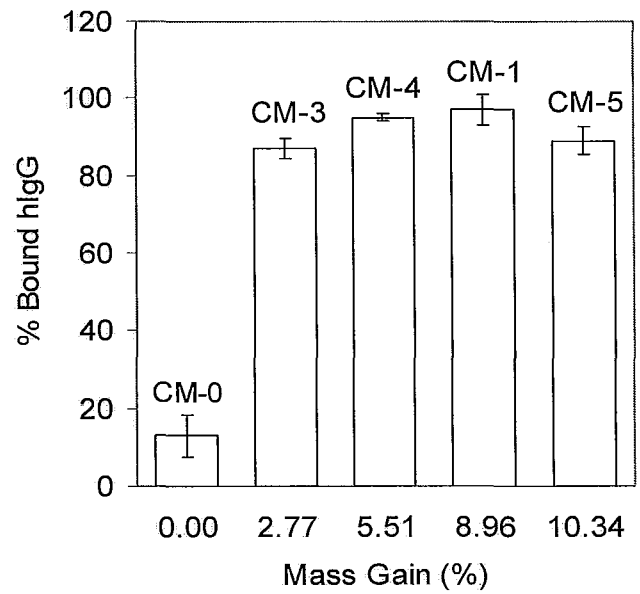

Figure 3.7 Dynamic binding capacity of composite membranes with different mass gains (IgG concentration in feed: 0.1 mg/ml; feed volume: 2 ml; flow rate: 1.0 ml/min; membrane bed volume: 0.140 ml, number of disc: 3).

The increase in IgG binding capacity at low polymer loading was due to the clear pore morphology in the membranes, as discussed in Chapter 2, which facilitated the access of ligands to IgG molecules in aqueous phase. However, the binding capacity was compromised at higher polymer coating because of the pore occlusion due to severe polymer coating. Hence, only a portion of ligands was able to reach out for the IgG molecules while the rest of the ligands were shielded from interacting with the antibodies. These results were in good agreement with the early work reported in literature[23, 25].

In order to achieve an excellent antibody binding in membrane chromatography, the ideal membrane adsorber must possess great permeability and high antibody binding

capacity. Based on Figure 3.7, the optimal polymer loading to achieve the maximum binding capacity of IgG was 8.96 % mass gain (CM-1 membranes). This membrane, however, was previously reported to have relatively low permeability (high operating pressure). CM-4 membrane, on the other hand, met both requirements and, henceforth, was selected for conducting the saturated binding capacity and antibody purification experiments.

3.3.4 Saturation Antibody Binding Capacity of pVCLM-paper Membrane

Figure 3.8 shows the breakthrough and elution curve of IgG binding on three CM-4 discs using the optimal salt concentration in binding buffer. The membranes were first saturated with IgG until the antibody concentration in permeate stream became identical to that in feed, as indicated in UV absorbance plot. The membranes were then washed with binding buffer to remove any loosely bound IgG. The bounded IgG was recovered in the final step by using salt-free buffer. The sharp eluted peak in the chromatogram corresponded to the recovery of bound IgG from the composite membrane.

