APPLICATION OF MEMBRANE CHROMATOGRAPHY IN BIOPROCESSING

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ABSTRACT

Improved and efficient bioprocessing technology is a key requirement in the manufacture of biopharmaceuticals. The increasing need to reduce biopharmaceutical cost is driven by the business challenges such as the emerging biogenerics. The great advances in upstream technologies make bioseparation the major cost in bioprocessing. Developing efficient bioseparation technologies is therefore strongly desired. Membrane chromatography is a promising bioseparation technology which combines the advantages of membrane technology and chromatography, thus making high-throughput and high-resolution bioprocesses feasible. This thesis focuses on the novel applications and improvements of membrane chromatography: bioprocessing of transgenic tobacco derived monoclonal antibodies and PEGylated proteins, integrated bioprocessing for antibody fragmentation, study of antibody binding on membranes and the development of novel membranes.

Membrane chromatography based bioprocesses were developed for purification of monoclonal antibody (mAb) from transgenic tobacco, primarily addressing the challenge of low mAb abundance in the feed material. PEGylated proteins were purified, demonstrating that high-throughput and high-resolution purification of low-binding-propensity proteins was feasible using membrane chromatography.

Membrane chromatography based reactant adsorptive membrane bioreactor separator (RAMBS) systems were developed to integrate enzymatic fragmentation of human IgG with the purification of target fragment. This novel system facilitated the process intensification and led to higher IgG digestion than in liquid phase reaction.

The mechanism of hydrophobic interaction based IgG binding on synthetic membranes was studied using the RAMBS system. The results showed that the binding took place primarily through a combination of the hinge and C_H2 domain of Fc. This study provides a new approach for studying antibody interaction with membranes and surfaces and could help design membrane-based antibody purification, immunoassay and biomaterials.

PEG grafted filter paper was developed as an inexpensive alternative to commercial synthetic membranes. These novel membranes possessed high permeability and low fouling tendency and demonstrated good selectivity and reusability in monoclonal antibody purification.

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PREFACE

This PhD thesis is organized in a sandwich style based on the following published, submitted or prepared articles:

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3. Yu D, Ghosh R. 2009. Purification of PEGylated protein using membrane chromatography. Submitted to Journal of Pharmaceutical Science. This article was made into Chapter 5.
4. Yu D, Ghosh R. 2009. Integrated fragmentation of human IgG and purification of Fab using a reactant adsorptive membrane bioreactor separator system. Biotechnology and Bioengineering 104:152-161. This article was made into Chapter 6.
5. Yu D, Ghosh R. 2009. Cation-exchange based reactant adsorptive membrane bioreactor separator for integrated bioprocessing of $F(ab')_2$. Pending submission to Biotechnology Progress. This article was compiled into Chapter 7.

6. Yu D, Ghosh R. 2009. Integrated bioprocessing of Fab using anion-exchange based reactant adsorptive membrane bioreactor separator. Pending submission to Journal of Immunological Methods. This article was compiled into Chapter 7.
7. Yu D, Ghosh R. 2009. Method for studying Immunoglobulin G binding on hydrophobic surfaces. Langmuir. DOI: 10.1021/la902395v. In press. This article was made into Chapter 8.
8. Yu D, Chen X, Pelton RH, Ghosh R. 2008. Paper-PEG based membranes for hydrophobic interaction chromatography: purification of monoclonal antibody. Biotechnology and Bioengineering 99: 1434-1442. This article was made into Chapter 9.

These articles were prepared by Deqiang Yu based on the research projects including purification of mAbs from transgenic tobacco using membrane-based bioprocesses, purification of PEGylated proteins using membrane chromatography, development of RAMBS system for integrated bioprocessing of antibody fragments, study of antibody binding mechanism and the development of PEG-paper membrane for antibody purification. Dr. R. Ghosh provided guidance in research direction and idea development and reviewed and helped prepare the manuscripts. Dr. J.C. Hall and Dr. M.D. Mclean provided transgenic and wide-type tobacco leaves, participated in the discussions and reviewed the manuscript of two papers in the project of purification of mAbs from transgenic tobacco. Dr. R.H. Pelton and Dr. X. Chen provided the PEG-filter papers and the SEM images and reviewed the paper manuscript in the project of the development of PEG-paper membrane for antibody purification.

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NOMENCLATURE

AMC	affinity membrane chromatography
BSA	bovine serum albumin
CBD	cellulose binding domain
CHO	Chinese hamster ovary
COG	cost of goods
DSP	downstream processing
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EPO	erythropoietin
FDA	Food and Drug Administration
G-CSF	granulate-colony stimulating factor
hIgG	human immunoglobulin G
HIMC	hydrophobic interaction membrane chromatography
HPLC	high performance liquid chromatography
HPTFF	high resolution tangential flow filtration
HSA	human serum albumin
IEC	ion exchange column chromatography
IEMC	ion exchange membrane chromatography
IPN	interpenetrating polymer network
Kav	gel phase distribution coefficient
kDa	kilo Dalton
KUSD	kilo US dollars
mAb	monoclonal antibody
Mr	molecular weight (Dalton)
MUSD	million US dollars
PACC	Protein A column chromatography
PAMC	Protein A membrane chromatography

PEG	polyethylene glycol
PES	polyethersulfone
pI	isoelectric point
PVDF	polyvinylidene fluoride
RAMBS	reactant adsorptive membrane bioreactor separator
Rh	hydrodynamic radius
RT	retention time
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
TFF	tangential flow filtration
TPA	tissue plasminogen activator
UF	ultrafiltration
USP	upstream processing
V_e	retention volume
V_o	void volume
V_t	total bed volume

Chapter 1

Introduction

1.1 Bioprocessing challenges facing biopharmaceutical industry

1.1.1 Modern biopharmaceutical industry

The modern biopharmaceutical industry started in the 1980s based on the breakthroughs of recombinant DNA and hybridoma cell technologies developed in the mid-1970s. In 1982, the first recombinant protein drug, recombinant human insulin developed by Genentech was commercialized by Eli Lilly. Since then, many natural protein drugs with limited availability and potential infection risks due to their human or animal sources have been replaced by their recombinant forms, including interferon-α, -β, -γ, interleukin-2, growth hormone, erythropoietin (EPO), tissue plasminogen activator (TPA) and blood factors. These recombinant protein drugs as well as murine monoclonal

antibodies produced by hybridoma cell technology have unaltered amino acid sequence from their natural forms and most were approved in 1980s and early 1990s. They are called the first generation of modern biopharmaceuticals (Walsh 2004). Second generation biopharmaceuticals are engineered protein drugs that emerged from 1990s and continue to increase in the 21st century. These protein drugs are altered in amino acid sequence by gene mutation or conjugated with special chemicals by chemical reaction. They are advantageous over replacement recombinant protein drugs in terms of higher biological activity, increased half life in the human body and reduced immunogenicity. Typical examples include engineered insulin, mutated interleukin-2, consensus interferon, PEGylated proteins and humanized monoclonal antibodies.

PEGylated proteins have prolonged in vivo half-life, decreased enzyme degradation and reduced immunogenicity compared with their native protein counterparts (Chapman et al. 1999). Motivated by the commercial success of PEGylated interferons and G-CSF, development of PEGylated protein drugs has gained substantial interest. Monoclonal antibodies have developed into the predominant class of therapeutic proteins. Antibody humanization circumvented the immunogenicity problem of murine monoclonal antibodies. The use of humanized or fully human antibodies expressed by recombinant DNA technology is continuously increasing these years. In 2007, six of the world top 10 biotech drugs were monoclonal antibodies (Harris et al. 2008). Except for the protein based drugs, gene- and cell- based therapies like DNA vaccine, gene therapy and stem cell therapy are emerging but are still in very early stages of development. In the research described in this thesis, bioprocessing of monoclonal antibodies and PEGylated

proteins was studied. These two important classes of protein drugs have shown commercial success and will be extensively developed.

1.1.2 Business challenges of biopharm development and manufacturing

The global biopharmaceutical market reached $83 billion in 2007 and will soon advance to $100 billion before the end of this decade (Harris et al. 2008). Despite the bright future, the efficiency and cost of biopharmaceutical is continuously increasing due to the changes in drug development, biomanufacturing and commercialization. For biotherapeutics development, the R&D cycle is prolonged and the cost is increased. The advantages of biotherapeutics over chemical drugs are the shorter R&D cycle, lower cost and higher success rate. These advantages are mainly associated with the first generation biopharmaceuticals due to the established efficacy of their natural form counterparts. However, second generation biopharmaceuticals consist of new, unproven therapeutics require longer development cycle and higher cost with lower success rate. The development time for biotherapeutics has increased to 7-8 years on average, very close to that of chemical drugs (Outlook 2005). Furthermore, the success rate of biotherapeutics has declined to around 30%, getting closer to 15-20% for new chemical entities.

The emergence of biogenerics or biosimilars is putting further economic pressure on biomanufacturing. Some big biopharm companies in North America and Europe have joined in this area to manufacture and commercialize such drugs and are getting legislation support such as FDA's abbreviated application process (Hess 2009). This phenomenon is accompanied by the patent expiration of many first generation biotech drugs. Furthermore, the biopharm market experiences the growing need of governments

and society to reduce the healthcare cost. Consequently biopharm manufacturers are being pushed to decrease development and manufacturing costs while maintaining the quality of biopharmaceuticals. The economic pressure on biomanufacturing is clearly demonstrated by the US government calling for proposals on "advanced manufacturing of pharmaceuticals" (Defense Advanced Research Projects Agency). Such a system requires producing three million doses of antibody or vaccine against a previously unknown agent within 12 weeks with the cost of goods (COG) of < $1/dose for a vaccine or < $10/dose for a mAb. This poses huge challenges to biomanufacturing technologies since at current optimal conditions, product development from cell line to pilot production takes 18-24 months with COG of $100-300/dose (Hagel et al. 2008).

1.1.3 Bioseparation - the major cost in bioprocessing

Production scale could significantly affect process economy. The production scale greatly increases from the first generation to the second generation of biopharmaceuticals. First generation protein drugs, mainly recombinant cell factors used at dosage level ranging from μg to mg require annual production of hundreds of grams to tens of kilograms with sales per gram of 0.5-10 MUSD (Million US dollars). Second generation protein drugs, mainly mAbs are used in the dosage range of mg to gram, requiring production quantity of hundreds of kilograms to several tons with sales per gram of 2-50 KUSD (Kilo US dollars) (Hagel et al. 2008). On one hand, the sharp increase of production capacity accompanied by the decrease of unit price poses the need of cost-effective technologies in both upstream processing (USP, or bioreaction) and downstream processing (DSP, or bioseparation). On the other hand, with the scale up of production

capacity, the ratio of DSP cost over USP cost is increased, thus bioseparation is becoming the major component of COG per gram (Farid 2009). In the bioprocess scale up, the growth ratio of bioreactors investment is much lower than the scale up factor of process capacity. Furthermore, the production bioreactors used in small scale could be directly reused as the seed bioreactors at large scale. Conversely, the bioseparation scale up is limited by the dimensions of equipments, e.g. the maximum 2 m of column diameter and thus investment of repetitive columns and/or multiple cycles of column runs are needed.

Significant progresses in molecular biology and cell culture technologies greatly promote the USP development. The expression systems have expanded from predominantly E.Coli to yeast, mammalian cell, transgenic animal and transgenic plant. These systems can accommodate the expression of a wide range of proteins with various biochemical properties and expression requirements like posttranslational modifications. The expression levels for these systems have been significantly improved. MAb expression by CHO (Chinese Hamster Ovary) cell culture has greatly increased from 0.02-0.05 g mAb/L cell culture in 1985 to 0.5-2.0 g/L in 1995 and more recently 3.0-5.0 g/L in 2005 (Li et al. 2005; Farid 2009). Further optimization of cell culture may reach 10-15 g/L in the next decade (Werner 2005; Birch and Racher 2006).

Compared with the development of upstream technology, great progress is not seen in bioseparation, especially at industrial scale. To match the upstream advances in order to reduce COG of biopharmaceutical, bioseparation needs substantial improvement in productivity and cost. For mAb production, the high titers in mammalian cell culture increase protein load and thus require higher process capacity and material cost in

bioseparation. Accordingly, the DSP/USP cost ratio increases with the titer growth. For example, increase of fermentation titer from 0.1 to 1 g/L changed the DSP/USP cost ratio from 55:45 to 30:70 (Sommerfeld and Strube 2005). Furthermore, the combination of production scale increase with the growth of titers enhanced the DSP/USP cost ratio in COG per gram. Consequently, in current biopharmaceutical industry, bioseparation becomes the major contributor to COG per gram and requires significant improvement in productivity and cost reduction.

1.2 Emerging bioseparation technologies

Various bioseparation technologies have been used for downstream processing of biopharmaceuticals. Currently some traditional technologies such as precipitation and crystallization arouse the interests for reexamination due to their large processing capacity but their improvements in resolving power and expanded use in biopharmaceutical are still a long way to go (Thommes and Etzel 2007). Therefore, both column chromatography and membrane technology are still the workhorses in bioseparation. Due to its high resolution advantage, column chromatography is widely used for protein capture, intermediate purification and polishing. However, its diffusion based mass transport and soft gel limit the flow rate, product throughput and scale up. Many efforts have focused on its improvement but real successes are rarely found. Different operation modes of column chromatography have been demonstrated but are not widely used in bioprocessing due to their complexities. For example, displacement chromatography could achieve high load but require the development and introduction of

displacers which then need further removal and related quality control (Zhao and Sun 2007). Simulated moving bed could continuously separate a simple two-component mixture but requires significant modification of chromatography system and very complex operation (Paredes et al. 2005). The gel development using new matrix or ligands focuses on improving binding capacity, flow rate and stability. For example, rigid resins are developed to improve the flow rate of compressible agarose gel (Sofer and Chirica 2006). However, their use could cause large buffer volume, high back pressure, packing complications and shear stress. Despite the improvements, the fundamental problem of column chromatography has not yet been solved, i.e. the diffusion based mass transport in porous gel particles. The dead channel diffusion in gel not only limits the flow rate to maintain binding capacity, but also broadens the peaks and weakens the resolution.

The intraparticle diffusion limitation has been reduced in perfusion chromatography. The POROS perfusible particles feature the bimodal pore network in which the 0.6-0.8 μm through-pores are interconnected by 0.08-0.15 μm diffusive pores (Regnier 1991; Fulton et al. 1992). The convective through-pores reduce the intraparticle diffusion path so high flow rate could be achieved without sacrificing resolution. However, the increase of porousity in particles reduces the surface area and binding capacity. Monolithic column also improved the mass transport in chromatography by achieving the bimodal pore network composed of macropores and mesopores (Svec and Frechet 1996). Different from particle based column, monolith represents a single piece continuous bed made of porous polymer or silica. It further avoids the interstitial volume

between particles and may reduce turbulen mixing. However, the in situ polymerization to make monolith has the limitation in column scale up and batch reproducibility. Similarly to perfusion chromatography, the large pores also reduce the binding capacity (Zou et al. 2002).

Membrane technologies including microfiltration, ultrafiltration and nanofiltration have been widely used for cell recovery, buffer exchange, protein concentration, and microorganism / virus removal in biopharmaceutical industry. The main advantages of membrane technology are high throughput and large processing capacity but with low resolving power. New improvements in membrane technology are very promising to develop both high throughput and high resolution advantages. Two of the new membrane technologies are membrane chromatography and high resolution tangential flow filtration (HPTFF). Traditional TFF based on size difference can only separate proteins with more than 10 folds of molecular weight difference. HPTFF with charged membranes exploits both size and charge difference of proteins to achieve high resolving power. Using HPTFF, proteins with low size difference or even similar in size could be separated based on charge difference (Zydney and van Reis 2001; van Reis and Zydney 2007). However, HPTFF performance relies on the co-current filtrate recirculation for constant transmembrane pressure and a precise filtrate pump controlling transmembrane flux, which is not compatible with industrial TFF system. The solution for operation without co-current filtrate loop is being explored, including membrane with lower permeability (Fontes and van Reis 2009).

1.3 Membrane chromatography - a promising bioseparation technology

1.3.1 Basic concept of membrane chromatography

Membrane chromatography is an integrated technology of column chromatography and membrane technology. It utilizes functionalized microporous membrane as membrane adsorbers for bioseparation, combining the advantages of both high resolution and high throughput. Membrane chromatography can utilize the same chemistries and operational modes as column chromatography and thus achieve high selectivity and resolving power. The advantages of membrane chromatography over column chromatography are based on the predominant convective mass transport in membrane adsorber. As shown in Figure 1.1, the main limitation of the gel particle is the diffusion in dead channels. Most of functional groups are inside these channels due to their large surface area. Therefore, in column chromatography, large biomolecules need to diffuse in these channels for binding and diffuse out for elution. On one hand, this slow diffusion significantly limits the flow rate to keep the good binding and recovery of biomolecules. On the other hand, the diffusion could broaden the peaks and lower the resolution. Conversely, dead channel diffusion does not happen in membrane adsorber. Although film diffusion exists on the surface for both membrane adsorber and gel particle, it does not affect mass transport significantly since the film is thin. Therefore, the predominant mass transport in membrane adsorber is convective flow through the open channel (Thommes and Kula 1995). This convective mass transport guarantees the high

throughput and productivity in membrane chromatography since the flow rate does not affect the binding capacity in a wide range (Ghosh 2002). In addition, dominant convection could reduce diffusion caused peak broadening and thus sharp peak and high resolution could be achieved even using a very thin membrane bed (Haber et al. 2004). The thin bed of membrane adsorbers combined with convective mass transport further guarantees high throughput, low pressure drop and easy scale up. Except for the merits of high throughput, high resolution and easy scale up, membrane chromatography possesses the power to integrate multi-unit operations in bioprocessing. Some potential applications include integrated purification / microorganism removal using 0.1 or 0.22 μm membrane adsorber, and integrated clarification / protein capture using membrane module like Sartobind Direct. Furthermore, membrane chromatography is easily accepted by industry since it uses the same equipments and operation modes as column chromatography, and, therefore, reduces new equipment investment and technical training.

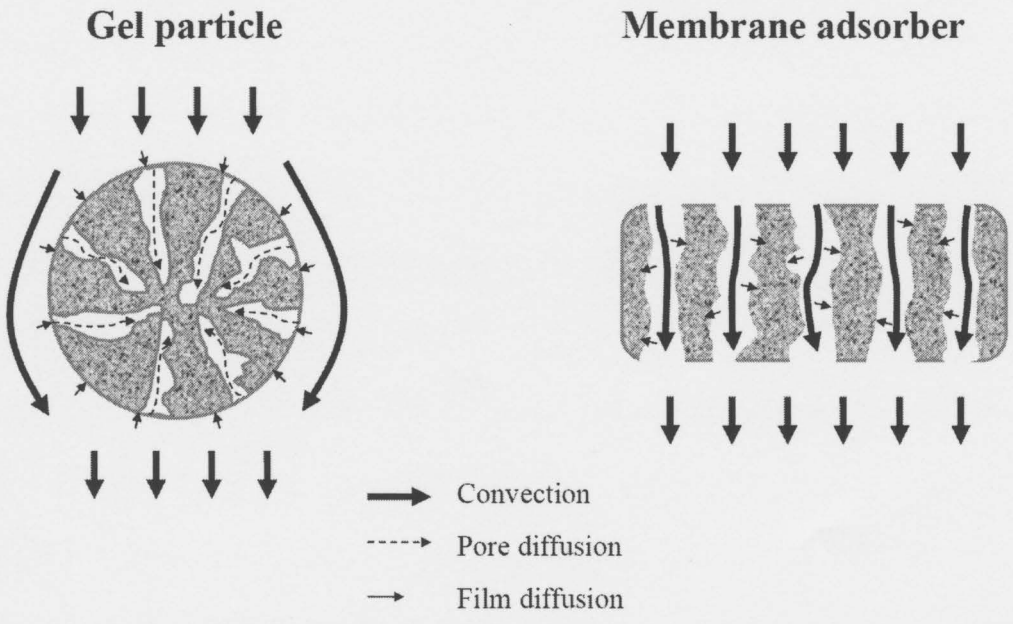


Figure 1.1 Mass transport in gel particle and membrane adsorber.

1.3.2 Membranes and modules for membrane chromatography

Synthetic porous membranes are widely used for membrane chromatography. Membrane materials generally could be either organic or inorganic. Membrane adsorbers are normally made from organic polymers such as regenerated cellulose, cellulose acetate, polysulfone, polyethersulfone (PES), polyvinylidene fluoride (PVDF) and polyamide due to their easy modification and functionalization with various ligands. Porous membranes with pore size of 0.1, 0.2, 0.45, 0.8 μm and > 3μm are normally used as base matrix for membrane adsorbers. The pore structure in membrane provides convective flow with negligible diffusion limitation. Sartobind membrane adsorbers from Sartorius use 0.45 or > 3μm stabilized reinforced cellulose membrane as chromatography matrix. Mustang ion exchange membranes from Pall are made from 0.8 μm PES membrane. PVDF membranes with 0.1 and 0.2 μm pore size were developed as hydrophobic interaction membrane (Ghosh 2001 and 2005). Since 0.1 and 0.2 μm membranes are originally used for virus and bacteria filtration, they are manufactured under very strict quality control and thus have excellent pore size uniformity, one of important properties of membrane adsorbers.

Two types of membrane module are widely used for membrane chromatography: flat sheet membrane module and radial flow membrane cartridge. Flat sheet membranes are normally cut into discs and stacks of discs with height in several millimeters are held in the membrane module. In addition to increasing membrane volume, using stacks of membrane could reduce membrane unevenness and increase binding capacity, which will be further discussed in 1.3.5. Radial flow membranes are prepared by spirally winding

flat sheet membrane around a porous cylindrical core where the flow is pumped into the cartridge and then distributed into the membrane in a radially outward direction. Such kind of cartridge holding 5 L membrane adsorber with bed height less than 1 cm has been commercialized by Sartorius and Pall. Larger membrane volume for industrial bioseparation can be achieved by connecting a number of cartridges in parallel and/or in series. While flat sheet membrane modules are mostly used in laboratory, radial flow membrane cartridges are claimed to be suitable for industrial applications (Ghosh 2002). Although different in flow mode, both kinds of membrane module use very thin membrane bed for bioseparation, providing high flow rate with low pressure drop. In the example of protein polishing, an anion-exchange membrane with 4 mm height was used at flow rates of more than 600 cm/h, while a column with 15 cm height had to be used to achieve the same function (Gottschalk, 2008). The thin membrane bed combined with convective flow could considerably reduce buffer consumption and shorten the process time.

1.3.3 Chemistries of membrane chromatography

Various chemistries have been utilized for membrane chromatography, including affinity membrane chromatography (AMC), ion exchange membrane chromatography (IEMC), hydrophobic interaction membrane chromatography (HIMC) and reversed phase membrane chromatography. For example, Protein A based AMC has been used for purification of human immunoglobulin (hIgG) (Zou et al. 2001; Castilho et al. 2002). IEMC has been reported for monoclonal antibody purification (Knudsen et al. 2001; Philips et al. 2004). HIMC using hydrophilized synthetic membrane has successfully

purified mAbs from serum and CHO cell culture (Ghosh 2001 and 2005; Ghosh and Wang, 2006). The HIMC adsorbers used in these reports are not traditionally functionalized for membrane chromatography. Rather, they are hydrophobic membrane (like PVDF) modified with hydrophilic polymers and thus suitable for microfiltration with less protein binding. Based on salt conditions, HIMC membranes possess tunable hydrophobicity and thus the property of reversible protein binding, i.e. binding protein at high salt and elution at low salt. Due to its mild hydrophobicity, high recovery of proteins could be observed with competitive binding capacity. HIMC is especially suitable for antibody purification since antibody is normally more hydrophobic than the proteins in its environments such as serum albumin. Commercial membrane adsorbers with various chemistries have been developed by membrane companies, such as Mustang ion exchange membranes by Pall Life Science, Sartobind Protein A/G, metal chelate and ion exchange membrane by Sartorius. Sartorius recently commercialized HIMC membrane called Sartobind Phenyl, which showed comparable binding capacity to Sepharose Phenyl but can be used at much higher flow rate (Fraud et al. 2008).

1.3.4 Operation modes of membrane chromatography

Both flow-through mode and bind-elute mode have been developed for membrane chromatography. Flow-through mode membrane chromatography has been utilized for trace impurities removal from monoclonal antibody (Knudsen et al. 2001; Philips et al. 2004). Most of these studies were done by industrial researchers and this technology has been used for commercial production of CAMPATH-1H (Gallher and Fowler 2001). In mAb production, most trace impurities including host cell DNA, virus, endotoxin and

many host cell proteins are negatively charged at neutral pH while mAbs are normally positively charged. This provides the basis to use anion-exchange chromatography for binding trace impurities while allowing mAb product to flow through. However, column chromatography needs a large diameter and a minimum bed height to overcome the limitations in volumetric flow rate and flow distribution, thus making the column oversized (Knudsen et al. 2001). Membrane chromatography is a better option for trace impurities removal from antibody since the convective flow provides high flow rate without affecting binding capacity. Flow distribution is not a limitation for industrial membrane cartridge with axial flow mode. Furthermore, the binding capacity of large biomolecules like DNA and virus is higher in membrane chromatography than in column chromatography (Teeters et al. 2003; Etzel 2009). Finally, the disposable use of membrane cartridge could save plenty of cost in column packing, cleaning, storage and validation of these activities. Zhou and Tressel (2006) from Amgen analyzed the cost of flow-through mode chromatography for trace impurity removal. The results showed 5 log removal of the model virus with antibody process capacity of 10.5 Kg/L Q membrane adsorber. Ten-year operation cost is lower on membrane chromatography even if the gel is reused for 100 cycles.

Bind-elute mode membrane chromatography is currently developed at laboratory and pilot scale. This mode should be the mainstream application of membrane chromatography. However, it has not been widely used due to the relatively lower binding capacity which will be specially discussed in the next section. By binding the target protein on the membrane adsorber followed by desorption using one step or

gradient elution, the resolving power of membrane chromatography could be fully exploited. Affinity membrane chromatography has been widely developed. A comprehensive review introduced membrane material and coupling of various affinity ligands on membrane (Zou et al. 2001). The most widely used affinity membranes include Protein A/G or synthetic ligands on membrane for antibody purification, immuno-affinity membrane for antibody or antigen purification and metal affinity for various proteins (Castilho et al. 2002; Denizli 2002; Ruckenstein and Guo 2001). AMC in bind-elute mode showed both high selectivity and high throughput. Plasmid DNA or virus for gene therapy or vaccine could be purified using IEMC in bind-elute mode. For example, separation efficiencies of Mustang Q for 6.1 kilo base pair plasmid was reported to be 200 plates / cm, over ten folds higher than that of the 15 μm gel particles (Teeters et al. 2003). A two step IEMC process for plasma protein fractionation was established and scaled up to pilot scale, yielding higher productivity than Sepharose based process as well as excellent reproducibility and stability (Gebauer et al. 1997). Except for affinity and ion exchange, HIMC was widely used in bind-elute mode, especially for high resolution antibody purification from serum and CHO cell culture (Ghosh 2001 and 2005; Ghosh and Wang 2006).

1.3.5 Binding capacity of membrane adsorber

Despite the high-throughput and high-resolution advantage, membrane chromatography was criticized for its low binding capacity. However, this disadvantage need be carefully examined. For large biomolecules like DNA, virus and endotoxin, membrane chromatography actually demonstrated much higher binding capacity than

column chromatography. When anion-exchange membrane chromatography was used for plasmid DNA purification, the binding capacity was reported to be 10 mg/ml, an order of magnitude higher than typical values of porous beads (Teeters et al. 2003). In another similar study, dynamic binding capacity was 20-25 times higher and flow rate was 55-550 higher than the values for beads (Endres et al. 2003). In gel beads, macrobiomolecule meets difficulty in diffusing into dead channels so they mostly bind on external surface of beads while convective flow in membrane adsorber could directly transport macrobiomolecule to pore surfaces. Furthermore, the specific surface area of membranes is generally smaller than that of gel particles but much larger than that of external gel surface. For example, a typical microporous membrane with 0.65 μm pore size, 0.7 void fraction and 140 μm thickness has internal pore surface of 1.1 m^2/mL while a column packed with 90 μm beads has about 0.11 m^2/mL external surface (Soltys and Etzel 2000; Etzel 2009). Therefore, the binding capacity of macrobiomolecules like plasmid DNA and virus particle is undoubtedly larger in membrane chromatography than column chromatography.

The binding capacity of small proteins in membrane chromatography is relatively lower than in column chromatography because the small proteins could diffuse into the pore structure of beads. For example, a chitosan anion-exchange membrane was reported to bind respectively 11.6, 19 and 20.8 mg/ml membrane for human serum albumin, ovalbumin and soybean trypsin inhibitor (Zeng and Ruckenstein 1998). The binding capacity of small proteins like lysozyme and serum albumin was 22-29 mg/ml with commercial Sartobind ion exchange membranes (supplier data from Sartorius). However,

for large proteins beyond 100 kDa, size exclusion effect exists in gel particles with 30-50 nm pore size (Karlsson et al. 1989). For example, thyroglobulin, a 660 kDa protein, had 18 mg/ml binding capacity in Sartobind Q while only 3.5 mg/ml in Q gel (Sartorius-Stedim Biotech). The proteins studied in this research, mainly antibodies (>150 kDa) and PEGylated proteins, would have reasonable binding capacity in membrane chromatography due to their relatively large size. For antibody binding in HIMC, the 10% breakthrough binding capacity could reach 35.2 and 42.8 mg antibody/mL respectively for 0.22 μm and 0.1 μm PVDF membranes (Ghosh 2005; Ghosh and Wang 2006). PEGylated proteins were much larger in size than proteins with the same molecular weight due to the high viscosity radius of PEG (Fee and van Alstine 2004). Therefore, they are also suitable for purification using membrane chromatography.

Many other factors also play vital roles in binding capacity, including flow distribution, the uniformity of pore size and membrane thickness, and membrane surface modification. Improvements of these factors have been achieved to enhance protein binding in membrane adsorbers. The flow distribution concern actually exists for both membrane and column chromatography. A new design of stacked membrane module was reported by Ghosh and Wong (2006). The improved flow distribution increased lysozyme binding capacity at 10% breakthrough from 28.9 to 45.2 mg/ml with cation-exchange PVDF membrane. For large scale module using radial flow through spinally wound membrane, flow distribution has much less effect. Multi-layer membrane adsorber could effectively reduce the adverse effect of uneven pore size and membrane thickness and thus improve binding capacity. The antibody breakthrough binding capacity of a cation-

exchange membrane was found to be independent of flow rate but increased sharply with layer numbers, from 7 mg/ml with one layer to 30 mg/ml with 15 layers and 40 mg/ml with 60 layers (Knudsen et al. 2001). More recently, the new progress in membrane surface modification has significantly improved binding capacity of membrane adsorbers. New metal affinity membranes were prepared by growing polymer brush on porous substrate followed by Cu^{2+} or Ni^{2+} functionalization (Sun et al. 2006; Jain et al. 2007). The new membrane reached high binding capacity of 150 mg bovine serum albumin (BSA)/ml membrane or 120 mg polyhistidine-tagged ubiquitin/ml membrane. A gel-filled Q type microporous membrane was found to bind 290-300 mg human serum albumin (HSA)/ml at saturating conditions, apparently higher than traditional gel medium (Kanani et al. 2007). Three-dimensional polymer layer was grafted on hydrophilized polypropylene membrane and this anion-exchange membrane adsorber could bind 80 mg/ml for BSA and 120 mg/ml for trypsin inhibitor (He and Ulbricht 2008). Surface-initiated atom transfer radical polymerization was used to grow the cation-exchanger poly (acrylic acid) on regenerated cellulose membrane. Lysozyme binding increased with modification times and reached 98.5 mg/ml for static binding capacity and 71.2 mg/ml for dynamic binding capacity (Singh et al. 2008). This method was further improved by first functionalizing initiator on membrane followed by polymerization of 2-dimethylaminoethyl methacrylate, preparing membrane adsorber with 130 mg BSA/ml at high linear flow rate (>350cm/h) with low pressure drop (<3 bar) (Bhut and Husson 2009). These researches significantly improved the binding capacity of membrane adsorbers which are close or even higher than those of new generation gel medium, e.g.

120 mg HSA /ml of new Q resin from GE Healthcare Life Sciences. Although these membrane preparation technologies are currently in laboratory research, further development will greatly promote the applications of membrane chromatography in biopharmaceutical industry.

1.4 Bioprocessing of antibodies and PEGylated proteins

1.4.1 Bioprocessing of antibodies and antibody fragments

Antibodies or immunoglobulins are host proteins produced in response to foreign molecules or organisms in the body. They are important immunochemical tools, diagnostic reagents and therapeutic protein drugs. All immunoglobulins have the common H2L2 structure composed of four polypeptide chains: two identical, glycosylated heavy (H) chains and two identical, non-glycosylated light (L) chains (Figure 1.2). Two heavy chains are joined together by disulfide bonds in a flexible region called the hinge. The light chain is also joined with the heavy chain by a disulfide bond. According to the H chain components, immunoglobulins are divided into five major classes, IgG, IgM, IgA, IgD and IgE, the most abundant of which is IgG. IgG is further divided into four subclasses: IgG1, IgG2, IgG3 and IgG4, which have minor differences in amino acid sequence. IgG could be enzymically cleaved at the hinge region into two Fab fragment and one Fc fragment by papain or into one $F(ab')_2$ fragment and some sub-Fc peptides by pepsin (Figure 1.2). Antibody fragments including Fab and $F(ab')_2$ have created potential for new applications. In diagnostic tests, using Fab or $F(ab')_2$ to detect cell surface antigen could avoid the interference caused by Fc receptor on cells (Andrew and Titus

1997). In the context of antibody based cancer therapy, the Fab fragment, due to its smaller size is thought to have higher tumor uptake ratio, faster blood clearance and reduced immunogenicity when compared with intact IgG (Covell et al. 1986; Cortez-Retamozo et al. 2004).

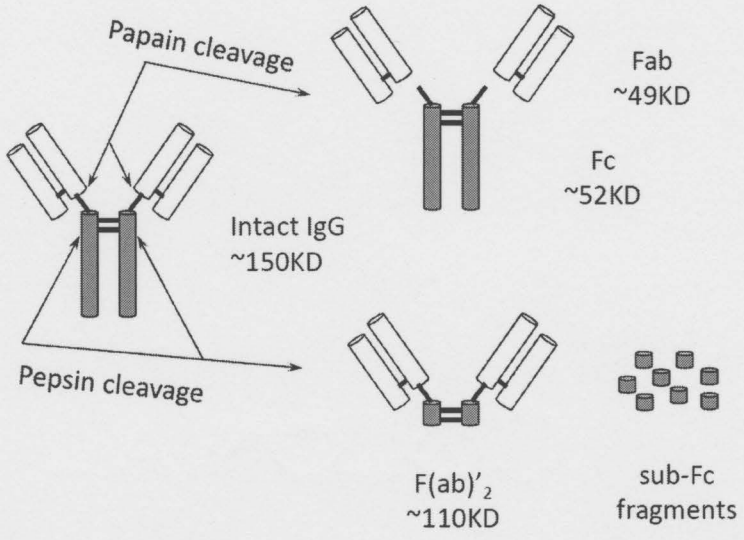


Figure 1.2 Antibody structure and antibody fragmentation.

Polyclonal antibodies are a group of antibodies that recognize different epitopes on an antigen. They could be purified from animal serum after antigen immunization and normally used as immunochemical reagents. Monoclonal antibodies are highly specific antibodies originally produced from hybridoma cells. Currently humanized monoclonal antibodies are a very successful class of therapeutic drugs. The typical bioprocess includes expression by CHO cell culture followed by purification using multi-steps of column chromatography and membrane technology. CHO cell culture is widely used for mAb production due to the correct glycosylation and high expression level. However, the high mAb titres up to 10 g/L and the large production scales of 50-100 Kg/batch make column chromatography based downstream process the manufacturing bottleneck due to

the low throughput, high column pressure and column size limit (Thommes and Etzel 2007; Gottschalk 2008). Alternative bioseparation technologies such as membrane chromatography are promising solutions.

New mAb expression systems are emerging, such as transgenic animals and transgenic plants (Houdebine 2009; Hiatt et al. 1989; McLean et al. 2007). Transgenic plants are not known to carry any human and animal pathogen and therefore safer than CHO cell culture and transgenic animals. Transgenic tobacco is even safer than the crop plants since it is not present in the food chain for human and animals. It could be easily scaled up by field plantation. However, this promising expression system proposes the big challenge to downstream processing due to the low mAb expression (Schillberg et al. 2003), which may be addressed by the high-resolution high-throughput membrane chromatography.

The bioprocessing of antibody fragments is different from that of mAb. Antibody fragments could be produced either by enzymatic fragmentation or by recombinant expression. Fab and $F(ab')_2$ fragments do not need glycosylation and thus could be expressed by simple E.Coli system at large scale. However, to produce antibody fragments for diagnosis and antitoxin applications, the approach of antibody fragmentation has to be used since plenty of different antibody fragments are needed for specific interaction with various antigens or toxins (Burnouf et al. 2004; Leon et al. 2007). This approach traditionally includes three unit operations: IgG purification from serum of immunized animals or from hybridoma cell culture, homogeneous liquid phase reaction, followed by multi-step purification to remove the enzyme and byproducts

(Karlson-Stiber et al. 1997; Jones and Landon 2002; Coleman and Mahler 2003; Ljunglöf et al. 2007). These complex processes are time-consuming and expensive with low overall productivity. Some innovative methods are needed for the process improvements. Membrane chromatography has the potential to integrate the reaction and separation and thus achieve process intensification.

1.4.2 Bioprocessing of PEGylated proteins

PEGylated protein drugs are considered as one important class in second generation biopharmaceutical (Walsh 2004). As illustrated in Figure 1.3, protein PEGylation could covalently conjugate protein drugs with PEG to improve serum half life and decrease immunogenicity and enzymatic degradation (Roberts et al. 2002). These enhancements of clinical efficacies are primarily based on the high hydrodynamic radius and non-immunogenicity of PEG.

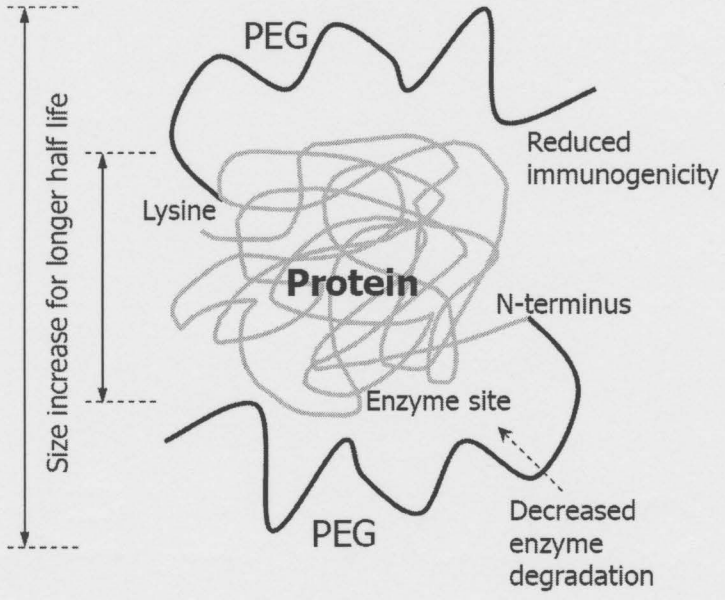


Figure 1.3 Cartoon image of a PEGylated protein showing two PEG molecules conjugated at N-terminus and a lysine residue of the protein and the enhanced clinical efficacies by PEGylation.

Bioprocessing of PEGylated proteins include the PEGylation reaction conjugating activated PEG to protein and the purification process to separate target PEG-protein from unreacted protein, PEG and unwanted PEG-protein forms. Various chemistry reactions have been developed for PEGylation. PEG conjugation at lysine amino group leads to heterogeneous product due to the prevalence of lysine residues, which makes purification and quality control difficult (Roberts et al. 2002). N-terminus protein PEGylation provides largely selective conjugation of PEG at N-terminus α-amino group and could apply to most proteins (Wong 1991; Hermanson 1996). Special approaches conjugating PEG with scarce groups such as thiol, amide and hydroxyl group are largely site-specific but only applicable to specific proteins (Abuchowski et al. 1977; Basu et al. 2006). Site-specific PEGylation with disulfide bonds is an interesting method for protein having paired disulfide but without free cysteine (Shaunak et al. 2006). This approach limits the application to few proteins and needs multistep complex reactions.

Column chromatography methods including size exclusion and ion exchange are currently used for purification of PEGylated proteins. However, the processing capacity of size exclusion chromatography is limited by the small feed volume and low flow rate. For ion exchange column chromatography, the dead channel diffusion in gel beads significantly limits the flow rate and the binding capacity of PEGylated proteins since PEG provides high hydrodynamic radius (Fee and van Alstine 2004 and 2006). Membrane chromatography is a promising alternative for purification of PEGylated proteins due to the large pore size and convective mass transport in membrane adsorbers.

1.5 References

Abuchowski A, van Es T, Palczuk NC, Davis FF. 1977. Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J Biol Chem 252:3578-3581.

Andrews BA, Nielsen S, Asenjo JA. 1996. Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. Bioseparation 6:303-313.

Basu A, Yang K, Wang M, Liu S, Chintala R, Palm T, Zhao H, Peng P, Wu D, Zhang Z, Hua J, Hsieh MC, Zhou J, Petti G, Li X, Janjua A, Mendez M, Liu J, Longley C, Zhang Z, Mehlig M, Borowski V, Viswanathan M, Filpula D. 2006. Structure-function engineering of interferon-a1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. Bioconjugate Chem 17:618-630.

Bhut BV, Husson SM. 2009. Dramatic performance improvement of weak anion-exchange membranes for chromatographic bioseparations. J Membr Sci 337:215-223.

Birch JR, Racher AJ. 2006. Antibody production. Adv Drug Deliv Rev 58:671-685.

Burnouf T, Griffiths E, Padilla A, Seddik S, Stephano MA, Gutiérrez JM. 2004. Assessment of the viral safety of antivenoms fractionated from equine plasma. Biologicals 32:115-128.

Castilho LR, Anspach FB, Deckwer WD. 2002. Comparison of affinity membranes for the purification of immunoglobulins. J Membr Sci 207:253-264.

Chapman AP, Antoniw P, Spitali M, West S, Stephens S, King DJ. 1999. Therapeutic antibody fragments with prolonged in vivo half-lives. Nat Biotechnol 17:780-783.

Coleman L, Mahler SM. 2003. Purification of Fab fragments from a monoclonal antibody papain digest by Gradiflow electrophoresis. Protein Expr Purif 32:246-251.

Cortez-Retamozo V, Backmann N, Senter PD, Wernery U, Baetselier PD, Muyldermans S, Revets H. 2004. Efficient Cancer Therapy with a Nanobody-Based Conjugate. Cancer Res 64:2853–2857.

Covell DG, Barbet J, Holton OD, Black CD, Parker RJ, Weinstein JN. 1986. Pharmacokinetics of monoclonal immunoglobulin G1, $F(ab')_2$, and Fab' in mice. Cancer Res 46:3969–3978.

Defense Advanced Research Projects Agency (DARPA). BAA 06-31, accelerated manufacturing of pharmaceuticals (AMP). US Government Federal Business Opportunities. http://www.fbo.gov/spg/ODA/DARPA/CMO/BAA06-31/Attachments.html (accessed July 7, 2009).

Denizli A. 2002. Preparation of immuno-affinity membranes for cholesterol removal from human plasma. J Chromatogr B 772:357-367.