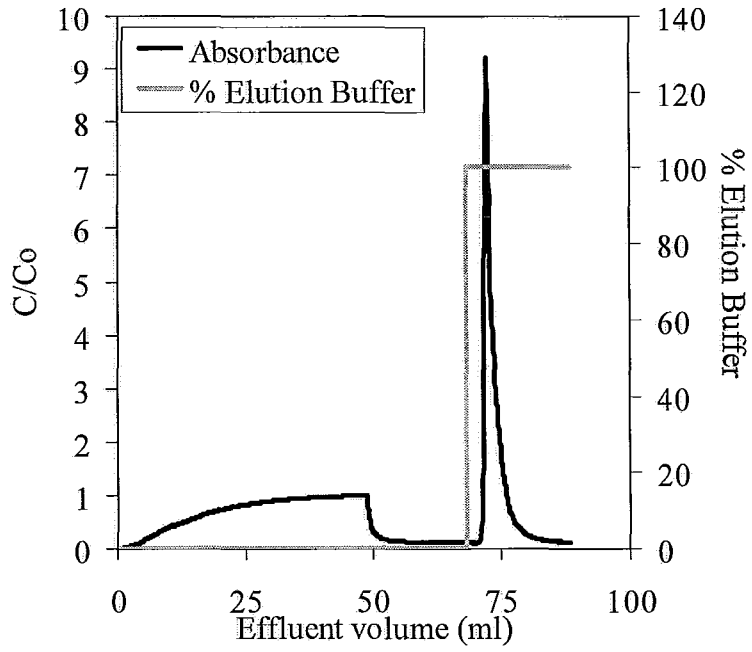

Figure 3.8 Chromatograms of saturation IgG binding on CM-4 membrane (IgG concentration in feed: 0.1 mg/ml; flow rate: 1 ml/min; membrane bed volume: 0.140 ml; number of disc: 3; binding buffer: 1.1 M ammonium sulphate in 20 mM phosphate buffer at pH 7.0; eluting buffer: 20 mM phosphate buffer at pH 7.0).

Table 3.1 summarizes the saturated IgG binding capacity of CM-4 membrane obtained using different disc numbers. The numerical values were computed from the area under the eluted peak, based on appropriate calibration. From the table, the saturated IgG binding capacity decreases with the number of membrane discs. This was probably because the increase in IgG deposition on the membrane created pore constriction and, possibly, pore occlusion, which severely impeded the liquid permeation. Gradually, the pressure drop across the disc stack developed over time and the membrane discs became severely compressed. As a result, the ligand accessibility became poor as they were being suppressed from interacting with IgG molecules. The highest saturated IgG binding

capacity was 12 mg/ml of dried membrane bed volume, obtained using three CM-4 membrane discs. This saturated binding value was significantly higher than that of untreated paper (about 2 mg/ml of dried membrane bed volume).

Table 3.1 Effect of disc number on saturated IgG binding capacity of CM-4 composite membrane.

Disc number	Binding capacity (mg/ml)
3	11.97
5	10.69
10	7.82

3.3.5 pVCLM-paper Membrane Performance in Antibody Purification

The feasibility study of IgG separation from simulated CHO cell culture supernatant was investigated at different feed conditions (i.e changing IgG concentration). Previous discussions had concluded that the selectivity of composite membrane was found to be the most pronounced at 1.1 M ammonium sulphate. Hence, the IgG binding was carried out in binding buffer at this salt concentration. To accommodate sufficient sample volumes for subsequent sample analysis, a stack of six CM-4 discs was mounted in the module.