Endres HN, Johnson JA, Ross CA, Welp JK, Etzel MR. 2003. Evaluation of an ion-exchange membrane for the purification of plasmid DNA. Biotechnol Appl Biochem 37:259-266.

Etzel MR. 2009. Charged ultrafiltration and microfiltration membranes in antibody purification. In Gottschalk U, editor. Process scale purification of antibodies. Hoboken: John Wiley & Sons Inc. p 325-348.

Farid SS. 2009. Process economic drivers in industrial monoclonal antibody manufacture. In: Gottschalk U, editor. Process scale purification of antibodies. Hoboken: John Wiley & Sons Inc. p 239-262.

Fee CJ, van Alstine JM. 2004. Prediction of the viscosity radius and the size exclusion chromatography behavior of PEGylated proteins. Bioconjugate Chem 15:1304-1313.

Fee CJ, van Alstine JM. 2006. PEG-proteins: Reaction engineering and separation issues. Chem Eng Sci 61:924-939.

Fontes N, van Reis R. 2009. Advances in technology and process development for industrial scale monoclonal antibody purification. In Gottschalk U, editor. Process scale purification of antibodies. Hoboken: John Wiley & Sons Inc. p 203-221.

Fraud N, Faber R, Kiss C. 2008. Novel Membrane Adsorber for Hydrophobic Interaction Chromatography. Presentation at 236th ACS National Meeting, August 17-21, in Philadelphia, USA.

Fulton SP, Shahidi AJ, Gordon NF, Afeyan NB. 1992. Large-scale processing and high-throughput perfusion chromatography. Bio/Technology 10:635-639.

Gallher P, Fowler E. 2001. Validation of impurity removal by the CAMPATH-1H biomanufacturing process. Presentation at IBC's Biopharmaceutical Production Week, November 12-15, in San Diego, USA.

Gebauer KH, Thommes J, Kula MR. 1997. Plasma protein fractionation with advanced membrane adsorbents. Biotech Bioeng 54:181-189.

Ghosh R, Wang L. 2006. Purification of monoclonal antibody by hydrophobic interaction membrane chromatography. J Chromatogr A 1107:104-109.

Ghosh R, Wong T. 2006. Effect of module design on the efficiency of membrane chromatographic separation processes. J Membr Sci 281:532-540.

Ghosh R. 2001. Separation of proteins using hydrophobic interaction membrane chromatography. J Chromatogr A 923:59-64.

Ghosh R. 2002. Protein separation using membrane chromatography: opportunities and challenges. J Chromatogr A 952:13-27.

Ghosh R. 2005. Fractionation of human plasma proteins by hydrophobic interaction membrane chromatography. J Membr Sci 260:112-118.

Gottschalk U. 2008. Bioseparation in Antibody Manufacturing: The Good, The Bad and The Ugly. Biotechnol Prog 24:496-503.

Haber G, Skupsky J, Lee A, Lander R. 2004. Membrane Chromatography of DNA: Conformation-Induced Capacity and Selectivity. Biotech Bioeng 88:26-34.

Hagel L, Jagschies G, Sofer G. 2008. Handbook of process chromatography: Development, manufacturing, validation and economics. San Diego: Academic Press. 27 p.

Harris M, Lyle A, Kite-Powell K. 2008. The top 25 biotech drugs in the future of biotech: The 2010 guide to emerging markets and technology. Atlanta: Bioworld Today. p 68-73.

He D, Ulbricht M. 2008. Preparation and characterization of porous anion-exchange membrane adsorbers with high protein-binding capacity. J Membr Sci 315:155-163.

Hermanson GT. 1996. Bioconjugation Techniques. San Diego: Academic Press. 141 p.

Hess G. Biosimilars debate. 2009. Chemical and Engineering News. April 6: 23-25.

Hiatt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants. 1989. Nature 342:76-78.

Houdebine LM. 2009. Production of pharmaceutical proteins by transgenic animals. Comp Immunol Microbiol Infect Dis 32:107-121.

Jain P, Sun L, Dai J, Baker GL, Bruening ML. 2007. High-capacity purification of his-tagged proteins by affinity membranes containing functionalized polymer brushes. Biomacromolecules 8:3102-3107.

Jones RGA, Landon L. 2002. Enhanced pepsin digestion: a novel process for purifying antibody $F(ab')_2$ fragments in high yield from serum. J Immunol Methods 263:57-74.

Kanani DM, Komkov E, Wong T, Mika A, Childs RF, Ghosh R. 2007. Separation of human plasma proteins HSA and hIgG using high-capacity macroporous gel-filled membranes. Biochem Eng J 35:295-300.

Karlson-Stiber C, Persson H, Heath A, Smith D, al-Abdulla IH, Sjostrom L. 1997. First clinical experiences with specific sheep Fab fragments in snake bite. Report of a multicentre study of Vipera berus envenoming. J Intern Med 241:53-58.

Karlsson E, Rydén L, Brewer J. 1989. In Janson JC, Rydén L, editor. Protein purification: Principles, high resolution methods and applications. Weinheim: VCH. 123 p.

Knudsen HL, Fahrner RL, Xu Y, Norling LA, Blank GS. 2001. Membrane ion-exchange chromatography for process-scale antibody purification. J Chromatogr A 907:145-154.

Leon G, Monge M, Rojas E, Lomonte B, Gutierrez JM. 2001. Comparison between IgG and $F(ab')_2$ polyvalent antivenoms: neutralization of systemic effects induced by

Bothrops asper venom in mice, extravasation to muscle tissue, and potential for induction of adverse reactions. Toxicon 39:793-801.

Li F, Zhou X, Yang X, Tressel T, Lee B. 2005. Current therapeutic antibody production and process optimization. Bioprocessing Journal 4:23-30.

Ljunglöf A, Lacki KM, Mueller J, Harinarayan C, van Reis R, Fahrner R, Van Alstine JM. 2007. Ion exchange chromatography of antibody fragments. Biotechnol Bioeng 96:515-524.

McLean MD, Almquist KC, Niu Y, Kimmel R, Lai Z, Schreiber JR, Hall JC. 2007. A human anti-pseudomonas aeruginosa O6ad IgG1 expressed in transgenic tobacco is capable of recruiting immune system effector function in vitro. Antimicrob Agents Chemother 51:3322-3328.

Outlook 2005. Tufts Center for the Study of Drug Development, January 2005.

Paredes G, Stadler J, Makart S, Morbidelli M, Mazzott M. 2005. SMB operation for three - fraction separations: Purification of plasmid DNA. Adsorption 11:841- 845.

Phillips M, Cormier J, Ferrence J, Dowd C, Kiss R, Lutz H, Carter J. 2004. Performance of a membrane adsorber for trace impurity removal in biotechnology manufacturing, J Chromatogr A 1078:74-82.

Regnier FE. 1991. Perfusion chromatography. Nature 350:634-635.

Roberts MJ, Bentley MD, Harris JM. 2002. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev 54:459-476.

Ruckenstein E, Guo W. 2001. Crosslinked mercerized cellulose membranes and their application to membrane affinity chromatography. J Membr Sci 193:131-140.

Sartorius-Stedim Biotech. http://www.sartorius-stedim.com/en/biotechnology/laboratory/products_applications/membrane_adsorbers/literature/pdfs/SartobindMembraneAdsorbers.pdf (accessed July 2, 2009).

Schillberg S, Fischera R, Emans N. 2003. 'Molecular farming' of antibodies in plants, Naturwissenschaften 90:145-155.

Shaunak S, Godwin A, Choi J, Balan S, Pedone E, Vijayarangam D, Heidelberger S, Teo I, Zloh M, Brocchini S. 2006. Site-specific PEGylation of native disulfide bonds in therapeutic proteins. Nature Chem Biol 2:312-313.

Singh N, Wang J, Ulbricht M, Wickramasinghe SR, Husson SM. 2008. Surface-initiated atom transfer radical polymerization: A new method for preparation of polymeric membrane adsorbers. J Membr Sci 309:64-72.

Sofer G, Chirica LC. 2006. Improving productivity in downstream processing. BioPharm International 19:30-35.

Soltys PJ, Etzel MR. 2000. Equilibrium adsorption of LDL and gold immunoconjugates to affinity membranes containing PEG spacers. Biomaterials 21:37-48.

Sommerfeld S, Strube J. 2005. Challenges in biotechnology production - Generic processes and process optimization for monoclonal antibodies. Chem Eng Process 44:1123-1137.

Sun L, Dai J, Baker GL, Bruening ML. 2006. High-capacity, protein-binding membranes based on polymer brushes grown in porous substrates. Chem Mater 18:4033-4039.

Svec F, Frechet JMJ. 1996. New designs of macroporous polymers and supports: from separation to biocatalysis. Science 273: 205-211.

Teeters MA, Conrardy SE, Thomas BL, Root TW, Lightfoot EN. 2003. Adsorptive membrane chromatography for purification of plasmid DNA. J Chromatogr A 989:165-173.

Thommes J, Etzel M. 2007. Alternatives to Chromatographic Separations. Biotechnol Prog 23:42-45.

Thommes J, Kula MR. 1995. Membrane chromatography-An integrative concept in the downstream processing of protein. Biotechnol Prog 11:357-367.

van Reis R, Zydney A. 2007. Bioprocess membrane technology. J Membr Sci 297:16-50.

Walsh G. 2004. Second-generation biopharmaceuticals. Eur J Pharm Biopharm 58:185-196.

Wang L, Hale G, Ghosh R. 2006. Non-size-based membrane chromatographic separation and analysis of monoclonal antibody aggregates. Anal Chem 78:6863-6867.

Werner RG. 2005. The development and production of biopharmaceuticals: Technological and economic success factors. BioProcess International 3:6-15.

Wong SS. 1991. Reactive groups of proteins and their modifying agents in: Chemistry of Protein Conjugation and Cross-linking. Boston: CRC Press. 13 p.

Zeng X, Ruckenstein E. 1998. Cross-linked macroporous chitosan anion-exchange membranes for protein separations. J Membr Sci 148:195-205.

Zhao G, Sun Y. 2007. Displacement chromatography of proteins on hydrophobic charge induction adsorbent column. J Chromatogr A 1165:109-15.

Zhou JX, Tressel T. 2006. Basic concepts in Q membrane chromatography for large-scale antibody production. Biotechnol Prog 22:341-349.

Zou H, Huang X, Ye M, Luo Q. 2002. Monolithic stationary phases for liquid chromatography and capillary electrochromatography. J Chromatogr A 954:5-32.

Zou H, Luo Q, Zhou D. 2001. Affinity membrane chromatography for the analysis and purification of proteins. J Biochem Biophys Methods 49:199-240.

Zydney A, van Reis R. 2001. High performance tangential flow filtration. In Wang WK, editor. Membrane separations in biotechnology. New York: Marcel Dekker. p 277-298.

Chapter 2

Research Objectives, Background and Thesis outline

2.1 Research objectives

The key aim of the research described in this thesis is to promote the application of membrane chromatography in bioprocessing by exploring novel application areas in bioseparation and bioreaction, studying the mechanism of protein binding on membrane and developing new membrane adsorbers. Therefore, as shown in Figure 2.1, this research will be generally categorized into four areas. While the main research areas are novel applications in bioseparation and bioreaction, better understanding of binding mechanism and development of new membrane adsorbers will greatly help achieve the research objective.

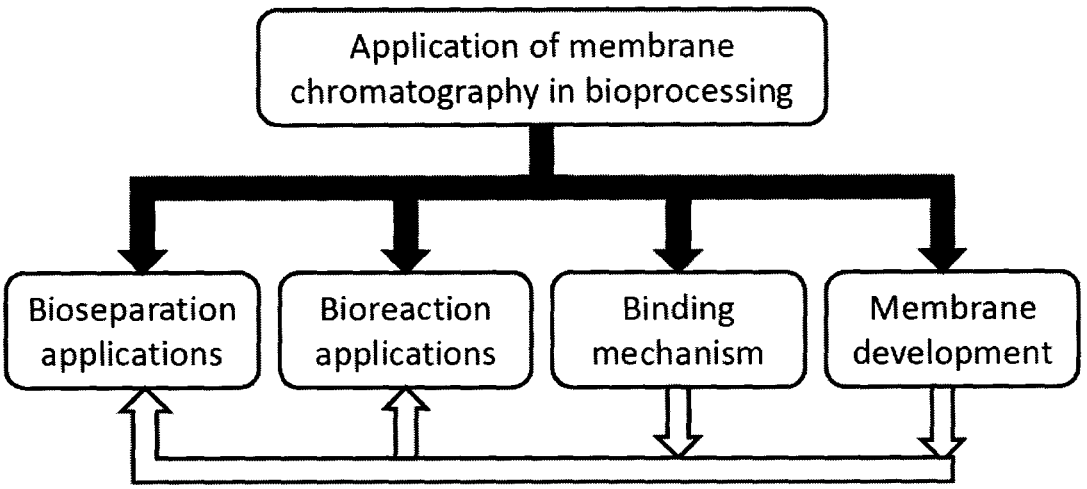


Figure 2.1 Schematic diagram of the research areas described in this thesis.

Membrane chromatography is a promising bioseparation technology. However, as a new technology, membrane chromatography still needs plenty of improvements. Firstly, although the high-throughput advantage of membrane chromatography has been well studied (as discussed in Chapter 1), the high-resolution advantage of membrane chromatography needs to be further exploited. Secondly, membrane chromatography is currently limited to the applications in bioseparation. Exploring new application areas such as bioreaction will definitely promote its development. Thirdly, the protein binding mechanism on membrane adsorbers has not been well studied and requires more explorations. Furthermore, application of commercial membrane adsorbers is limited by the high cost and fouling tendency. The advantage of membrane chromatography would be enhanced by developing new membrane adsorbers with low cost and less fouling problem. By addressing these issues, this research aims at the following specific objectives:

1. To develop the high resolution advantage of membrane chromatography in novel bioseparation applications: purification of transgenic tobacco expressed mAb and purification of PEGylated proteins.
2. To develop membrane chromatography based bioreactor-separator for integration of enzymatic fragmentation of IgG and purification of IgG fragments.
3. To study the mechanism of hydrophobic interaction based antibody binding on membrane.
4. To develop filter paper based new membrane adsorber with low cost and less fouling.

Since these objectives span highly diverse areas, the background for each area are introduced as follows.

2.2 Research background

2.2.1 Purification of monoclonal antibodies from transgenic tobacco using membrane chromatography based bioprocesses

Monoclonal antibodies are traditionally expressed by mammalian cell culture. Molecular farming, the technology for protein expression with transgenic plants provides a new source of mAb expression (Hiatt et al. 1989; Ma et al. 1995; Schillberg et al. 2005; Olea-Popelka et al. 2005; Almquist et al. 2006; McLean et al. 2007). As discussed in 1.4.1, transgenic plants are much safer than CHO cell culture and transgenic animals and furthermore, transgenic tobacco is even safer than other crop plants. It is easy to scale up with low cost by large scale plantation. Despite the advantages of molecular farming over

CHO cell culture, transgenic tobacco faces great challenges in purification. The expression level of mAb in transgenic tobacco is normally in the range of 0.1% of total soluble protein (Schillberg et al. 2003). Furthermore, tobacco leaves have a plethora of plant proteins and various chemicals such as cellulose, alkaloids, pigments, polyphenols. Combining these two factors, large amount of tobacco leaves need be treated and plenty of impurities need be removed to produce mAb from transgenic tobacco. Therefore techniques used for mAb purification from CHO cell culture, such as column chromatography might not be suitable for purifying tobacco-sourced mAbs. Currently very few researches focus on mAb purification from transgenic tobacco. Some of them study the preliminary recovery of proteins from tobacco extract using precipitation or aqueous two-phase extraction (e.g. Zhang et al. 2005). Others just use column based processes (e.g. Ramirez et al. 2003). However, column chromatography has apparent limitations for transgenic tobacco sourced mAb purification. The soft gel, high column bed and diffusion-limited mass transport cause low throughput and high back pressure, which further decrease the productivity and make scale-up difficult.

Membrane chromatography featuring the high-throughput high-resolution advantages could be very useful to purify transgenic tobacco sourced mAb. Membrane chromatography has been examined for purification of CHO cell culture based mAbs (Knudsen et al. 2001; Gallher and Fowler 2001; Zhou and Tressel 2006; Ghosh and Wang 2006). However, its application for mAb purification from transgenic plant has not been reported. The current research explores the purification of mAbs from transgenic tobacco leaves using high-resolution membrane chromatography based bioprocesses. The tobacco

leaves were first pretreated by blending and homogenization for mAb release followed by centrifugation and microfiltration for clarification. The clarified tobacco juice was then used for mAb purification. The process of ion exchange membrane chromatography followed by Protein A membrane chromatography was established using hIgG1-CD4 (Isaacs et al. 1997) as a model antibody. This process was then utilized for purification of *Pseudomonas aeruginosa* O6ad hIgG1 (McLean et al. 2007) from transgenic tobacco. Furthermore, a non-Protein A based membrane process was established, overcoming the Protein A related limitations such as high cost, immunotoxicity and antibody aggregation due to acidic elution (Carlson 2005; Arakawa et al. 2004). This process utilized IEMC followed by HIMC and ultrafiltration for purification of hIgG1-CD4 from spiked tobacco juice.

2.2.2 Purification of PEGylated proteins using membrane chromatography

PEGylated protein drugs are one important class in second generation biopharmaceuticals (Walsh 2004). As discussed in Chapter 1, the PEGylated proteins have enhanced clinical efficacies compared with their native proteins. Some FDA approved PEGylated protein drugs have achieved significant success, such as PEG-interferon α2a (Pegsys®), PEG-interferon α2b (PEG-Intron®) and PEG-G-CSF (Neulasta®) (Reddy et al. 2002; Wang et al. 2002; Kinstler et al. 2002).

As discussed in 1.4.2, early protein PEGylation using protein lysine amino group as PEG conjugation site leads to heterogeneous products, making purification and quality

control difficult (Roberts et al. 2002). N-terminus protein PEGylation provides largely selective conjugation of PEG at α-amino group based on reductive alkylation (Wong 1991; Hermanson 1996). Some recombinant proteins have been PEGylated using this approach, including G-CSF, insulin, interferon β-1b and epidermal growth factor (Kinstler et al. 2002; Dou et al. 2007; Basu et al. 2006; Lee et al. 2003). However, the reaction conditions still need be optimized for each protein.

Purification of PEGylated proteins using column chromatography has apparent limitations. Size exclusion chromatography is mainly used for analysis due to low flow rate and small processing capacity. Ion exchange column chromatography, due to the diffusion based mass transport shows limited flow rate and binding capacity, especially for PEGylated proteins possessing high hydrodynamic radius (Fee and van Alstine 2004). IEC based purification of PEGylated proteins typically uses flow rates around 100 cm/h (Yun et al. 2005; Piquet et al. 2002; Foser et al. 2003; Pabet et al. 2007). The binding capacities of PEGylated proteins in IEC are normally 10 folds lower than that of native proteins (Pabst et al. 2007; Fee and van Alstine 2006). This phenomenon was explained by both the charge shielding and high hydrodynamic radius caused by PEG.

Membrane chromatography could overcome the limitations that column chromatography faces for PEGylated protein separation. The binding capacity of membrane chromatography is not affected by the flow rate in a wide range due to the predominant convective mass transport (Ghosh 2002). The large pore size of membrane adsorbers in micrometer level is especially suitable for binding PEGylated proteins. Therefore, the high-throughput and high-resolution membrane chromatography could be a

useful technique for purification of PEGylated proteins. To the best of my knowledge, this area has not been studied before this work.

2.2.3 IgG fragmentation using reactant adsorptive membrane bioreactor separator

Antibody fragments including Fab and $F(ab')_2$ are important diagnostic reagents and therapeutic drugs (Yamaguchi et al. 1995; Cohen et al. 2000; Ljunglöf et al. 2007). IgG fragmentation is a useful tool for producing antibody fragments, characterizing antibodies and studying antibody interactions with antigens (Parham et al. 1982; Parham 1983; Jones and Landon 2002). IgG is composed of Fab and Fc portions which are responsible for antigen binding and cell receptor binding respectively (Harlow and Lane 1998). In diagnostic tests, using Fab or $F(ab')_2$ to detect cell surface antigen could avoid the interference caused by Fc receptor on cells (Andrew and Titus 1997). For antivenom therapy, using Fab or $F(ab')_2$ could reduce adverse reactions caused by the Fc portion (Leon et al. 2001; Theakston et al. 2003). Therapeutic Fab fragments have been extensively developed and commercialized, e.g. Crotalidae polyvalent immune Fab (Consroe 1995), digoxin immune Fab (Bateman 2004), Abciximab (Gabriel and Oliveira 2006) and Ranibizumab (Dugel 2006).

As mentioned in 1.4.1, the enzymatic fragmentation of IgG is traditionally carried out by three unit operations: IgG purification from serum or cell culture, liquid phase reaction, followed by multistep purification of target fragment (Karlson-Stiber et al. 1997; Jones and Landon 2002; Coleman and Mahler 2003; Ljunglöf et al. 2007). These complex

processes overall are time and cost-consuming with low productivity. IgG purification using salt precipitation and affinity chromatography is likely to cause aggregate formation and product activity loss (Friesen 1987). The enzymatic reactions are usually performed in buffered solutions at 30-40°C for 2-24 hours (Rousseaux et al. 1983; Cresswell et al. 2005). Complete antibody fragmentation is rarely achieved except by long time reaction such as 48-144 hours of pepsin digestion (Bennich and Turner 1969; Parham 1983; Jones and Landon 2002). However, long time reaction may cause microbial contamination and significant product activity loss (Morais et al 1994; Morais and Massaldi 2005). Purification of antibody fragments from reaction mixture needs pretreatment using dialysis and ultrafiltration followed by various column chromatographic methods based on affinity, ion exchange and size exclusion (Harlow and Lane 1988; Roque et al. 2004; Ljunglöf et al. 2007). Affinity chromatography is often used for this step but is expensive and susceptible to ligand leakage. Antibody fragmentation could also be carried out using immobilized enzymes on agarose beads but these reagents are very expensive and normally used in laboratory only. Despite the merits of reusability and separation of products from immobilized enzyme, the desired antibody fragments still need be purified from undigested antibody and byproducts. Immobilized enzymes are normally used in packed bed column where the mass transport of large substrates like IgG could potentially be affected by diffusion limitations and high back pressure (Bommarius and Ribel 2004; Mateo et al. 2002). Furthermore, the economic advantages of using immobilized enzymes are not significant for applications like antibody fragmentation, where the substrate is significantly more expensive than the enzyme.

Membrane bioreactors have been extensively utilized for enzymatic reactions. Currently used reactors could be classified into two types but they all sequester enzymes within the reactor and the reactant is allowed to flow through for reaction (Gekas 1986; Heath and Belfort 1990; Giorno and Drioli 2000). The first type of membrane reactors that retain soluble enzyme by ultrafiltration membrane could not provide selective separation in IgG fragmentation since the substrate, the enzyme and the products are all macromolecules. In the second type of reactors, enzyme is immobilized on a membrane by physical entrapment, adsorption or covalent binding. It could provide the separation of enzyme from other components but further purification of the desired fragment from undigested IgG and byproducts is still needed.

A novel membrane bioreactor separator system proposed here utilizes the principle of membrane chromatography. In this reactant adsorptive membrane bioreactor separator system (RAMBS), the reactant i.e. the antibody is reversibly immobilized on a stack of microporous membranes while the enzyme flows through, carrying out the reaction. In the meantime, the target product, byproduct, unreacted IgG and enzyme could be separated based on their different interactions with membrane. In this work, RAMBS systems based on different chemistries, ion exchange or hydrophobic interaction were developed. The HIMC based RAMBS was utilized for integrated separation-reaction-separation to produce Fab directly from serum. Cation-exchange based RAMBS was utilized to integrate papain digestion of hIgG and purification of Fab. Anion-exchange based RAMBS was utilized for pepsin digestion of hIgG and purification of $F(ab')_2$. The RAMBS system allows reaction and separation to be carried out using a single unit

operation within a single device to achieve process intensification. High enzymatic conversion is also expected based on the convective mass transport in membrane adsorber.

2.2.4 Hydrophobic interaction based IgG binding on membrane

IgG binding on membrane through hydrophobic interaction is important for membrane chromatography, membrane immunoassay and biomedical devices such as artificial kidney and blood dialyzer. On one hand, antibody binding on HIMC or immunoassay should be reasonably strong enough to provide the processing capacity or detection sensitivity. On the other hand, the binding of IgG in microfiltration, ultrafiltration and dialysis should be reduced as much as possible. Therefore, the study of how IgG binds on the membrane is very useful for designing efficient membrane adsorbers and conversely, minimizing IgG binding in applications that require less binding. In hydrophobic interaction based binding, IgG binds on membranes through its hydrophobic patches. However, the orientation of the bound IgG molecule and the key IgG region for binding are not well understood. Many researchers thought that the Fc region of the IgG molecule interacts with the binding surface while the two Fab domains protrude for antigen interaction (Bujis et al. 1996; Bujis et al. 1997; Erp et al. 1992; Nagaoka et al. 2000; Nagaoka et al. 2001). These thoughts are especially typical when IgG binds on the plate in ELISA. Some researchers suggested that Fab rather than Fc binds to a Teflon surface since they found Fab is more hydrophobic than Fc (Vermeer and Norde 2000 and 2000; Vermeer et al. 2001). Other researchers thought IgG binding is random (Konig and Skerra 1998).

In most previous studies, the materials for IgG binding such as polystyrene and Teflon were very hydrophobic and therefore, it is difficult to compare the relative hydrophobicity of the different domains of the IgG. Their true hydrophobicity may be revealed by using materials with tunable hydrophobicity, such as the environment-responsive synthetic membranes used for HIMC (Ghosh 2001; Wang et al. 2006). Such materials like hydrophilized PVDF membranes are prepared by grafting hydrophilic polymers on the surface of hydrophobic membranes (Hester and Mayes 2002). These hydrophilized membranes are very hydrophilic at low salt condition due to the hydration of grafted polymer but become hydrophobic at high salt due to the dehydration and collapse of grafted polymer, thus providing tunable hydrophobicity for reversible IgG binding.

The IgG binding on hydrophilized PVDF membrane in the presence of ammonium sulfate will be studied using newly developed RAMBS system. Such a system could integrate enzymatic catalysis and membrane bioseparation into a single unit operation. Membrane-bound hIgG was fragmented by pulsing proteolytic enzymes pepsin and papain and the fragments were separated by gradient elution. By analyzing the composition of the flow-through and eluate fractions from the membrane, the manner of hIgG binding was conclusively verified.

2.2.5 Paper-PEG based membrane for antibody purification

Protein A column chromatography is widely used for purifying monoclonal antibodies from CHO cell culture. In addition to the general limitations caused by soft gel beads such as low throughput and high back pressure, Protein A column has the problems

of high cost, potential leakage of immunotoxic ligand and antibody aggregation due to acidic elution (Carlson 2005; Arakawa et al. 2004).

Membrane chromatography with high-throughput and high-resolution advantages is promising to overcome the limitations of column chromatography. Membrane chromatography using non-protein A approaches such as ion exchange and hydrophobic interaction have been examined for monoclonal antibody purification (e.g. Knudsen et al, 2001; Philips et al, 2004; Ghosh, 2001; Ghosh and Wang, 2006). However, commercial membrane adsorbers are very expensive and some of them show fouling tendencies. To enhance the competitive edge of membrane chromatography, the current work attempts to improve the cost and fouling problems of membrane adsorber by developing PEG grafted filter paper. These new membranes were prepared by forming interpenetrating polymer network (IPN) of PEG and cellulose. The advantages of filter paper as base material include low cost and high hydraulic permeability. Furthermore, filter paper has less fouling tendency due to the large pore structure and high compatibility with biomolecules. Chitosan coated filter paper linked to protein A via spacer arm was utilized as affinity membrane adsorber for IgG purification (Yang and Chen 2002). HIMC with PVDF membranes was reported for purification of monoclonal antibody from simulated CHO cell culture supernatant (Ghosh and Wang 2006). To the best of my knowledge the use of filter paper based hydrophobic interaction media has not yet been reported in the literature.

PEG is a very hydrophilic polymer. However, in the presence of antichaotropic salts such as ammonium sulphate, PEG can undergo phase separation and become mildly

hydrophobic (Ananthapadmanabhan and Goddard, 1987). This reversible change in hydrophobicitiy provides the basis for protein separation using hydrophobic interaction. The PEG grafted filter paper is postulated to bind proteins at high salt conditions but release the bound proteins at low salt conditions. This new membrane adsorber would provide better release and thus higher recovery of proteins than traditional hydrophobic interaction media which use naturally hydrophobic ligands such as phenyl, butyl and octyl groups. Furthermore, Interaction of proteins with PEG does not affect their biological activity (Wang et al, 2006). PEG-salt aqueous two phase extraction uses protein interaction with PEG for bioseparation (Ananthapadmanabhan and Goddard, 1987; Andrews et al, 1996; Huddleston et al, 1996). PEG modified beads have been used for column chromatography (Ling and Mattiasson 1983). The current research will utilize PEG-filter paper as a novel hydrophobic interaction membrane for monoclonal antibody purification.

2.3 *Thesis outline*

The research objectives will be achieved in the thesis corresponding to the four research areas. Chapters 3 to 5 focus on application of high resolution membrane chromatography for purification of monoclonal antibodies and PEGylated proteins. Chapter 6 and 7 focus on the development of membrane chromatography based bioreactor-separator. Chapter 8 explores the mechanism of hydrophobic interaction based IgG binding on membrane. Chapter 9 describes the PEG-grafted filter paper as a novel membrane for hydrophobic interaction membrane chromatography.

Chapter 3 presents the purification of a hIgG1 monoclonal antibody from transgenic tobacco using membrane chromatographic processes. This work discusses the use of cation-exchange and Protein A affinity-based membrane chromatographic techniques, singly and in combination for the purification of anti-Pseudomonas aeruginosa O6ad hIgG1 mAb from transgenic tobacco. The process of ion exchange membrane chromatography followed by Protein A membrane chromatography was successfully utilized to achieve highly purified mAb with high recovery and high permeability. This work led to the publication in Journal of Chromatography A.

Chapter 4 describes the purification of monoclonal antibody from tobacco extract using membrane-based bioseparation techniques. This non-Protein A based process tried to address the limitations associated with Protein A ligand, including high cost, immunotoxicity, and antibody aggregation during elution. The three-step process utilized IEMC for primary capture and preliminary purification of the mAb from tobacco juice, HIMC for high-resolution purification followed by ultrafiltration for polishing, desalting and buffer exchange. Membrane chromatography is generally considered unsuitable for resolving bound proteins by gradient elution. This study showed that the gradient elution process can be optimized in HIMC to obtain high resolution and high purity of target product. This work led to a publication in Journal of Membrane Science.

Chapter 5 studies the purification of PEGylated lysozyme using membrane chromatography. N-terminus PEGylation was used for site-specific lysozyme PEGylation. The reaction conditions were optimized for production of mono-PEGylated lysozyme. Ion exchange membrane chromatography was utilized for purification of

mono-PEGylated lysozyme from reaction mixture. The convective mass transport of membrane chromatography was demonstrated to be advantageous for PEGylated protein binding. The resolution and binding capacity increased with the increase of flow rate. This work was submitted to Journal of Pharmaceutical Science.

Chapter 6 discusses the development of reactant adsorptive membrane bioreactor-separator for integration of IgG fragmentation and purification of Fab fragment. The hydrophobic interaction membrane chromatography based RAMBS was designed for integrated separation-reaction-separation to produce Fab fragment directly from hIgG in human serum. Under optimized conditions, the Fab was recovered from reaction flow through while other components were bound on membrane and subsequently eluted. The RAMBS system achieved the process intensification and higher catalytic efficiency of papain than that of liquid phase reaction. This work was published in Biotechnology and Bioengineering.

Chapter 7 applies ion exchange membrane chromatography based RAMBS system for integrated bioprocessing of antibody fragments. Ion exchange membrane adsorber was utilized for IgG binding followed by flow through of enzyme to digest bound IgG into fragments. The fragments were then separated based on their different interaction with the membrane adsorber. Cation-exchange based RAMBS was utilized to integrate papain digestion of IgG and purification of Fab. Anion-exchange based RAMBS was utilized to integrate pepsin digestion of IgG and purification of $F(ab')_2$. The ion exchange based RAMBS systems successfully intensified the processes of Fab and $F(ab')_2$ production and achieved much higher catalytic efficiency than that in liquid phase

reaction. This work will be separated into two papers and respectively submitted to Biotechnology Progress and Journal of Immunological Methods.

Chapter 8 explores the mechanism of hydrophobic interaction based IgG binding on synthetic membrane using newly developed RAMBS system. Membrane bound hIgG was digested by papain and pepsin respectively followed by gradient elution. The product profiles from the two enzyme digestions showed that hIgG bound to the membrane through its middle region. Analysis of eluate samples verified that the binding of hIgG took place primarily through a combination of the hinge and C_H2 domain of Fc. The result from this study is useful for developing membrane chromatography, immunoassay and blood contact biomedical devices. This work was in press by Langmuir.

Chapter 9 discusses the development of PEG-grated filter paper as a new hydrophobic interaction membrane for mAb purification. These PEG-paper membranes prepared by interpenetrating polymer network address the limitations of high cost and fouling tendency of commercial membrane adsorbers. Using this membrane, a single-step hydrophobic interaction membrane chromatography could produce mAb from simulated CHO cell culture with high purity and high recovery. PEG-paper membrane also demonstrated high hydraulic permeability and good reusability. This work was published in Biotechnology and Bioengineering.

As a whole, the research reported in this thesis could lead to eight scholarly publications. Four papers had been published (Yu et al. 2008a, 2008b, 2008c; Yu and Ghosh 2009a), one was in press (Yu and Ghosh 2009b), one submitted and two more papers are pending submission to journals.

2.4 References

Almquist KC, McLean MD, Niu Y, Byrne G, Olea-Popelka FC, Murrant C, Barclay J, Hall JC. 2006. Expression of an anti-botulinum toxin A neutralizing single chain Fv recombinant antibody in transgenic tobacco. Vaccine 24:2079-2086.

Ananthapadmanabhan KP, Goddard ED. 1987. Aqueous biphase formation in polyethylene oxide-inorganic salt systems. Langmuir 3:25-31.

Andrew SM, Titus JA. 1997. Current Protocols in Immunology. New York: John Wiley & Sons. p 2.8.1-2.8.10.

Andrews BA, Nielsen S, Asenjo JA. 1996. Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. Bioseparation 6:303-313.

Arakawa T, Philo JS, Tsumoto K, Yumioka R, Ejima D. 2004. Elution of antibodies from a Protein-A column by aqueous arginine solutions. Protein Expression Purif 36:244-248.

Basu A, Yang K, Wang M, Liu S, Chintala R, Palm T, Zhao H, Peng P, Wu D, Zhang Z, Hua J, Hsieh MC, Zhou J, Petti G, Li X, Janjua A, Mendez M, Liu J, Longley C, Zhang Z, Mehlig M, Borowski V, Viswanathan M, Filpula D. 2006. Structure-function engineering of interferon-α1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. Bioconjugate Chem 17:618-630.

Bateman DN. 2004. Digoxin-specific antibody fragments: how much and when? Toxicol Rev 23:135–143.

Bennich H, Turner MW. 1969. The release of Fc' and pFc' fragments from human immunoglobulin G following enzymic digestion: a time study. Biochim Biophys Acta 175:388-395.

Bommarius AS, Ribel BR. 2004. Biocatalysis, Fundamentals and Application. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA. p 109-110.

Buijs J, Berg P, Lichtenbelt J, Norde W, Lyklema J. 1996. Adsorption dynamics of IgG and its $F(ab')_2$ and Fc fragments studied by reflectometry. Coll Interf Sci 178:594-605.

Buijs J, White DD, Norde W. 1997. The effect of adsorption on the antigen binding by IgG and its $F(ab')_2$ fragments. Colloids Surf B 8:239-249.

Carlson KL. 2005. Flexibility—the guiding principle for antibody manufacturing. Nat Biotechnol 23:1054-1058.

Chapman AP, Antoniw P, Spitali M, West S, Stephens S, King DJ. 1999. Therapeutic antibody fragments with prolonged in vivo half-lives. Nat Biotechnol 17:780-783.

Cohen SA, Trikha M, Mascelli MA. 2000. Potential future clinical applications for the GPIIb/IIIa antagonist, abciximab in thrombosis, vascular and oncological indications. Pathol Oncol Res 6:163-174.

Coleman L, Mahler SM. 2003. Purification of Fab fragments from a monoclonal antibody papain digest by Gradiflow electrophoresis. Protein Expr Purif 32:246-251.

Consroe P, Egen NB, Russell FE, Gerrish K, Smith DC, Sidki A, Landon JT. 1995. Comparison of a new ovine antigen binding fragment (Fab) antivenin for United

States Crotalidae with the commercial antivenin for protection against venom-induced lethality in mice. Am J Trop Med Hyg 53:507-510.

Cresswell C, Newcombe AR, Davies S, Macpherson I, Nelson P, O'Donovan K, Francis R. 2005. Optimal conditions for the papain digestion of polyclonal ovine IgG for the production of bio-therapeutic Fab fragments. Biotechnol Appl Biochem 42:163-168.

Dou H, Zheng M, Zhang Y, Yin C. 2007. Synthesis and purification of mono-PEGylated insulin. Chem Biol Drug Des 69:132-138.

Dugel PU. 2006. Ranibizumab treatment of patients with ocular diseases. Int Ophthalmol Clin 46:131-140.

Erp R, Linders YEM, Sommeren APG, Gribnau TCJ. 1992. Characterization of monoclonal antibodies physically adsorbed onto polystyrene latex particles. J Immunol Meth 152:191-199.

Fee CJ, van Alstine JM. 2004. Prediction of the viscosity radius and the size exclusion chromatography behavior of PEGylated proteins. Bioconjugate Chem 15:1304-1313.

Fee CJ, van Alstine JM. 2006. PEG-proteins: Reaction engineering and separation issues. Chem Eng Sci 61:924-939.

Foser S, Schacher A, Weyer KA, Brugger D, Dietel E, Marti S, Thomas Schreitmuller T. 2003. Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon α-2a (PEGASYS). Protein Expr Purif 30:78–87.

Friesen AD. 1987. Chromatographic methods of fractionation. Dev Biol Stand 67:3-13.

Gabriel HM, Oliveira EI. 2006. Role of abciximab in the treatment of coronary artery disease. Expert Opin Biol Ther 6:935-942.

Gallher P, Fowler E. 2001. Validation of impurity removal by the CAMPATH-1H biomanufacturing process. Presentation at IBC's Biopharmaceutical Production Week, November 12-15, in San Diego, USA.

Gekas VC. 1986. Artificial membranes as carriers for the immobilization of biocatalysts. Enzyme Microb Technol 8:450-460.

Ghosh R, Wang L. 2006. Purification of monoclonal antibody by hydrophobic interaction membrane chromatography. J Chromatogr A 1107:104-109.

Ghosh R, Wong T. 2006. Effect of module design on the efficiency of membrane chromatographic separation processes. J Membr Sci 281:532-540.

Ghosh R. 2001. Separation of proteins using hydrophobic interaction membrane chromatography. J Chromatogr A 923:59-64.

Ghosh R. 2002. Protein separation using membrane chromatography: opportunities and challenges. J Chromatogr A 952:13-27.

Ghosh R. 2005. Fractionation of human plasma proteins by hydrophobic interaction membrane chromatography. J Membr Sci 260:112-118.

Giorno L, Drioli E. 2000. Biocatalytic membrane reactors: applications and perspectives. Trends Biotechnol 18:339-349.

Harlow E, Lane D. 1988. Antibodies, A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press. p 626-629.

Harlow E, Lane D. 1998. Using Antibodies: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press. 5 p.

Heath C, Belfort G. 1990. Membranes and bioreactors. Int J Biochem 22:823-825.

Hermanson GT. 1996. Bioconjugation Techniques. San Diego: Academic Press. 141 p.

Hester JF, Mayes AM. 2002. Design and performance of foul-resistant poly(vinylidene fluoride) membranes prepared in a single-step by surface segregation. J Membr Sci 202:119-135.

Hiatt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants. 1989. Nature 342:76-78.

Huddleston J, Abelaira JC, Wang R, Lyddiatt A. 1996. Protein partition between the different phases comprising poly(ethylene glycol)-salt aqueous two-phase systems, hydrophobic interaction chromatography and precipitation: a generic description in terms of salting-out effects. J Chromatogr B Biomed Appl 680:31-41.

Isaacs JD, Burrows N, Wing M, Keogan MT, Rebello PR, Watts RA, Pye RJ, Norris P, Hazelman BL, Hale G, Waldmann H. 1997. Humanized anti-CD4 monoclonal antibody therapy of autoimmune and inflammatory disease. Clin Exp Immunol 110:158-166.

Jones RGA, Landon L. 2002. Enhanced pepsin digestion: a novel process for purifying antibody $F(ab')_2$ fragments in high yield from serum. J Immunol Methods 263:57-74.

Karlson-Stiber C, Persson H, Heath A, Smith D, al-Abdulla IH, Sjostrom L. 1997. First clinical experiences with specific sheep Fab fragments in snake bite. Report of a multicentre study of Vipera berus envenoming. J Intern Med 241:53-58.

Kinstler O, Molineux G, Treuheit M, Ladd D, Gegg C. 2002. Mono-N-terminal poly(ethylene glycol)–protein conjugates. Adv Drug Deliv Rev 54:477-485.

Knudsen HL, Fahrner RL, Xu Y, Norling LA, Blank GS. 2001. Membrane ion-exchange chromatography for process-scale antibody purification. J Chromatogr A 907:145-154.

König T, Skerra A. 1998. Use of an albumin-binding domain for the selective immobilisation of recombinant capture antibody fragments on ELISA plates. J Immunol Methods 218:73-83.

Lee H, Jang H, Ryu SH, Park TG. 2003. N-Terminal site-specific mono-PEGylation of epidermal growth factor. Pharm Res 20:818-825.

Leon G, Monge M, Rojas E, Lomonte B, Gutierrez JM. 2001. Comparison between IgG and $F(ab')_2$ polyvalent antivenoms: neutralization of systemic effects induced by Bothrops asper venom in mice, extravasation to muscle tissue, and potential for induction of adverse reactions. Toxicon 39:793-801.

Ling TGI, Mattiasson B. 1983. Poly(ethylene glycol)- and poly(vinyl alcohol)-substituted carbohydrate gels for "mild" hydrophobic chromatography. J Chromatogr A 254:83-89.

Ljunglöf A, Lacki KM, Mueller J, Harinarayan C, van Reis R, Fahrner R, Van Alstine JM. 2007. Ion exchange chromatography of antibody fragments. Biotechnol Bioeng 96:515-524.

Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, Van Dolleweerd C, Mostov K, Lehner T. 1995. Generation and assembly of secretory antibodies in plants. Science 268:716-719.

Mateo C, Abian O, Fernandez-Lorente G, Pedroche J, Fernandez-Lafuente R, Guisan JM, Tam A, Daminati M. 2002. Epoxy sepabeads: A novel epoxy support for stabilization of industrial enzymes via very intense multipoint covalent attachment. Biotechnol Prog 18:629-634.

McLean MD, Almquist KC, Niu Y, Kimmel R, Lai Z, Schreiber JR, Hall JC. 2007. A human anti-pseudomonas aeruginosa O6ad IgG1 expressed in transgenic tobacco is capable of recruiting immune system effector function in vitro. Antimicrob Agents Chemother 51:3322-3328.