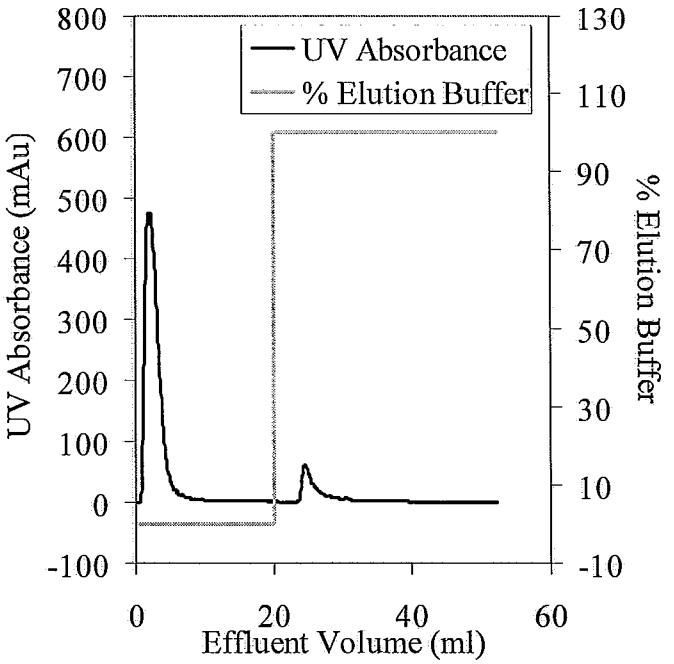

Figure 3.9 Chromatograms of separation of IgG from simulated CHO cell culture supernatant (flow rate: 1.5 ml/min; number of disc: 6; membrane bed volume: 0.277 ml; IgG concentration in feed: 0.1 mg/ml; CHO media proteins concentration in feed: 0.48 μg/ml; feed volume: 1.0 ml; binding buffer: 1.1 M ammonium sulphate in 20 mM phosphate buffer at pH 7.0; eluting buffer: 20 mM phosphate buffer at pH 7.0).

Figure 3.9 shows the chromatograms obtained from the separation experiments, in which 1 ml of feed solution, containing respective IgG and media protein concentration of 0.5 mg/ml and 0.48μg/ml, was injected into the module during the binding phase. The first peak corresponded to flow-through peak which was due to the unbound impurities from the feed. The second peak, occurring at higher volume retention, represented eluent peak which was due to the recovery of bound IgG by eluting buffer. Figure 3.10 shows the Protein-A affinity chromatogram of purified IgG sample, collected from the separation. The first and second peaks were due to impurities and pure IgG, respectively.

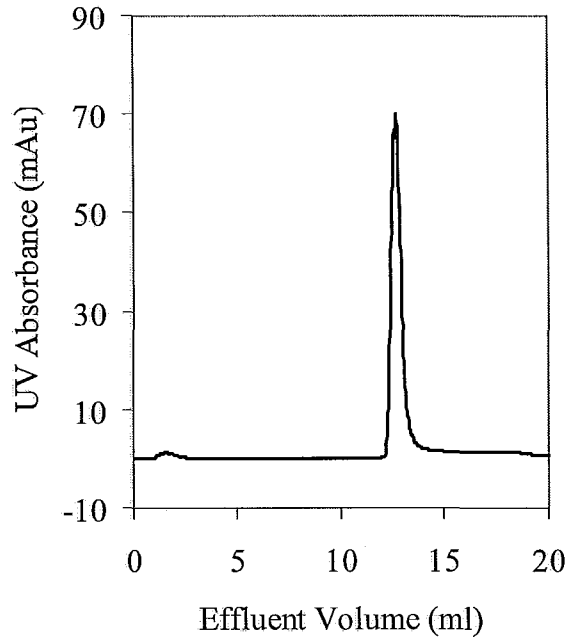

Figure 3.10 Protein A affinity chromatogram of purified IgG sample obtained from IgG purification (feed volume: 500 µl; flow rate: 1.0 ml/min).

Table 3.2 Summary of IgG separation results at different feed conditions evaluated in terms of IgG purity and recovery.

IgG concentration in CHO cell culture medium (mg/ml)	Feed Volume (ml)	IgG purity (%)	IgG recovery (%)
0.50	1	97	98
0.25	1	91	96

Table 3.2 presents a complete summary of the separation experiments. The concentration of media proteins was kept the same throughout the separation experiments. The purity and recovery of IgG were found to decrease when the IgG concentration in feed samples was reduced by half. The decrease in purity and recovery of IgG was due to the increase in mass ratio of media proteins to IgG when the amount of IgG in feed

solution was reduced. Therefore, the CHO media proteins competed with the IgG for the binding sites on the membrane. The results of the separation studies suggested that the selectivity of the composite membrane was somewhat compromised when low concentration of IgG was purified from the CHO cell culture solution.

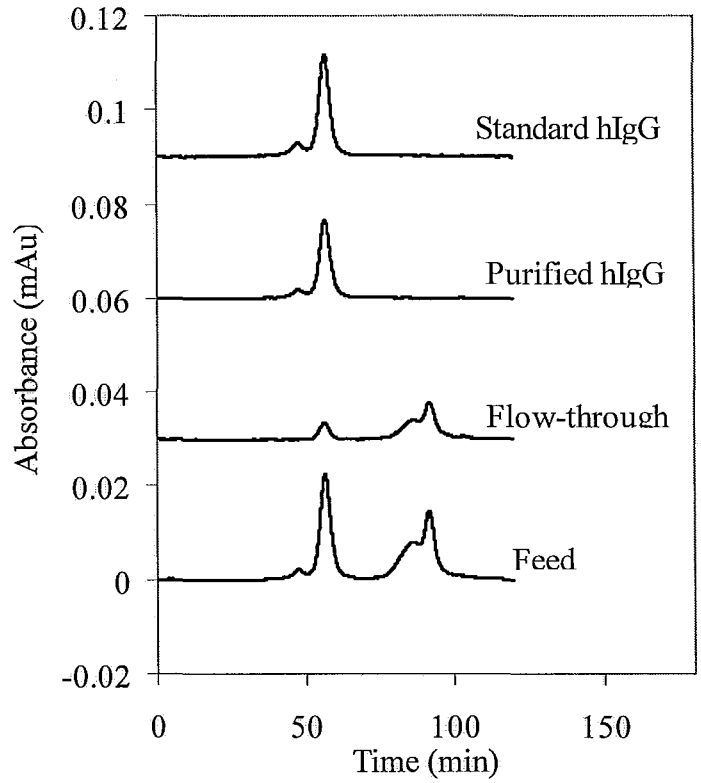

Figure 3.11 HPLC chromatograms of samples collected from IgG separation experiment (flow rate: 0.2 ml/min; run duration: 150 min; feed volume: 250 ml).

SEC chromatograms of feed, flow through, and purified IgG samples are presented in Figure 3.11. The first peak appearing within the retention time range of 45 to 60 min corresponded to IgG while the second and the third peaks, which overlapped one another at higher time retention, corresponded to the media proteins of CHO cell culture

medium. The occurrence of the media protein peaks was consistent with the molecular size range (< 10 kDa) of the media proteins provided by the supplier[43]. Despite a minor loss of IgG in the flow-through sample, the SEC chromatograms suggested that an absolutely pure IgG was obtained in purified IgG. This high purity value of purified IgG analyzed in SEC was, however, grossly overestimated in comparison to the much accurate technique, as reported in Protein-A affinity analysis. Such discrepancy in SEC analysis could be justified by the restriction of the equipment sensitivity to pick up the faint signal of low molecular weight UV absorbing impurities, which existed in unusually minute amount as in the case of purified IgG sample. Based on material balance, the IgG recovery calculated from SEC chromatograms was consistent with that determined in Protein-A analysis.

3.3.6 Fouling Study of pVCLM-paper Membrane

Figure 3.12 presents the health profile of the pVCLM-paper membranes as a function of run number. The transmembrane pressure profile of original filter paper is also included in the figure for comparison. These membranes were kept in membrane modules throughout the study. After seven runs, the original filter paper was found to have negligible fouling, as reflected by the flat profile of transmembrane pressure. By contrast, the pressure in composite membrane increased gradually with number of runs. This implied that a minor fouling had occurred in the membrane throughout the study, possibly, due to non-specific and permanent protein adsorption on the membrane[24].