Morais JF, De Freitas MCW, Yamaguchi IK, Dos Santos MC, Dias da Silva W. 1994. Snake antivenoms from hyperimmunized horses: comparison of the antivenom activity and biological properties of their whole IgG and $F(ab')_2$ fragments. Toxicon 32:725-734.

Morais V, Massaldi H. 2005. Effect of pepsin digestion on the antivenom activity of equine immunoglobulins. Toxicon 46:876–882.

Nagaoka S, Ashiba K, Kawakami H. 2000. Interaction between biocomponents and surface modified fluorinated polyimide. Mat Sci Eng C 20:181-185.

Nagaoka S, Kanno M, Kawakami HJ. 2001. Evaluation of blood compatibility of fluorinated polyimide by immunolabeling assay. Artif Organs 4:107-112.

Olea-Popelka FC, McLean MD, Horsman J, Almquist K, Brandle JE, Hall JC, 2005. Increasing expression of an anti-picloram single-chain variable fragment (ScFv) antibody and resistance to picloram in transgenic tobacco (Nicotiana tabacum). J Agric Food Chem 53:6683-6690.

Pabst TM, Buckley JJ, Ramasubramanyan N, Hunter AK. 2007. Comparison of strong anion-exchangers for the purification of a PEGylated protein. J Chromatogr A 1147:172–182.

Parham P, Androlewicz MJ, Brodsky FM, Holmes NJ, Ways JP. 1982. Monoclonal antibodies: purification, fragmentation and application to structural and functional studies of class I MHC antigens. J Immunol Methods 53:133–173.

Parham P. 1983. On the fragmentation of monoclonal IgG1, IgG2a, and IgG2b from BALB/c mice. J Immunol 131:2895–2902.

Phillips M, Cormier J, Ferrence J, Dowd C, Kiss R, Lutz H, Carter J. 2004. Performance of a membrane adsorber for trace impurity removal in biotechnology manufacturing, J Chromatogr A 1078:74-82.

Piquet G, Gatti M, Barbero L, Traversa S, Caccia P, Esposito P. 2002. Set-up of large laboratory-scale chromatographic separations of poly (ethylene glycol) derivatives of the growth hormone-releasing factor 1–29 analogue. J Chromatogr A 944:141–148.

Ramirez N, Rodriguez M, Ayala M, Cremata J, Perez M, Martinez A, Linares M, Hevia Y, Paez R, Valdes R, Gavilondo JV, Selman-Housein G. 2003. Expression and characterization of an anti-(hepatitis B surface antigen) glycosylated mouse antibody in transgenic tobacco (Nicotiana tabacum) plants and its use in the immunopurification of its target antigen. Biotechnol Appl Biochem 38: 223-230.

Reddy KR, Modi MW, Pedder S. 2002. Use of peginterferon alfa-2a (40 KD) (Pegasys) for the treatment of hepatitis C. Adv Drug Deliv Rev 54:571–586.

Roberts MJ, Bentley MD, Harris JM. 2002. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev 54:459-476.

Roque AC, Lowe CE, Taipa MA. 2004. Antibodies and genetically engineered related molecules: Production and purification. Biotechnol Prog 20:639–654.

Rousseaux J, Rousseaux-Prévost R, Bazin H. 1983. Optimal Conditions for the Preparation of Fab and $F(ab')_2$ Fragments from Monoclonal IgG of Different Rat IgG Subclasses. J Immunol Methods 64:141–146.

Schillberg S, Fischera R, Emans N. 2003. 'Molecular farming' of antibodies in plants, Naturwissenschaften 90:145-155.

Schillberg S, Twymanb RM, Fischera R. 2005. Opportunities for recombinant antigen and antibody expression in transgenic plants—technology assessment, Vaccine 23:1764-1769.

Theakston RDG, Warrell DA, Griffiths E. 2003. Report of a WHO workshop on the standardization and control of antivenoms. Toxicon 41:541-557.

Vermeer AWP, Giacomelli CE, Norde W. 2001. Adsorption of IgG onto hydrophobic teflon. Differences between the Fab and Fc domains. Biochemica et Biophysica Acta 1526:61-69.

Vermeer AWP, Norde W. 2000. The thermal stability of immunoglobulin: Unfolding and aggregation of a multi-domain protein. Biophys J 78:394-404.

Vermeer AWP, Norde W. 2000. The unfolding/denaturation of immunogammaglobulin of isotype 2b and its Fab and Fc fragments. Biophys J 79:2150-2154.

Walsh G. 2004. Second-generation biopharmaceuticals. Eur J Pharm Biopharm 58:185-196.

Wang L, Hale G, Ghosh R. 2006. Non-size-based membrane chromatographic separation and analysis of monoclonal antibody aggregates. Anal Chem. 78:6863-6867.

Wang RW, Zhang Y, Ma GH, Su ZG. 2006. Modification of poly(glycidyl methacrylate-divinylbenzene) porous microspheres with polyethylene glycol and their adsorption property of protein. Colloids and Surf B: Biointerfaces 51:93-99.

Wang YS, Youngster S, Grace M, Bausch J, Bordens R, Wyss DF. 2002. Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications. Adv Drug Deliv Rev 54:547-570.

Wong SS. 1991. Reactive groups of proteins and their modifying agents in: Chemistry of Protein Conjugation and Cross-linking. Boston: CRC Press. 13 p.

Yamaguchi Y, Kim H, Kato K, Masuda K, Shimada I, Arata Y. 1995. Proteolytic fragmentation with high specificity of mouse immunoglobulin G. J Immunol Methods 181:259-267.

Yang L, Chen P. 2002. Chitosan/coarse filter paper composite membrane for fast purification of IgG from human serum. J Membr Sci 205:141-153.

Yu D, Chen X, Pelton RH, Ghosh R. 2008a. Paper-PEG based membranes for hydrophobic interaction chromatography: purification of monoclonal antibody. Biotech Bioeng 99: 1434-1442.

Yu D, Ghosh R. 2009a. Integrated fragmentation of human IgG and purification of Fab using a reactant adsorptive membrane bioreactor separator system. Biotech Bioeng 104:152-161.

Yu D, Ghosh R. 2009b. Method for studying Immunoglobulin G binding on hydrophobic surfaces. Langmuir. DOI: 10.1021/la902395v. In press.

Yu D, McLean MD, Hall JC, Ghosh R. 2008b. Purification of a human immunoglobulin G1 monoclonal antibody from transgenic tobacco using membrane chromatographic processes. J Chromatogr A 1187: 128-137.

Yu D, McLean MD, Hall JC, Ghosh R. 2008c. Recovery and purification of monoclonal antibody from tobacco plant using membrane-based bioseparation techniques. J Membr Sci 323: 159-166.

Yun Q, Yang RE, Chen T, Bi J, Ma G, Su Z. 2005. Reproducible preparation and effective separation of PEGylated recombinant human granulocyte colony-stimulating factor with novel "PEG-pellet" PEGylation mode and ion-exchange chromatography. J Biotechnol 118:67-74.

Zhang C, Lillie R, Cotter J, Vaughan D. 2005. Lysozyme purification from tobacco extract by polyelectrolyte precipitation. J Chromatogr A 1069:107-112.

Zhou JX, Tressel T. 2006. Basic concepts in Q membrane chromatography for large-scale antibody production. Biotechnol Prog 22:341-349.

Chapter 3

Purification of a Human Immunoglobulin G1 Monoclonal Antibody from Transgenic Tobacco Using Membrane Chromatographic Processes

This chapter is organized based on a paper published in Journal of Chromatography A, 1187: 128-137 (2008) by Deqiang Yu, Michael D. McLean, J. Christopher Hall, and Raja Ghosh.

3.1 Abstract

Efficient purification of protein biopharmaceuticals from transgenic plants is a major challenge, primarily due to low target protein expression levels, and high impurity content in the feed streams. These challenges may be addressed by using membrane chromatography. This paper discusses the use of cation-exchange and Protein A affinity-

based membrane chromatographic techniques, singly and in combination for the purification of an anti-*Pseudomonas aerugenosa* O6ad human IgG1 monoclonal antibody from transgenic tobacco. Protein A membrane chromatography on its own was unable to provide a pure product, mainly due to extensive non-specific binding of impurities. Moreover, the Protein A membrane showed severe fouling tendency and generated high back-pressure. With cation-exchange membrane chromatography, minimal membrane fouling and high permeability were observed but high purity could not be achieved using one step. Therefore, by using a combination of the cation-exchange and Protein A membrane chromatography, in that order, both high purity and recovery were achieved with high permeability. The antibody purification method was first systematically optimized using a simulated feed solution. Anti-*P. aeruginosa* human IgG1 type monoclonal antibody was then purified from transgenic tobacco juice using this optimized method.

3.2 Introduction

Monoclonal antibodies (mAbs) are traditionally produced by mammalian cell culture (Chu and Robinson 2001; Zhou et al. 2005), which is technically demanding, difficult to scale up and expensive due to high capital and operating costs. Also, products synthesized by mammalian cell culture have the potential of being contaminated by animal viruses and other pathogens so their removal is needed to comply with regulatory requirements (Zhou and Tressel 2006; Zhou et al. 2006). Molecular farming, which refers to the production of recombinant proteins in genetically modified plants, provides a new

option for monoclonal antibody production (Schillberg et al. 2003; Schillberg et al. 2005; Ma et al. 2005; Ko and Koprowski 2005). The potential for this technology was first demonstrated by the expression of a functional antibody in tobacco (Hiatt et al. 1989). More recently, antibody fragments such as an anti-picloram single-chain Fv Ab and an anti-botulinum toxin A neutralizing single-chain Fv Ab were also expressed in tobacco (Olea-Popelka et al. 2005; Almquist et al. 2006). Some of the problems associated with mammalian cell culture can be solved using plants. For example, expression of proteins in transgenic plants is much safer than by CHO cell culture or expression in transgenic animals since plants are not known to carry any human or animal pathogens. Transgenic tobacco is considered safer than using edible crop plants since the transgenic protein will not end up in the food chain. Furthermore, protein production based on large-scale transgenic plant agriculture should be economically viable because of the low cost of plant growth and harvest as well as the large scale of biomass production (Almquist 2006; Ma et al. 1995; Daniell et al. 2001; Farid 2006; Stoger et al. 2005).

Separation and purification is a major challenge facing the manufacturers of plant derived antibodies. The expression level of a mAb in transgenic tobacco is normally about 0.1% of total soluble protein (TSP) (Schillberg et al. 2003). Moreover, tobacco leaves contain chemicals such as cellulose, alkaloids, pigments, polyphenols and a plethora of plant proteins from which the antibodies have to be selectively purified. Therefore, column chromatographic techniques used for purifying mAbs from CHO cell culture supernatant may not be suitable for purifying tobacco based mAbs. The major limitations with column chromatography are high back pressure, low product throughput

and scale-up problems. These limitations result mainly from the use of soft gel based chromatographic media within which diffusion based mass transport predominates.

Membrane chromatography is a fast growing bioseparation technique which combines the advantages of membrane technology and chromatography, thereby leading to the possibility of developing high-throughput and -resolution purification methods. Due to the predominance of convective mass transport, the binding capacity of membranes is generally independent of flow rate (Ghosh 2002). Higher productivities and easier scale-up relative to column-based processes are therefore possible with membrane chromatography. Membrane chromatography is particularly well-suited for application where large volumes of dilute feed solutions need to be processed. For example, Protein A membrane chromatography (PAMC) has been used for purification of human immunoglobulin (Gastilho et al. 2002). Monoclonal antibody purification from CHO cell culture supernatant has also been studied using membrane chromatography (Knudsen et al. 2001; Philips et al. 2004; Ghosh and Wang 2006; Yu et al. 2008). To the best of our knowledge the use of membrane chromatography for mAb purification from transgenic plant has not yet been reported. Membrane chromatography is expected to be suitable for this application because large volumes of tobacco juice containing very low concentrations of mAb are used, which makes conventional packed bed chromatography unsuitable for mAb purification.

The current research explores the purification of mAbs from transgenic tobacco leaves using various membrane chromatographic techniques: PAMC, ion-exchange membrane chromatography (IEMC), and PAMC and IEMC in combination. The two

mAbs used in this study were human (h)IgG1-CD4 (which is a CHO cell-culture derived antibody) (Issacs et al. 1997), and anti-*Pseudomonas aeruginosa* O6ad hIgG1 (expressed in transgenic tobacco) (McLean et al. 2007); hIgG1-CD4 is a humanized IgG1 type antibody which has been shown to be quite promising in the treatment of refractory psoriasis and rheumatoid arthritis (Issacs et al. 1997); anti-*P. aeruginosa* hIgG1 is a fully human antibody with protective action against this pathogenic bacterium (McLean et al. 2007; Lai et al. 2005). Wild type tobacco juice was first spiked with hIgG1-CD4, i.e. simulated feed solution. This solution was used to systematically optimize operating conditions for the membrane chromatographic purification processes. After establishing the separation and purification methodology for mAb purification, anti- *P. aeruginosa* hIgG1 mAb was purified from transgenic tobacco using this method.

3.3 Experimental

3.3.1 Materials

Humanized monoclonal antibody hIgG1-CD4 (batch 12) was kindly donated by the Therapeutic Antibody Center, University of Oxford, UK. The samples in vials were shipped in dry ice and used as received. Chemical reagents, including sodium phosphate (mono- and di-basic), sodium citrate, citric acid and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All buffers were prepared using ultra-pure water (18.2 MΩ-cm) obtained from a Diamond Nanopure water purification unit (Barnstead International, Dubuque, IA, USA) and vacuum-filtered using 0.45 μm cellulose acetate membrane (Nalgene Nunc, Rochester, NY, USA; order number

DS0210-4045). Sartobind S cation-exchange membrane sheets (thickness = 275 μm, catalogue number 94IEXS42-001) for IEMC and Sartobind Protein A 75 membrane module (catalogue number 93PR-A06DB-12--V) for PAMC were purchased from Sartorius AG (Goettingen, Germany). A Protein A chromatographic column (1 ml rProtein A FF, catalogue number 17-5079-02) was purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). A homozygous transgenic tobacco line was bred for uniform production of the anti-*P. aeruginosa* hIgG1 by standard genetic breeding procedures from a primary transgenic plant [produced in ref. (McLean et al. 2007)] identified with a single T-DNA locus [performed according to procedures outlined in ref. (Horseman et al. 2007); data not shown]. Transgenic and wild-type tobacco [cultivar 81v9; ref. (Miki et al. 1999)] were grown in a greenhouse at the University of Guelph, Ontario, Canada and the leaves were stored in plastic bags at -20 degrees centigrade following their harvest.

3.3.2 Pretreatment of tobacco leaves

The overall bioseparation method for obtaining pure mAb from tobacco leaves was composed of two processing segments: pretreatment (i.e., recovery and isolation) and purification (see Fig. 3.1). Tobacco leaves were removed from the freezer and immediately crushed into small pieces within the plastic bag. The crushed leaves were mixed with extraction buffer (40 mM pH 7.0 phosphate buffer containing 50mM ascorbic acid as antioxidant, 50 μM leupeptin as protease inhibitor, and 10 mM sodium EDTA), using 2 L buffer per kg of leaves, and ground in a 51BL32 blender (Waring Commercial, Torrington, CT, USA) for three minutes (three pulses of 1 minute each with 30 second

between each pulse). The grinding process reduced the particle size of the tobacco leaves to ca. 1 mm. The ground material was homogenized for three minutes (three pulses of 1 minute each with 30 second intervals between each pulse) using a flow through ULTRA TURRAX T25 Basic homogenizer (IKA Works, Staufen, Germany) operated at a flow rate of 21.5 litres/min. This homogenization reduced the particle size to the 1-10 μm range. The size of the plant cells in the tobacco leaves are reported to be ca. 100 μm (Chen et al. 2001), hence the mAb would be expected to be in the solution phase after the homogenization process. The homogenate was pre-filtered through cheese cloth to remove large cellulose fibers, and centrifuged for 30 minutes at 10000 rpm using an Allegra X22R centrifuge (Beckman Coulter, Fullerton, CA, USA). The homogenate was then passed through a 0.45 μm pore size PES membrane disc (42 mm diameter: Pall Co., East Hills, NY, USA) contained in a custom-designed stirred cell. The microfiltration was carried out at a constant flux of 3.0×10^{-5} $m^3/m^2.s$ to reduce fouling of the chromatographic membranes. Aliquots of the microfiltered tobacco juice was stored at -20℃ and used as required. The final volumes of microfiltered tobacco juices were 2.75 and 2.64 litres per kg of wild type and transgenic tobacco leaves, respectively. The simulated transgenic tobacco juice was prepared by spiking wild type tobacco juice with 10 mg of hIgG1-CD4 mAb per kg of frozen leaves.

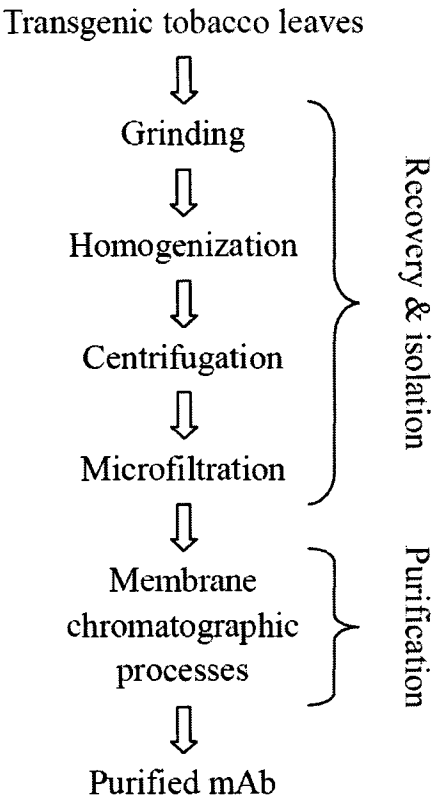


Figure 3.1 Scheme for purifying mAb from transgenic tobacco leaves.

3.3.3 Membrane chromatography system

For the PAMC experiments, the commercial Sartobind Protein A membrane module, which contained 15 discs each having a 25-mm diameter, was connected to an AKTA Prime liquid chromatography system (GE Healthcare Bio-Sciences). For the IEMC experiments, the Sartobind S cation-exchange membrane sheets were cut into discs each having a 18-mm diameter and 15 such discs were placed in a custom-designed membrane module (Ghosh and Wong 2006); this membrane module was connected to the AKTA prime system. The effluent from both PAMC and IEMC units was continuously monitored for UV absorbance at 280 nm, pH and conductivity; the data was logged into a

computer using Prime View software (GE Healthcare Bio-Sciences). The system pressure was also continuously monitored and recorded using the pressure transducer of the AKTA Prime system.

3.3.4 Protein A membrane and column chromatography

The binding (A) and eluting (B) buffers used were the same for both types of chromatography. Buffer A consisted of 20 mM sodium phosphate (pH 7.0) while 100 mM sodium citrate (pH 3.0) served as buffer B. In the purification experiments, 45 ml of feed solution, i.e., transgenic tobacco juice or spiked wild-type tobacco juice (containing 0.164 mg hIgG1-CD4), was injected into the membrane module or column (1ml rProtein A F.F column, GE Healthcare Bio-Sciences) after equilibration with buffer A. After feed injection, the membrane module or column was washed with buffer A to remove unbound material and the bound material was eluted with buffer B. The eluted samples were immediately neutralized using 0.1 M Tris-HCl (pH 9.0) to minimize degradation and aggregation of the antibody. The unbound and eluted bound proteins were collected and analyzed by affinity and size exclusion chromatography, and SDS-PAGE.

3.3.5 Ion-exchange membrane chromatography

The binding buffer (buffer A, as above) was 20 mM sodium phosphate while the eluting buffer (Buffer B) was prepared by adding 0.5 M NaCl to Buffer A. Spiked wild-type tobacco juice (simulated feed solution prepared using 10 mg of hIgG1-CD4 per kg of leaves) was diluted 1:1 with Buffer A. After equilibrating the membrane module with binding buffer, 45 ml of tobacco juice was injected into the membrane module, followed

by washing with buffer A. The bound material was then eluted from membrane using buffer B. The flow-through and elution-peak samples were collected and analyzed using affinity analysis and SDS-PAGE. It is important to note that the buffer solutions were used at various pHs to determine at which pH IEMC was optimized.

3.3.6 IEMC-PAMC combination

The eluate from the IEMC obtained as described above was diluted with 20 mM sodium phosphate buffer (pH 7.0) at a ratio of 1:1 and used as the feed for PAMC. Eluates obtained from the PAMC experiments were collected and analysed using size exclusion chromatography and SDS-PAGE. All feed, flow-through, and eluate samples from the IEMC and PAMC experiments were analyzed by SDS-PAGE.

3.3.7 Sample analysis

A HiTrap 1-ml Protein A HP column (GE Healthcare Bio-Sciences) was used to determine mAb purity in plant extracts at various stages in the purification process. These affinity chromatography experiments were run at 1.0 ml/min flow rate with 20 mM sodium phosphate buffer (pH 7.0) as binding buffer and 100 mM sodium citrate buffer (pH 3.0) as eluting buffer.

The purified mAb samples were analyzed by size exclusion chromatography (SEC) using a Superdex 200 10/300 GL column (GE Healthcare Bio-Sciences) fitted to a HPLC system (Varian Inc., Palo Alto, CA, USA). The mobile phase was 20 mM phosphate buffer (pH 7.0) containing 150 mM NaCl, running at a flow rate of 0.2 ml/min.

The feed, flow through and eluate samples were also analyzed by SDS-PAGE (Lammli 1970). Both reducing (7.5%) and non-reducing (12.5%) gels were obtained using a Hoefer MiniVE device (GE Healthcare Bio-Sciences).

3.4 Results and discussion

The purification methods were chosen based on the biochemical properties of the mAbs and the other components present in the transgenic tobacco juice. IgG1 type mAbs are large macromolecules with molecular weight in the range of 146-155 kDa, having a Y-shaped structure composed of two heavy and two light chains. Protein A based column chromatography is widely used for purifying mAbs from mammalian cell culture supernatant (Hober et al. 2007; Shukla et al. 2007; Ghose et al. 2007). The suitability of PAMC as a single-step purification process was therefore first examined in our study. However, we anticipated that PAMC on its own would not be suitable for producing pure mAb since tobacco juice is significantly more complex (i.e. it has more chemical components) in nature than mammalian cell-culture supernatant. Moreover, we anticipated other problems such as severe membrane fouling and non-specific binding. Most IgG can be neutral or basic in nature with isoelectric point in the range of 6.0-9.0 (Amersham Pharmacia Biotech 2002). The binding test of tobacco proteins on Sartobind S membrane showed that pH increase from 5.0 to 6.0 greatly decrease the binding percentage, while further pH increase from 6.0 to 7.0 kept the binding at a low level. This indicated that tobacco proteins were mostly acidic in nature, which was also reported by other researchers (Balasubramaniam et al. 2003). Based on this observation we

hypothesized that IEMC could be utilized for mAb purification in combination with PAMC.

We first examined the possibility of using PAMC for purifying mAb from tobacco juice. Figure 3.2 shows the chromatogram obtained during the purification of hIgG1-CD4 from spiked tobacco juice, while Figure 3.3, shows the purification of anti-*Pseudomonas* mAb from transgenic tobacco juice. Both mAbs were eluted with 135 to 140 ml of effluent. Although the same feed volume was injected, the mAb elution peak obtained with transgenic tobacco juice was smaller than that with the spiked tobacco juice. The mAb recovery from spiked tobacco juice based on overall material balance was 93.3%. The recovery of mAb from the transgenic tobacco juice could not be accurately determined since the concentration in the feed was low and pre-concentration was required before analysis. However, assuming that specific absorbance of the two mAbs are similar, the mAb content in the transgenic tobacco, based on amount recovered, was approximately 0.42 mg per kg of leaves. Since the mAb concentration in the transgenic tobacco juice were near the limit of quantification, it was difficult to determine the effects of operating conditions on mAb purity and recovery. Hence, experiments to optimize the separation process were done using hIgG1-CD4 spiked tobacco juice.

The purity of the mAb samples from the PAMC experiments was determined using analytical Protein A chromatography (results not shown; see Table 3.1). The purity of mAb obtained from spiked tobacco juice was 92.7% while that from the transgenic tobacco juice was only 78.0%. The lower mAb purity obtained from transgenic tobacco indicated that purity was influenced by the low mAb concentration in starting material.

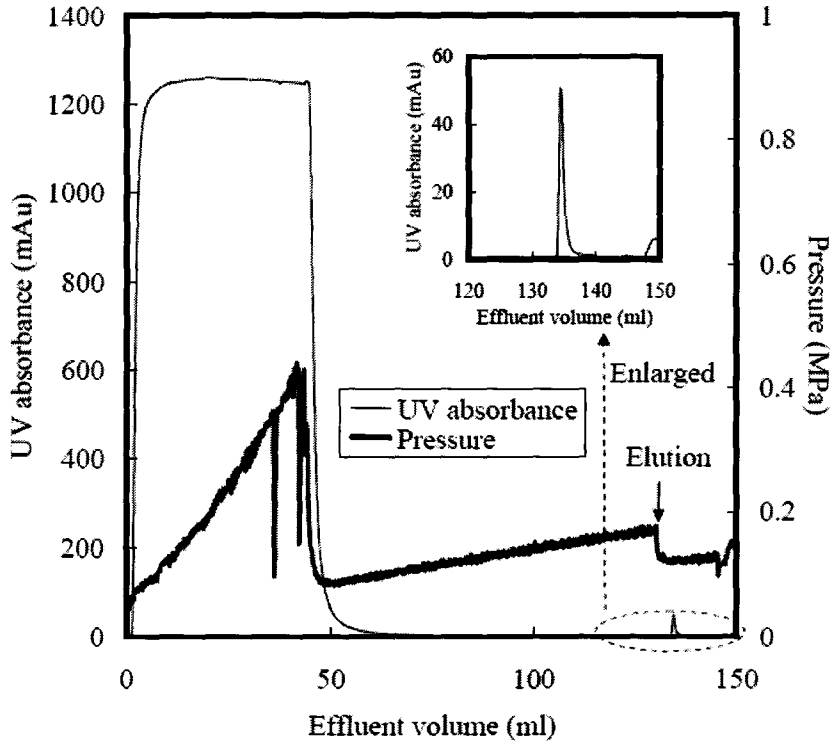


Figure 3.2 Protein-A membrane chromatography based single step purification of hIgG1-CD4 from spiked wild type tobacco juice (Binding buffer: 20 mM sodium phosphate, pH 7.0; eluting buffer: 100 mM citrate, pH 3.0; flow rate: 1 ml/min; inset: enlarged eluted peak).

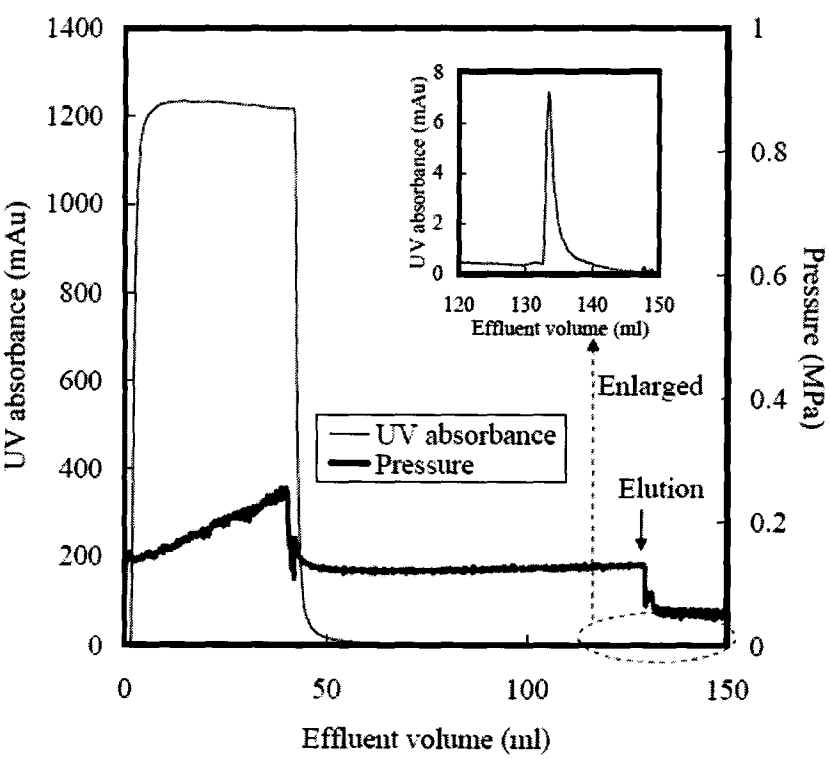


Figure 3.3 Protein-A membrane chromatography based single step purification of anti-*P.aeruginosa* mAb from transgenic tobacco juice (Binding buffer: 20 mM sodium phosphate, pH 7.0; eluting buffer: 100 mM citrate, pH 3.0; flow rate: 1 ml/min; inset: enlarged eluted peak).

Table 3.1 Comparison of single step PAMC and single step PACC mAb purification processes.

	Support material for protein A ligand	Binding capacity*	Recovery of hIgG1-CD4 from spiked sample	Purity of hIgG1-CD4 from spiked sample	Purity of anti *P. aeruginosa* from transgenic sample
PAMC	0.45μm cellulose membrane	2.9mg hIgG/ml	93.3%	92.7%	78%
PACC	Cross-linked 4% agarose beads	30mg hIgG/ml	79.4%	87.4%	73%

* Manufacturers specifications from GE Healthcare for rProtein A Sepharose 4 Fast Flow and Sartorius AG for Sartobind Protein A 75.

The flow-through and purified mAb samples from the PAMC experiments were further analyzed using SDS-PAGE. The non-reducing gel of the spiked tobacco juice shows the purified mAb as a single major band of ca. 150 kDa (Figure 3.4). The impurities which were eluted in the flow-through have low molecular weights, in the 25 to 45 kDa range. The reducing gel shows the purified mAb as two bands, corresponded to the 50 kDa heavy chains and the 25 kDa light chains (Figure 3.4). Figure 3.5 shows the reducing SDS-PAGE obtained with the flow-through and purified mAb from the transgenic tobacco juice PAMC experiment. Since the mAb concentration in the purified sample was low, the gel was developed using silver staining. The purified mAb shows the

characteristic heavy chain and light chain bands along with some other bands which are either impurities or degradation products.

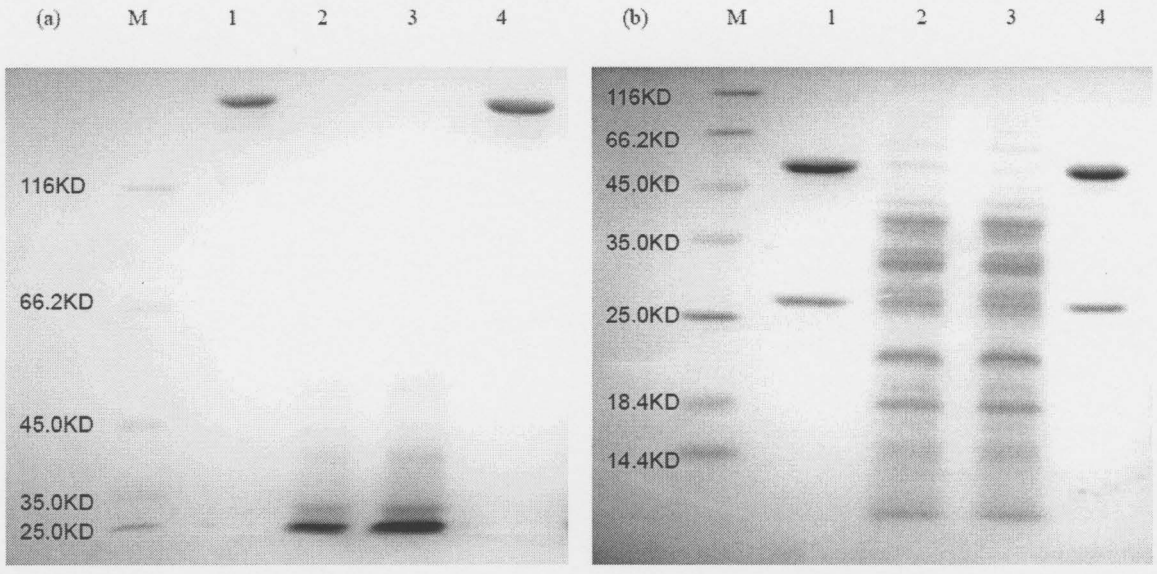


Figure 3.4 Coomassie blue stained SDS-PAGE of samples obtained in the PAMC process for purifying hIgG1-CD4 from spiked wild type tobacco juice: (a) 7.5% non-reducing gel, (b) 12.5% reducing gel (for both (a) and (b): M -marker; 1 - standard mAb; 2 - wild type tobacco juice spiked with mAb; 3- flow through; 4 - purified mAb).

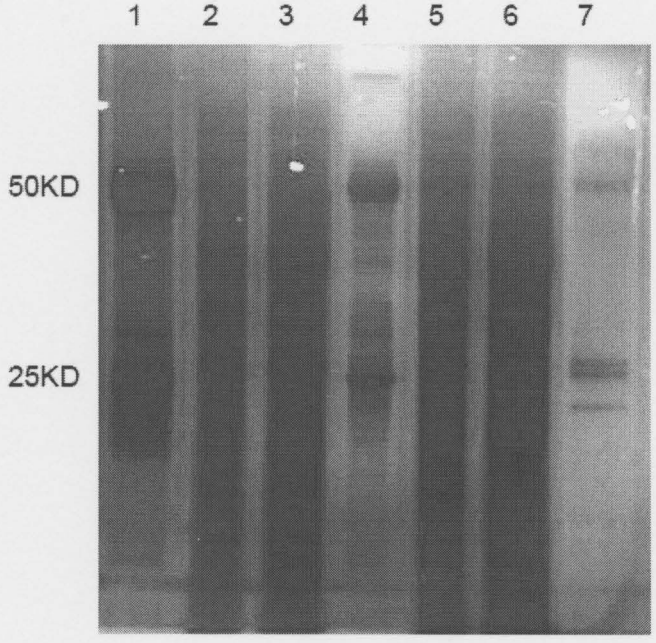


Figure 3.5 Silver stained 12.5% reducing SDS-PAGE of samples obtained in the PAMC processes for purifying hIgG1-CD4 and anti-*P. aeruginosa* mAb from spiked and transgenic tobacco juice (M - marker; 1 - standard mAb; 2, 3, 4 -feed, flow through and purified mAb respectively from spiked tobacco juice experiment; 5, 6, 7 - feed, flow through and purified mAb respectively from transgenic tobacco juice experiment.

PACC-based mAb purification experiments were also done using the spiked and transgenic tobacco juices. The operating conditions and feed volumes were similar to those used in the PAMC experiments. The mAb purified by PAMC from spiked tobacco juice and transgenic tobacco was purer than that obtained by PACC (Table 3.1). This was probably due to the higher specific surface area available on the agarose beads relative to that on the membrane, leading to higher non-specific adsorption of impurities. Furthermore, recovery of mAb from the spiked tobacco juice was higher with PAMC (93.3%) than with PACC (79.4%) (Table 3.1). This was probably caused by poorer elution from the Protein A beads. In the case of PAMC, elution is governed mainly by convective protein transport while with PACC there are significant amount of diffusion, particularly dead channel diffusion. This was confirmed by challenging both the Protein A column and the Protein A membrane module with same amount of pure hIgG1-CD4 (i.e. not in tobacco juice) as used in the purification experiments with spiked tobacco juice. The PAMC recovery was almost complete, i.e., 99.8%, while the PACC recovery was 89.5%.

On the basis of these results, one can conclude that PAMC was more efficient than PACC, i.e. the recovery and purity of mAb was greater with PAMC, particularly when spiked tobacco juice was used. However, there were two major disadvantages of using PAMC for this particular application, high-pressure drop and the tendency for the membrane to foul. Figures 3.2 and 3.3 show that the pressure drop was enhanced when tobacco juice was injected into the membrane module. This problem was particularly severe with the spiked tobacco juice. Also, the pressure drop increased quite significantly

from one run to the next, clearly indicating fouling. Fouling of chromatographic media by plant material is a commonly reported problem (Menkhaus et al. 2004). When the same membrane module was challenged with pure hIgG1-CD4, the pressure drop was reduced and fouling was not observed. Therefore, the Protein A membrane appears to be fouled by non-mAb proteins present in the tobacco juice. Based on these observations we concluded that PAMC was not suitable for purifying mAb directly from tobacco juice. However, if the tobacco juice was pre-processed using another technique, the Protein A membrane may be used without major pressure drop or fouling problems. Therefore, we decided to investigate IEMC as a first purification step to be followed by PAMC. The IEMC step would capture the mAb and remove many other tobacco proteins, thus reducing fouling of the Protein A membrane.

The purification of hIgG1-CD4 from spiked wild-type tobacco juice using IEMC was first examined. Binding tests carried out using pure hIgG1-CD4 showed that the mAb bound well to the Sartobind S membrane at pH 6.0 but binding decreased significantly at pH 7.0, while at pH 8.0 there was no binding. These results indicate that the isoelectric point of the mAb is between 7.0 and 8.0. It was also observed that a conductivity value lower or equal to 4 mS/cm favored binding. Ideal binding pH and conductivity could be achieved by one-fold dilution of the tobacco juice with 20 mM phosphate buffer (pH 6.0). Consequently, IEMC experiments were done using spiked tobacco juice adjusted accordingly (Figure 3.6). Most impurities flowed through the membrane without binding, while the mAb and some impurities bound to the membrane and were eluted using buffer B. The flow-through and partially purified mAb (i.e., the eluate) were analyzed using

analytical Protein A affinity chromatography (Figure 3.6 insets). It was thus determined that by using IEMC, the mAb purity could be increased from about 0.1% (in the feed) to 13-26% (in the eluate) with a recovery in excess 90%.

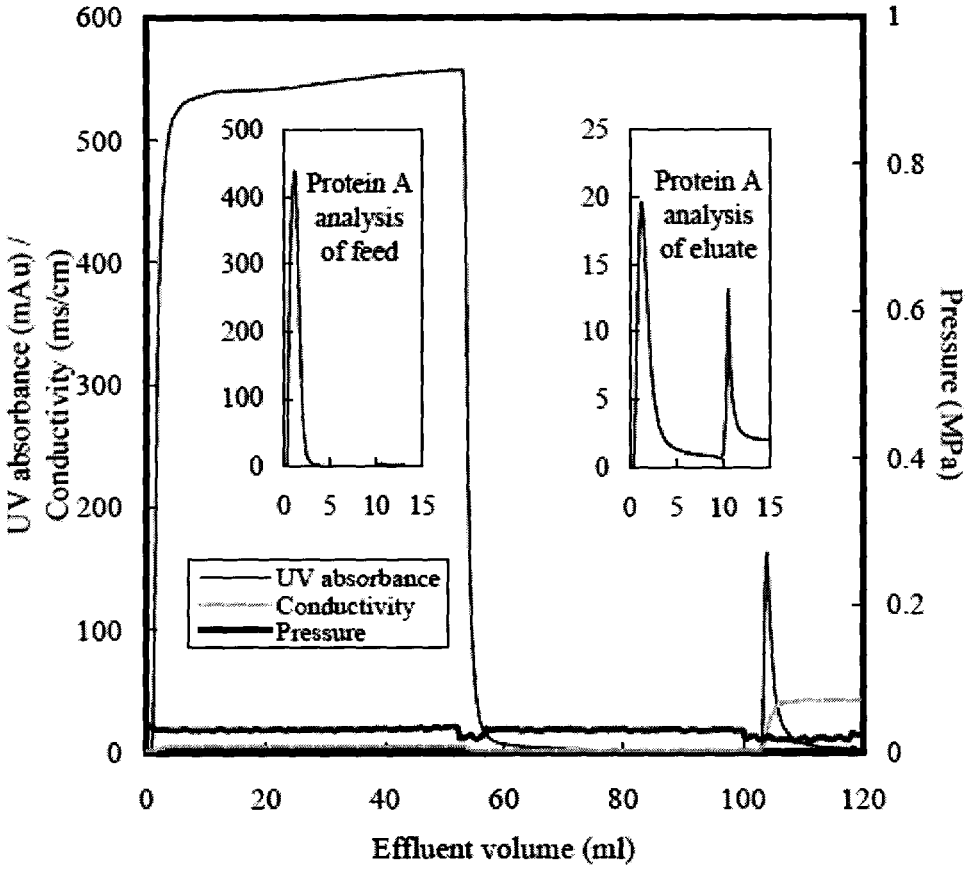


Figure 3.6 IEMC chromatogram for purification of hIgG1-CD4 from spiked tobacco juice (flow rate: 2 ml/min; binding buffer: 20 mM sodium phosphate buffer, pH 6.0; eluting buffer: binding buffer + 0.5 M NaCl; inset: analytical protein–A chromatograms for flow through and eluted peaks).

The isoelectric point of the anti-*P. aeruginosa* mAb expressed in the transgenic tobacco was theoretically calculated using Compute pI/MW tool (http://expasy.org/tools/pi_tool.html) to be ca. 8.25. The binding conditions used with the hIgG1-CD4 may not be very suitable for this mAb. Separate pH screening experiments using the Sartobind S membranes were therefore done with the transgenic tobacco juice.

The purity and relative recovery at various pH values are shown in Table 3.2. The relative recovery was calculated by assigning the highest recovery in the experimental range as 100%. Quite clearly, higher mAb purity was obtained at pH 7.0 than at pH 6.0 and 6.5. However, the maximum recovery was obtained at pH 6.0. Since the purpose of the IEMC step was to partially purify the mAb prior to the PAMC step, recovery was given priority. Thus, IEMC experiments were conducted using transgenic tobacco juice adjusted to pH 6.0. By using IEMC, the purity of anti *P. aeruginosa* mAb was increased from ca. 0.03% (in the juice) to ca. 11% (in the eluate) (See Figure 3.7). Furthermore, there was only a minor pressure drop with the Sartobind S membrane during sample injection, and after 20 purification cycles, the pressure drop remained below 0.05 MPa, indicating that there was little fouling. The life of the Sartobind S membrane was prolonged even further, i.e. beyond 20 purification cycles, by cleaning procedures using 0.1 M NaOH and 6M guanidine hydrochloride.

Table 3.2 pH influence on IEMC purification of anti P. aeruginosa mAb from transgenic tobacco.

pH	Purity of Purified mAb	Relative recovery of purified mAb
6.0	11.0%	100.0%
6.5	16.7%	61.0%
7.0	37.6%	64.0%

Number of Sartobind S discs: 15; Effective diameter: 18mm
Binding buffer: 20mM phosphate buffer (various pH); Elution buffer: binding buffer + 1 M NaCl

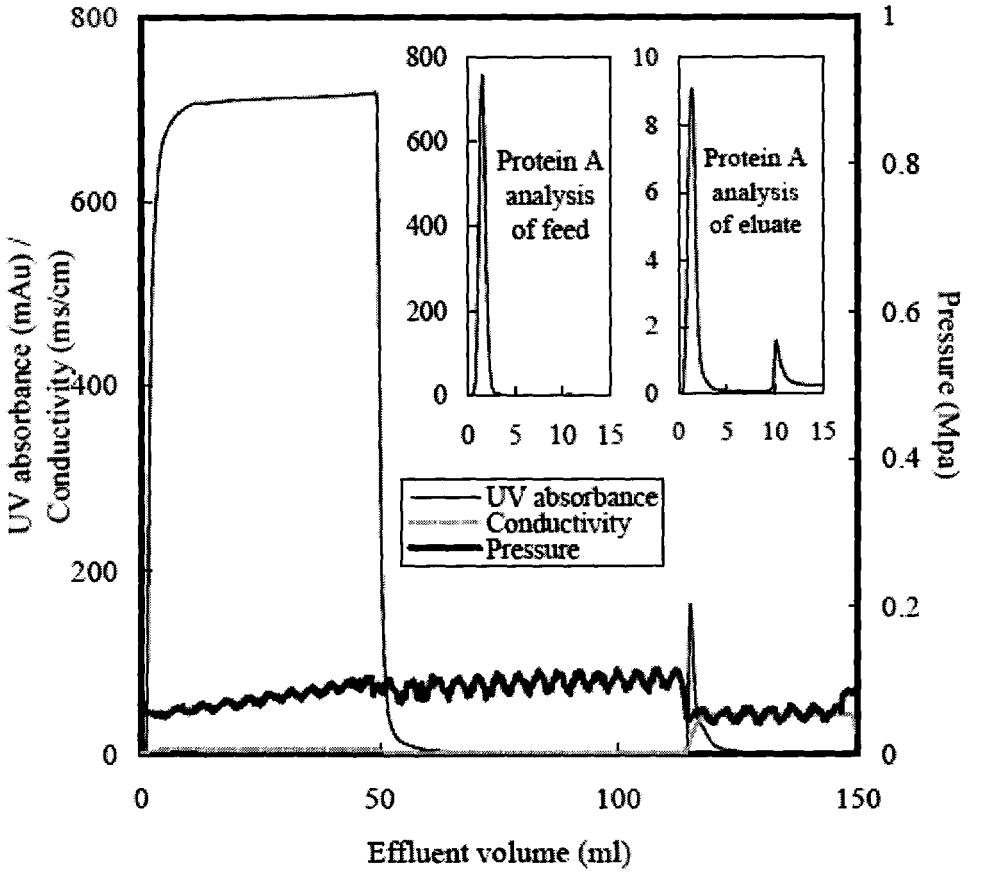


Figure 3.7 IEMC chromatogram for purification of anti-*P. aeruginosa* from transgenic tobacco juice (flow rate: 2 ml/min; binding buffer: 20 mM sodium phosphate buffer, pH 6.0; eluting buffer: binding buffer + 0.5 M NaCl; inset: analytical protein–A chromatograms for flow through and eluted peaks).

The eluate obtained from IEMC (i.e., from both spiked and transgenic tobacco juice) was further purified using PAMC (Figures 3.8 and 3.9). The pressure drop was low (ca. 0.02 MPa) in both experiments and did not increase during sample injection. Moreover, there was little or no pressure increase when tobacco juice was subjected to PAMC. Therefore, by using IEMC as the first purification step, the major problems associated with processing tobacco juice directly with PAMC, i.e., high pressure drop and membrane fouling, could be overcome.

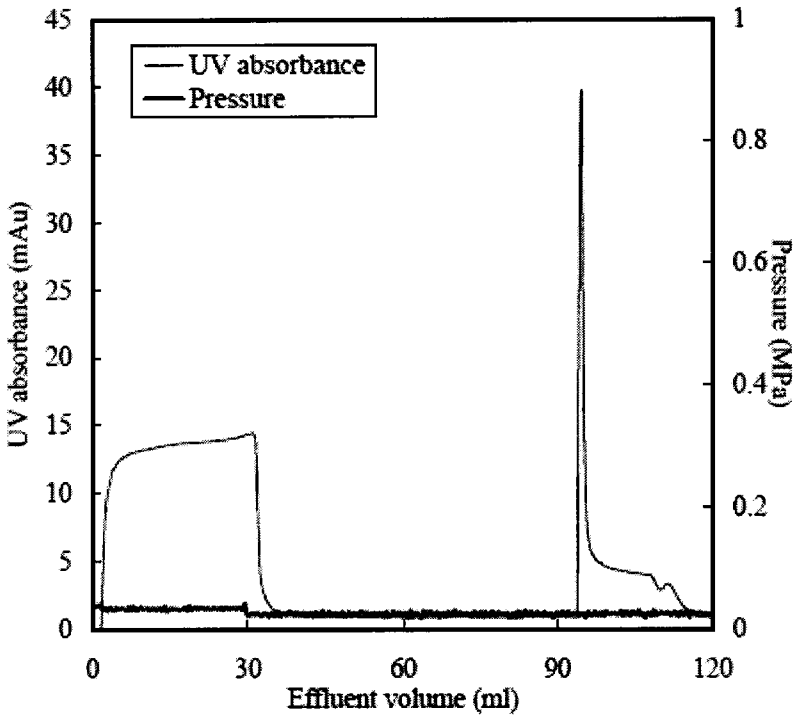


Figure 3.8 PAMC chromatogram for purification of hIgG1-CD4 from partially purified material obtained by IEMC (flow rate: 1 ml/min; binding buffer: 20 mM sodium phosphate buffer, pH 7.0; eluting buffer: 100 mM sodium citrate buffer, pH 3.0).

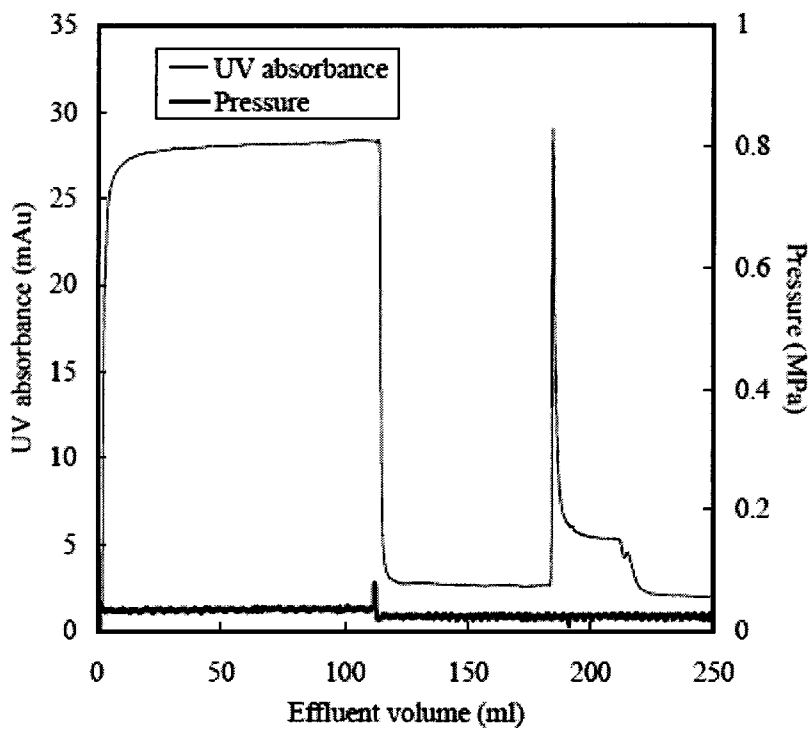


Figure 3.9 PAMC chromatogram for purification of anti-*P. aeruginosa* from partially purified material obtained by IEMC (flow rate: 1 ml/min; binding buffer: 20 mM sodium phosphate buffer, pH 7.0; eluting buffer: 100 mM sodium citrate buffer, pH 3.0).

Figure 3.10 shows the SEC-HPLC chromatograms obtained with the purified mAb samples obtained from both the spiked and transgenic tobacco juice using the two-step IEMC-PAMC process, i.e. juice was first subjected to IEMC after which PAMC was performed on the eluate from IEMC. The hIgG1-CD4 obtained from the spiked juice, after this two-step process, presented as a single peak, with a retention time (RT=56.2 minute) similar to that of the hIgG1-CD4 standard (RT=56.4 minute). The purified anti-*P. aeruginosa* mAb obtained from the transgenic tobacco juice sample presented as one major peak (RT=55.7 minute) along with a minor impurity or interference peak, indicating that the mAb was not completely pure following the two-step IEMC-PAMC process. The lower RT for the anti-*P. aeruginosa* mAb indicates that its molecular weight was greater than that of hIgG1-CD4.

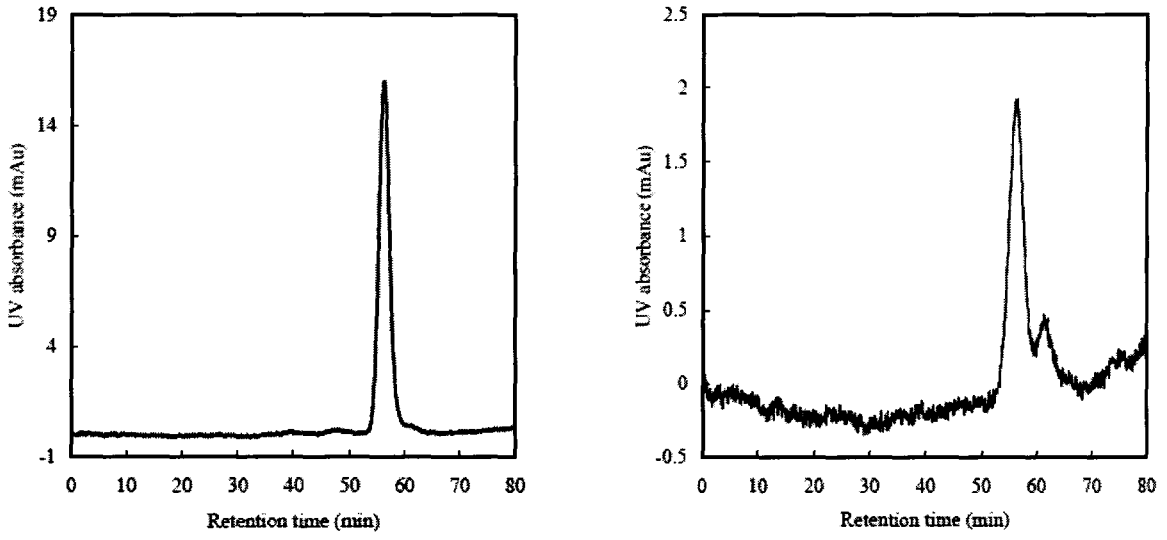


Figure 3.10 SEC chromatograms for purified mAb obtained by IEMC-PAMC two-step method (Left) hIgG1-CD4 obtained from spiked tobacco juice. (Right) anti-Pseudomonas obtained from transgenic tobacco juice (column: Superdex 75 10/300 GL; flow rate: 0.2 ml/min; mobile phase: 20 mM phosphate buffer, pH 7.0 + 150 mM NaCl.)

A molecular weight calibration for the SEC column was carried out using SEC standard proteins and a correlation for the partition coefficient (K_{av}) in terms of the molecular weight (M_r, Da) was obtained:

$$K_{av} = -1.4535 \, \mathrm{Log}(M_r) + 10.693 \qquad (1)$$

The square of the regression coefficient (R^2) for the above correlation was 0.9961. The K_{av} can be experimentally determined as shown below:

$$K_{av} = (V_e - V_o) / (V_t - V_o) \qquad (2)$$

Where V_e, V_o and V_t separately represent retention volume (ml), void volume (ml) and total bed volume (ml).

The molecular weights of hIgG1-CD4 and anti-*P. aeruginosa* mAb as determined using equations (1) and (2) were 201,443 Da and 210,617 Da, respectively. The predicted molecular weight of a human IgG1 type antibody is typically in the range of 150,000-160,000 Da, but this does not take post-translational modifications such as glycosylation into account (McLean et al. 2007). Another reason for the discrepancy is that the SEC calibration standard proteins used were regular shaped globular proteins while antibodies have their typical Y-shaped structure which makes it harder for them to penetrate the pores of the SEC medium, resulting in lower retention volumes than globular proteins having same molecular weights, thus biasing the molecular weight calibration.

Figure 3.11 shows the non-reducing (a) and reducing (b) SDS-PAGE results obtained with samples from the IEMC-PAMC purification experiments using spiked tobacco juice. By comparing lanes 1, 2 and 3 of both gels it can be seen that the IEMC step removed most of the impurities and resulted in high mAb recovery. The impurities

left behind in the IEMC eluate, which were mostly low molecular weight proteins, were removed following PAMC (see lanes 3, 4 and 5 of both gels; Figure 3.11). In Figure 3.11a the purified mAb appears as a single major band with some degradation products which may have been generated during sample preparation for electrophoresis. In Figure 3.11b the purified mAb appears as two bands, one corresponding to the heavy chain and the other for the light chain.

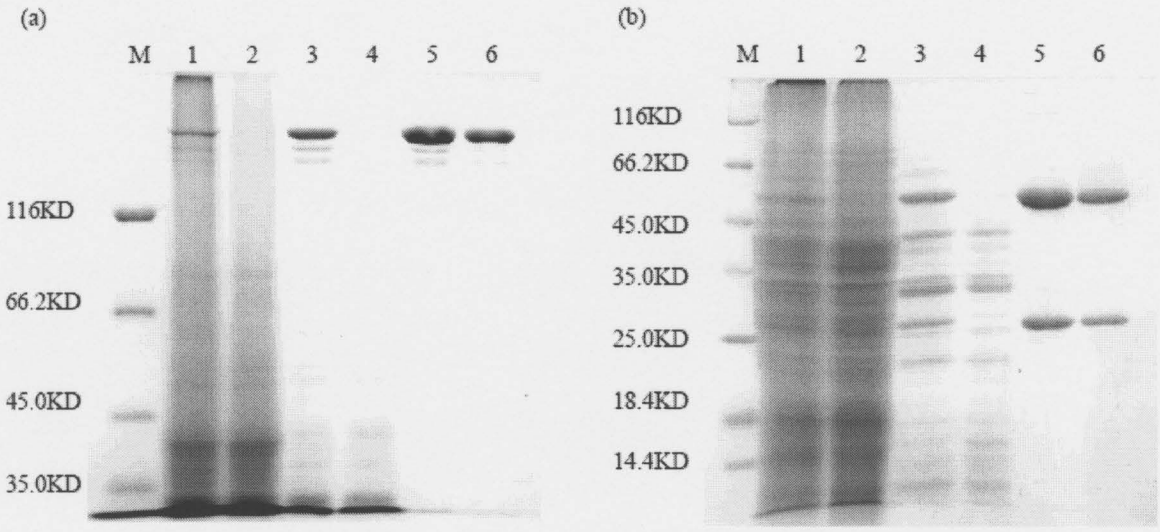


Figure 3.11 Coomassie blue stained SDS-PAGE of samples obtained in the IEMC-PAMC process for purifying hIgG1-CD4 from spiked tobacco juice: (a) 7.5% non-reducing gel, (b) 12.5% reducing gel (for both (a) and (b): M - marker; 1, 2 and 3 are feed, flow through and eluate respectively from IEMC experiment; 4 and 5 are flow through and eluate from PAMC).

Figure 3.12 shows the corresponding results obtained with samples from the IEMC-PAMC experiments using the transgenic tobacco juice. Lanes 1 of both Figure 3.12a and Figure 3.12b do not show the mAb band since its concentration in the starting material was very low. On the non-reducing gel (Figure 3.12a), the purified mAb obtained after PAMC presents as a single band (lane 5), which has higher molecular weight than standard hIgG1-CD4 mAb (lane 6). On the reducing gel (Figure 3.12b), the

purified mAb is reduced to the heavy chain and light chain (lane 5), which both show higher molecular weight than the corresponding bands in lane 6. The higher molecular weight of transgenic mAb than standard hIgG1-CD4 mAb in the gel is consistent with the SEC results discussed earlier. Plant protein glycosylation differ from mammalian protein glycosylation in terms of both sugar content and pattern (Shah et al. 2003). Tobacco expressed mAbs are normally glycosylated with high mannose carbohydrate content than mammalian derived Abs (McLean et al. 2007; Cabanes-Macheteau et al. 1999; Ko et al. 2003; Sriraman et al. 2004; Triguero et al. 2005). Therefore, the SEC-HPLC indicates that the glycosylation could increase the molecular weight by up to 10 kDa.

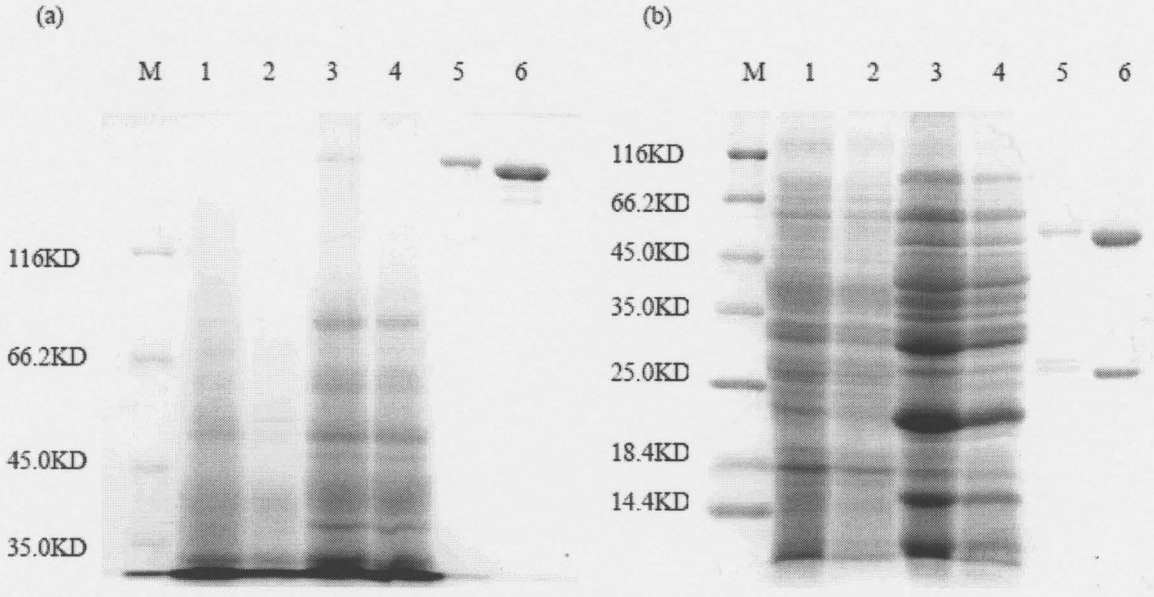


Figure 3.12 Coomassie blue stained SDS-PAGE of samples obtained in the IEMC-PAMC process for purifying anti-*P. aeruginosa* from transgenic tobacco juice: (a) 7.5% non-reducing gel, (b) 12.5% reducing gel (for both (a) and (b): M - marker; 1, 2 and 3 are feed, flow through and eluate respectively from IEMC experiment; 4 and 5 are flow through and eluate from PAMC).

The results described above clearly demonstrate that by using the two-step IEMC-PAMC process highly pure hIgG1-CD4 and anti-*P. aeruginosa* can be obtained from spiked tobacco and transgenic tobacco juice, respectively. The mAb recovery was calculated based on area under the curve obtained from SEC chromatograms. For hIgG1-CD4 the overall recovery from spiked tobacco juice was 80.4%. The corresponding value for anti-*P. aeruginosa* mAb was 88.9%. However, it should be noted that transgenic tobacco samples frequently had to be pre-concentrated by centrifugal ultrafiltration and this could potentially introduce errors in calculation. The higher recovery of anti-*P. aeruginosa* relative to hIgG1-CD4 may result from greater binding of anti-*P. aeruginosa* to the ion-exchange membrane because of its higher pI. The IEMC-PAMC purification process was demonstrated to be flexible, reproducible and potentially scalable.

Detailed characterization of purified mAb is essential to ensure activity and product safety. This includes tests for immunological activity of mAb, determination of extent of product degradation by proteases, tests for presence of tobacco DNA, proteins and alkaloid. IEMC used in our process would affect DNA removal which on account of negative charged would not bind on the cation-exchange membrane. The elimination of alkaloid by Protein A gel chromatography has been reported (Ko et al. 2004). A fast process, which characterizes membrane chromatography, would minimize action of proteases.

3.5 Conclusions

A comparison of PAMC and PACC showed the former to be better for mAb purification from tobacco juice, both in terms of product purity and recovery. The mAb purity obtained from spiked tobacco juice was greater than that obtained from transgenic tobacco juice, indicating that the mAb content of the starting material affected the purity of the final product. Overall, PAMC gave good purity and recovery of mAb but it was not suitable as an one-step process for purifying mAb directly from tobacco juice, primarily due to substantial pressure drop and membrane fouling problems caused by non-mAb tobacco proteins. IEMC was found to be suitable as a first purification step prior to PAMC. There was little or no pressure drop with IEMC and it resulted in substantial purification and high recovery of both hIgG1-CD4 and anti *P. aeruginosa* from tobacco juice. Thus, pretreatment of the juice by IEMC greatly reduced the impurity burden and fouling tendency during PAMC. The combination of IEMC and PAMC gave both high mAb purity and recovery from spiked as well as transgenic tobacco juice with low pressure drops, demonstrating that a flexible, reproducible and scalable bioprocess can be developed.

3.6 References

Almquist KC, McLean MD, Niu Y, Byrne G, Olea-Popelka FC, Murrant C, Barclay J, Hall JC. 2006. Expression of an anti-botulinum toxin A neutralizing single chain Fv recombinant antibody in transgenic tobacco. Vaccine 24:2079-2086.

Amersham Pharmacia Biotech. 2002. Antibody Purification Handbook. Uppsala. 55 p.

Balasubramaniam D, Wilkinson C, Cott KV, Zhang C. 2003. Tobacco protein separation by aqueous two-phase extraction J Chromatogr A 989:119-129.

Cabanes-Macheteau M, Fitchette-Laine AC, Joutelier-Bourhis C, Lange C, Vine NC, Ma JKC, Lerouge P, Faye L. 1999. N-Glycosylation of a mouse IgG expressed in transgenic tobacco plants. Glycobiology 9:365-372.

Castilho LR, Anspach FB, Deckwer WD. 2002. Comparison of affinity membranes for the purification of immunoglobulins. J Membr Sci 207:253-264.

Chen JG, Shimomura S, Sitbon F, Sandberg GR, Jones AM. 2001. The role of auxin-binding protein 1 in the expansion of tobacco leaf cells. Plant J 28:607-617.

Chu L, Robinson DK. 2001. Industrial choices for protein production by large-scale cell culture. Curr Opin Biotechnol 12:180-187.

Daniell H, Streatfield SJ, Wycoff K. 2001. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6:219-226.

Farid SS. 2006. Established bioprocesses for producing antibodies as a basis for future planning. Adv Biochem Eng Biotechnol 101:1-42.

Ghose S, Hubbard B, Cramer SM. 2007. Binding capacity differences for antibodies and Fc-fusion proteins on protein A chromatographic materials. Biotechnol Bioeng 96:768-779.

Ghosh R, Wang L. 2006. Purification of monoclonal antibody by hydrophobic interaction membrane chromatography. J Chromatogr A 1107:104-109.

Ghosh R, Wong T. 2006. Effect of module design on the efficiency of membrane chromatographic separation processes. J Membr Sci 281:532-540.

Ghosh R. 2002. Protein separation using membrane chromatography: opportunities and challenges. J Chromatogr A 952:13-27.

Hiatt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants. 1989. Nature 342:76-78.

Hober S, Nord K, Linhult M. 2007. Protein A chromatography for antibody purification. J Chromatogr B 848:40-47.

Horsman J, McLean MD, Olea-Popelka FC, Hall JC. 2007. Picloram Resistance in Transgenic Tobacco Expressing an Anti-Picloram scFv Antibody Is Due to Reduced Translocation. J Agric Food Chem 55:106-112.

Isaacs JD, Burrows N, Wing M, Keogan MT, Rebello PR, Watts RA, Pye RJ, Norris P, Hazelman BL, Hale G, Waldmann H. 1997. Humanized anti-CD4 monoclonal antibody therapy of autoimmune and inflammatory disease. Clin Exp Immunol 110:158-166.

Knudsen HL, Fahrner RL, Xu Y, Norling LA, Blank GS. 2001. Membrane ion-exchange chromatography for process-scale antibody purification. J Chromatogr A 907:145-154.

Ko K, Koprowski H. 2005. Plant biopharming of monoclonal antibodies. Virus Res 111:93-100.

Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S, Hanlon CA, Rupprecht C, Dietzschold B, Golovkin M, Koprowski H. 2003. Function and glycosylation of

plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci USA 100:8013-8018.

Ko K, Wei X, Crooks PA, Koprowski H. 2004. Elimination of alkaloids from plant-derived human monoclonal antibody. J Immunol Methods 286:79-85.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lai Z, Kimmel R, Petersen S, Thomas S, Pier G, Bezabeh B, Luo R, Schreiber JR. 2005. Multi-valent human monoclonal antibody preparation against Pseudomonas aeruginosa derived from transgenic mice containing human immunoglobulin loci is protective against fatal pseudomonas sepsis caused by multiple serotypes. Vaccine 23:3264-3271.

Li F, Zhou X, Yang X, Tressel T, Lee B. 2005. Current therapeutic antibody production and process optimization. Bioprocessing Journal 4:23-30.

Ma JK, Drake PM, Chargelegue D, Obregon P, Prada A. 2005. Antibody processing and engineering in plants, and new strategies for vaccine production. Vaccine 23:1814-1818.

Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, Van Dolleweerd C, Mostov K, Lehner T. 1995. Generation and assembly of secretory antibodies in plants. Science 268:716-719.

McLean MD, Almquist KC, Niu Y, Kimmel R, Lai Z, Schreiber JR, Hall JC. 2007. A human anti-pseudomonas aeruginosa O6ad IgG1 expressed in transgenic tobacco is

capable of recruiting immune system effector function in vitro. Antimicrob Agents Chemother 51:3322-3328.

Menkhaus TJ, Bai Y, Zhang C, Nikolov ZL, Glatz CE. 2004. Considerations for the Recovery of Recombinant Proteins from Plants. Biotechnol Prog 20:1001-1014.

Miki B, McHugh SG, Labbe H, Ouellet T, Tolman JH, Brandle JE, 1999. Transgenic tobacco: gene expression and applications. Biotechnol Agric For 45:336-354.

Olea-Popelka FC, McLean MD, Horsman J, Almquist K, Brandle JE, Hall JC, 2005. Increasing expression of an anti-picloram single-chain variable fragment (ScFv) antibody and resistance to picloram in transgenic tobacco (Nicotiana tabacum). J Agric Food Chem 53:6683-6690.

Phillips M, Cormier J, Ferrence J, Dowd C, Kiss R, Lutz H, Carter J. 2004. Performance of a membrane adsorber for trace impurity removal in biotechnology manufacturing, J Chromatogr A 1078:74-82.

Schillberg S, Fischera R, Emans N. 2003. 'Molecular farming' of antibodies in plants, Naturwissenschaften 90:145-155.

Schillberg S, Twymanb RM, Fischera R. 2005. Opportunities for recombinant antigen and antibody expression in transgenic plants—technology assessment, Vaccine 23:1764-1769.

Shah MM, Fujiyama K, Flynn CR, Joshi L. 2003. Sialylated endogenous glycoconjugates in plant cells. Nature Biotech 21:1470-1471.

Shukla AA, Hubbard B, Tressel T, Guhan S, Low D. 2007. Downstream processing of monoclonal antibodies—Application of platform approaches. J Chromatogr B 848:28-39.

Sriraman R, Bardor M, Sack M, Vaquero C, Faye L, Fischer R, Finnern R, Lerouge P. 2004. Recombinant anti-hCG antibodies retained in the endoplasmic reticulum of transformed plants lack core-xylose and core-α(1,3)-fucose residues. Plant Biotech J 2:279-287.

Stoger E, Sack M, Nicholson L, Fischer R, Christou P. 2005. Recent progress in plantibody technology. Curr Pharm Des 11:2439-2457.

Triguero A, Cabrera G, Cremata JA, Yuen CT, Wheeler J, Ramirez NI. 2005. Plant-derived mouse IgG monoclonal antibody fused to KDEL endoplasmic reticulum-retention signal is N-glycosylated homogeneously throughout the plant with mostly high-mannose-type N-glycans. Plant Biotech J 3:449-457.

Yu D, Chen X, Pelton RH, Ghosh R. 2008. Paper-PEG based membranes for hydrophobic interaction chromatography: purification of monoclonal antibody. Biotech Bioeng 99: 1434-1442.

Zhou JX, Tressel T, Gottschalk U, Solamo F, Pastor A, Dermawan S, Hong T, Reif O, Mora J, Hutchison F, Murphy M. 2006. New Q membrane scale-down model for process-scale antibody purification. J Chromatogr A 1134:66-73.

Zhou JX, Tressel T. 2006. Basic concepts in Q membrane chromatography for large-scale antibody production. Biotechnol Prog 22:341-349.

Chapter 4

Purification of Monoclonal Antibody from Tobacco Extract Using Membrane-Based Bioseparation Techniques

This chapter is organized based on a paper published in Journal of Membrane Science, 323: 159-166 (2008) by Deqiang Yu, Michael D. McLean, J. Christopher Hall, and Raja Ghosh.

4.1 Abstract

Transgenic plants offer a promising system for large-scale production of therapeutic proteins such as monoclonal antibodies (mAbs). This paper describes a membrane-based process suitable for purification of a humanized mAb expressed in tobacco. Most monoclonal antibody purification schemes rely on the use of Protein A as the affinity ligand for antibody capture. The main objective of our work was to develop non-Protein A-based purification methods to avoid some of the problems and limitations

associated with this ligand, e.g. cost, immunotoxicity, and antibody aggregation during elution. Ion exchange membrane chromatography (IEMC) was used for primary capture and preliminary purification of the mAb from tobacco juice. Hydrophobic interaction membrane chromatography (HIMC) was then used for high resolution purification, followed by ultrafiltration for polishing, desalting and buffer exchange. Using this scheme, both high mAb purity (single peak in size exclusion chromatogram, i.e. ca 100% purity) and high recovery (77% of mAb spiked into the tobacco extract) were achieved. Membrane chromatography is generally considered unsuitable for resolving bound proteins by gradient elution and is therefore commonly used in the bind and elute mode with a single step change of mobile phase. We show that the gradient elution process in the HIMC step can be optimized to increase the resolution and thereby obtain product of high purity.

4.2 Introduction

Molecular farming of recombinant proteins by expression in genetically modified plants provides an alternative production route for biopharmaceuticals such as monoclonal antibodies (mAb) (Schillberg et al. 2005; Ma et al. 1995 and 2005; Ko and Koprowski 2005; Hiatt et al. 1989; McLean et al. 2007; Olea-Popelka et al. 2005; Almquist et al. 2006). This approach offers reduced contamination risks from animal viruses, potential for reduced cost and simplified scale-up of the manufacturing process (Daniell et al. 2001; Farid 2006; Stoger et al. 2005). Bioseparation is recognized as the bottleneck in molecular farming since the target protein has to be separated from

impurities such as starch, cellulose, sugars, alkaloids, pigments, polyphenols, native plant proteins, and nucleic acids which are present in the plant homogenate (Schillberg et al. 2003). Furthermore, recombinant protein purification from genetically modified plants is challenging due to the low expression levels of transgenic proteins *in planta* (i.e. mg/kg), resulting in the need for processing huge quantities of biomass. This implies that the overall purification process must combine high-resolution with high-throughput.

Protein A- and/or Protein G-based column chromatography is widely used for purifying mAbs from mammalian cell-culture supernatant as well as polyclonal antibodies from serum. The major limitations with column chromatography, particularly those processes employing soft gel-based media are the need to use low flow-rates to avoid back pressure, bed compaction, high buffer consumption, low product throughput, and scalability problems. Protein A/G based affinity media is widely available in the soft-gel bead format. In such chromatographic media, diffusion-based mass transport within the gel beads predominates and this makes the separation process slow. Furthermore, specific problems associated with the use of Protein A include the leakage of this ligand, which is immunotoxic, into the product (Carlson et al. 2005) and the need to elute column bound mAb under acidic conditions, which may lead to antibody aggregation (Arakawa et al. 2004).

Membrane-based bioseparation processes can be made to combine high-resolution separation with high product throughput, which may be conducted under relatively benign operating conditions thereby ensuring product stability (Ghosh 2002). With membrane chromatography this is achieved by combining the advantages of membrane technology

(which gives high throughput) with those of chromatography (which gives high resolution). In column chromatography, both binding efficiency and separation are dependent on diffusive transport, thus mobile phase flow rate may restrict productivity and make scale-up difficult. In contrast, binding and separation in membrane chromatography depend upon convective transport (i.e., are generally independent of flow-rate), over a wide operating range (Ghosh 2002). High flow-rates, shorter process times and easy scale-up are therefore achievable in membrane chromatography.

Different types of membrane chromatographic processes have been successfully used for purification of both serum-based polyclonal and cell culture-based monoclonal antibodies (i.e., Protein A based affinity membrane chromatography (Zou et al. 2001; Castilho et al. 2002), ion exchange membrane chromatography (IEMC) (Knudsen et al. 2001; Philips et al. 2004), and hydrophobic interaction membrane chromatography (HIMC) (Ghosh 2001 and 2005; Ghosh and Wang 2006; Yu and Chen et al. 2008). However, only one paper discusses purification of recombinant monoclonal antibodies from plants using membrane chromatography (combination of protein A and ion exchange membrane chromatography) (Yu and McLean et al. 2008). This previous paper described the purification of mAb from transgenic tobacco leaves using IEMC in combination with Protein A membrane chromatography (PAMC). As discussed earlier, the use of a Protein A leads to some limitations and there is a growing trend in the bio-industry to examine and develop alternatives to Protein A based purification methods. The current work deals with purification of humanized mAb from tobacco extract using a combination of three non-Protein A-based methods i.e. IEMC, HIMC and ultrafiltration

(UF). A simulated transgenic tobacco juice feedstock was used in our current work. This was prepared by spiking juice obtained from wild-type tobacco leaves with humanized monoclonal antibody hIgG1-CD4. In our purification scheme IEMC was used to capture and enrich the mAb, HIMC was then used to further purify the mAb, while ultrafiltration was used as the last step to polish and desalt the purified protein. Each of these separation steps were extensively optimized to obtain high resolution. The current work examines the possibility of fine tuning the gradient elution process in the HIMC for increasing the separation efficiency.

4.3 Experimental

4.3.1 Materials

Humanized mAb hIgG1-CD4 (batch 12) by CHO cell culture was kindly donated by the Therapeutic Antibody Center, University of Oxford, UK. The samples were shipped in dry ice (each vial containing 10 mg mAb in 2.1 mL buffer) and were used as received. Wild-type tobacco plants were grown in a greenhouse (University of Guelph, Ontario, Canada), the leaves were harvested from healthy plants and stored in a freezer at -20℃. Chemicals including sodium phosphate (mono- and di-basic), ammonium sulphate, sodium citrate, citric acid and sodium chloride were purchased from Sigma-Aldrich, St. Louis, MO, USA. All buffers and protein solutions were prepared using ultra-pure water (18.2MΩ-cm) obtained from a Diamond Nanopure water purification unit (Barnstead International, Dubuque, IA, USA) and micro-filtered through 0.45 μm membranes (product# DS0210-4045; Nalgene Nunc, Rochester, NY, USA). Sartobind S membranes

(catalogue number 94IEXS42-001) used for IEMC were purchased from Sartorius AG, Goettingen, Germany; polyvinylidine fluoride (PVDF) membrane discs (0.22 μm GVWP14250) from Millipore, Billerica, MA, USA; and 70 kDa polyethersulfone (PES) ultrafiltration membrane (part number OT070) from Pall, East Hills, NY, USA.

4.3.2 Preparation of tobacco juice

The frozen wild type leaves were manually crushed into small pieces within their plastic storage bags and then mixed with extraction buffer (40 mM phosphate buffer, pH 7.0, containing 50 mM ascorbic acid as antioxidant, 50 μM leupeptin as protease inhibitor, and 10 mM disodium EDTA as chelating agent). The leaves were ground in a jar type blender (51BL32, Waring Commercial, Torrington, CT, USA) for three minutes and ground further in a homogenizer (ULTRA TURRAX T25 Basic, IKA Works, Staufen, Germany) for another three minutes. The homogenate was pre-filtered through cheese cloth to remove large particles, centrifuged at 10528 × g using a Allegra refrigerated centrifuge (X22R, Beckman, Fullerton, CA, USA) for 30 minutes and micro-filtered through a 42 mm diameter membrane disc (0.45 μm pore size, PES) housed in a custom-designed stirred cell at a permeate flux value of 3.0×10^{-5} m^3/m^2 s. The clarified tobacco juice was stored at -20℃ and thawed just prior to purification.

4.3.3 Ion-exchange membrane chromatography

The membrane discs were housed in a custom-designed membrane module (Ghosh and Wong 2006) having a 18 mm effective diameter. The module was integrated with an AKTA prime liquid chromatography system (GE Healthcare Biosciences,

Uppsala, Sweden). The effluent from the membrane module was continuously monitored for absorbance (280 nm), pH and conductivity; the data was logged into a computer using Prime View software (GE Healthcare Biosciences). The system pressure was also continuously monitored and recorded by the AKTA prime system.

IEMC was carried out using a membrane module which contained a stack of 15 Sartobind S discs, each having an effective diameter of 18 mm. The pH of the feed solution was first optimized using 20 mM sodium phosphate buffer (PB) adjusted to different pH values. The corresponding elution buffers were prepared by adding 0.5 M NaCl to the binding buffer. The effect of pH on mAb binding was studied by injecting 5 mL of 18.9 μg/mL hIgG1-CD4 solution prepared in the appropriate binding buffer. The binding of tobacco proteins on the ion exchange membrane was tested by injecting 5 mL of tobacco juice diluted 1:1 (v/v) with the binding buffer. The effect of feed conductivity on mAb and tobacco protein binding on the membrane was examined at the optimized pH value, with the conductivity of the buffer being adjusted by addition of sodium chloride. The flow rate used in the pH and conductivity optimization experiments was 1 mL/min. Simulated transgenic tobacco feed solution was prepared by spiking wild-type tobacco juice with hIgG1-CD4 (10 mg of hIgG1-CD4 per kg of frozen leaves). Prior to the purification experiments, the tobacco simulated feed solution was diluted 1:1 (v/v) with the appropriate binding buffer (i.e. at optimized pH and conductivity). Different volumes of the simulated transgenic tobacco feed were injected and eluted to determine mAb binding efficiency from the corresponding feed solution. The flow rate used in these experiments was 2 mL/min since this gave good separation at reasonably low

transmembrane pressure. In these experiments, the membrane module was first equilibrated with binding buffer followed by injection of the feed. The unbound material was then removed from the membrane module by washing with binding buffer followed by removal of bound material using eluting buffer. The effects of applying different types of NaCl gradients on the purity of eluted mAb were examined. The flow-through and "elution peak" samples from the IEMC experiments were collected and analyzed by analytical Protein A affinity chromatography and SDS-PAGE.

4.3.4 Hydrophobic interaction membrane chromatography

Chromatography system used for HIMC was the same as IEMC. Membrane modules for HIMC had both 18-mm and 42-mm effective diameters. Feed solutions for HIMC were prepared by mixing in 1:1 ratio the IEMC eluate with concentrated ammonium sulfate solution of the appropriate molar concentration, adjusted to the appropriate pH. Initial experiments for optimizing of ammonium sulfate concentration and pH in the binding buffer for HIMC purification were conducted using a membrane module fitted with stacks of 15 PVDF membrane discs, each having a 18-mm effective diameter. All subsequent HIMC experiments were done using a larger membrane module which housed 15 or 30 membrane discs each having a 42-mm effective diameter. The flow rates used were 1 mL/min and 5 mL/min, respectively, for the 18-mm and 42-mm membrane module. All the feed solutions were prepared using the same ammonium sulfate concentration and pH as used for the binding buffers. The bound mAb was eluted from the PVDF membrane using ammonium sulfate free 20 mM PB. As in the IEMC purification experiments, the effect of gradient type on the purity of eluted mAb were

examined. The flow-through and eluted peak samples from the HIMC experiments were analyzed by Protein A affinity chromatography and SDS-PAGE.

4.3.5 Ultrafiltration

A custom-designed stirred cell module fitted with a 70 kDa PES membrane disc having an effective diameter of 18 mm was used in ultrafiltration experiments. The volume of the stirred cell was 8 mL and the stirring rate within it was maintained at 600 rpm. The permeate was drawn by suction using a peristaltic pump (101U, Watson Marlow) and was passed through the on-line detection suite of an AKTA Prime system for monitoring UV absorbance, conductivity and pH. The HIMC-purified mAb was fed into the stirred cell and diafiltered at constant volume by using 20 mM sodium phosphate buffer (pH 7.0) to replace volume lost in the permeate. When the conductivity of the permeate decreased to the same level as the replacement buffer, further buffer addition was stopped and the content of the stirred cell was concentrated to the minimum volume that could still be stirred. The UF desalted and polished sample was analyzed by SDS-PAGE and high performance size exclusion chromatography (HPSEC).

4.3.6 Sample analysis

A HiTrap Protein A HP affinity column (GE Healthcare Biosciences) was used for analyzing the purity of the mAb samples obtained by membrane chromatography. These affinity chromatography experiments were run at 1.0 mL/min flow rate with 20 mM phosphate buffer (pH 7.0) as binding buffer and 100 mM sodium citrate buffer (pH 3.0) as elution buffer. The samples obtained from IEMC, HIMC and UF experiments were

analyzed by 12.5% reducing SDS-PAGE (Laemmli et al. 1970). These electrophoresis experiments were run using a Hoefer MiniVE device (GE Healthcare Biosciences). The UF purified samples were analyzed by HPSEC using a Superdex 200 10/300 GL column (GE Healthcare Biosciences) fitted to a Varian HPLC system (Varian Analytical Instruments). The HPSEC mobile phase was 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl. The flow rate was maintained at 0.2 mL/min.

4.4 Results and discussion

The scheme for mAb purification from tobacco juice is shown in Fig. 4.1. These separation and purification methods were chosen based on the physicochemical properties of the mAb and the other constituents present in tobacco juice (i.e. impurities). Tobacco proteins are mostly known to be acidic, i.e. with low pI values, while mAbs are largely neutral or basic proteins, hIgG1-CD4 having a pI at ca. 7.5 (Yu and McLean et al. 2008; Balasubramaniam et al. 2003). Therefore, IEMC of the cation-exchange type could potentially be used to capture the mAb while allowing most tobacco proteins to pass through the membrane. Cation-exchange membrane having a pore size of 3 μm was chosen to minimize membrane fouling (Yu and McLean et al. 2008). Our previous work on HIMC (Ghosh 2005; Ghosh and Wang 2006; Yu and Chen et al. 2008) has shown that antibodies are quite hydrophobic in nature and can, therefore, be purified from serum and cell culture supernatant based on differences in hydrophobicity. The partially purified mAb obtained from the IEMC was further purified using HIMC. Since most potential foulants were removed by the IEMC, a PVDF membrane having 0.22 μm pore size was

used for the HIMC experiments. Our previous work has shown that tobacco proteins are, for the most part, much smaller molecules than mAb (Yu and McLean et al. 2008). Therefore, UF was used as the third purification step to remove salts (added by the HIMC method) as well as small proteins and their fragments. A polyethersulfone (PES) ultrafiltration membrane having a 70 kDa molecular weight cut-off was used for the UF experiments since this membrane should retain the mAb while allowing the impurities to pass through the membrane.