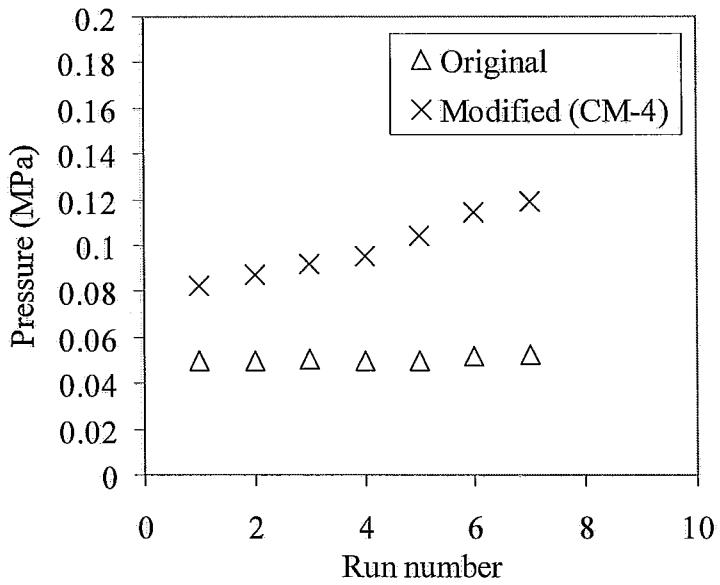

Figure 3.12 Comparison of fouling profile between CM-4 membrane and untreated paper (flow rate: 1 ml/min; number of disc: 3; binding buffer: 1.1 M ammonium sulphate in 20 mM phosphate buffer at pH 7.0; eluting buffer: 20 mM phosphate buffer at pH 7.0).

3.4 Conclusions

Based on the experimental results, the significant findings can be concluded as follows.

(1) Paper-based composite membranes with salt-responsive property were successfully prepared via IPN technique. The resulting membranes were found to exhibit hydrophobic and hydrophilic characteristic in the presence and the absence of salt, respectively.

(2) The study of salt concentration effect on antibody solubility revealed that above 95% of antibody in 0.1 mg/ml antibody concentration was completely dissolved over the range of salt concentration examined. Above 0.1 mg/ml antibody concentration, the antibody solubility decreased significantly as the salt concentration increased.

(3) The coating of pVCLM hydrogel on filter paper could increase the antibody binding capacity of original filter paper by 8 to 11 folds. The highest antibody binding capacity was found at 1.1 M ammonium sulphate concentration and, thereafter, the binding capacity became independent of salt concentration between 1.1 and 1.5 M ammonium sulphate concentration.

(4) An optimal polymer loading of 5.51 ± 0.91 % mass gain was determined where deviation from this net mass gain led to undesirable properties, such as increasing transmembrane pressure and/or decreasing protein binding capacity.

(5) The saturated IgG binding capacity of the optimal membrane was found to be 12 mg/ml of dried membrane bed volume with the value decreasing when more than three membrane discs were used. This value is six times higher than that of untreated filter paper.

(6) The optimal composite membrane could demonstrate selective separation and reversible binding of IgG at a much lower salt concentration (1.1 M) than those reported in literature, thereby further alleviating the binding condition for IgG molecules. High IgG purity (> 91 %) and recovery (> 96 %) were obtained using the optimal membrane and salt concentration.

(7) The composite membrane also experienced a trivial fouling with insignificant compressibility in membrane after seven continuous runs.

(8) Overall results of the feasibility study concluded that the paper-based membrane with environment-responsive properties was shown as a promising material in performing low-cost, bio-friendly, high-throughput, and high-resolution hydrophobic interaction chromatographic separations.

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CHAPTER 4

CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

The overall objective of the study was to synthesize an environment-responsive paper-based membrane adsorber, which is cost-effective and bio-compatible, for use in rapid chromatographic separations of proteins by HIMC. In this study, smart polymeric gels coated on paper-based membrane were used as the adsorbent media for HIMC. The following were the desired attributes of ideal ER gels coated on membrane.

1) Easy to synthesize smart gel and coat them on filter paper in controlled manner by UV-initiated radical polymerization.
2) Able to self-regulate the pore size (permeability) and the surface property (hydrophobicity) of the composite membrane.
3) Significantly improve the capture efficiency and product recovery of the membrane.
4) Complete recovery of bound protein without causing membrane to foul.

Based on these criteria, the prospect of smart gel application in HIMC was evaluated.

In Chapter 2, the preparation of bio-compatible composite membrane with environment responsive properties was successful. The SEM characterization study showed that the coating of polymeric gel on filter paper could change the morphology of

membrane support, depending on the coating extent. The mass gain of composite membrane was found to increase with the percentage mass of monomer and cross-linker in reaction media. The modified membranes were shown to be salt-responsive, such that the membrane permeability was high in salt-present environment and vice-versa. The average permeability of the composite membrane was one order of magnitude higher than those commercial membrane adsorbers. The dynamic change of membrane permeability in composite membrane was found to be both reversible and repeatable over the studied duration. For application in HIMC, CM-4 membrane, with an average net mass gain of 5.51 %, was found suitable as it demonstrated high permeability and noticeable pressure change at reasonably low operating pressure (0.055 – 0.065 MPa) throughout the study.