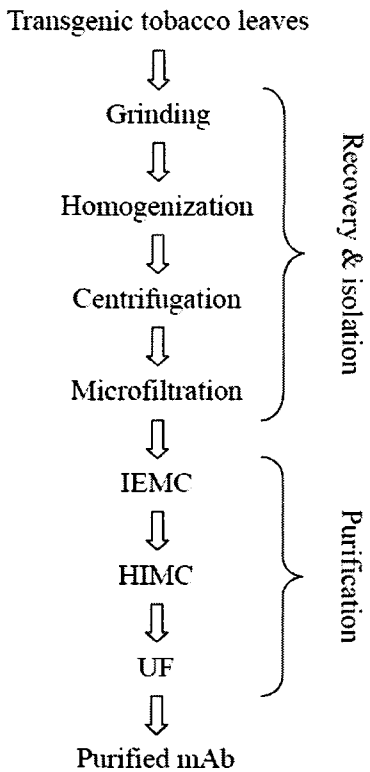


Figure 4.1 Scheme for mAb purification from transgenic tobacco leaves. The mAb was added after "Recovery & Isolation" for obtaining the simulated transgenic tobacco extract.

The effect of pH (pH 5 to 7 range) on binding of mAb and tobacco proteins on the Sartobind S membrane used for IEMC was investigated. The quantities of bound protein or mAb were expressed as percentage of total protein injected. Furthermore, the quantity

of protein injected in these rapid experiments was in excess of the binding capacity of the membrane stack, to examine the effect of pH on reversible binding of the mAb and other proteins to the membrane (Fig. 4.2). The percentage of injected mAb bound was found to be ca. 97% when the pH ranged from 5.0 to 6.0 but decreased to 66.5% at pH 6.5 and from there to 9.1% at pH 7.0. These data suggest that the positive charge on hIgG1-CD4 diminishes as the pH approaches 7.5, which is the pI of the mAb. Furthermore, the percentage of tobacco proteins bound to the membrane decreased sharply from pH 5.0 to 6.0 and remained low between pH 6.0 and 7.0. These results indicate that most tobacco proteins are acidic, with pIs less than pH 6.0. A pH value of 6.0 was therefore chosen for the IEMC since this pH resulted in high mAb and low tobacco protein binding.

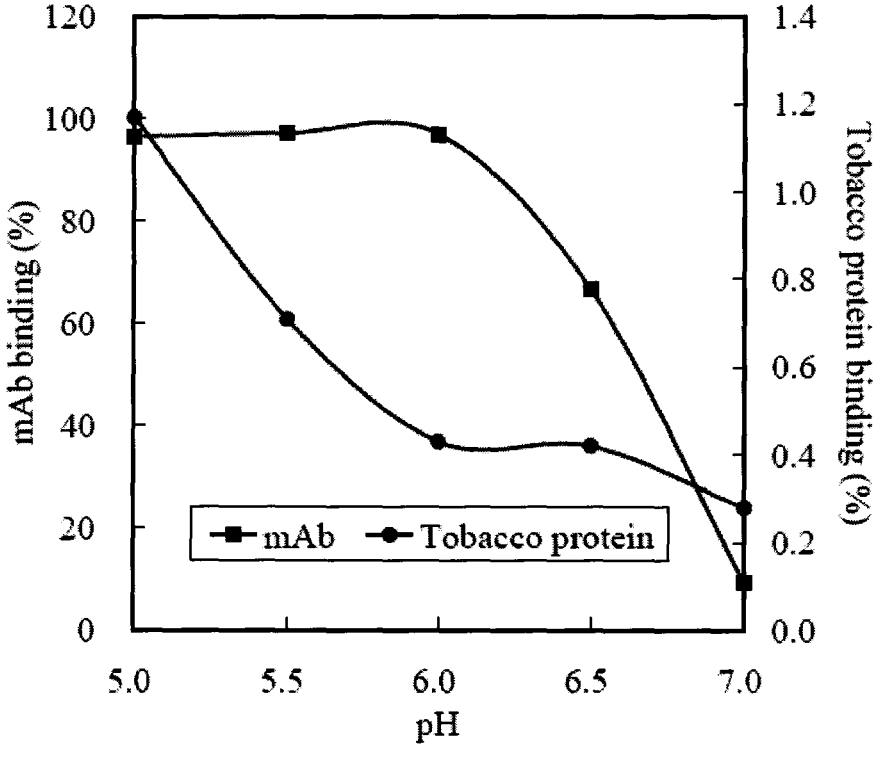


Figure 4.2 Effect of pH on the binding of hIgG1-CD4 and tobacco proteins to a Sartobind S cation-exchange membrane. The percentage of mAb or tobacco proteins bound was determined from the unbound and eluted peak areas.

The effect of conductivity of the feed solution on the binding of mAb to the Sartobind S membrane during IEMC was examined at pH 6.0. As shown in Fig. 4.3, mAb binding remained close to 95% in the 1.5 to 4.0 mS/cm range, but decreased thereafter, indicating that a conductivity value higher than 4.0 mS/cm shielded the electrostatic interactions between the mAb and the membrane and thereby reduced binding. Thus a conductivity value of 4.0 mS/cm was chosen. This conductivity was achieved by mixing tobacco juice and 20 mM sodium phosphate buffer (pH 6.0) in a 1:1 ratio.

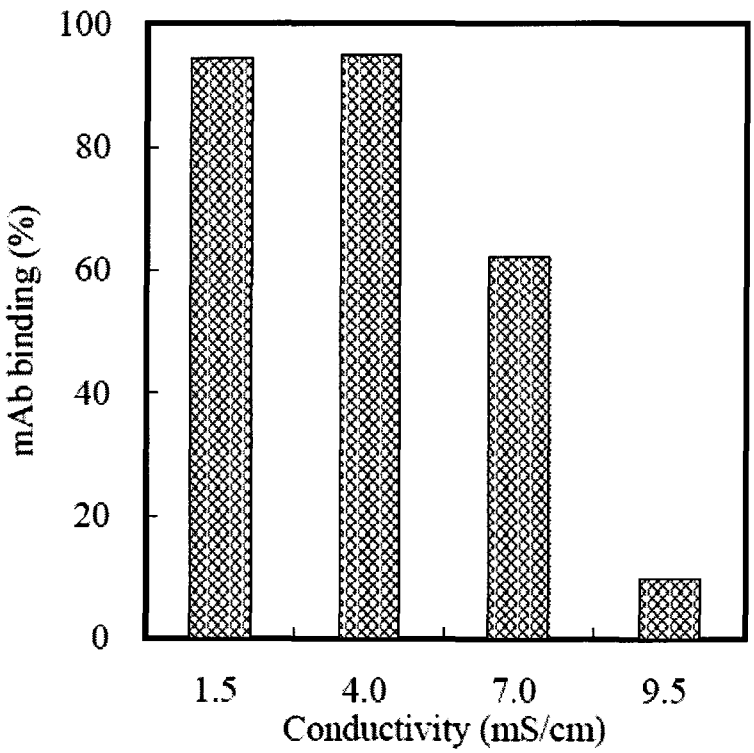


Figure 4.3 Effect of conductivity on binding of hIgG1-CD4 to a Sartobind S ion exchange membrane. The percentage of mAb bound was determined from the unbound and eluted peak areas.

Different volumes of simulated tobacco feed solution containing 1.89 μg hIgG1-CD4 /ml were injected to determine the effect of feed volume on mAb binding on the Sartobind S membrane. For feed volumes of 15, 30 and 45 mL the purity and recovery remained unchanged, i.e. 20% and 90%, respectively, but at feed volumes greater than 45 mL, both recovery and purity diminished. Therefore in all subsequent IEMC purification experiment, 45 mL of feed was used. Fig. 4.4 shows the IEMC chromatogram obtained using a short linear gradient elution in which the switchover from binding to eluting buffer was made over 20 mL of mobile phase flow. Three fractions were collected during mAb elution as indicated in Fig. 4.4. Each of the three eluate samples (E1, E2, E3) were subjected to analysis by Protein A affinity chromatography; the purity of the mAb in the respective samples were 26%, 35% and 10% (see Fig. 4.5). The initial purity of hIgG1-CD4 in the simulated feed as determined using Protein A affinity chromatography was 0.1%. Therefore, the purification factors obtained by IEMC in the three eluate samples were 260, 350 and 100, respectively. Since there was a significant quantity of mAb present in each of the three fractions, they were pooled, adjusted to appropriate salt concentration and pH, and used as feed for the HIMC experiments. Thus the mean purification factor obtained with IEMC was 130 and the overall mAb purity of the feed solution used in the HIMC experiments was 13%.

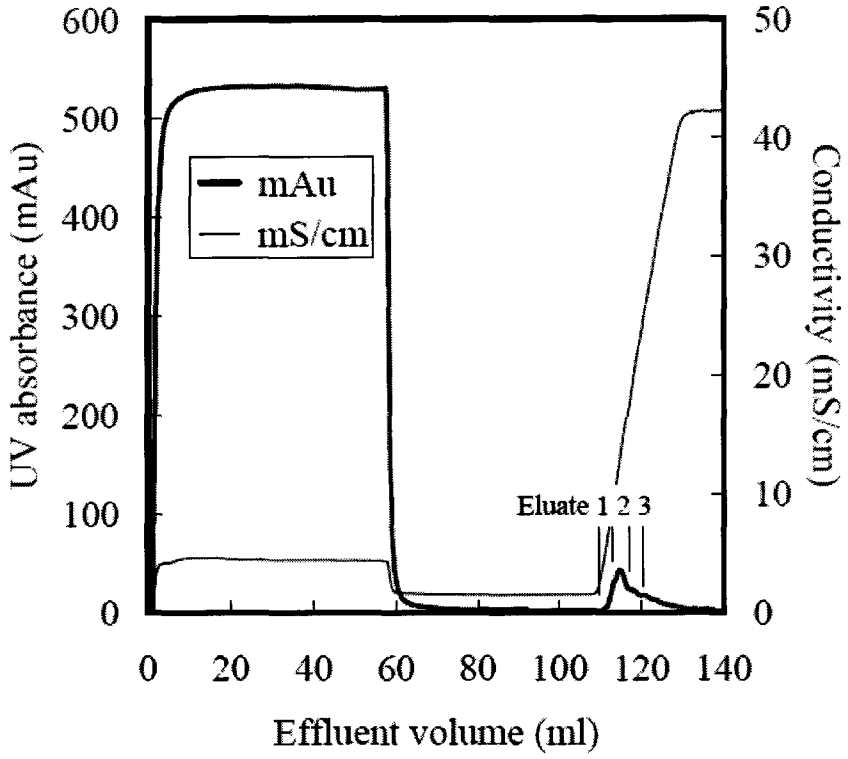


Figure 4.4(Up) Purification of hIgG1-CD4 from simulated transgenic tobacco feed by IEMC with linear gradient elution (eluate samples E1, E2, and E3 are indicated on the chromatogram).

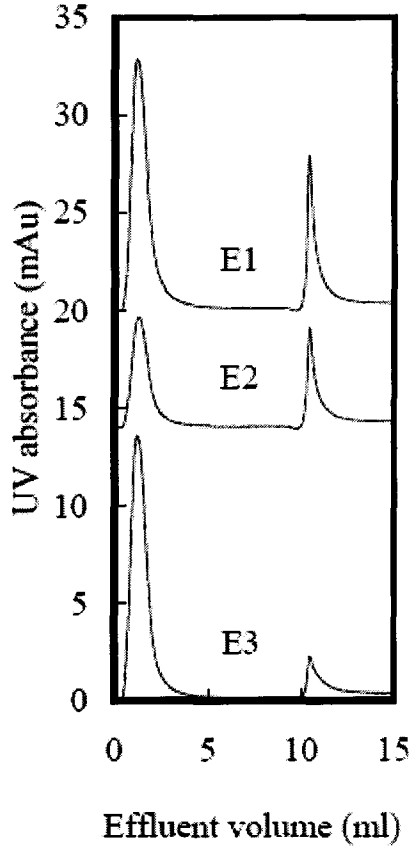


Figure 4.5(Right) Protein A affinity analysis of eluate fractions E1, E2 and E3 obtained from the IEMC purification experiment. The purity of mAb in these samples were determined by comparing the areas of the first peak (impurities) with the second peak (mAb).

Hydrophilized PVDF membrane (0.22 μm GVWP) was chosen for carrying out the HIMC experiments based on initial screening experiments (not discussed). Membranes made from PVDF are intrinsically hydrophobic but can be hydrophilized using appropriate surface treatment methods (Hester et al. 2002). Such membranes are marketed by membrane manufactures as "hydrophilic" PVDF membranes. Ghosh (2001) showed that hydrophilic PVDF membranes were suitable for monoclonal antibody purification using HIMC since these membranes were hydrophobic in the presence of anti-chaotropic salts such as ammonium sulfate but were quite hydrophilic in the absence of such salts. In the research presented here, feed solutions used in the HIMC experiments were prepared by mixing (1:1 v/v) the IEMC eluate with concentrated ammonium sulfate solution of the appropriate molar concentration. The salt concentration in the feed solution was systematically changed to determine its effect on purity and recovery. The relative recovery was calculated by assigning the highest recovery value in the experimental range as 100%, i.e. 1.9 M = 100%. As shown in Fig. 4.6, both the purity and recovery continuously increased when the ammonium sulfate concentration was increased from 1.4 M to 1.7 M. Between 1.7 and 1.9 M ammonium sulfate concentration, there was little or no increase in recovery but the purity tended to decrease significantly. Furthermore, there was a continuous increase of mAb binding as salt increased from 1.4 to 1.7 M; little increase thereafter. Conversely, the binding of most tobacco proteins present in the IEMC eluate began at 1.7 M.

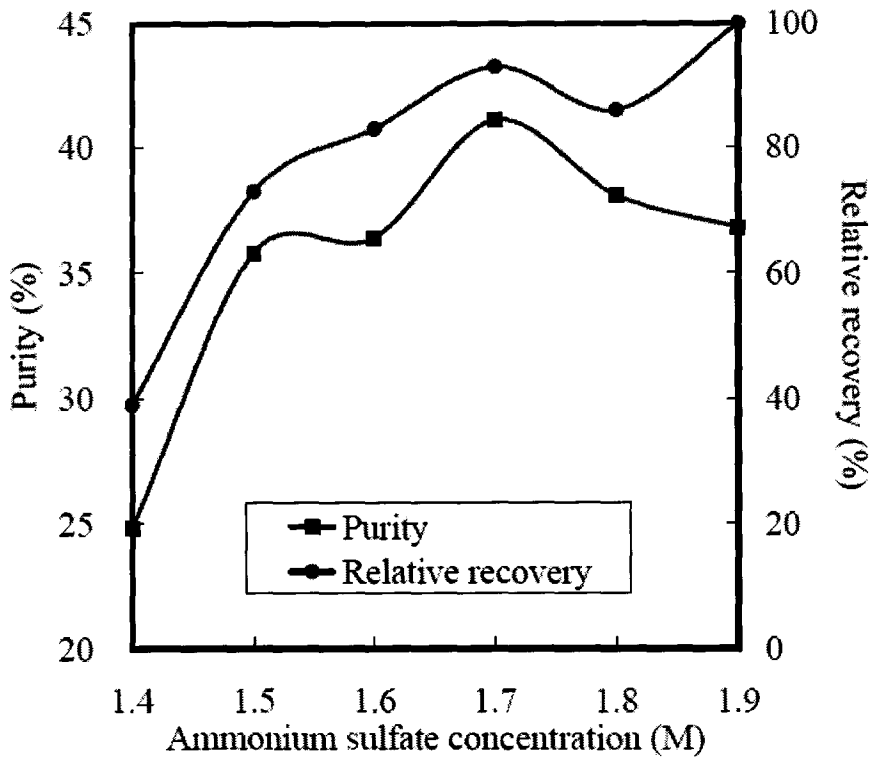


Figure 4.6 Effect of ammonium sulfate concentration in the feed on purification of hIgG1-CD4 by HIMC from eluate obtained from the IEMC purification experiment.

The pH values examined for HIMC were 6.0 to 7.5, pH 6.0 being the original pH of the IEMC eluate and pH 7.5 being the pI of hIgG1-CD4. Fig. 4.7 shows that at both ammonium sulfate concentrations examined (i.e. 1.7 M and 1.9 M), the mAb purity was higher at pH 7.5 (with an average increase of 12%). Based on the results of the salt concentration and pH optimization studies pH 7.5 and 1.7 M ammonium sulfate concentration were selected for the HIMC purification experiments. The binding buffer was a 1.7 M solution of ammonium sulfate prepared in the elution buffer which was 20 mM sodium phosphate (pH 7.5). Figure 4.8 (a) shows the chromatogram obtained from an HIMC purification experiment where one-step elution was employed for recovering the membrane bound mAb. The membrane module contained 15 discs each having 42

mm effective diameter. The feed solution was injected in the form of two pulses, resulting in two peaks at ~15 and ~43 mL, respectively. The purity of the mAb obtained by one-step elution was 28%, making the purification factor 2.15. Fig. 4.8 (b) shows the HIMC chromatogram from an experiment which was similar to the one just discussed except that a 40-mL linear gradient was employed during elution. Sample collected from the major elution peak had a mAb purity of 57%. Fig. 4.8 (c) shows the chromatogram from an HIMC experiment where 30 membrane discs were used and an 80-mL linear gradient was employed for elution; mAb purity obtained in the major elution peak was ca. 80%. Thus MAb purity improved significantly by increasing both the volume of the linear gradient and the number of membranes used.

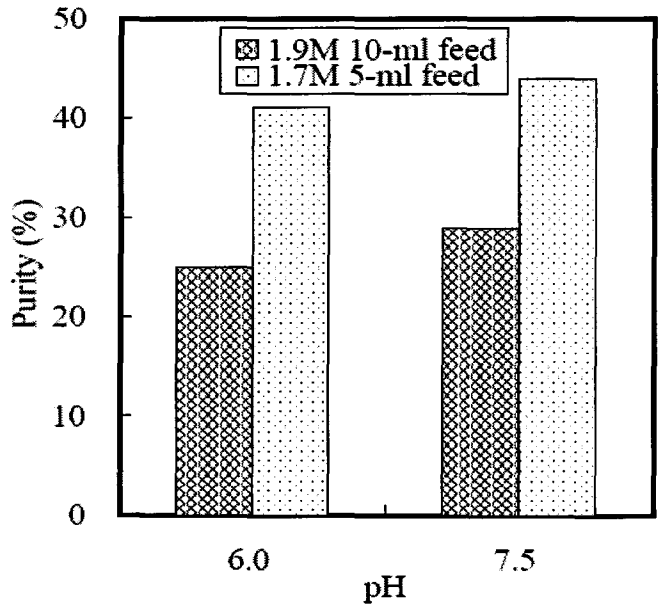


Figure 4.7 Effect of pH and ammonium sulfate concentration combination on purification of hIgG1-CD4 by HIMC.

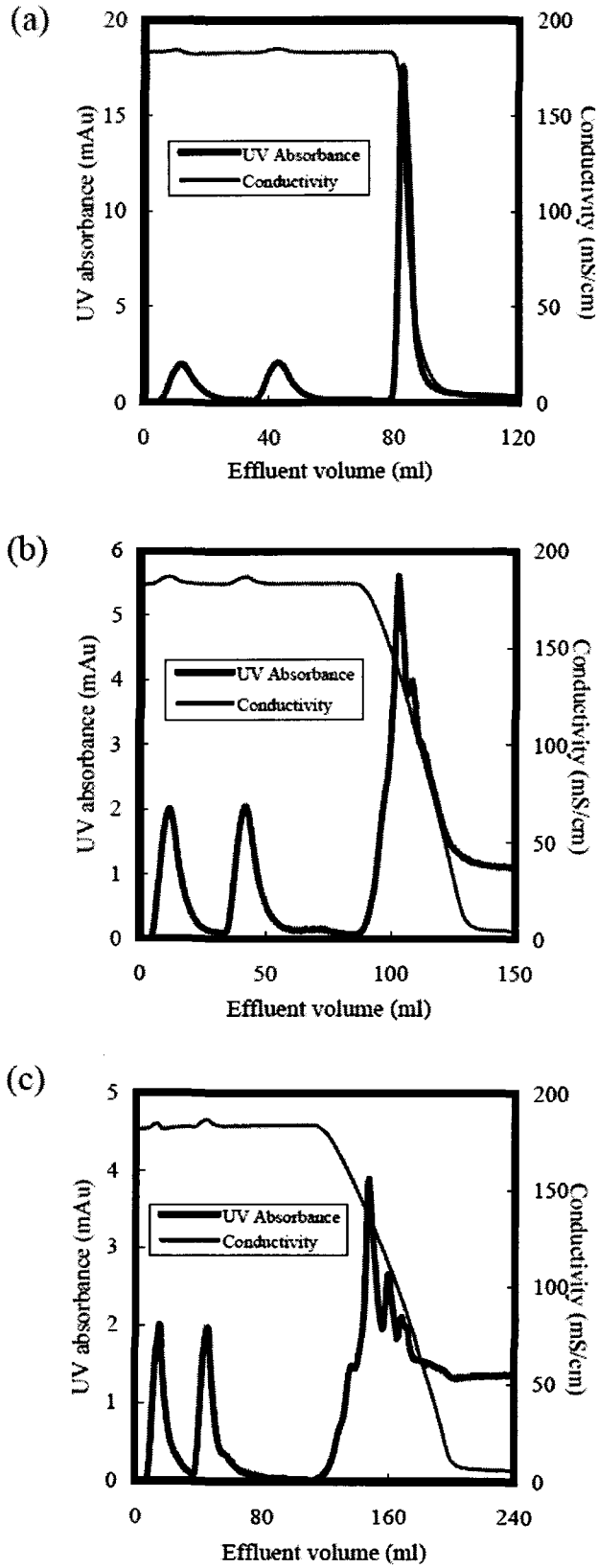
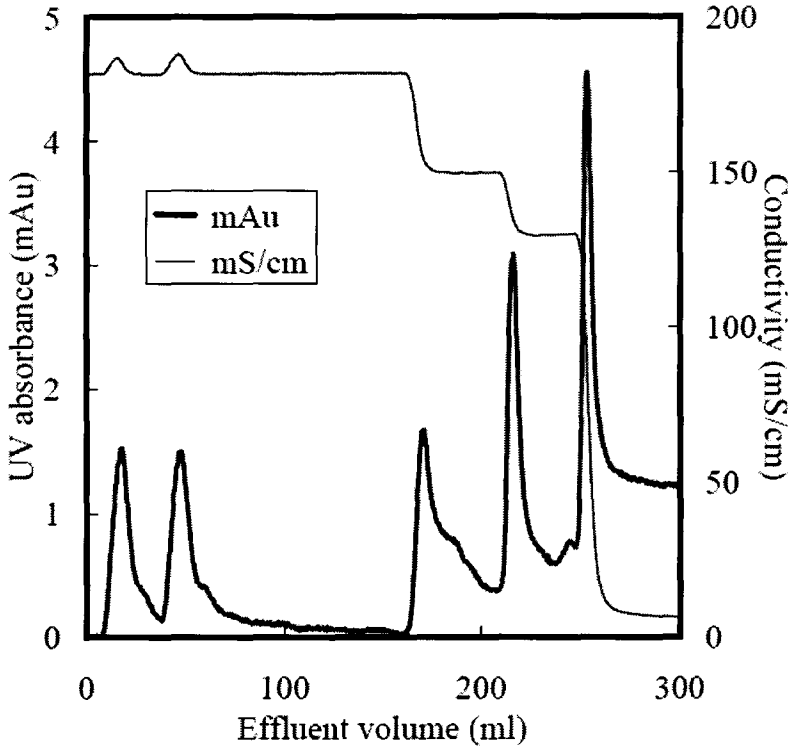


Figure 4.8 Effect of the mode of elution on purification of hIgG1-CD4 by HIMC from eluate obtained from the IEMC purification experiment: (a) one-step elution (carried out with 15 membrane discs), (b) 40-mL linear gradient (carried out with 15 membrane discs), and (c) 80-mL linear gradient (carried out with 30 membrane discs).

By analyzing the UV and conductivity profiles obtained in the above experiment a HIMC purification scheme based on sequential step gradients was developed. Fig. 4.9 shows the chromatogram obtained from this experiment in which a stack of 30 membrane discs were employed. A step change to 28% elution buffer was first used to remove the contaminating proteins that are less hydrophobic than hIgG1-CD4; a second step change to 43% elution buffer was used to elute the bound mAb from membrane; a third step change to 100% elution buffer was used to remove all contaminants and thereby regenerate the membrane. Fig. 4.10 shows a summary of the Protein A chromatograms obtained from both the feed and purified mAb samples after the IEMC-HIMC purification process. There was little mAb in the simulated tobacco feed as indicated by small antibody peak. The pooled eluate obtained from the IEMC step had an overall purity of 13% and this was used as the feed for the HIMC step. The eluate obtained from the HIMC experiment has a mAb purity of 85%.

The eluate obtained from the HIMC step was ultrafiltered at a volumetric permeate flux of 8.9×10^{-6} m^3/m^2 s, in a stirred cell fitted with a 70 kDa MWCO membrane, at a constant volume mode to remove the trace impurities and salts. The permeate flux value was selected based on preliminary screening experiments which indicated that at this flux there was least mAb permeation and greatest impurity permeation through the membrane. Once the salt was removed from the stirred cell, the addition of replacement buffer was discontinued and the retentate was concentrated to 2 mL at the same flux. The retentate obtained from the stirred cell represented purified mAb.



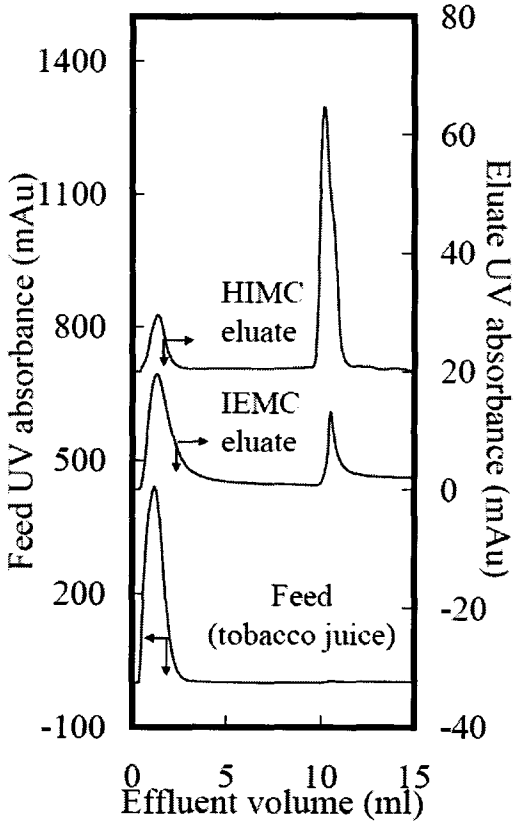


Figure 4.9(Up) Purification of hIgG1-CD4 by HIMC using multiple-step elution process developed based on the earlier gradient elution experiments (the three step changes were 28%, 43% and 100% elution buffer, respectively).

Figure 4.10(Left) Protein A affinity chromatographic analysis of simulated transgenic tobacco feed, IEMC eluate, and HIMC eluate (the first and second peaks represent impurities and mAb, respectively).

The samples from the different steps (i.e. IEMC, HIMC) of the overall purification process were analyzed by reducing SDS-PAGE (see Fig. 4.11). On a reducing gel, the standard mAb shows two bands (lane 1), the 50 kDa band representing the heavy chain and the 25 kDa band representing the light chain. There was little mAb in the simulated tobacco feed (Fig. 4.11, lane 2). The mAb purity was significantly enhanced following IEMC (lane 4) and enhanced again following HIMC (lane 6). The ultrafiltered mAb sample was run on lane 7 of the gel. The absence of mAb bands in the flow through from both IEMC and HIMC (lanes 3 and 5, respectively), indicates high recovery of mAb in each of these steps. Using material balance based on calibrated HPSEC analysis, the overall recovery of hIgG1-CD4 was found to be 77%. Fig. 4.12 shows the HPSEC chromatogram obtained with the UF purified mAb sample. A single peak at a retention time of 56.6 minutes confirms the high purity of the mAb obtained from the (IEMC-HIMC-UF) purification process. When compared with the previously discussed IEMC-PAMC process for purifying mAb from tobacco extract (Yu and McLean et al. 2008), the current IEMC-HIMC-UF process resulted in similar purity, i.e. a single peak in HPSEC with slightly lower recovery, i.e. 77% as opposed to 80%. This slight decrease in recovery could be attributed to the additional purification step i.e. ultrafiltration. The little decrease of recovery could be caused by the further purification step of ultrafiltration but it did not affect the feasibility of IEMC-HIMC-UF as a non-Protein A based membrane process for mAb purification.

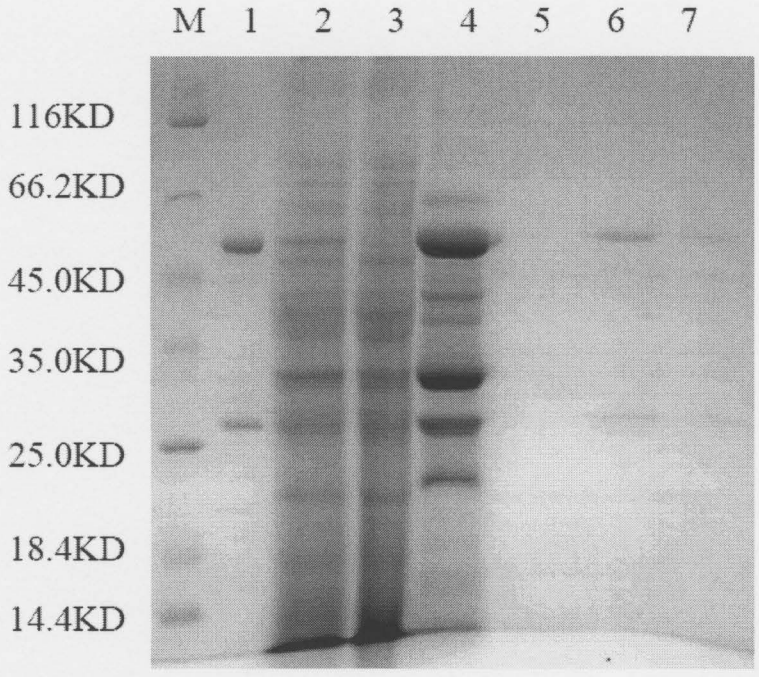


Figure 4.11(Up) SDS-PAGE (reducing 12.5%) analysis of samples obtained from the overall membrane-based mAb purification method (M: marker; lane 1: standard hIgG1-CD4; lanes 2, 3 and 4: simulated transgenic tobacco feed, flow-through and eluate from IEMC experiment; lanes 5 and 6: flow-through and eluate from HIMC experiment; lane 7: retentate from UF experiment). The gel was stained with Coomassie blue dye.

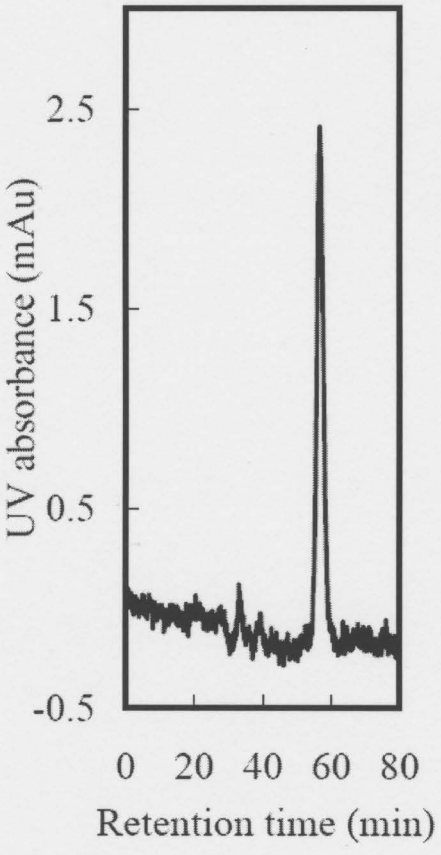


Figure 4.12(Left) HPSEC analysis of purified mAb obtained by UF (column: Superdex 200 10/300 GL; flow rate: 0.2 mL/min; running buffer: 20 mM sodium phosphate buffer + 150 mM NaCl, pH 7.0).

4.5 Conclusions

A membrane-based process for purifying monoclonal antibody from tobacco juice was successfully demonstrated. IEMC using Sartobind S membrane proved to be a very efficient method for mAb capture and preliminary purification from tobacco juice. A pH value of 6.0 and a conductivity value of 4 mS/cm were found to be optimal for the IEMC purification. The purity of mAb in the different eluted fractions from the IEMC method varied, suggesting that the purification process could be further optimized; however, doing this compromised mAb recovery and therefore the eluate which contained mAb was collected and used as feed for the next purification step. The purification factor obtained in the IEMC step was 130. The second purification step using the optimized HIMC method with a 0.22 micron hydrophilized PVDF membrane resulted in a mAb that was 85% pure. An ammonium sulfate concentration of 1.7 M at pH 7.5 was found to be optimal for this method. Furthermore, the HIMC experiments demonstrate that the nature of the negative salt gradient during the elution process has a significant influence on the purity of the product. The purified mAb obtained by HIMC was further polished using ultrafiltration and presented as a single peak following size exclusion chromatography. Furthermore, after reducing SDS-PAGE two characteristic bands corresponding to the IgG heavy and light chains could be seen. Total recovery of hIgG1-CD4 from spiked sample was 77% using IEMC-HIMC-UF process. Overall, the membrane-based process resulted in both high resolution and recovery and, therefore, can be considered as a viable alternative to Protein A-based purification methods.

4.6 References

Almquist KC, McLean MD, Niu Y, Byrne G, Olea-Popelka FC, Murrant C, Barclay J, Hall JC. 2006. Expression of an anti-botulinum toxin A neutralizing single chain Fv recombinant antibody in transgenic tobacco. Vaccine 24:2079-2086.

Arakawa T, Philo JS, Tsumoto K, Yumioka R, Ejima D. 2004. Elution of antibodies from a Protein-A column by aqueous arginine solutions. Protein Expression Purif 36:244-248.

Balasubramaniam D, Wilkinson C, Cott KV, Zhang C. 2003. Tobacco protein separation by aqueous two-phase extraction J Chromatogr A 989:119-129.

Carlson KL. 2005. Flexibility—the guiding principle for antibody manufacturing. Nat Biotechnol 23:1054-1058.

Castilho LR, Anspach FB, Deckwer WD. 2002. Comparison of affinity membranes for the purification of immunoglobulins. J Membr Sci 207:253-264.

Daniell H, Streatfield SJ, Wycoff K. 2001. Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6:219-226.

Farid SS. 2006. Established bioprocesses for producing antibodies as a basis for future planning. Adv Biochem Eng Biotechnol 101:1-42.

Ghosh R, Wang L. 2006. Purification of monoclonal antibody by hydrophobic interaction membrane chromatography. J Chromatogr A 1107:104-109.

Ghosh R, Wong T. 2006. Effect of module design on the efficiency of membrane chromatographic separation processes. J Membr Sci 281:532-540.

Ghosh R. 2002. Protein separation using membrane chromatography: opportunities and challenges. J Chromatogr A 952:13-27.

Ghosh R. 2005. Fractionation of human plasma proteins by hydrophobic interaction membrane chromatography. J Membr Sci 260:112-118.

Ghosh R. 2001. Separation of proteins using hydrophobic interaction membrane chromatography. J Chromatogr A 923:59-64.

Hester JF, Mayes AM. 2002. Design and performance of foul-resistant poly (vinylidene fluoride) membranes prepared in a single-step by surface segregation. J Membr Sci 202:119-135.

Hiatt A, Cafferkey R, Bowdish K. Production of antibodies in transgenic plants. 1989. Nature 342:76-78.

Knudsen HL, Fahrner RL, Xu Y, Norling LA, Blank GS. 2001. Membrane ion-exchange chromatography for process-scale antibody purification. J Chromatogr A 907:145-154.

Ko K, Koprowski H. 2005. Plant biopharming of monoclonal antibodies. Virus Res 111:93-100.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Ma JK, Drake PM, Chargelegue D, Obregon P, Prada A. 2005. Antibody processing and engineering in plants, and new strategies for vaccine production. Vaccine 23:1814-1818.

Ma JK, Hiatt A, Hein M, Vine ND, Wang F, Stabila P, Van Dolleweerd C, Mostov K, Lehner T. 1995. Generation and assembly of secretory antibodies in plants. Science 268:716-719.

McLean MD, Almquist KC, Niu Y, Kimmel R, Lai Z, Schreiber JR, Hall JC. 2007. A human anti-pseudomonas aeruginosa O6ad IgG1 expressed in transgenic tobacco is capable of recruiting immune system effector function in vitro. Antimicrob Agents Chemother 51:3322-3328.

Olea-Popelka FC, McLean MD, Horsman J, Almquist K, Brandle JE, Hall JC, 2005. Increasing expression of an anti-picloram single-chain variable fragment (ScFv) antibody and resistance to picloram in transgenic tobacco (Nicotiana tabacum). J Agric Food Chem 53:6683-6690.

Phillips M, Cormier J, Ferrence J, Dowd C, Kiss R, Lutz H, Carter J. 2004. Performance of a membrane adsorber for trace impurity removal in biotechnology manufacturing, J Chromatogr A 1078:74-82.

Schillberg S, Fischera R, Emans N. 2003. 'Molecular farming' of antibodies in plants, Naturwissenschaften 90:145-155.

Schillberg S, Twymanb RM, Fischera R. 2005. Opportunities for recombinant antigen and antibody expression in transgenic plants—technology assessment, Vaccine 23:1764-1769.

Stoger E, Sack M, Nicholson L, Fischer R, Christou P. 2005. Recent progress in plantibody technology. Curr. Pharm Des 11:2439-2457.

Yu D, Chen X, Pelton RH, Ghosh R. 2008. Paper-PEG based membranes for hydrophobic interaction chromatography: purification of monoclonal antibody. Biotech Bioeng 99:1434-1442.

Yu D, McLean MD, Hall JC, Ghosh R. 2008. Recovery and purification of monoclonal antibody from tobacco plant using membrane-based bioseparation techniques. J Membr Sci 323:159-166.

Zou H, Luo Q, Zhou D. 2001. Affinity membrane chromatography for the analysis and purification of proteins. J Biochem Biophys Methods 49:199-240.

Chapter 5

Purification of PEGylated Protein Using Membrane Chromatography

This work will be submitted to the Journal of Pharmaceutical Sciences by Deqiang Yu and Raja Ghosh.

5.1 Abstract

Reaction conditions were systematically optimized for production of mono-PEGylated lysozyme by N-terminus specific PEGylation. However, some di- and tri-PEGylated proteins were also produced due to side chain reaction. The reaction products were characterized by chromatographic and electrophoretic methods. Commercial cation exchange membrane Sartobind S was used for chromatographic purification of PEGylated lysozyme, the basis of separation being the shielding of protein charge by PEG. Using the membrane chromatographic method, lysozyme and mono-, di- and tri-PEGylated

lysozyme could be resolved into separate peaks. Increasing the superficial velocity during chromatographic separation from 24 cm/h to 240 cm/h increased both protein binding capacity and resolution due to enhancement of protein mass transfer coefficient.

5.2 Introduction

Protein PEGylation refers to the modification of proteins or peptides by conjugation with polyethylene glycol (PEG). PEGylation prolongs the *in-vivo* half-life of protein drugs and reduces enzymatic degradation and immunogenicity, thereby greatly enhancing the clinical efficacy and acceptability of therapeutic proteins. PEGylated proteins comprise an emerging class of biopharmaceuticals. Several PEGylated proteins have been approved by FDA and some have achieved significant clinical and market success, e.g. PEG-interferon α2a (Pegsys®), PEG-interferon α2b (PEG-Intron®) and PEG-G-CSF (Neulasta®) (Reddy et al. 2002; Wang et al. 2002; Kinstler et al. 2002). Many PEGylated proteins such as PEGylated antibody fragments are in different stages of development.

Approaches used for protein PEGylation can be classified into two categories, a) general and b) site-specific. The general approach involves random PEGylation, mainly at lysine residues (Roberts et al. 2002). This results in the formation of a heterogeneous mixture of proteins with varying degrees of PEGylation necessitating complicated purification processes for fractionating differently PEGylated forms. Where a heterogeneous product is acceptable, batch to batch reproducibility of PEGylation sites and composition becomes a crucial regulatory issue. There have been attempts to

conjugate PEG with free cysteine or oxidized carbohydrate (Chapman et al. 1999; Chen et al. 2003; DeFrees et al. 2006). Due to the scarcity of these residues these approaches are site-specific but not widely applicable. PEGylation with disulfide bond is suitable for proteins with paired disulfide bonds and without free cysteine (Shaunak et al. 2006). This approach is site-specific but requires complex reactions. N-terminus protein PEGylation which is site-specific to the α-amino group can be carried out with all proteins. This approach utilizes the pKa difference between the α-amino group (pKa 7.6-8.0) and the ε-amino group of lysine residues (pKa 10.0-10.2) (Wong 1991). The reaction is based on reductive alkylation. At acidic condition, mPEG-aldehyde can react largely selectively with the N-terminal α-amino group to produce a Schiff base intermediate (Hermanson 1996). This intermediate can then be reduced to conjugate PEG with the protein through a secondary amide bond. The use of this approach has been reported for production of PEG-G-CSF (Neulasta®) and PEGylated epidermal growth factor (EGF), insulin, interferon β-1b and consensus interferon (Kinstler et al. 2002; Lee et al. 2003; Dou et al. 2007; Basu et al. 2006; Kinstler 2005).

Most PEGylated proteins are separated by column chromatography, primarily size exclusion chromatography (SEC) and ion exchange chromatography (IEC). SEC which is based on size difference is limited by the amount of sample that can be loaded as well as in terms of mobile phase flow rate restrictions, and therefore mainly used for analytical separations. IEC is based on charge differences between the PEGylated proteins, native protein and PEG. PEG being uncharged shields the intrinsic charge on the protein, the extent of shielding increasing with the degree of PEGylation. However, column based

IEC based on porous particulate chromatography is diffusionally limited and this restricts the speed of separation. Typical superficial velocities reported during separation of PEGylated proteins using IEC include 90 cm/h for purification of PEG-G-CSF, 100 cm/h for purification of PEG-GRF (1-29) and 21-136 cm/h for purification of PEG-IFN (Yun et al. 2005; Piquet et al. 2002; Foser et al. 2003). The binding capacity of PEGylated proteins on IEC media is normally an order of magnitude lower than that of native proteins (Pabst et al. 2007; Fee and Van Alstine 2006). This phenomenon has been explained both in terms of charge shielding and increase in hydrodynamic radius (leading to decrease in diffusivity). While this is convenient for separation of native proteins from PEGylated proteins in general, it makes it difficult to fractionate for example mono-, di- and tri- PEGylated forms. The current work shows that even with a site specific approach, minor quantities of other PEGylated forms are produced and need to be separated from the desired form. Some researchers have used monolithic chromatographic media such as CIM^{TM} to overcome limitations of particulate media based chromatography (Hall et al. 2004). Although high flow rates could be used with the CIM^{TM} media, high back pressure was generated as well, e.g. at 300 cm/h, SO_3-CIM^{TM} gave a backpressure of 3.90 MPa while QA-CIM^{TM} could not be operated due to overpressure. Severe peak broadening was also observed particularly with SO_3-CIM^{TM}.

Membrane chromatography which uses stacks of microporous membranes as chromatographic media is suitable for rapid separation of proteins and other bio-macromolecules, a feature made possible by the predominance of convective mass transport (Ghosh 2002). The large flow-through pores present on an adsorptive membrane

would make it particularly suitable for processing large bio-macromolecules such as PEGylated proteins at relatively low backpressure. In this paper, membrane chromatography is therefore suggested as an alternative to both column and monolith chromatography for purification of PEGylated proteins. Lysozyme is used as a model protein and N-terminus PEGylation of lysozyme is systematically optimized. The separation on mono-PEGylated lysozyme from native lysozyme and other PEGylated forms is then attempted using ion exchange membrane chromatography (or IEMC). IEMC has been successfully applied for purification of other therapeutic proteins (Knudsen et al. 2001; Philips et al. 2005). To the best of our knowledge, this is the first paper that discusses the use of membrane chromatography for purification of PEGylated proteins. The effects of key process parameters such as flow rate on membrane binding capacity, separation and backpressure are studied.

5.3 Experimental methods

5.3.1 Materials

Lysozyme (L6876), glycine (G8898), Trizma base (T1503), sodium chloride (S7653), sodium cyanoborohydride (156159), barium chloride (202738), iodine (326143), hydrochloride acid (258148), 25% glutaraldehyde solution (G6257) and 70% perchloric acid (77227) were purchased from Sigma-Aldrich (St. Louis, MO). Sodium acetate trihydrate (SX0255-1) and glacial acetic acid (AX0073-6) were purchased from EMD (Gibbstown, NJ). Potassium iodide (74210-140) was purchased from Anachemia (Montreal, QC, Canada). mPEG-Propionaldehyde 5,000 and 10,000 (P1PAL-5 and

P1PAL-10) were purchased from Sunbio Inc. (Anyang, South Korea). Low molecular weight (LMW) protein calibration kit (28-4038-41) was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). High quality purified water (18.2 MΩ cm) obtained from a Diamond^{TM} NANOpure (Barnstead, Dubuque, IA) water purification unit was used to prepare all the test solutions and buffers. Sartobind S cation-exchange membrane (94IEXS42-001, 275 μm thickness) was purchased from Sartorius (Goettingen, Germany).

5.3.2 Lysozyme PEGylation

PEGylation reactions were carried out in the small flasks with continuous magnetic stirring at room temperature. The reaction mixture consisted of lysozyme, mPEG-aldehyde and sodium cyanoborohydride in 100 mM acetate buffer. The concentration of lysozyme was kept fixed at 1 mg/mL. To study the effect of pH on PEGylation, reactions were carried out for 5 hrs using acetate buffer at different pH values i.e. pH 4.0, 4.5, 5.0, 5.5, and 6.0 while maintaining the mPEG/lysozyme molar ratio at 4:1 and the sodium cyanoborohydride concentration at 10 mM. The effect of mPEG/lysozyme molar ratio (i.e. 2:1, 4:1 and 6:1) on PEGylation was examined at the optimized pH value and the samples were taken at 5 hrs and 20 hrs. A reaction was also carried out at molar ratio of 4:1 using 20 mM sodium cyanoborohydride to examine the effect of this reagent on PEGylation. To study the effect of reaction time, 4:1 mPEG/lysozyme molar ratio and 10 mM sodium cyanoborohydride concentration were used at the optimized pH value, samples being taken at 0 hr and after 2 hr, 4 hr, 6 hr, 8.5

hr, 10 hr and 20 hr of reaction. 1.0 M glycine solution was added to all samples (to obtain a final concentration of 10 mM) to stop the reaction. These were then analyzed by SEC.

5.3.3 SEC analysis

SEC analysis was carried out using a Superdex 200 10/300 GL column (GE Healthcare Bio-Sciences) fitted to a Varian HPLC system (Palo Alto, CA). The mobile phase used was 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl, at a flow rate of 0.2 mL/min.

5.3.4 Determination of degree of PEGylation

The degree of PEGylation of lysozyme was determined using the approach reported by Fee and Van Alstine (2004). The universal calibration curve for hydrodynamic radius (R_h) versus the gel phase distribution coefficient (K_{av}) was established by SEC analysis of PEG 5000, PEG 10000 and a low molecular weight protein calibration kit for SEC. K_{av} was determined using the following equation:

$$K_{av} = (V_e - V_0)/(V_t - V_0) \qquad (1)$$

Where V_e, V_o and V_t respectively represent retention volume (ml), void volume (ml) and total bed volume (ml).

R_h of proteins was calculated using Equation 2 while Equation 3 was used for determining the R_h for PEG.

$$R_{h,prot} \approx (0.82 \pm 0.02) M_r^{1/3} \qquad (2) \text{ (Hagel 1998)}$$

Where $R_{h,prot}$ and M_r are respectively the viscosity radius (Angstrom) and molecular weight (Dalton) of protein.

$$R_{h,PEG} = 0.1912 M_r^{0.559} \quad (3) \text{ (Kuga 1981)}$$

Where $R_{h,PEG}$ and M_r are respectively the viscosity radius (Angstrom) and molecular weight (Dalton) of PEG.

The hydrodynamic radius component of the PEG layer of PEGylated lysozyme was then determined using Equation 4 while the molecular weight of the conjugated PEG was calculated using Equation 3.

$$R_{h,PEG} = \frac{(R_{h,PEGprot}^{\ 3} - R_{h,prot}^{\ 3})}{R_{h,PEGprot}^{\ 2}} \quad (4)$$

Equation 4 is a reciprocal form of an equation described in a previous report (Fee and Van Alstine 2004), where $R_{h,PEGprot}$ is the viscosity radius (Angstrom) of PEGylated proteins.

5.3.5 Membrane chromatography system

Discs having 18 mm diameter were cut out from the Sartobind S cation-exchange membrane sheets and 15 such discs were stacked and housed within a custom-designed membrane module (Ghosh and Wong 2006). The module was then integrated with an AKTA prime system (GE Healthcare Bio-Sciences). The effluent from membrane module was continuously monitored for UV absorbance at 280 nm, pH and conductivity; the data was logged into a computer using Prime View software (GE Healthcare Bio-Sciences). The system pressure was also continuously monitored using the pressure transducer of the AKTA Prime system.

5.3.6 Hydraulic testing

The effect of superficial velocity of backpressure generated was studied by passing binding buffer, i.e. 50 mM acetate buffer (pH 5.0) through the membrane module described above at different flow rates. The experiment was started at the lowest flow rate examined and the flow rate was then increased in steps. The step volume was ca. 5 ml for superficial velocities of 24, 96 and 144 cm/h and ca. 10 ml for superficial velocities of 240 cm/h. The pressure drop across the membrane module was recorded and logged into a computer.

5.3.7 Separation of PEGylated lysozyme using IEMC

The separation of PEGylated lysozyme was examined using a stack of 15 membrane discs housed in the membrane module, the effective bed volume being 1.05 mL. The binding buffer used was 50 mM acetate (pH 5.0) while the eluting buffer consisted of the binding buffer adjusted to 1 M NaCl concentration. The feed consisted of 1 ml of the reaction mixture pooled from the reactions carried out using PEG/lysozyme ratios of 2:1, 4:1 and 6:1 in 10 mM sodium cyanoborohydride for 20 hrs. A series of flow rates from 24 to 96, 144 and 240 cm/h were used to study the flow rate effect on separation. After sample injection, the membrane module was washed with binding buffer till the UV absorbance reached the baseline. A 40 mL gradient from 0 to 100% eluting buffer was then used to fractionate the bound proteins. The peak fractions collected in each experiment were then analyzed by SEC and SDS-PAGE.

5.3.8 SDS-PAGE

SDS-PAGE experiments were carried out according to the work of Laemmli (1970). 15% non-reducing gels were run in duplicate using a Hoefer MiniVE system (GE Healthcare Bio-Sciences). One gel was stained by Coomassie blue dye to visualize the protein bands. The second gel was stained using a modified protocol based on a published method (Kurfurst 1992) for visualizing the PEG component of PEGylated proteins. The separated bands on the gel were first fixed in 50 mL of 5% glutaraldehyde solution for 15 min at room temperature. Then the gel was transferred to 20 mL of 0.1 M perchloric acid for 15 min, followed by addition of 5 mL of 5% barium chloride solution and 2 mL of 0.1 M iodine/potassium iodide solution. After 15 min of incubation, the staining solution was replaced by water and the stained gel was photographed.

5.3.9 Lysozyme and mono-PEGylated lysozyme binding capacity determination

The smaller membrane module which housed 15 discs of Sartobind S membrane each having effective diameter of 8 mm (0.207 mL bed volume) was used for determining the protein binding capacity. The binding and eluting buffers were the same as those used in the separation experiments. The binding capacities were determined in the breakthrough mode at a flow rate of 0.8 mL/min, i.e. corresponding to a superficial velocity of 96 cm/h. The lysozyme binding capacity was determined by injecting 20 mL of 1 mg/mL lysozyme solution prepared in binding buffer followed by washing and single step (0 to 100%) elution. The mono-PEGylated lysozyme binding capacity was

determined using pooled purified material obtained from the separation experiments described above. SEC analysis showed that the pooled material contained ca. 93% mono-PEGylated lysozyme and ca. 7% di-PEGylated lysozyme. Prior to the binding capacity determination experiments, buffer exchange using 50 mM acetate (pH 5.0) was carried out using a centrifugal ultrafilter (3 kDa MWCO) and the final concentration was adjusted to 0.783 mg/mL (based on UV absorbance). 5 ml of this material was injected into the membrane module followed by washing and elution.

5.4 Results and discussion

Figure 5.1 shows the SEC chromatogram obtained with the reaction mixture from a typical lysozyme PEGylation reaction. Comparison of the retention time of the peaks P1, P2 and P3 with molecular weight calibration standards showed that these were tri-, di- and mono-PEGylated lysozyme respectively while P4 was native lysozyme. Figure 5.2 shows the universal calibration curve of K_{av} values obtained by SEC analysis of PEG and protein standards (using Equation 1) versus R_h (obtained using Equation 2 and 3 for protein and PEG respectively). The R_h values for PEGylated lysozyme and native lysozyme were calculated using the experimentally derived K_{av} values for P1, P2, P3 and lysozyme and the regression equation for the universal calibration curve (see Table 5.1). The R_h values of the PEG layer of P1, P2 and P3 were then calculated by Equation 4 and the corresponding M_r was calculated using Equation 3. The average molecular weight of the PEG used for PEGylation was 5 kDa and therefore dividing the calculated M_r by 5000 gave the degree of PEGylation. Table 5.1 shows that P1, P2 and P3 contained

respectively 3.12, 2.02 and 1.00 molecules of PEG5000 thus confirming that these were indeed tri-, di- and mono-PEGylated lysozyme.

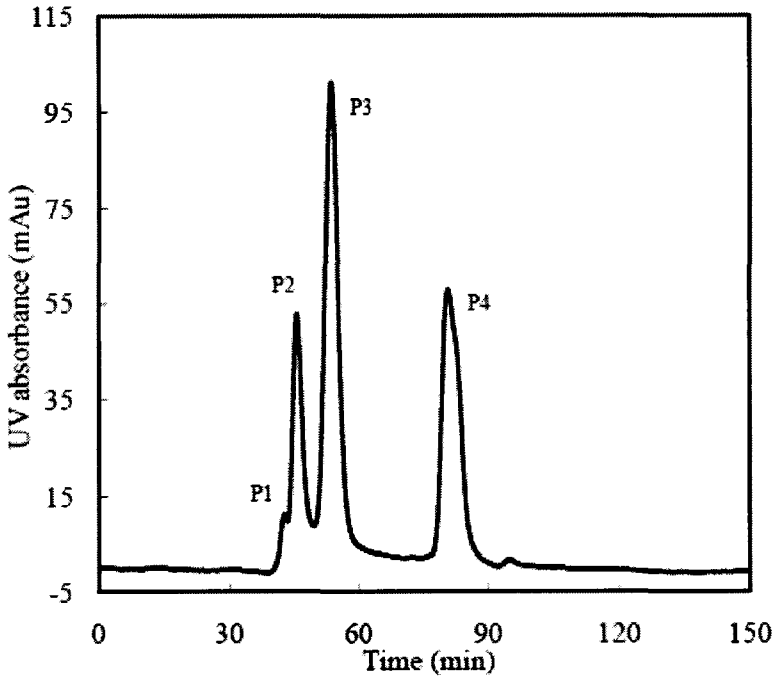


Figure 5.1 SEC analysis of lysozyme PEGylation mixure (pH 5.0, PEG/lyosyzyme = 4:1, 10 mM sodium cyanoborohydride, 20 hrs reaction).

Table 5.1 Determination of PEG molecular weight in PEGylated lysozyme.

Protein	$R_{hPEGylated\ lysozyme}$ (Angstrom)	R_{hPEG} (Angstrom)	M_{rPEG} (Dalton)	$M_{rPEG}/5000$
P1	43.62	42.22	15602.57	3.12
P2	35.21	33.06	10076.08	2.02
P3	26.25	22.38	5014.49	1.00
$R_{h\ Lysozyme}$ = 13.86 Angstrom				

Error range: ± 1.0% (the average deviation divided by the average value from two batches of standard proteins and PEGs)

The R_h values for PEGylated lysozyme and native lysozyme were calculated using the SEC analysis derived K_{av} values and the regression equation for the universal calibration curve (Fig. 5.2). The R_h values of the PEG layer of P1, P2 and P3 were then calculated by Equation 4 and the corresponding M_r was calculated using Equation 3.

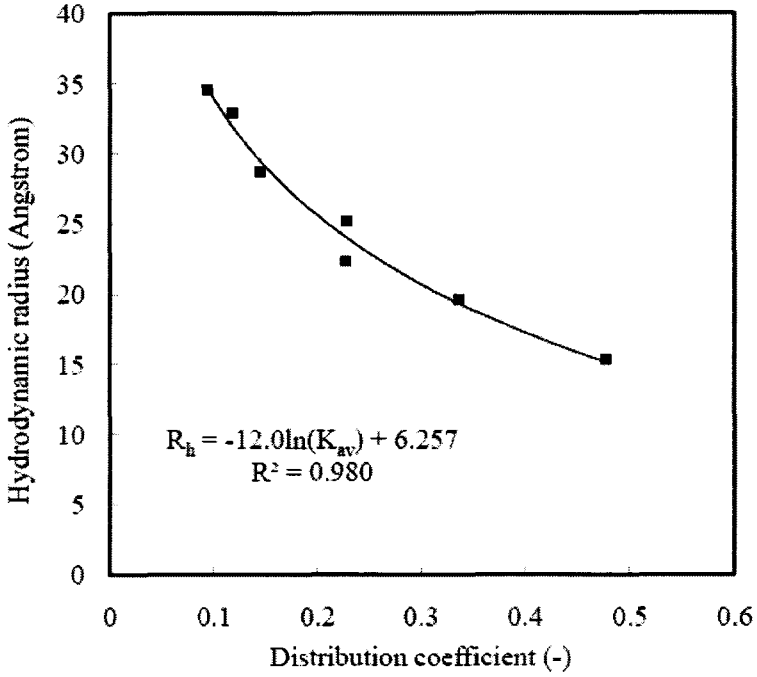


Figure 5.2 Universal calibration curve of hydrodynamic radius (Rh) versus gel phase distribution coefficient (Kav) established by analysis of PEG standards and LMW protein standards using SEC column.

Figure 5.3 shows the effect of pH on the extent and degree of PEGylation in reactions carried out for 5 hours. At pH values 4.0, 4.5, 5.0, 5.5 and 6.0 the percentages of total PEGylated lysozyme in the reaction mixture were respectively 35.6%, 32.7%, 31.9%, 35.5% and 39.7% with mono-PEGylated lysozyme constituting 85.4%, 87.8%, 88.2%, 85.6% and 81.9% of total PEGylated lysozyme respectively (Error range ± 1.6%). The extent of PEGylation increased as pH was increased but selectivity of synthesis of mono-PEGylated protein was better at lower pH values reflecting the difference in pKa between the α-amino group of N-terminus (pKa 7.6-8.0) and the ε-amino group of lysine residues (pKa 10.0-10.2) (Wong 1991; Hermanson 1996; Kinstler 2005). The degradation of lysozyme under acidic conditions was monitored using SEC (data not shown). Some

degradation was observed at pH 4.0 and 4.5 but at pH 5.0 and above there was no apparent degradation. Thus pH 5.0 was chosen for N-terminus PEGylation of lysozyme.

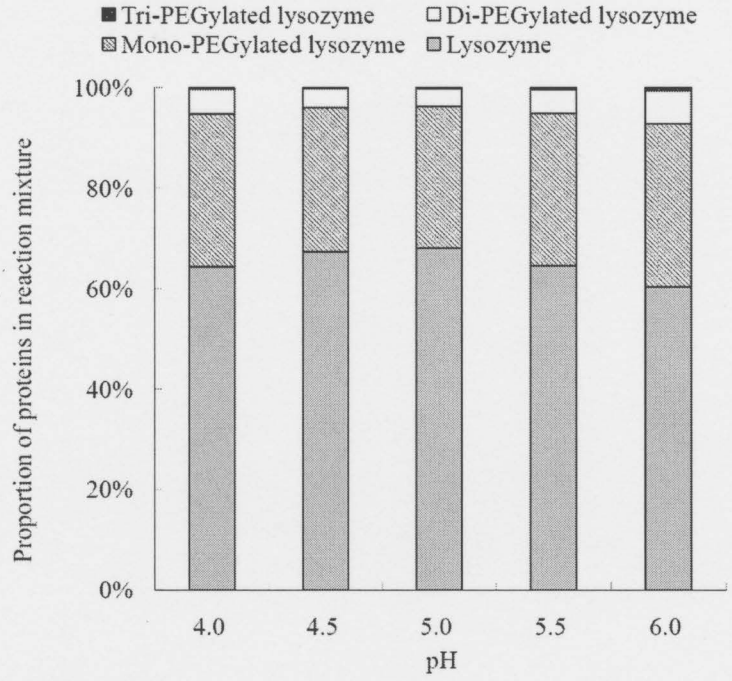


Figure 5.3 Effect of pH on lysozyme PEGylation (pH varied from pH 4.0 to 6.0 with 0.5 interval, PEG/lysozyme = 4:1, 10 mM sodium cyanoborohydride, 5 hrs reaction, percentage of proteins in reaction mixture was achieved by SEC analysis).

The effect of PEG/lysozyme ratio and reductant concentration on extent and degree of lysozyme PEGylation was next examined. Figure 5.4 shows that in both reactions carried out for 5 hrs and 20 hrs using 10 mM reductant concentration, increase in PEG/lysozyme ratio increased the extent of total PEGylation. However, reactions carried out for 20 hrs showed that increasing the PEG/lysozyme ratio from 4:1 to 6:1 did not lead to increase in the formation of mono-PEGylated lysozyme (45.6% at 4:1 ratio as compared to 45.4% at 6:1 ratio). The increase in the extent of total PEGylation was due to formation of more di- and tri-PEGylated lysozyme. The increase in reductant

concentration from 10 mM to 20 mM did affect both the extent of total PEGylation and the formation of mono-PEGylated lysozyme in a 5 hr reaction but there was virtually no effect in a 20 hr reaction.

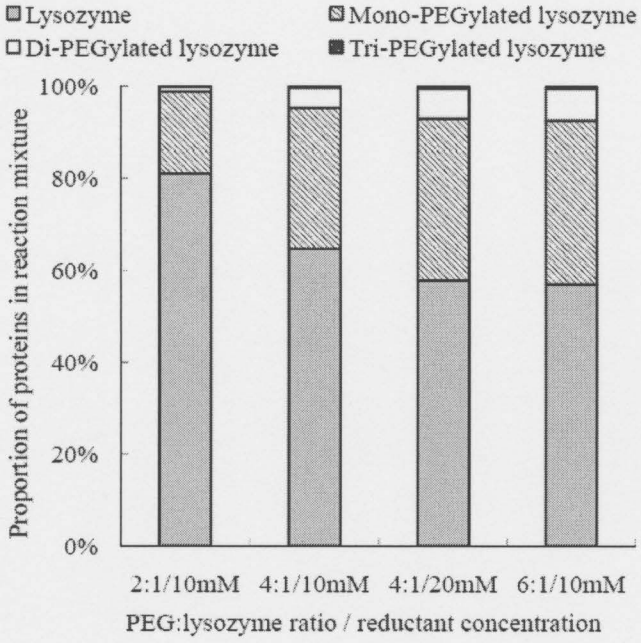


Figure 5.4 Effect of PEG/lysozyme ratio and reductant concentration on lysozyme PEGylation (Top: 5 hrs reaction; Bottom: 20 hrs reaction) (pH 5.0, PEG/lysozyme = 2:1, 4:1 or 6:1, 10 mM or 20 mM sodium cyanoborohydride, percentage of proteins in reaction mixture was achieved by SEC analysis).

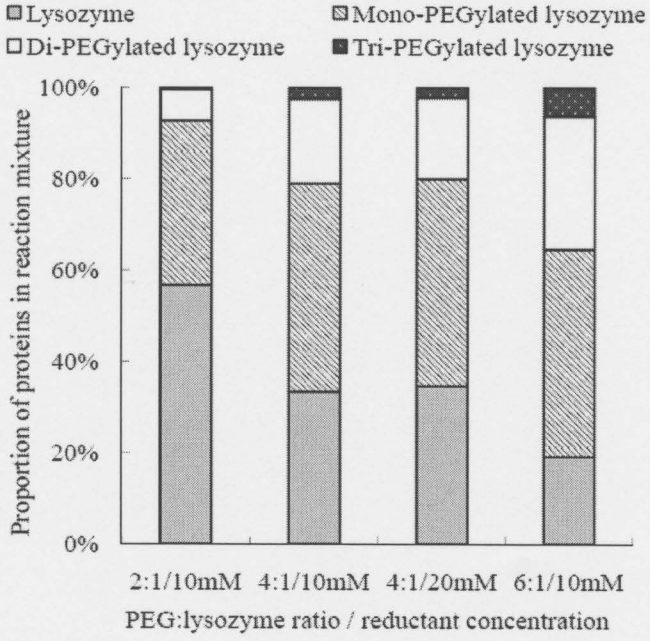


Figure 5.5 shows the progress of lysozyme PEGylation reaction with time carried out using 4:1 PEG/lysozyme ratio and 10 mM reductant concentration. Between 0 and 4 hrs the native lysozyme concentration sharply decreased to 57.9%, most of this being converted to mono-PEGylated lysozyme, shown by its sharp increase to 36.0%. Between 4 to 20 hrs, lysozyme content decreased while the mono-PEGylated lysozyme content increased more gradually to 34.5% and 45.6% respectively.

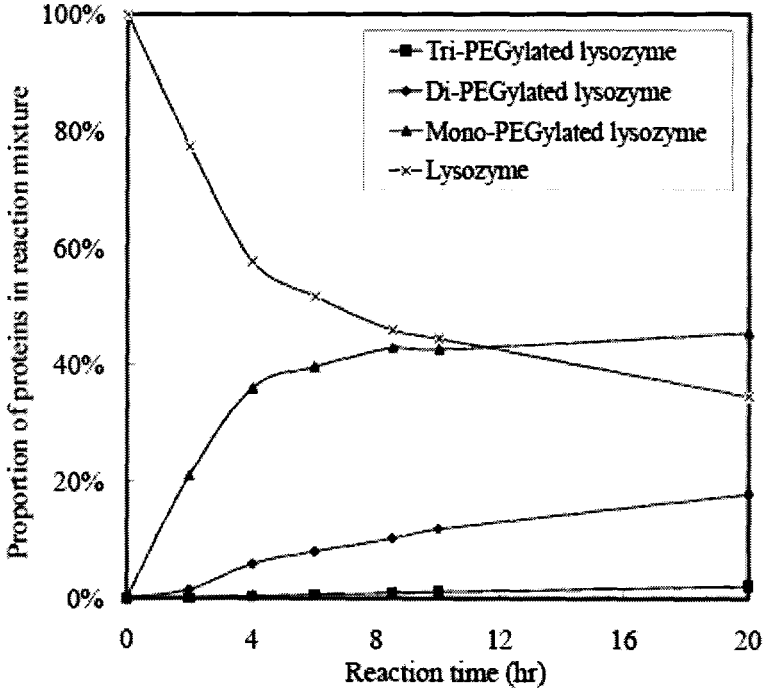


Figure 5.5 Effect of reaction time on lysozyme PEGylation (pH 5.0, PEG/lyosyzyme = 4:1, 10 mM sodium cyanoborohydride, sampling time at 0, 2, 4, 6, 8.5, 10 and 20 hrs reaction, percentage of proteins in reaction mixture was achieved by SEC analysis).

Figure 5.6 shows the effect of mobile phase superficial velocity on membrane back pressure. With each stepped increase in superficial velocity, the back pressure increased but remained reasonably stable after increasing. The average back pressures generated per unit superficial velocity per unit bed height was 0.00125 MPa h cm^{-2} which

was more than an order of magnitude lower than that reported for monoliths (0.014 MPa h cm^{-2}) (Hall et al. 2004). The constant pressure at each flow rate examined indicated that the membrane bed was stable and did not undergo significant compaction.

Figure 5.7 shows the chromatograms obtained during the separation of lysozyme PEGylation reaction mixture using ion exchange membrane chromatography at different superficial velocities. The chromatograms look largely similar, each with a small flow through peak (FT) and four eluate peaks (E1, E2, E3 and E4). The best separation (i.e. resolution) of E1 and E2 and sharpest E2 and E3 peaks were obtained at 240 cm/h, the highest superficial velocity examined. This is contrary to what is observed in particulate media based chromatography where both resolution and peak sharpness decrease at high superficial velocities. The transport of species in membrane chromatography takes place predominantly by convection. However, some diffusion takes place within the stagnant film very close to the pore wall. Increasing the superficial velocity decreased the thickness of this film, resulting in better mass transport and hence better resolution and peak sharpness. Better mass transport at higher superficial velocity also contributed towards better utilization of the binding capacity of the membrane. This is evident from Table 5.2 where the effect of superficial velocity on the area of the flowthrough peak is summarized. Increasing the superficial velocity resulted in a smaller flowthrough peak indicating greater binding of injected material.

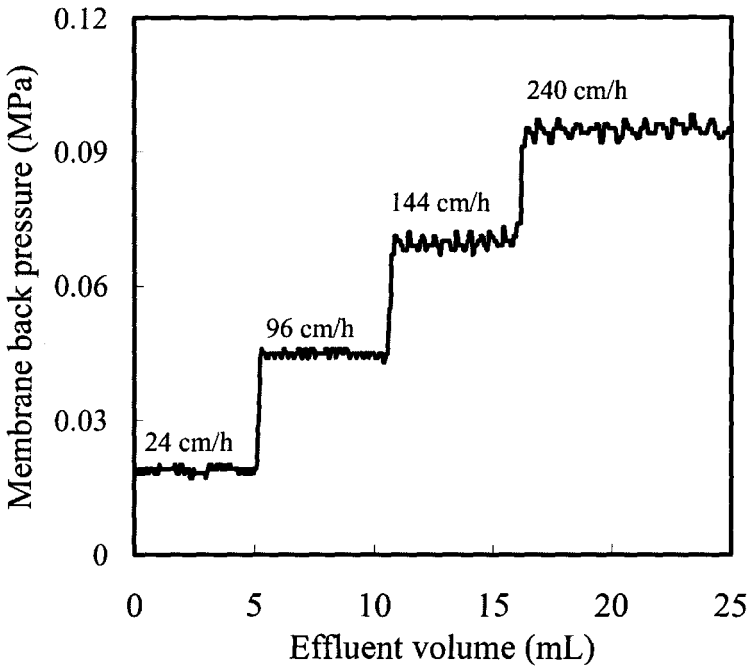


Figure 5.6 Effect of superficial velocity on membrane backpressure (Membrane module contained 15 layers of Sartobind S with 18 mm i.d. Flow rate increased from 24 to 240 cm/h. Running buffer was 50 mM pH 5.0 acetate buffer. 5 ml effluent volume was kept for 24, 96 and 144 cm/h and 10 ml for 240 cm/h.)

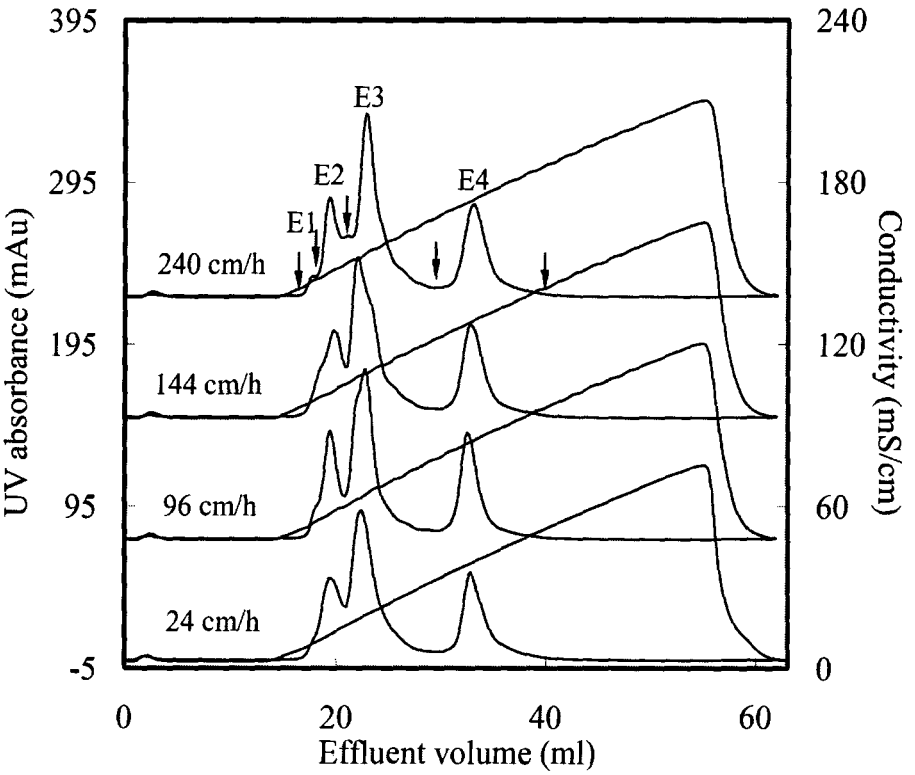


Figure 5.7 Effect of superficial velocity on purification of PEGylated lysozyme separation using IEMC (Membrane module contained 15 layers of Sartobind S with 18 mm i.d. Flow rate increased from 24 to 240 cm/h. 40 ml gradient from 0 to 100% elution buffer was used for separation. The feed was 1 ml of the reaction mixture from 20 hrs reactions with PEG/lysozyme ratio of 2:1, 4:1 and 6:1 at 10 mM sodium cyanoborohydride. The arrows show the start and end of each fraction collection.)

Table 5.2 The flow through peak area and its percentage in total peak areas at various flow rates.

Linear flow rate (cm/h)	24	96	144	240
Flow through peak area (mAu*ml)	3.7505	2.6731	1.6673	1.5468
Flow through peak area / total peak areas	1.23%	0.75%	0.48%	0.49%

Error range: ± 1.5% (the average deviation divided by the average value from the total peak areas at various flow rates)
The flow through peak areas and total peak areas were integrated from the chromatograms in Fig. 5.7 using PrimeView software.

The flow through and elution peaks from the separation experiment at 240 cm/h along with the feed were analyzed by SEC and SDS-PAGE (see Fig. 5.8). The SEC chromatograms show that E1 contained ca. 72.7% tri-PEGylated lysozyme, E2 containing ca. 94.4% di-PEGylated lysozyme, E3 contained ca. 93.0% mono-PEGylated lysozyme while E4 contained only lysozyme. Quite clearly, the IEMC method gave complete separation of lysozyme from PEGylated lysozyme and nearly complete separation of mono-PEGylated lysozyme from di- and tri-PEGylated lysozyme. The above results also clearly demonstrate that PEG shielded the positive charge on lysozyme, the shielding increasing with degree of PEGylation. It is interesting that IEMC separation (Fig. 5.7, 240 cm/h) showed similar chromatography profile to SEC analysis (Fig. 5.8, Feed). However, the processing capacity of IEMC (1 ml feed / 1 ml bed volume / 0.1 hrs) was 6000 folds higher than that of SEC (0.1 ml feed / 24 ml bed volume / 2.5 hrs), which clearly demonstrated the suitability of IEMC for large scale separation. Although IEC was not tested in this study, its generally low flow rate (21-136 cm/h) and high column bed (7.5-20 cm) (Yun et al. 2005; Piquet et al. 2002; Foser et al. 2003) would make its processing

capacity apparently lower than that of IEMC (flow rate of 240 cm/h with the bed height of 0.4 cm in this study).

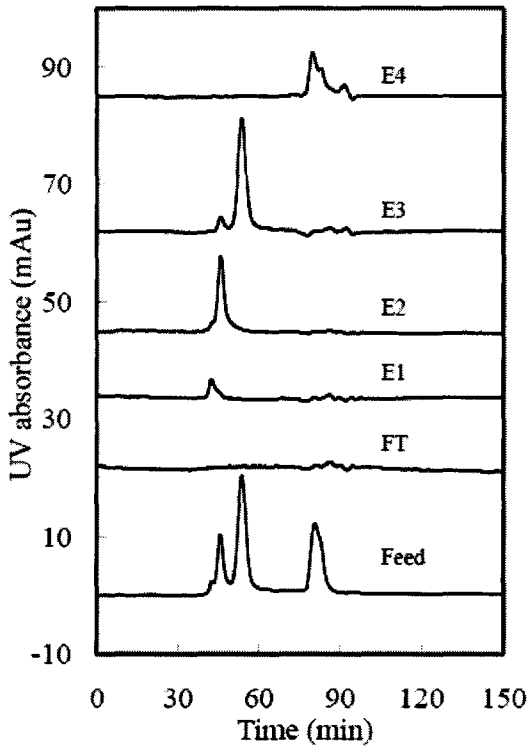


Figure 5.8 SEC analysis of samples obtained by IEMC separation at 240 cm/h (Superdex 200 10/300 GL column, the mobile phase was 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl, at a flow rate of 0.2 mL/min).

The flow through and elution peaks from the separation experiment carried out at 240 cm/h along with the feed were also analyzed by SDS-PAGE (see Fig. 5.9). Figure 5.9A shows the gel stained for PEG detection while Figure 5.9B shows that stained for protein detection. The band corresponding to native lysozyme can only be seen on the gel stained for protein, while that corresponding to PEG5000 can only be seen on the gels stained for PEG. Mono-, di- and tri-PEGylated lysozyme can be seen on both gels. The

SDS-PAGE results confirm that the IEMC method separated gave excellent separation of lysozyme from and its PEGylated forms.

The lysozyme and mono-PEGylated lysozyme binding capacities at 10% breakthrough were respectively 24.6 mg/mL and 11.1 mg/mL. Therefore the dynamic binding capacity ratio of lysozyme to mono-PEGylated lysozyme was 2.22. The typical binding capacity ratios reported in particulate media based chromatography is 10 (Pabst et al. 2007; Fee and Van Alstine 2006). While a high ratio is desirable when total PEGylated proteins need to be separated from native proteins, it is not particularly suitable for the fractionating PEGylated form e.g. mono-PEGylated protein from the native protein and other PEGylated forms. The low binding capacity ratio of the Sartobind S membrane was therefore ideal for the purification mono-PEGylated lysozyme. The high binding capacity of mono-PEGylated lysozyme on Sartobind S could be attributed to the predominance of convective mass transport.

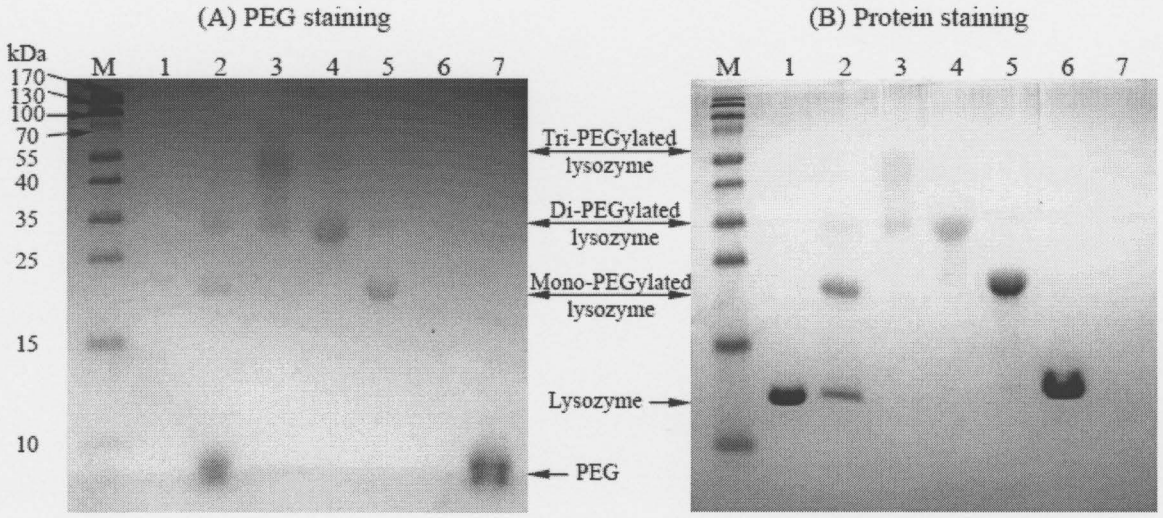


Figure 5.9 SDS-PAGE analysis of samples obtained by IEMC separation at 240 cm/h (15% non-reducing gel. M: maker, 1: standard lysozyme, 2: feed, 3-6: respectively E1, E2, E3 and E4 from IEMC gradient elution, all loaded with 4 μg in terms of lysozyme content, 7: PEG5000).

5.5 Conclusions

The optimization of N-terminus specific PEGylation of lysozyme resulted in the formation of a higher proportion of mono-PEGylated protein. The optimized reaction conditions were pH 5.0, a PEG/lysozyme ratio of 4:1 and 10 mM sodium cyanoborohydride and the reaction leveled off after 20 hrs. However, significant amounts of di- and tri-PEGylated lysozyme were also found to be synthesized along with the mono-PEGylated form. The differently PEGylated forms of lysozyme were characterized using SEC and SDS-PAGE. Ion exchange membrane chromatography using Sartobind S membrane gave excellent separation of mono-PEGylated lysozyme, other PEGylated forms and native lysozyme, the purity achieved being 93% (as determined by SEC). The resolution and peak sharpness increased with increase in superficial velocity, this being due to the enhancement of mass transport.

5.6 References

Basu A, Yang K, Wang M, Liu S, Chintala R, Palm T, Zhao H, Peng P, Wu D, Zhang Z, Hua J, Hsieh MC, Zhou J, Petti G, Li X, Janjua A, Mendez M, Liu J, Longley C, Zhang Z, Mehlig M, Borowski V, Viswanathan M, Filpula D. 2006. Structure-function engineering of interferon-α1b for improving stability, solubility, potency, immunogenicity, and pharmacokinetic properties by site-selective mono-PEGylation. Bioconjugate Chem 17:618-630.

Chapman AP, Antoniw P, Spitali M, West S, Stephens S, King DJ. Therapeutic antibody fragments with prolonged in vivo half-lives. 1999. Nat Biotechnol 17:780-783.

Chen T, Small, David A, Wu, L, Rubloff, GW, Ghodssi R, Vazquez-Duhalt R. 2003. Nature-inspired creation of protein-polysaccharide conjugate and its subsequent assembly onto a patterned surface. Langmuir 19:9382-9386.

DeFrees S, Wang ZG, Xing R, Scott AE, Wang J, Zopf D, Gouty DL, Sjoberg ER, Panneerselvam K, Brinkman-Van der Linden EC, Bayer RJ, Tarp MA, Clausen H. 2006. GlycoPEGylation of recombinant therapeutic proteins produced in Escherichia coli. Glycobiology 16:833-843.

Dou H, Zheng M, Zhang Y, Yin C. 2007. Synthesis and purification of mono-PEGylated insulin. Chem Biol Drug Des 69:132-138.

Fee CJ, Van Alstine JM. 2004. Prediction of the viscosity radius and the size exclusion chromatography behavior of PEGylated proteins. Bioconjugate Chem 15:1304-1313.

Hagel L. 1998. Gel filtration. In Janson JC, Ryden L, editors. Protein purification, 2nd ed., New York: John Wiley & Sons.

Fee CJ, Van Alstine JM. 2006. PEGylated proteins: Reaction engineering and separation issues. Chem Eng Sci 61:924-939.

Foser S, Schacher A, Weyer KA, Brugger D, Dietel E, Marti S, Thomas Schreitmuller T. 2003. Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon α-2a (PEGASYS). Protein Expr Purif 30:78-87.

Ghosh R, Wong T. 2006. Effect of module design on the efficiency of membrane chromatographic separation processes. J Membr Sci 281:532-540.

Ghosh R. 2002. Protein separation using membrane chromatography: opportunities and challenges. J Chromatogr A 952:13-27.

Hall T, Wood DC, Smith CE. 2004. Preparative and analytical chromatography of pegylated myelopoietin using monolithic media. J Chromatogr A 1041:87-93.

Hermanson GT. 1996. Bioconjugation Techniques. Academic Press. 141 p.

Kinstler O, Molineux G, Treuheit M, Ladd D, Gegg C. 2002. Mono-N-terminal poly(ethylene glycol)–protein conjugates. Adv Drug Deliv Rev 54:477-485.

Kinstler OB. 2005. N-terminally chemically modified protein compositions and methods. US Patent US 6956027, Amgen.

Knudsen HL, Fahrner RL, Xu Y, Norling LA, Blank GS. 2001. Membrane ion-exchange chromatography for process-scale antibody purification. J Chromatogr A 907:145-154.

Kuga S. 1981. Pore size distribution analysis of gel substances by size exclusion chromatography. J Chromatogr 206:449-461.

Kurfurst MM. 1992. Detection and molecular weight determination of polyethylene glycol-modified hirudin by staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Anal Biochem 200:244-248.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Lee H, Jang H, Ryu SH, Park TG. 2003. N-Terminal site-specific mono-PEGylation of epidermal growth factor. Pharm Res 20:818-825.

Pabst TM, Buckley JJ, Ramasubramanyan N, Hunter AK. 2007. Comparison of strong anion-exchangers for the purification of a PEGylated protein. J Chromatogr A 1147:172-182.

Phillips M, Cormier J, Ferrence J, Dowd C, Kiss R, Lutz H, Carter J. 2005. Performance of a membrane adsorber for trace impurity removal in biotechnology manufacturing. J Chromatogr A 1078:74-82.

Piquet G, Gatti M, Barbero L, Traversa S, Caccia P, Esposito P. 2002. Set-up of large laboratory-scale chromatographic separations of poly (ethylene glycol) derivatives of the growth hormone-releasing factor 1–29 analogue. J Chromatogr A 944:141-148.

Reddy KR, Modi MW, Pedder S. 2002. Use of peginterferon alfa-2a (40 KD) (Pegasys®) for the treatment of hepatitis C. Adv Drug Deliv Rev 54:571-586.

Roberts MJ, Bentley MD, Harris JM. 2002. Chemistry for peptide and protein PEGylation. Adv Drug Deliv Rev 54:459-476.

Shaunak S, Godwin A, Choi J, Balan S, Pedone E, Vijayarangam D, Heidelberger S, Teo I, Zloh M, Brocchini S. 2006. Site-specific PEGylation of native disulfide bonds in therapeutic proteins. Nat Chem Bio 2:312-313.

Wang YS, Youngster S, Grace M, Bausch J, Bordens R, Wyss DF. 2002. Structural and biological characterization of pegylated recombinant interferon alpha-2b and its therapeutic implications. Adv Drug Deliv Rev 54:547-570.

Wong SS. 1991. Reactive groups of proteins and their modifying agents in: Chemistry of Protein Conjugation and Cross-linking. CRC Press, Boston. p 13.

Yu D, McLean MD, Hall JC, Ghosh R. 2008. Purification of a human immunoglobulin G1 monoclonal antibody from transgenic tobacco using membrane chromatographic processes. J Chromatogr A 1187:128-137.