In Chapter 3, the potential application of the ER membranes in purification of human Immunoglobulin G by HIMC was evaluated. The ER membrane (CM-4) was found to exhibit hydrophobic and hydrophilic behaviour in the presence and the absence of salt, respectively. By grafting pVCLM hydrogel on filter paper, the antibody binding capacity of original filter paper was dramatically improvement by 8 to 11 folds with the highest value achieved at 1.1 M ammonium sulphate concentration. The optimal polymer loading was found to be 5.51 ± 0.91 % mass gain (CM-4 membrane). Deviation from this net mass gain led to transmembrane pressure increase and/or protein binding capacity decrease. The saturated IgG binding capacity of the optimal membrane was found to be 12 mg/ml of dried membrane bed volume.

High IgG purity (> 91 %) and recovery (> 96 %) was achieved from IgG purification studies using optimal membrane and salt concentration. Despite the slight fouling issue, the overall feasibility study concluded that the environment-responsive paper-based membrane was shown as a promising material in performing low-cost, bio-friendly, high-throughput, and high-resolution hydrophobic interaction chromatographic protein separations.

The results presented in this study are novel in that no prior studies have reported on filter paper coated with bio-compatible pVCLM hydrogel as hydrophobic interaction-based membrane. Certainly, a similar type of membrane adsorber made of cellulose filter paper support has recently been reported in the literature but the smart hydrogel was based on pNIPAAM, which is known to produce toxic and charged small molecular weight amine[1-3]. Such by-products are highly undesirable when used in bio-material and bio-separation application[4]. Most of the paper-supported membrane adsorbers reported in literature do not used hydrogel as adsorbent but rely on either affinity or ion-exchange ligands to separate particles or bio-molecules.

As demonstrated, the novel membranes possess high capture efficiency, binding capacity and product recovery. However, low non-specific binding was detected on these membranes over the runs and is consistent with those reported in literature on using pVCLM as adsorbent[5]. As a result, these modified membranes failed in achieving a

complete recovery of bound proteins. Nevertheless, the advantages of these membranes significantly mask the negative side of the membranes.

4.2 Recommendations

Based on the results of the study, there are several aspects which are recommended for further studies. The fouling issue arose in the pVCLM-based ER membrane was due to non-specific protein binding. pVCLM is inherently a hydrophobic material based on its 7-membered lactam rings. Therefore, hydrophilic polymer chains, such as, poly(ethylene glycol), PEG, and poly(ethylene oxide), PEO, can be incorporated into the smart gel to ease its hydrophobicity. A recent publication[6] showed that using a PEG-pNIPAAM hydrogel coated on paper-supported membrane could yield a complete recovery of bound proteins with little or no membrane fouling after 25 uses. The mole ratio of the PEO to pVCLM and the PEG molecular weight can be tailored accordingly to yield a ER membrane which is hydrophobic enough to induce mild hydrophobic interaction at above its LCST and becomes very hydrophilic below its LCST to release the bound molecules completely.

The novel membrane is also appeared to exhibit relatively slow phase transition as indicated by the broad elution peak. As such, the slow hydrophobic-to-hydrophilic transition can be exploited in potential multi-component separation based on hydrophobicity differences. An example of such separation is the segregation of antibody, its dimer and higher order aggregates with increasing order of hydrophobicity[7-8].

Another potential application of paper-supported ER membrane is membrane chromatographic immunoassay technique[9-10]. Such membrane can be used to first separate antibody from serum by hydrophobic interaction. The membrane bound with antibody becomes an affinity media on which different antigens are pulsed and screened. The matching antigen-antibody can then be inferred from the antigen chromatogram peak profiles. Since paper-based membrane is inherently micro-porous, diagnosis and assessment can be carried out, in a rapid manner, using these low-cost novel membranes.

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