Yu D, McLean MD, Hall JC, Ghosh R. 2008. Recovery and purification of monoclonal antibody from tobacco plant using membrane-based bioseparation techniques. J Membr Sci 323:159-166.

Yun Q, Yang RE, Chen T, Bi J, Ma G, Su Z. 2005. Reproducible preparation and effective separation of PEGylated recombinant human granulocyte colony-sitmulating factor with novel "PEG-pellet" PEGylation mode and ion-exchange chromatography. J Biotechnol 118:67-74.

Chapter 6

Integrated Fragmentation of Human IgG and Purification of Fab Using a Reactant Adsorptive Membrane Bioreactor Separator System

This chapter is organized based on a paper published in Biotechnology and Bioengineering, 104:152-161 (2009) by Deqiang Yu and Raja Ghosh.

6.1 Abstract

This paper discusses an integrated separation-reaction-separation scheme for producing Fab fragment directly from human immunoglobulin G (hIgG) present in serum feed. The novel reactant adsorptive membrane bioreactor separator (or RAMBS) system used in the current study consisted of a stack of microporous adsorptive membranes held within a temperature controlled module. The membrane stack, in the presence of salt,

selectively and reversibly adsorbed hIgG by hydrophobic interaction while allowing most other serum proteins to flow through. The bound hIgG was then fragmented by pumping a solution of papain through the reactor at controlled temperature and flow rate. The salt concentration and pH for reaction and separation were systematically optimized using pure hIgG as reactant. The Fab fragment was separated from undigested hIgG and other byproducts such as Fc fragment based on their differences in hydrophobicity. Under optimal conditions, Fab was obtained in the reaction flow through while the other proteins remained bound to the membrane, these being subsequently eluted by lowering the salt concentration. The RAMBS system in addition to being convenient from process integration and intensification points of view also showed higher catalytic efficiency of papain in comparison to that in liquid phase reactions.

6.2 Introduction

Immunoglobulin G (IgG) fragments are widely used for various diagnostic and therapeutic applications (Yamaguchi et al. 1995; Cohen et al. 2000; Ljunglöf et al. 2007). In tests based on the binding of antibodies to antigens present on cells, using Fab or $F(ab)_2$ would prevent interference due to non-specific binding of Fc to receptors. Fab fragments are used in biosensors (Cornell et al. 1997) and for tumor imaging (Moffat et al. 1999). With therapeutic equine and ovine antibodies, removal of Fc portion reduces adverse reactions in patients (Leon et al. 2001; Theakston et al. 2003). Fab fragments have been extensively used as antidotes, e.g. Crotalidae polyvalent immune Fab (Consroe 1995) and digoxin immune Fab (Bateman 2004). Therapeutic monoclonal Fab such as

Abciximab and Ranibizumab (Gabriel and Oliveira 2006; Dugel 2006) have also been commercialized in recent years. Fab fragments are more effective than intact IgG in cancer therapy due to their higher tumor uptake ratio and reduced immunogenicity (Covell et al. 1986; Cortez-Retamozo et al. 2004).

IgG is first purified, followed by homogeneous liquid phase enzymatic fragmentation, eventually followed by multi-step purification to remove the enzyme and byproducts (Karlson-Stiber et al. 1997; Jones and Landon 2002; Coleman and Mahler 2003; Ljunglöf et al. 2007). These processes tend to be slow, complex and expensive with low overall productivity. Papain based digestion is carried out in buffered solutions at 30-40°C for 2-24 hours (Rousseaux et al. 1983; Cresswell et al. 2005). A longer reaction time guarantees higher conversion but complete antibody fragmentation is rarely achieved. The reaction mixture is then dialysed or ultrafiltered prior to purification by column chromatography (Harlow and Lane 1988; Roque et al. 2004; Ljunglöf et al. 2007). Protein-A affinity chromatography which is commonly used for purification of antibody fragments is expensive and susceptible to ligand leakage. Antibody fragmentation could also be carried out using enzymes immobilized on beads but these are expensive. The use of immobilized enzymes facilitates its reuse but the antibody fragments still have to be separated from undigested antibody and byproducts. Immobilized enzyme processes are affected by diffusion limitations, particularly with macromolecular substrates like IgG. Furthermore, immobilized enzymes are advantageous only in applications where the enzyme is significantly more expensive than the substrate e.g. antibiotic intermediate production using Penicillin G Acylase (Bruggink et al. 1998; Kallenberg et al. 2005).

Membrane bioreactors could be broadly classified into two types depending on the nature of enzyme retention (Gekas 1986; Heath and Belfort 1990; Giorno and Drioli 2000). In one type, the soluble enzyme is retained by an ultrafiltration membrane while in the other type the enzyme is immobilized on a membrane. In both types, the enzyme is sequestered within the reactor while the reactant is allowed to flow through for bioconversion. The first type is suitable only for low molecular weight substrates. With the second type, further purification of the fragments from undigested IgG and byproducts is required. In our membrane bioreactor the substrate i.e. the antibody is reversibly immobilized on a stack of environment responsive microporous membranes as in membrane chromatography followed by flow through enzymatic reaction. To the best of our knowledge, this is the first report describing such a reactant adsorptive membrane bioreactor separator system, which henceforth shall be abbreviated as RAMBS.

The main advantages of membrane chromatography over competing separations techniques result from the predominance of convective mass transport. Hydrophobic interaction membrane chromatography (HIMC) with environment responsive membranes has been shown to be suitable for antibody purification (Ghosh 2001; Ghosh 2005). The current work deals with an HIMC based RAMBS where three functions are integrated into one unit operation as shown in Figure 6.1: 1. human immunoglobulin G (hIgG) purification from human serum, 2. hIgG fragmentation using papain, and 3. separation of the Fab fragment from undigested hIgG and byproducts such as the Fc. hIgG was first selectively adsorbed on to hydrophilized polyvinylidene fluoride (PVDF) membrane by hydrophobic interaction in the presence of salt while other serum proteins (mainly human

serum albumin or HSA) were obtained in the purification flow through. Papain solution was then introduced into the RAMBS system to digest the hIgG into Fab and Fc. Fab being less hydrophobic than either Fc or intact hIgG was obtained in the digestion flow through. The system could then be regenerated by eluting the undigested hIgG and Fc fragment using the salt-free buffer. The RAMBS process for producing pure Fab was systematically optimized and effects of operating and solution conditions on efficiency of both separation and catalysis were examined.

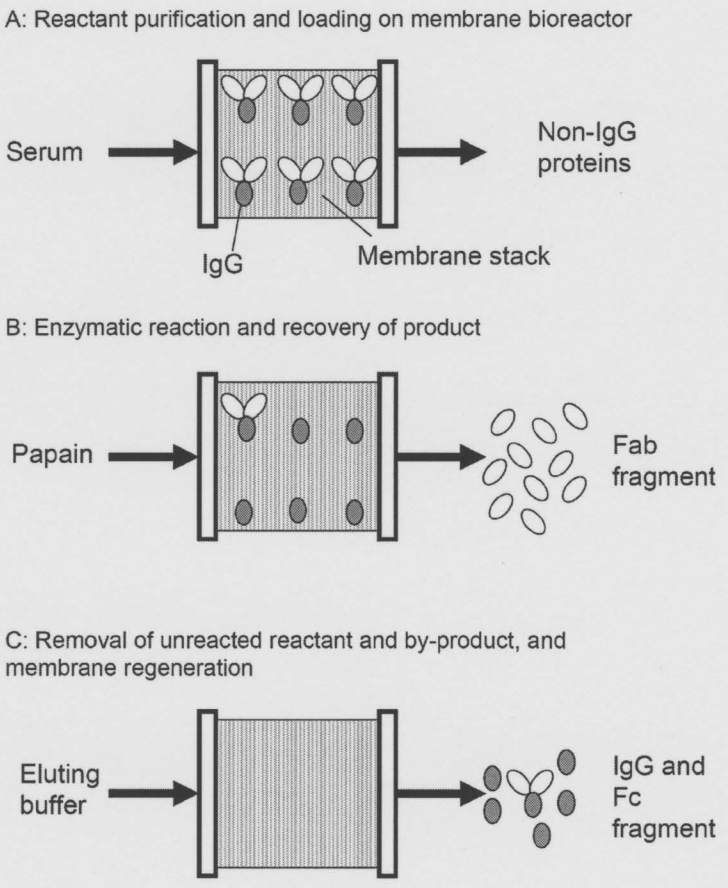


Figure 6.1 Schematic diagram for integrated hIgG purification from human serum, its enzymatic fragmentation followed by Fab purification using a RAMBS system.

6.3 *Materials and methods*

6.3.1 Materials

Human IgG (I4506), human serum (H4522), papain (P3125), anti-hIgG (Fab-specific) alkaline phosphatase conjugated antibody (A8542), anti-hIgG (Fc-specific) alkaline phosphatase conjugated antibody (A9544), BCIP®/NBT-purple liquid substrate for membranes (B3679), TWEEN® 20 (P5927), L-cysteine (30089), disodium EDTA salt dihydrate (E4884), iodoacetamide (16125), sodium phosphate (monobasic) (S0751) and sodium phosphate dibasic (S0876), ammonium sulfate (A4418), citric acid (C0759) and sodium citrate (S4641) were purchased from Sigma-Aldrich (St. Louis, MO). High quality deionised water (18.2 MΩ cm) obtained from a Barnstead Diamond™ NANOpure (Dubuque, IA) water purification unit was used to prepare all the solutions and buffers. 0.22 μm Hydrophilized PVDF membrane (GVWP) was purchased from Millipore (Billerica, MA). Hybond™-ECL™ nitrocellulose membrane was purchased from GE Healthcare Bio-Sciences, USA.

6.3.2 Membrane bioreactor

The RAMBS system (see schematic diagram in Figure 6.2) consisted of a custom-designed stacked membrane bioreactor, a MultiTemp™ III thermostatic hot water circulator and an AKTA Prime liquid chromatography system (the last two from GE Healthcare Bio-Sciences). The stacked membrane bioreactor consisted of a membrane stack provided with a heating water jacket for temperature control. The buffers, reactant and enzyme solutions were fed into this membrane bioreactor through a heat exchanger

by the AKTA system. The effluent from the membrane bioreactor was continuously monitored using the UV detector and conductivity and pH sensors of the AKTA system and the data was logged into a computer using Prime View software (GE Healthcare Bio-Sciences).

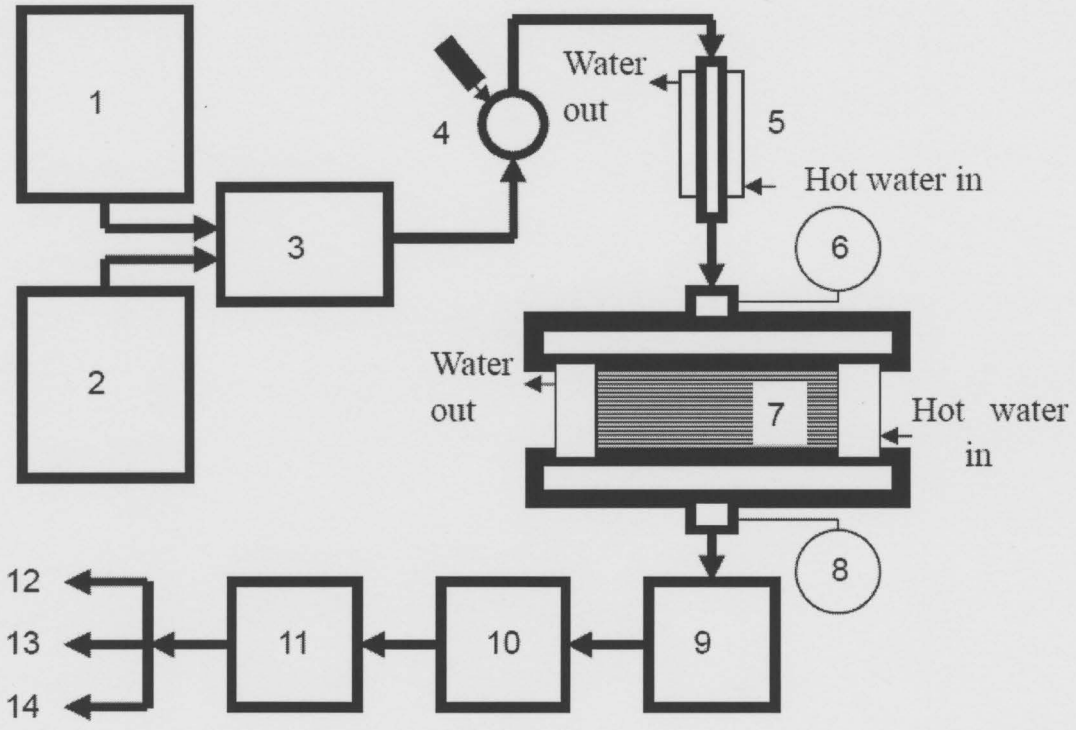


Figure 6.2 Experimental set-up used for the RAMBS experiments (1 binding buffer tank, 2 eluting buffer tank, 3 pump, 4 sample injector, 5 heat exchanger, 6 reactor inlet thermometer, 7 jacketed stacked membrane bioreactor, 8 reactor outlet thermometer, 9 UV monitor, 10 pH monitor, 11 conductivity monitor, 12 product, 13 by-product, and 14 waste).

6.3.3 Liquid phase enzymatic digestion of human IgG

This was carried out at 37°C for 1 hour in 1.5 mL plastic tubes containing 1 mg pure hIgG and 0.05 mg papain in 1 mL digestion buffer. The starting digestion buffer consisted of 20 mM pH 7.0 sodium phosphate buffer, 10 mM disodium EDTA and 10

mM L-cysteine. The reaction was terminated by adding 50 μL of 0.6 M iodoacetamide into the reaction mixture. The effects of pH and ammonium sulfate concentration on papain activity were studied. The reaction mixtures were analyzed by Size Exclusion Chromatography (SEC) for determining hIgG conversion (calculated based on reduction in IgG peak area using standard IgG calibration) and by SDS-PAGE for qualitative assessments.

6.3.4 Protein-A based separation of Fab and Fc

A 1 mL Protein-A HP column (GE Healthcare Bio-Sciences) was used to separate Fab from Fc and undigested hIgG using 20 mM sodium phosphate (pH 7.0) as binding buffer and 100 mM sodium citrate (pH 3.0) as eluting buffer, at 1 mL/min flow rate. The reaction mixture was diluted 1:10 in binding buffer prior to loading onto the column. The flow through and eluate samples were collected and analyzed by SEC and SDS-PAGE.

6.3.5 Digestion of pure hIgG and purification of Fab using RAMBS

Thirty PVDF membrane discs having 18 mm diameter were stacked within the membrane bioreactor to provide 0.95 mL bed volume. The eluting buffer used was 20 mM sodium phosphate (pH 7.5) while the binding buffers were prepared by adding appropriate amounts of ammonium sulfate to the eluting buffer. The membrane bioreactor was first equilibrated using binding buffer and the effluent temperature was maintained at 37 ± 0.5 °C. hIgG solution (0.2 mg/mL) prepared in the appropriate binding buffer was then fed into membrane reactor at followed by washing with binding buffer until a stable UV absorbance baseline was obtained. 1 mg of hIgG was used in the experiments for

studying the effect of salt concentration while 4 mg was used in those for studying the effect of enzyme/substrate ratio and flow rate. Papain solution prepared in the corresponding binding buffer (containing in addition, 10 mM cysteine and 10 mM EDTA) was then fed into membrane module to digest the bound hIgG and the digestion flow through was collected. The bound material was then removed from the membrane stack using eluting buffer. Feed, digestion flow through and eluate samples obtained from the experiments were analyzed by SEC, SDS-PAGE and western blotting.

6.3.6 Digestion of human serum and purification of Fab using RAMBS

This procedure was similar to that used for digestion of pure hIgG. The feed solution (0.2 mL human serum in 20 mL binding buffer) was fed into the membrane bioreactor for selective hIgG adsorption. After washing the membrane stack with binding buffer the papain solution was fed into membrane module to digest the bound hIgG. This was followed by elution of bound material. The feed, purification flow through, digestion flow through and eluate were collected and analyzed using SEC, SDS-PAGE and western blotting.

6.3.7 SEC analysis

SEC analysis was carried out at a flow rate of 0.2 mL/min using a Superdex 200 10/300 GL column (GE Healthcare Bio-Sciences) fitted to a Varian HPLC system (Palo Alto, CA), using 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl as mobile phase.

6.3.8 SDS-PAGE and Western Blotting

SDS-PAGE experiments were run according to the work of Laemmli (1970). 10% non-reducing gels were obtained using a Hoefer MiniVE system (GE Healthcare Bio-Sciences). After running the gels, the protein bands obtained were either directly stained by Coomassie blue or electro-transferred to a Hybond^{TM}-ECL^{TM} nitrocellulose membrane using a MiniVE blot module. The membranes were then blocked followed by immunoprobing with anti-hIgG (Fab-specific or Fc-specific) alkaline phosphatase conjugated antibody. The procedure used was slightly modified from the protocol described by Gallagher et al. (1998). BCIP®/NBT-purple liquid substrate was used to visualize the bands on the membranes.

6.4 Results and discussion

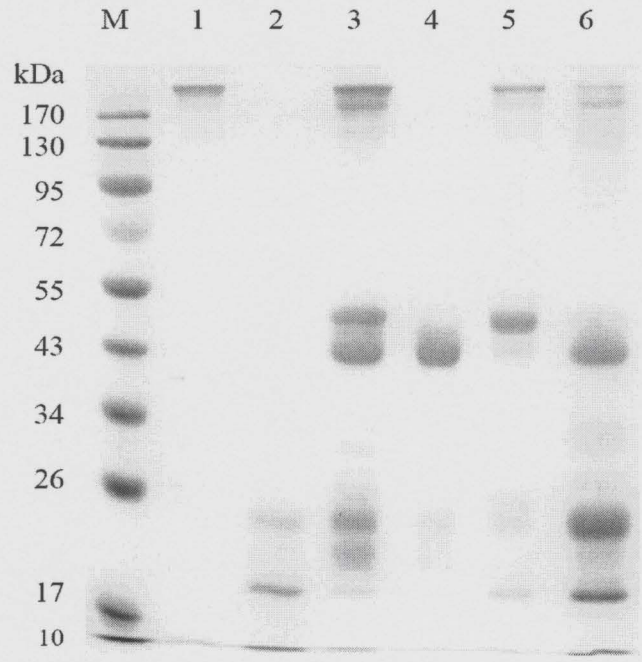


Figure 6.3 SDS-PAGE gel characterizing the key components in hIgG digestion (Lanes: M marker, 1 pure hIgG, 2 papain, 3 products of liquid phase hIgG digestion, 4 flow through of Protein-A chromatography, 5 eluate of Protein-A chromatography, 6 total reaction mixture obtained by hIgG digestion using RAMBS at 1.75 M ammonium sulfate concentration).

Figure 6.3 shows the key components obtained by papain digestion of hIgG. Standard hIgG (lane 1) gave a single band (slightly higher than the 170 kDa marker). The actual average molecular weight (MW) of hIgG is known to be c.a. 155 kDa. The main reason for this discrepancy is that antibodies due to their atypical shape have lower mobility in the gel medium. Standard papain (lane 2) with a MW of 23 kDa showed a thick band of approximately 20 kDa and a faint band of approximately 24 kDa. SEC analysis of papain gave a single symmetric peak (chromatogram not shown here) clearly indicating homogeneity. Presumably, a significant proportion of papain was degraded from 24 kDa to 20 kDa due to boiling with SDS. The reaction mixture obtained by liquid phase digestion (lane 3) contained undigested hIgG, two close bands between the 43 and 55 kDa markers and some low MW components. Protein-A column chromatography was able to separate the digestion mixture into Fab (in the flow through) and Fc and undigested hIgG (in the eluate). Fab (see lane 4) was obtained closer to the 43 kDa MW marker while Fc (see lane 5) was obtained closer to the 55 kDa marker. An equivalent RAMBS experiment was carried out in the presence of 1.75 M ammonium sulfate. At this salt concentration, all components including Fab, undigested hIgG and byproduct remained bound to the membrane and were eluted out together using salt free buffer. The eluate (lane 6) showed a higher degree of hIgG digestion than in the liquid phase reaction sample. In the RAMBS experiment, Fc degraded into half Fc fragments to a significantly greater extent. Digestion of Fc by papain into c.a. 27 kDa fragments in liquid phase reactions has been reported by other researchers (Andrew and Titus 1997; Bennich and Turner 1969; Nardella and Teller 1985). These half Fc fragments were recognized by

anti-hIgG Fc immunoprobing on western blots as described later on in the paper (Figure 6.10(b)). It should be noted that the fainter of the two papain bands had a similar position as half Fc on the gel. However, the amount of papain used in these digestion experiments was very small and it was hence unlikely to contribute towards the 24 kDa bands in lanes 3 and 6 in any significant way. The RAMBS experiment also demonstrated that the presence of ammonium sulfate did not inhibit papain based digestion of hIgG.

The effect of ammonium sulfate concentration on hIgG digestion in liquid phase reactions was studied. The 1.3 M to 1.8 M salt concentration range was chosen since preliminary experiments showed that hIgG binding on the hydrophilized PVDF became insignificant below 1.3 M salt concentration while at concentrations higher than 1.8 M significant hIgG precipitation occurred. The hIgG conversions for 0M, 1.3M, 1.4M, 1.5M, 1.6M, 1.7M and 1.8M salt concentration were 67.6%, 78.3%, 78.3%, 74.9%, 74.8%, 70.6% and 66.3% respectively with the average of standard error based on experiments carried out in duplicate being ± 0.6%. Papain based digestion of other proteins such as bovine serum albumin and heavy meromyosin have been found to be similarly affected by the ionic strength of the reaction medium (Sorrentino et al. 1982; Suzuki 1988). The presence of ammonium sulfate being the basis for hIgG binding on the membrane, one of the initial concerns was whether the presence of this salt would adversely affect catalysis. The above results clearly demonstrate that rather than being an impediment, the presence of the salt improved reaction efficiency, certainly in liquid phase reactions.

Figure 6.4 summarizes the effects of ammonium sulfate concentration on the composition of digestion flow through obtained from the RAMBS system. No apparent protein bands at 1.75 M concentration meant that all components including Fab, Fc and undigested hIgG remained bound to the membrane. At 1.6 M salt concentration a faint Fab band was observed with the band intensity increasing at 1.5 M concentration. No apparent impurity was observed in the flow through at both these conditions. When salt concentration was further decreased to 1.4 M, an Fc band was observed indicating that at this salt concentration it no longer remained completely bound to the membrane. However, no undigested hIgG was observed in the flow through. These results indicate that the hydrophobicity increased in the order: Fab < Fc < intact hIgG. An ammonium sulfate concentration of 1.5 M therefore appeared to be appropriate for carrying out hIgG digestion with papain using the HIMC based RAMBS system since it ensured high enzyme activity and made it possible to purify Fab.

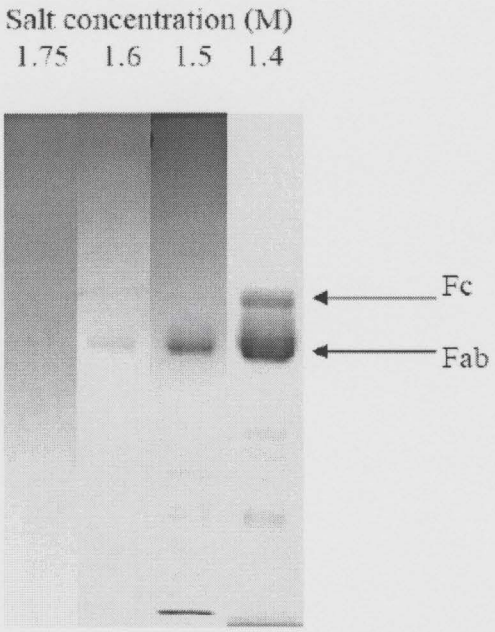


Figure 6.4 SDS-PAGE analysis of flow through obtained during hIgG digestion using the RAMBS system at different ammonium sulfate concentrations.

The effect of solution pH on hIgG digestion by papain in liquid phase reaction was examined in the presence of 1.5 M ammonium sulfate. The conversions for pH 5.8, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 were 76.1%, 81.8%, 82.9%, 85.1%, 83.9%, 79.3% and 80.2% respectively with the average standard error being ± 1.8%. The hIgG conversion clearly increased between pH 5.8 and pH 7.5 and then decreased at pH 9.0, being high in the pH 6.5 to 8.0 range. Earlier studies have shown that papain was most active between pH 6.0 and pH 7.0 (Kilara et al. 1977). The slight shift in the optimal pH range could be due to the presence of ammonium sulfate. All further experiments using the RAMBS system were therefore carried out at pH 7.5 and in the presence of 1.5 M ammonium sulfate, these solution conditions being optimal for both catalysis and separation.

Purified hIgG was digested by papain using the RAMBS system under optimized solution conditions to produce Fab. Figure 6.5 shows the UV absorbance and conductivity profiles of the bioreactor effluent. The hIgG injected was totally bound to the membrane stack as evident from the absence of purification flow through peaks following the two feed injections. When papain solution was introduced into the bioreactor, Fab was obtained in the digestion flow through as evident from the broad peak between 40 and 50 mL effluent volume. The membrane bound Fc and undigested hIgG were then eluted around 72 mL effluent volume by reducing the salt concentration. The feed, digestion flow through and eluate samples obtained from the above experiment were analyzed by SEC (see Figure 6.6(a)) and SDS-PAGE (see Figure 6.6(b)). The hIgG chromatogram in Figure 6.6(a) shows the monomer peak at a retention time (RT) of 56.6 min. The smaller peak at an RT of 47.9 min was due to hIgG dimer, this being verified by MW calibration.

The chromatogram obtained with the digestion flow through shows the Fab peak at an RT of 73.8 min, this corresponding to the Fab band closer to the 43 kDa MW marker (see lane 2 of Figure 6.6(b)). The peak at an RT of 95.7 min in the digestion flow through (see Figure 6.6(a)) was due to very small fragments, these being less than 6.5 kDa as determined by comparison with low MW marker proteins in SEC (chromatogram not shown here). The chromatogram obtained with the eluate (see Figure 6.6(a)) shows peaks corresponding to undigested hIgG (RT around 56 min) and Fc (RT of 72.0 min). The third peak on the chromatogram obtained with the eluate was due to half Fc and possibly trace amounts of papain. The presence of undigested hIgG, Fc and half Fc in the eluate was verified by the presence of corresponding bands on SDS-PAGE (see lane 4 of Figure 6.6(b)).

The RAMBS system was then used to obtain Fab directly from human serum which is a complex mixture of proteins, the two most abundant among these being HSA and hIgG, together making up more than 80% of total serum proteins (Putnam 1975). The UV absorbance and conductivity profiles of the reactor effluent obtained during this experiment are shown in Figure 6.7. hIgG was bound to the membrane during the two injections of human serum feed while most non-hIgG proteins including HSA were obtained in the purification flow through (corresponding to the two broad peaks following the two injections). When the enzyme solution was introduced into the reactor at controlled flow rate, Fab was obtained in the digestion flow through (i.e. around 65 mL effluent volume). Fc and undigested hIgG were eluted in the sharp peak around 87 mL effluent volume.

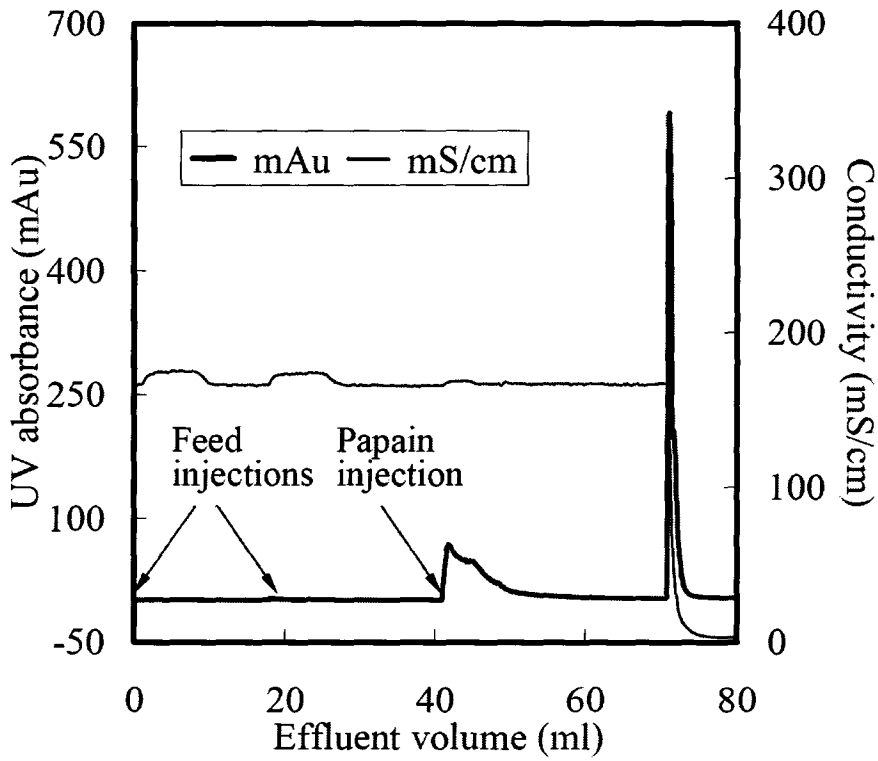


Figure 6.5 UV absorbance and conductivity profiles obtained with the bioreactor effluent during papain digestion of hIgG using RAMBS system (membrane: 0.22 micron hydrophilized PVDF, membrane diameter: 18 mm, number of discs in stack: 30, temperature: 37°C, eluting buffer: 20 mM sodium phosphate pH 7.5, binding buffer: eluting buffer + 1.5 M ammonium sulfate, flow rate: 1 mL/min for equilibration, washing and elution, 0.1 mL/min during IgG digestion, IgG loading: 4 mg; papain amount: 0.8 mg dissolved in 5 mL binding buffer containing in addition 10 mM cysteine and 10 mM EDTA).

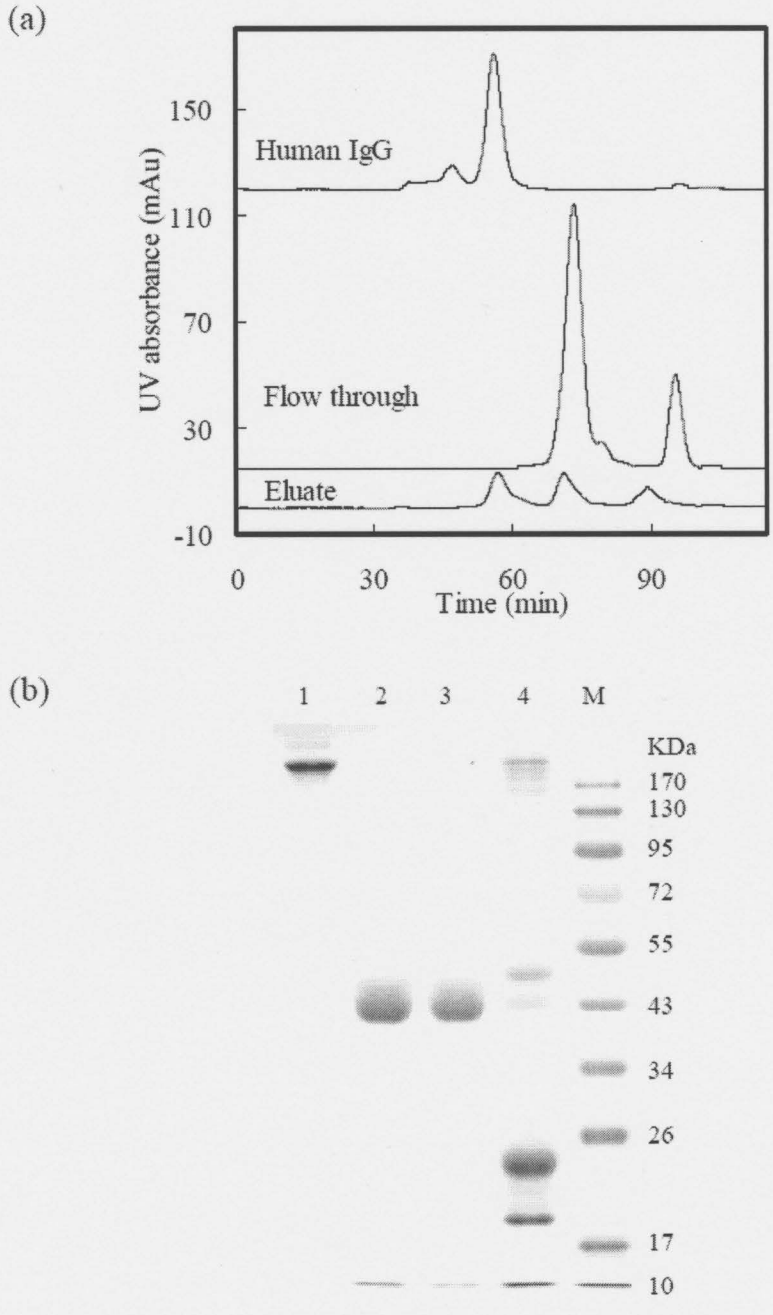


Figure 6.6 Analysis of feed, flow through and eluate obtained during hIgG digestion using RAMBS system. (a) SEC analysis (column: Superdex 200 10/300 GL, mobile phase: 20 mM sodium phosphate buffer containing 150 mM NaCl pH 7.0, flow rate: 0.2 mL/min); (b) Coomassie blue stained 10% non-reducing SDS-PAGE gel (Lanes: M marker, 1 IgG feed, 2 flow through obtained during papain digestion, 3 flow through processed by 30 kDa centrifugal ultrafiltration, 4 eluate from membrane bioreactor).

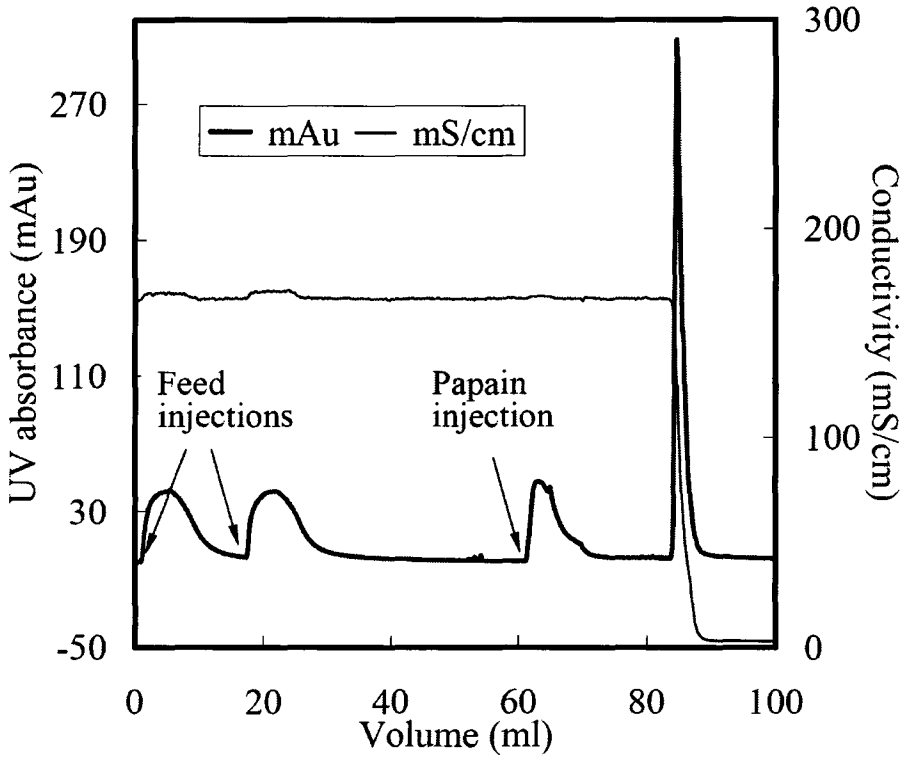


Figure 6.7 UV absorbance and conductivity profiles obtained with the bioreactor effluent during papain digestion of human serum using RAMBS system (membrane: 0.22 micron hydrophilized PVDF, membrane diameter: 18 mm, number of discs in stack: 30, temperature: 37°C, eluting buffer: 20 mM sodium phosphate pH 7.5, binding buffer: eluting buffer + 1.5 M ammonium sulfate, flow rate: 1 mL/min for equilibration, washing and elution, 0.1 mL/min during hIgG digestion, feed: 0.2 mL of human serum pre-mixed with 20 mL of binding buffer; hIgG loading: c.a. 4 mg; papain amount: 0.8 mg dissolved in 5 mL binding buffer containing in addition 10 mM cysteine and 10 mM EDTA).

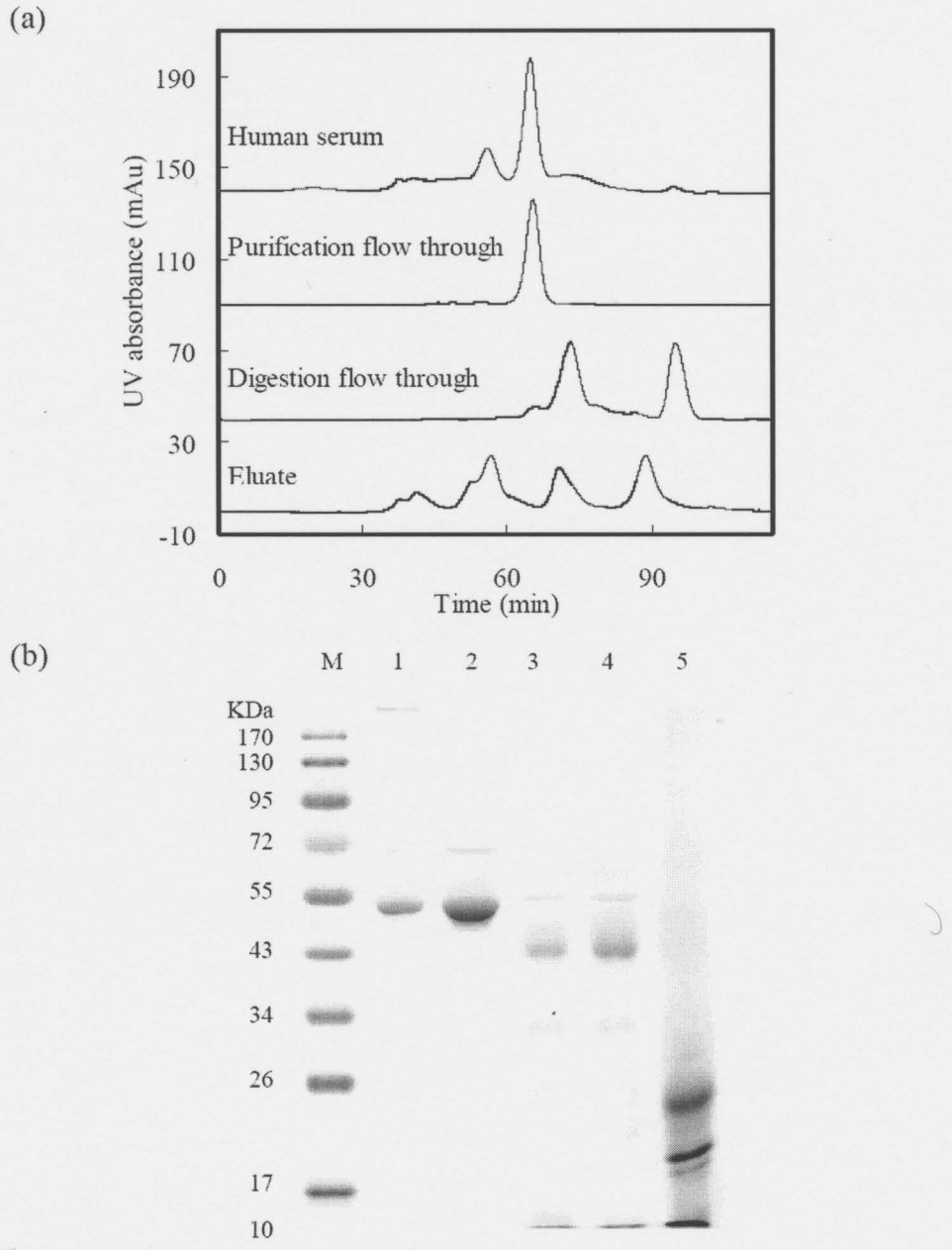


Figure 6.8 Analysis of feed, separation and reaction flow through samples and eluate obtained during digestion of IgG in human using RAMBS system. (a) SEC analysis (column: Superdex 200 10/300 GL, mobile phase: 20 mM sodium phosphate buffer containing 150 mM NaCl pH 7.0, flow rate: 0.2 mL/min); (b) Coomassie blue stained 10% non-reducing SDS-PAGE gel (Lanes: M marker, 1 human serum feed, 2 flow through obtained during hIgG purification from human serum feed, 3 second flow through, i.e. flow through obtained during papain digestion; 4 second flow through after processing by 30 kDa centrifugal ultrafiltration, 5 eluate from membrane stack).

The feed, the purification and digestion flow through and eluate samples from the above RAMBS experiment were analyzed by SEC (Figure 6.8(a)) and SDS-PAGE (Figure 6.8(b)). The human serum feed chromatogram (Figure 6.8(a)) showed the hIgG peak (at an RT of 56.7 min) and a big HSA peak (at an RT of 65.3 min), these corresponding to the band above the 170 kDa and 55 kDa markers respectively on the SDS-PAGE gel (see lane 1 of Figure 6.8(b)). The purification flow through consisted mainly of HSA, indicating the nearly complete binding of hIgG from the serum feed. The faint band in lane 2 (corresponding to the 72 kDa marker) of Figure 6.8(b) was due to transferrin, the third most abundant serum protein having a MW of 79.5 kDa (Putnam et al. 1975). The digestion flow through contained mainly Fab (at RT of 73.8 min), small amounts of fragments (at RT of 95.2 min) and small amounts of HSA. The eluate showed some high MW impurities (RT of 38-42 min) along with unreacted hIgG (RT of 57.4 min), Fc (RT of 71.9 min) and half-Fc (RT of 89.5 min).

Centrifugal ultrafiltration with 30 kDa molecular weight cut-off (MWCO) membrane was used to remove the small fragments and salts from the Fab samples obtained by the RAMBS system. As shown in Figure 6.9, Fab obtained from the pure hIgG was 96.5% pure with the impurities mainly consisting of degraded Fab (as shown in Figure 6.10, these low molecular weight fragments recognized by anti-hIgG Fab but not by anti-hIgG Fc), while that obtained from human serum was 82.1% pure with impurity mainly consisting of HSA.

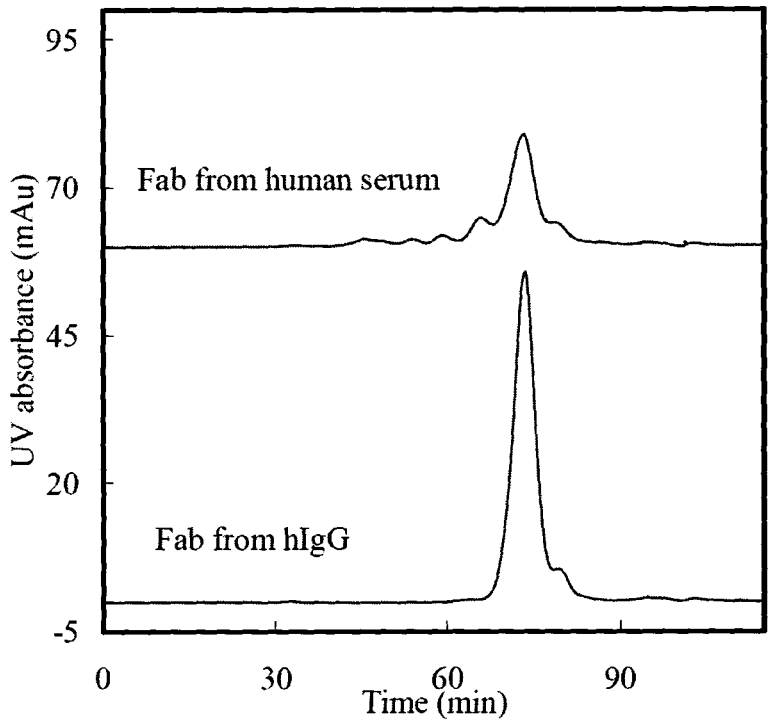


Figure 6.9 SEC analysis of Fab produced from hIgG and human serum using RAMBS, both samples having been processed by 30 kDa centrifugal ultrafiltration to remove small fragments (column: Superdex 200 10/300 GL, mobile phase: 20 mM sodium phosphate buffer containing 150 mM NaCl pH 7.0, flow rate: 0.2 mL/min).

Figure 6.10 (a) shows the western blot obtained by anti-hIgG Fab immunoprobing. hIgG present in the feed (lane 1) and Fab present in the digestion flow through (lanes 2-3) could be clearly identified. The eluate sample (lane 4) contained undigested hIgG and some amount of Fab. The results obtained with samples from the human serum digestion experiment were consistent with expectations. Figure 6.10 (b) shows the blot obtained by anti-hIgG Fc immunoprobing. All Fab containing samples were free from Fc. The eluate samples from the pure hIgG digestion experiment (lane 4) and the human serum digestion experiment (lane 8) each showed three major bands corresponding to intact hIgG, Fc and half Fc respectively. Some faint bands close to the

130 kDa MW marker (corresponding to actual MW of c.a. 100 kDa due to atypical shape) were detected with both anti-hIgG Fab and anti-hIgG Fc (lanes 4 and 8 of both blots). Papain digestion of hIgG is known to generate, albeit in small quantities, three molecular entities having 100 kDa molecular weight: $F(c)_2$, F(ab)c and $F(ab)_2$ (Michaelsen and Natvig 1972 and 1973).

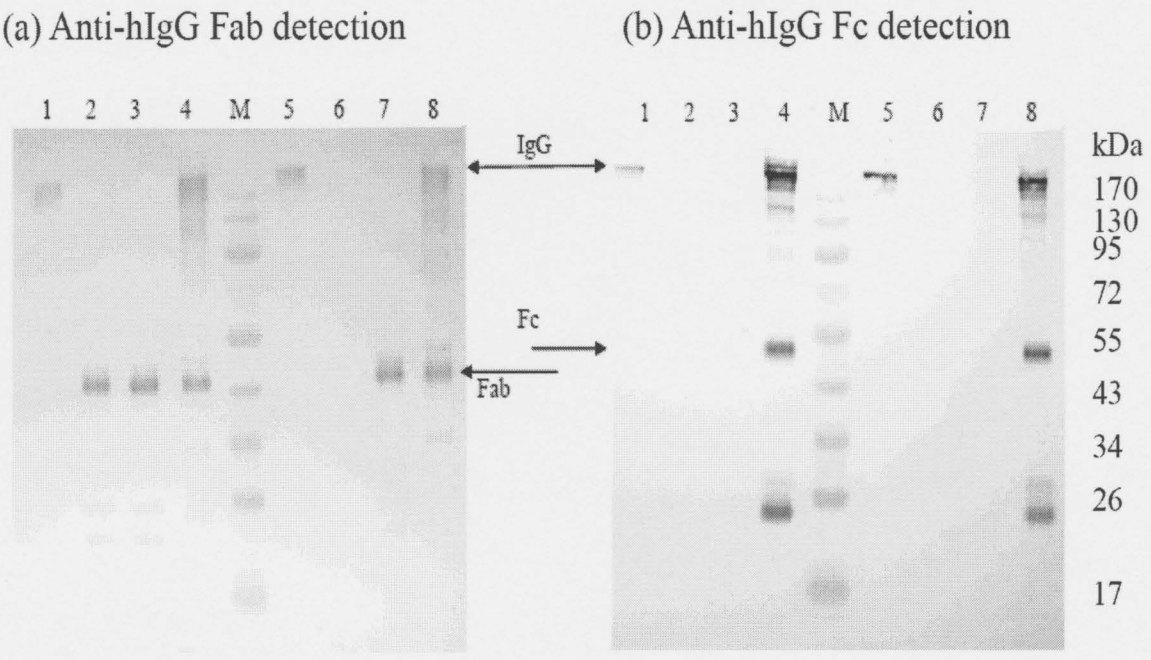


Figure 6.10 Western-blot analysis of sample obtained during digestion of IgG and human serum using RAMBS (a) anti-hIgG-Fab detection, (b) anti-hIgG-Fc detection (Lanes: M marker, 1 pure hIgG, 2 flow through from hIgG digestion experiment, same after 30 kDa centrifugal ultrafiltration, 4 eluate from hIgG digestion experiment, 5 human serum, 6 flow through obtained during purification of hIgG from human serum, 7 flow through from human serum digestion experiment, 8. eluate from human serum digestion experiment).

The conversion of pure hIgG in liquid phase reaction carried out at pH 7.5 and 37°C for 1 hour using an enzyme/substrate mass ratio of 1:20 was 85.1%. In the corresponding RAMBS experiment the conversion was 89.7%. The hIgG digestion rates for the RAMBS system and liquid phase reaction were therefore 2.4×10^{-6} and 1.9×10^{-6} mmol IgG/ mg papain/ min respectively. The digestion rate is an overall reaction parameter which is obtained by dividing the amount of IgG fragmented (in mmol) by the total amount of papain used (in mg) and the reaction time (in min). The actual catalytic efficiency of papain in the RAMBS system was much greater than that indicated by these numbers. In liquid phase reaction, the entire amount of enzyme in the reaction mixture was available for IgG digestion while with the RAMBS system only a small proportion of the enzyme injected was available at any given instant, the void volume of the membrane stack being only 0.5 mL as compared to 5 mL papain solution injected. The higher conversion and productivity of the RAMBS system could be partly attributed to the predominance of convective mass transport inherent with membrane chromatographic systems (Ghosh 2002). In liquid phase reaction, both enzyme and reactant were freely mobile whereas with the RAMBS system the reactant was held immobile on the membrane surface for enzymatic digestion. Presumably, the manner in which the reactant was presented to the enzyme contributed towards more efficient digestion. This phenomenon, i.e. the potential increase in the rate of enzymatic digestion of a macromolecular substrate by its immobilization on a solid support is currently being investigated.

When the enzyme/substrate molar ratio in the RAMBS experiment was increased to 1:5 by increasing the amount of enzyme while keeping the amount of hIgG the same, the conversion increased to 94.8%. However, increasing the enzyme solution flow rate from 0.1 to 0.2 mL/min decreased the conversion from 94.8% to 79.3%. While the enzyme mass transfer coefficient increased due to increase in flow rate, its residence time and hence effective reaction time decreased. However, the digestion rate increased from 0.63 $\times 10^{-6}$ mmol IgG/ mg papain/ min to 1.1 $\times 10^{-6}$ mmol IgG/ mg papain/ min with increase in flow rate. The hIgG conversion from human serum feed using the RAMBS system (using the same amount of hIgG and 1:20 enzyme/substrate mass ratio) was 73.9%. The eluate from the human serum feed experiment contained some large macromolecules corresponding to RT of 38, 42 and 53 min, presumably IgM (MW 950 kDa), α2-Macroglobulin (MW 725 kDa) and IgA (MW 300 kDa). These large proteins could potentially cause steric hindrance to papain-hIgG interaction and thereby reduce conversion.

The above results clearly demonstrate that hIgG purification, its digestion with papain and purification of Fab thus produced could be integrated into one unit operation using the novel RAMBS system. The membrane was used as a *molecular chopping board* for holding in place the hIgG molecules so that these could be fragmented by papain, followed by separation of the fragments thus generated by their sequential release. This system would be attractive in any application where relatively expensive macromolecular reactants such as antibodies are to be digested using relatively inexpensive enzymes such as papain and pepsin. The current work uses hydrophobic interaction for reversible

immobilization of the reactant on the membrane stack but other types of interactions such as ion-exchange could also be utilized. The production capacity of the system was not fully exploited in the current work since the primary goal was to provide proof-of-concept for a novel approach to enzymatic digestion. The amount of hIgG used in the current study resulted in an effective loading of 4.21 mg of antibody per mL of bed volume. The antibody binding capacities of the PVDF membranes being much higher (Ghosh and Wang 2006; Ghosh 2005), significantly greater amounts of reactants than those used in this study could potentially have been loaded.

6.5 Conclusions

The integrated purification-reaction-purification scheme used in the RAMBS system greatly simplified as well as intensified the process of Fab production. The presence of ammonium sulfate in the reaction medium was found to be beneficial for the hIgG digestion by papain. The pH of the reaction medium influenced the extent of hIgG digestion. The salt concentration affected the composition of the digestion flow through from the membrane bioreactor, thus making it possible to purify Fab. Fab was less hydrophobic than the other component of the reaction medium and under optimized conditions Fab was obtained in the digestion flow through. Undigested hIgG and byproducts could subsequently be eluted by lowering the salt concentration. The Fab purity and recovery in the RAMBS experiments using human serum feed were lower than the corresponding values obtained using pure hIgG feed, presumably due to the presence of bound impurities. For a given enzyme/substrate ratio, greater conversion of hIgG to

Fab was observed with the RAMBS system than in a liquid phase reaction. The catalytic efficiency of papain was therefore higher when hIgG was immobilized on a surface. The product profile obtained using the RAMBS system was also different from that obtained in a liquid phase reaction.

6.6 References

Andrew SM, Titus JA. 1997. Current Protocols in Immunology. New York: John Wiley & Sons. p 2.8.1-2.8.10.

Bateman DN. 2004. Digoxin-specific antibody fragments: how much and when? Toxicol Rev 23:135-143.

Bennich H, Turner MW. 1969. The release of Fc' and pFc' fragments from human immunoglobulin G following enzymic digestion: a time study. Biochim Biophys Acta 175:388-395.

Bruggink A, Roos EC, DeVroom E. 1998 Penicillin acylase in the industrial production of β -lactam antibiotics, Org Proc Res Dev 2:128-133.

Cohen SA, Trikha M, Mascelli MA. 2000. Potential future clinical applications for the GPIIb/IIIa antagonist, abciximab in thrombosis, vascular and oncological indications. Pathol Oncol Res 6:163-174.

Consroe P, Egen NB, Russell FE, Gerrish K, Smith DC, Sidki A, Landon JT. 1995. Comparison of a new ovine antigen binding fragment (Fab) antivenin for United States Crotalidae with the commercial antivenin for protection against venom-induced lethality in mice. Am J Trop Med Hyg 53:507-510.

Cornell BA, Braach-Maksvytis VLB, King LG, Osman PDJ, Raguse L, Wieczorek L, Pace RJ. 1997. A biosensor that uses ion-channel switches. Nature 387: 580-582.

Cortez-Retamozo V, Backmann N, Senter PD, Wernery U, Baetselier PD, Muyldermans S, Revets H. 2004. Efficient cancer therapy with a nanobody-based conjugate. Cancer Res 64:2853-2857.

Covell DG, Barbet J, Holton OD, Black CD, Parker RJ, Weinstein JN. 1986. Pharmacokinetics of monoclonal immunoglobulin G1, $F(ab')_2$, and Fab' in mice. Cancer Res 46:3969-3978.

Cresswell C, Newcombe AR, Davies S, Macpherson I, Nelson P, O'Donovan K, Francis R. 2005. Optimal conditions for the papain digestion of polyclonal ovine IgG for the production of bio-therapeutic Fab fragments. Biotechnol Appl Biochem 42(Pt 2):163-168.

Dugel PU. 2006. Ranibizumab treatment of patients with ocular diseases. Int Ophthalmol Clin 46:131-140.

Gabriel HM, Oliveira EI. 2006. Role of abciximab in the treatment of coronary artery disease. Expert Opin Biol Ther 6:935-942.

Gallagher S, Winston SE, Fuller SA, Hurrell JGR. 1998. Current Protocols in Immunology. New York: John Wiley & Sons. p 8.10.8-8.10.10.

Gekas VC. 1986. Artificial membranes as carriers for the immobilization of biocatalysts. Enzyme Microb Technol 8:450-460.

Ghosh R. 2001. Separation of proteins using hydrophobic interaction membrane chromatography. J Chromatogr A 923:59-64.

Ghosh R. 2002. Protein separation using membrane chromatography: opportunities and challenges. J Chromatogr A 952:13-27.

Ghosh R. 2005. Fractionation of human plasma proteins by hydrophobic interaction membrane chromatography. J Membr Sci 260:112-118.

Ghosh R, Wang L. 2006. Purification of monoclonal antibody by hydrophobic interaction membrane chromatography. J Chromatogr A 1107:104-109.

Giorno L, Drioli E. 2000. Biocatalytic membrane reactors: applications and perspectives. Trends Biotechnol 18:339-349.

Harlow E, Lane D. 1988. Antibodies, A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press. p 626–629.

Heath C, Belfort G. 1990. Membranes and bioreactors. Int J Biochem 22:823-825

Jones RGA, Landon L. 2002. Enhanced pepsin digestion: a novel process for purifying antibody $F(ab')_2$ fragments in high yield from serum. J Immunol Methods 263:57-74.

Kallenberg AI, van Rantwijk F, Sheldona RA. 2005. Immobilization of penicillin G acylase: the key to optimum performance, Adv Synth Catal 347:905-926.

Karlson-Stiber C, Persson H, Heath A, Smith D, al-Abdulla IH, Sjostrom L. 1997. First clinical experiences with specific sheep Fab fragments in snake bite. Report of a multicentre study of Vipera berus envenoming. J Intern Med 241:53-58.

Kilara A, Shahani KM, Wagner FW. 1977. Preparation and properties of immobilized papain and lipase. Biotechnol Bioeng 19:1703-1714.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Leon G, Monge M, Rojas E, Lomonte B, Gutierrez JM. 2001. Comparison between IgG and $F(ab')_2$ polyvalent antivenoms: neutralization of systemic effects induced by Bothrops asper venom in mice, extravasation to muscle tissue, and potential for induction of adverse reactions. Toxicon 39:793-801.

Ljunglöf A, Lacki KM, Mueller J, Harinarayan C, van Reis R, Fahrner R, Van Alstine JM. 2007. Ion exchange chromatography of antibody fragments. Biotechnol Bioeng 96:515-524.

Michaelsen TE, Natvig JB. 1972. Three new fragments, $F(ab)_2$, $F(c)_2$, and Fab-c, obtained by papain proteolysis of normal human IgG. Scand J Immunol 2:299-312.

Michaelsen TE, Natvig JB. 1973. Characterization of subclass-related $F(ab)_2$, Fab-c and Fch fragments obtainined by short papain digestion of human IgG myeloma proteins. Scand J Immunol 2:299-312.

Moffat FL Jr, Gulec SA, Serafini AN, Sfakianakis GN, Pop R, Robinson DS, Franceschi D, Boggs J, Livingstone AS. 1999. A thousand points of light or just a dim light bulb? Radiolabelled antibodies and colorectal cancer imaging. Cancer Invest 17:322-334.

Nardella FA, Teller DC. 1985. Fc intermediate (Fci), a papain-generated fragment of human IgG, intermediate in charge, molecular weight and cleavage between the Fc and Fc' fragments of IgG. Mol Immunol 22:705-713.

Putnam FW. 1975. The plasma proteins: Structure, function, and genetic control. New York: Academic Press. p 26-29.

Roque AC, Lowe CE, Taipa MA. 2004. Antibodies and genetically engineered related molecules: Production and purification. Biotechnol Prog 20:639-654.

Rousseaux J, Rousseaux-Prévost R, Bazin H. 1983. Optimal Conditions for the Preparation of Fab and F(ab')$_2$ Fragments from Monoclonal IgG of Different Rat IgG Subclasses. J Immunol Methods 64:141-146.

Sorrentino S, Yakovlev GI, Libonati M. 1982. Dimerization of deoxyribonuclease I, lysozyme and papain effects of ionic strength on enzymic activity. Eur J Biochem 124:183-189.

Suzuki H, Kondo Y, Carlos AD, Seidel JC. 1988. Effects of phosphorylation, MgATP, and ionic strength on the rates of papain degradation of heavy and light chains of smooth muscle heavy meromyosin at the 51-52 junction. J Bio Chem 263:10974-10979.

Theakston RDG., Warrell DA, Griffiths E. 2003. Report of a WHO workshop on the standardization and control of antivenoms. Toxicon 41:541-557.

Yamaguchi Y, Kim H, Kato K, Masuda K, Shimada I, Arata Y. 1995. Proteolytic fragmentation with high specificity of mouse immunoglobulin G. J Immunol Methods 181:259-267.

Chapter 7

Ion-exchange Based Reactant Adsorptive Membrane Bioreactor Separator System for Integrated Bioprocessing of Antibody Fragments

This work will be separated into two papers, tentatively titled as "Cation-exchange based reactant adsorptive membrane bioreactor separator for integrated bioprocessing of $F(ab')_2$" and "Integrated bioprocessing of Fab using anion-exchange based reactant adsorptive membrane bioreactor separator", and respectively submitted to Biotechnology Progress and Journal of Immunological Methods by Deqiang Yu and Raja Ghosh.

7.1 Abstract

This chapter discusses ion-exchange based novel reactant adsorptive membrane bioreactor separator systems (or RAMBS) for production of Fab and $F(ab')_2$ fragments

from human IgG. The RAMBS system utilized a stack of ion-exchange membrane held in a temperature controlled module for integrating enzymatic digestion of IgG and purification of fragments. Cation-exchange adsorption was used to bind IgG for pepsin digestion to produce $F(ab')_2$ while anion-exchange adsorption was used to bind IgG for papain digestion to produce Fab. The IgG bound on membrane was fragmented by pumping a solution of enzyme through the reactor. The desired IgG fragment was finally separated from other byproducts based on their differences in the electrostatic interactions with membrane. pH adjustment played significant role to increase Fab recovery and pepsin activity. High purity and high recovery were achieved for the Fab and $F(ab')_2$ fragments produced using RAMBS. The ion-exchange based RAMBS system successfully intensified the process by integrating reaction and separation and showed more than three folds higher digestion rate than liquid phase reaction. In addition, cation-exchange based RAMBS using gradient elution could be a useful approach for simultaneous production of hIgG1 $F(ab')_2$ and hIgG2 $F(ab')_2$.

7.2 Introduction

A reactant adsorptive membrane bioreactor separator system (RAMBS) was developed for integrated enzymatic reaction and separation (Yu and Ghosh 2009). Traditional membrane bioreactor focuses on the retention of enzyme in a membrane system while allowing substrate flow through the system (Gekas 1986; Giorno and Drioli 2000; Heath and Belfort 1990). The enzyme is either retained by ultrafiltration membrane or immobilized on the membrane. In RAMBS, the substrate is reversibly adsorbed by the

membrane adsorber while the enzyme is introduced into the system to catalyze the reaction. Using RAMBS, process integration and high catalytic efficiency could be achieved in a single unit operation using a single device.

Fab and $F(ab')_2$ fragment are important diagnostic reagents and therapeutic proteins (Yamaguchi et al. 1995; Cohen et al. 2000; Ljunglöf et al. 2007). In our previous work (Yu and Ghosh, 2009), hydrophobic interaction membrane chromatography based RAMBS was utilized for integration of IgG fragmentation using papain and purification of Fab. In our current work, ion exchange membrane chromatography based RAMBS was utilized for production of Fab and $F(ab')_2$ fragments.

IgG is normally digested by papain to produce Fab and by pepsin to produce $F(ab')_2$. The enzymatic reactions are usually performed in buffered solutions at 30-40°C for 2-24 hours (Rousseaux et al. 1983; Cresswell et al. 2005). Nearly complete antibody fragmentation can only be achieved by long time reaction such as 48-144 hours' pepsin digestion (Bennich and Turner 1969; Parham 1983; Jones and Landon 2002). However, long time reaction gives rise to unexpected activity loss of product, especially in the case of $F(ab')_2$ fragment production that needs low pH digestion (Morais et al 1994; Morais and Massaldi 2005). In addition, it increases the chance of microbial contamination. After liquid phase reaction, complex purification processes are needed including protein precipitation and various column chromatographic methods such as affinity separation, ion exchange and gel filtration (Harlow and Lane 1988; Raweerith and Ratanabanangkoon 2003; Roque et al. 2004; Ljunglöf et al. 2007). Immobilized papain or pepsin on agarose beads could be used in packed column but they are limited by the high

cost and diffusion limitations in beads (Bommarius and Ribel 2004; Mateo et al. 2002). After reaction, the desired antibody fragments still mix up with undigested antibody molecules and byproducts.

In our present work, ion-exchange based RAMBS was utilized to integrate IgG fragmentation and purification of IgG fragments. At suitable conditions, charged IgG could be bound on ion exchange membrane for the enzymatic reaction. After reaction, the pI difference between IgG fragments and IgG could be utilized for their separation to achieve purified IgG fragments. The anion-exchange based RAMBS was utilized for integration of IgG fragmentation by papain and purification of Fab. The cation-exchange based RAMBS was utilized for integration of IgG fragmentation by pepsin and purification of $F(ab')_2$. The reaction and separation conditions were optimized to achieve high conversion of IgG and high purity and recovery of Fab and $F(ab')_2$ fragment.

7.3 Experimental

7.3.1 Materials

Human IgG (I4506), human serum (H4522), papain (P3125), pepsin (P7000), anti-hIgG (Fab-specific) alkaline phosphatase conjugated antibody (A8542), anti-hIgG (Fc-specific) alkaline phosphatase conjugated antibody (A9544), BCIP®/NBT-purple liquid substrate system for membranes (B3679), TWEEN® 20 (P5927), L-cysteine (30089), disodium EDTA salt dihydrate (E4884), iodoacetamide (16125), sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), ammonium sulfate (A4418), citric acid (C0759) and sodium citrate (S4641) were purchased from Sigma-Aldrich (St.

Louis, MO). Sodium acetate trihydrate (SX0255-1) and glacial acetic acid (AX0073-6) were purchased from EMD (Gibbstown, NJ). High quality deionised water (18.2 MΩ cm) obtained from a Diamond™ NANOpure (Barnstead, Dubuque, IA) water purification unit was used to prepare all the test solutions and buffers. Sartobind S cation-exchange membrane sheets (thickness = 275 μm, catalogue number 94IEXS42-001) and Sartobind Q anion-exchange membrane sheets (thickness = 275 μm, catalogue number 94IEXQ42-001) were purchased from Sartorius (Goettingen, Germany). Hybond™-ECL™ nitrocellulose membrane was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ).

7.3.2 Membrane bioreactor

The RAMBS system used in the current work is similar to what was described before (Yu and Ghosh, 2009) except for the different membranes used here. Briefly, it consisted of a custom-designed stacked membrane bioreactor holding membrane and also being jacketed for heating water circulation, a MultiTemp™ III thermostatic hot water circulator and an AKTA Prime liquid chromatography system (the last two from GE Healthcare Bio-Sciences).

7.3.3 Liquid phase enzymatic digestion of human IgG

The papain digestion of hIgG in liquid phase was carried out in 1.5 mL micro centrifuge tubes containing 1 mg purified hIgG and 0.05 mg papain in 1 mL 50 mM pH 9.2 Tris-HCl buffer with 10 mM disodium EDTA and 10 mM L-cysteine. The pepsin digestion of hIgG in liquid phase was carried out in 1.5 mL eppendorf tubes containing 1

mg purified hIgG and 0.05 mg pepsin in 1 mL 50 mM acetate buffer at pH 4.0 and 4.5. These reactions were carried out at 37°C for 1 hour in a thermostatic water bath, the reactions being terminated by adding 50 μL of 0.6 M iodoacetamide into the papain digestion mixture or adding 200 μL 2 M Tris-Base into the pepsin digestion mixture. The reaction mixtures were analyzed by Size Exclusion Chromatography (SEC) and SDS-PAGE for determining the final compositions and the extents of hIgG fragmentation.

7.3.4 Papain digestion of hIgG and purification of Fab using anion-exchange based RAMBS

Sartobind Q membrane discs having 18 mm diameter were cut out from the membrane sheet and 15 of these were stacked within the membrane bioreactor. The effective bed volume of the membrane stack was 1.05 mL. The binding buffer was 50 mM Tris-HCl at different pHs. The eluting buffer was prepared by adding 1 M sodium chloride to the binding buffer. The membrane bioreactor was first equilibrated using binding buffer at 1 mL/min flow rate and its jacket was circulated with hot water to maintain the effluent temperature at 37 ± 0.5 °C. After equilibration and temperature stabilization, hIgG solution prepared in the binding buffer (1 mg / 2 mL) was fed into membrane reactor at 1 mL/min flow rate and the membrane stack was washed with binding buffer until a stable UV absorbance baseline was obtained. Papain solution (0.05 mg / 2 mL) prepared in the corresponding binding buffer (containing in addition, 10 mM cysteine and 10 mM EDTA) was then fed into membrane module at 0.1 mL/min flow rate to digest the bound hIgG. This was followed by eluting the bound material from the

membrane stack. The digestion flow through and eluate were collected and immediately added with 5% of collection volume of 0.6 M iodoacetamide to terminate reaction. The feed, digestion flow through and eluate samples from the RAMBS experiments were then analyzed by SEC, SDS-PAGE and western blotting. The digestion conversion was calculated based on SEC analysis and standard hIgG calibration.

7.3.5 Pepsin digestion of hIgG and purification of F(ab')$_2$ using cation-exchange based RAMBS

Sartobind S membrane discs having 18 mm diameter were cut out from the membrane sheet and 15 of these were stacked within the membrane bioreactor. The effective bed volume of the membrane stack was 1.05 mL. The binding buffer was 50 mM sodium acetate at pH 4.0. The eluting buffer was prepared by adding 1 M sodium chloride to the binding buffer. The membrane bioreactor was first equilibrated using binding buffer at 1 mL/min flow rate and its jacket was circulated with hot water to maintain the effluent temperature at 37 ± 0.5 °C. After equilibration and temperature stabilization, hIgG solution prepared in binding buffer (1 mg / 2 mL) was fed into the membrane reactor at 1 mL/min flow rate and the membrane stack was washed with binding buffer until a stable UV absorbance baseline was obtained. Pepsin solution (0.05 mg / 2 mL) prepared in the corresponding binding buffer was then fed into the membrane module at 0.1 mL/min flow rate to digest the bound hIgG. This was followed by eluting the bound material from the membrane stack using eluting buffer. The digestion flow through and eluate were collected and immediately added with 20% of collection volume

of 2 M Tris-Base to terminate the reaction. Feed, flow through and eluate samples were then analyzed by SEC, SDS-PAGE and western blotting. The digestion conversion was calculated based on SEC analysis and standard hIgG calibration.

7.3.6 SEC analysis

SEC analysis was carried out using a Superdex 200 10/300 GL column (GE Healthcare Bio-Sciences) fitted to a HPLC system (Varian Inc., Palo Alto, CA, USA). The mobile phase used in the SEC experiments was 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl, at a flow rate of 0.2 mL/min.

7.3.7 SDS-PAGE and Western Blotting

SDS-PAGE experiments were run according to the work of Laemmli (1970). 10% non-reducing gels were obtained using a Hoefer MiniVE system (GE Healthcare Bio-Sciences). After running the gels, the protein bands obtained were either directly stained by Coomassie blue dye or for western blotting, electro-transferred to Hybond^{TM}-ECL^{TM} nitrocellulose membranes using a MiniVE blot module. These membranes were then blocked followed by immunoprobing with anti-hIgG (Fab-specific) alkaline phosphatase conjugated antibody or anti-hIgG (Fc-specific) alkaline phosphatase conjugated antibody. The procedure used was slightly modified from the protocol described in the instruction manual of Hoefer MiniVE system, based on a published protocol (Gallagher et al. 1998). BCIP$^{®}$/NBT-purple liquid substrate was used to visualize the bands on the nitrocellulose membranes. The stained gels were imaged and analyzed by Molecular Imager Gel Doc XR+ System (Bio-Rad, Hercules, CA, USA).

7.4 Results and Discussion

Fig. 7.1 shows the influence of loading papain on hIgG binding on the anion-exchange membrane. When papain was fed into the membrane module at pH 9.2, it mostly flowed through the membrane with a small proportion bound and then eluted. When hIgG was fed into the membrane, most hIgG bound on the membrane without flow through. The same amount of papain then being fed into the membrane that bound hIgG, the papain flow through peak was quite similar to the one before hIgG binding. Bound hIgG was shown in the elution peak. Therefore, feeding papain did not affect the pre-bound hIgG. This result corresponds with the predicted charge behaviors from pI analysis. At pH 9.2, hIgG could bind well on anion-exchange membrane due to its pI of 7-8 (Tracy 1982), while papain with pI close to 9 (Smith et al. 1954) binds very less.

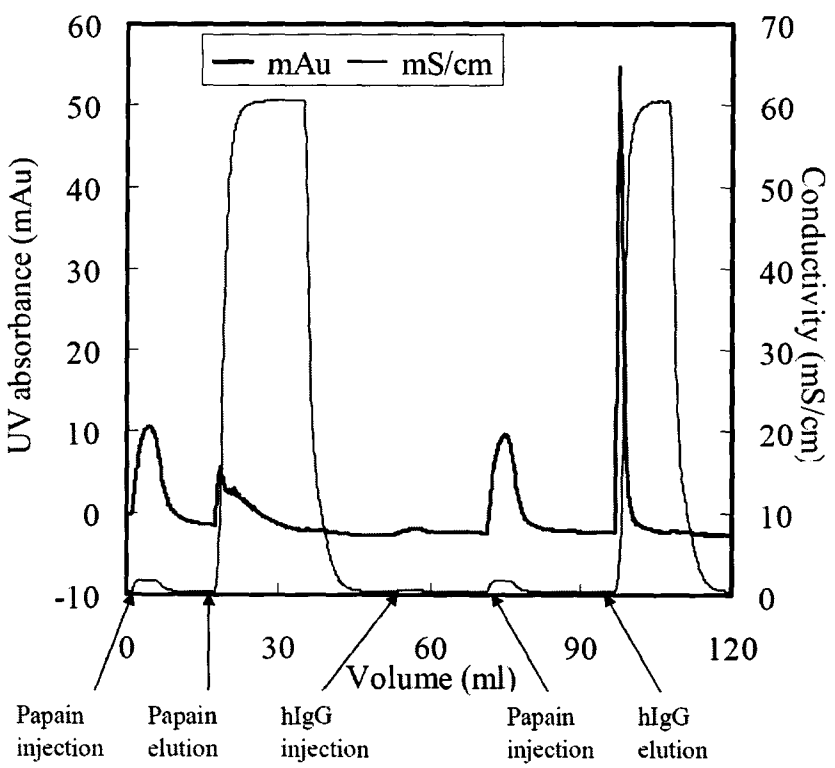


Figure 7.1 Test of papain influence on hIgG binding on anion-exchange membrane. Membrane: 15 layers of Sartobind Q membrane with 18 mm i.d. Binding buffer: 50 mM Tris-HCl, pH 9.2. Elution buffer: binding buffer added with 1.0 M NaCl. Flow rate: 1 ml/min. Papain feed: 0.01 mg/ml in 5 ml binding buffer (containing in addition, 10 mM cysteine and 10 mM EDTA). hIgG feed: 0.05 mg/ml in 5 ml binding buffer.

Anion-exchange based RAMBS was used to integrate papain digestion of hIgG and purification of Fab fragment. Preliminary RAMBS experiment at pH 9.2 was compared with liquid phase digestion by SDS-PAGE analysis (Fig. 7.2). The liquid phase digestion with salt free buffer (Lane 2) showed unreacted IgG band close to 170 kDa marker, Fab band close to 43 kDa marker, Fc band just above Fab band, and some faint bands of small fragments between 26 and 17 kDa. When the same salt free buffer at pH 9.2 and the same amounts of hIgG and papain were used for RAMBS based digestion, the flow through peak (Lane 3) contained Fab and a small fragment close to 17 kDa marker but no IgG showed. The bound and eluted material from membrane (Lane 4) showed unreacted IgG, Fc, a little Fab and small fragments. It appeared that unreacted hIgG from RAMBS was less than that from liquid phase digestion, indicating higher conversion in RAMBS. However, small proportion of Fab still bound on the membrane although most of them went into flow through.

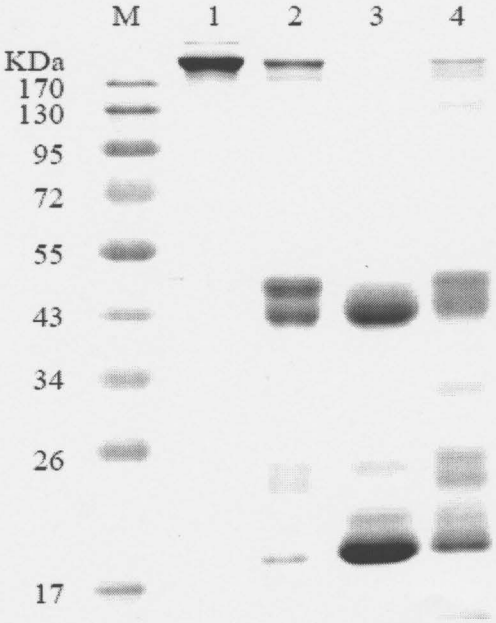
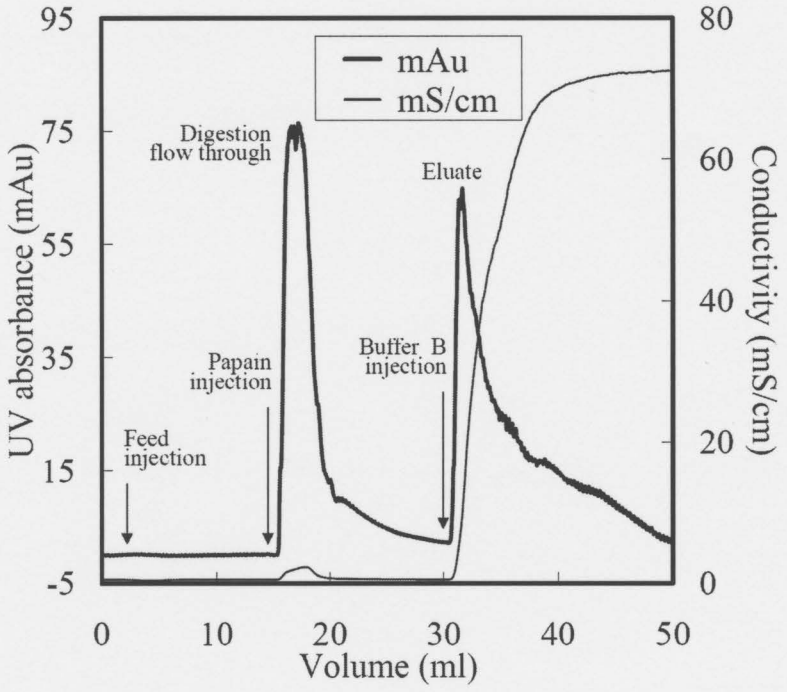


Figure 7.2 10% non-reducing SDS-PAGE for comparison of liquid phase and RAMBS based papain digestion of hIgG. M: marker. 1: hIgG. 2: liquid phase reaction mixture in 50 mM Tris-HCl buffer (pH 9.2). 3 and 4: Flow through and elution peak from anion-exchange based RAMBS. Binding and digestion buffer, 50 mM Tris-HCl buffer (pH 9.2); Elution buffer, binding buffer + 1.0 M NaCl.

Micro-adjustment of pH was tried for anion-exchange based RAMBS to recover more Fab in the flow through. The results in Fig. 7.2 actually showed pI of Fab is higher than hIgG and Fc. Since hIgG is composed of two Fab and one Fc, a pI order could be inferred, i.e. Fc < hIgG < Fab. Lowering pH could reduce the charge of Fab and thus release more Fab into flow through but big pH change may also weaken the binding of hIgG, Fc and other fragments on membrane, which could decrease Fab purity. Therefore, micro-adjustment to pH 9.0 was used in RAMBS (chromatogram shown in Fig. 7.3A). When IgG was fed into the membrane module, flow though peak was not shown, indicating complete binding of IgG. The papain solution was then injected to digest bound IgG on membrane at controlled flow rate and temperature. The digestion flow through and eluate were collected and analyzed by SDS-PAGE (Fig. 7.3B). The flow through (Lane 2) showed a strong Fab band and a small fragment band. This small fragment close to 17 kDa could be removed by centrifugal filter. The eluate (Lane 2) showed a Fc band and a Fab band which is apparently fainter than what is at pH 9.2 (Fig. 7.2, Lane 5). Therefore, the recovery of Fab was increased. Based on gel scanning, the recovery of Fab in flow through at pH 9.0 was c.a. 84.3%. SEC analysis (Fig. 7.8) showed the purity of Fab after 30 kDa filter treatment was 94.2% with minor impurities consisting of unreacted IgG and intermediate product. The conversion in RAMBS at pH 9.0 was 91.3% while at the same conditions (buffer and enzyme / substrate ratio), the one in liquid phase reaction was only 84.8%. The digestion rates for RAMBS and liquid phase reaction were respectively 6.1×10^{-6} mmol hIgG / mg papain / min and 1.9×10^{-6} mmol

hIgG / mg papain / min, clearly showing much higher catalytic efficiency in anion-exchange based RAMBS than that in liquid phase digestion.

(A)



(B)

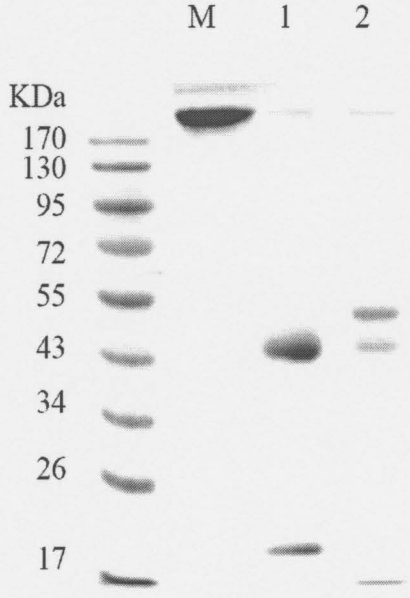


Figure 7.3 (A). UV absorbance and conductivity profiles obtained with the bioreactor effluent during papain digestion of hIgG using anion-exchange based RAMBS system (membrane: Sartobind Q, membrane diameter: 18 mm, number of discs in stack: 15, temperature: 37°C, binding buffer: 50 mM Tris-HCl pH 9.0, eluting buffer: binding buffer + 1.0 M NaCl, flow rate: 1 mL/min for equilibration, washing and elution, 0.1 mL/min during IgG digestion, IgG loading: 1 mg; papain amount: 0.05 mg dissolved in 2 mL binding buffer containing in addition 10 mM cysteine and 10 mM EDTA). (B). 10% non-reducing SDS-PAGE analysis of samples from (A). M: marker; 1: hIgG; 2, 3: respectively digestion flow through and eluate in (A).

Cation-exchange based RAMBS was used for pepsin digestion of hIgG and purification of $F(ab')_2$ fragment. pH effect on pepsin digestion of hIgG was first studied in liquid phase. SEC analysis in Fig. 7.4 clearly showed that pH significantly affected hIgG conversion. The conversion at pH 4.5 was only 16.1% while the respective value at pH 4.0 was 78.2%. Porcine pepsin was known to have high activity at low pH such as pH 2.2 or pH 3.5 but low pH could cause IgG aggregation and precipitation (Bohak 1969; Rea and Ultee 1993; Arakawa et al. 2004). Therefore, pH 4.0 was chosen as the pH for pepsin digestion. At this pH, hIgG could bind well on cation-exchange membrane while pepsin does not bind due to its low pI of 2-3 (Malamud 1978). Using the same pH and pepsin / hIgG ratio, digestion using cation-exchange based RAMBS was compared with that in liquid phase. As shown in SDS-PAGE analysis (Fig. 7.5), pepsin digestion mixture in pH 4.0 salt free buffer (Lane 2) contained the unreacted hIgG band close to 170 kDa marker, the $F(ab')_2$ band close to 130 kDa marker and some smaller fragments, possibly over-digested $F(ab')_2$. The flow through from RAMBS based digestion (Lane 3) did not show any bands since it contains very small peptides digested from Fc. The eluate from RAMBS (Lane 4) showed a very faint band of hIgG, a strong band of $F(ab')_2$ and no small fragment, clearly indicating the high conversion of hIgG and high purity of $F(ab')_2$ achieved by the RAMBS system.

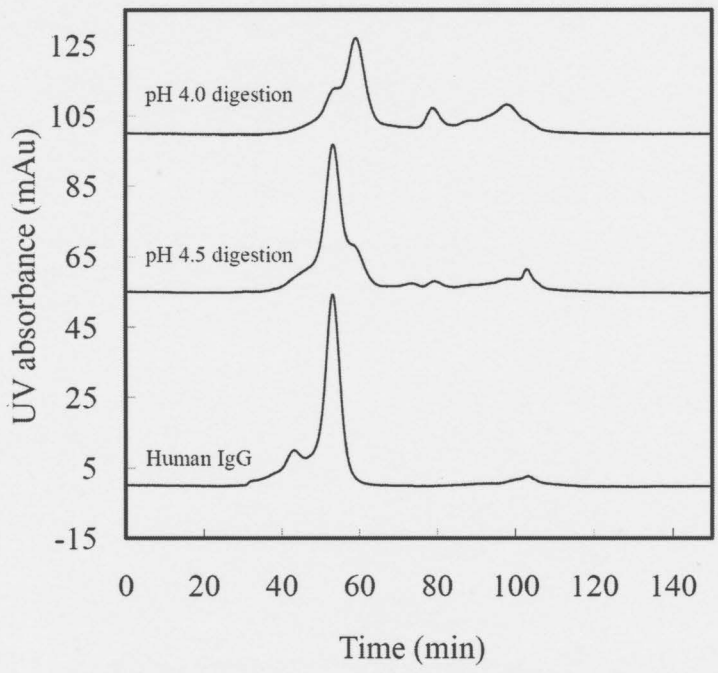


Figure 7.4 SEC analysis of pepsin digestion of hIgG in liquid phase at different pH (column: Superdex 200 10/300 GL, mobile phase: 20 mM sodium phosphate buffer containing 150 mM NaCl pH 7.0, flow rate: 0.2 mL/min).

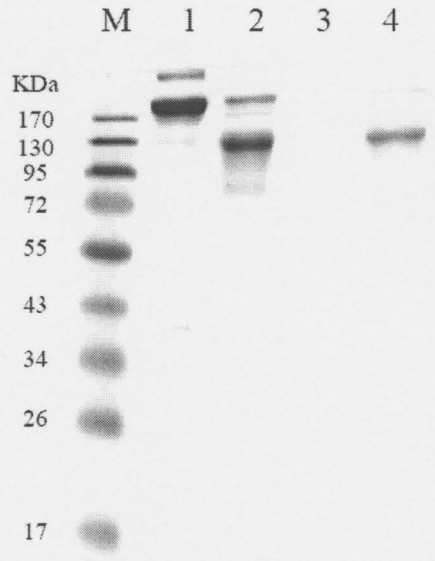
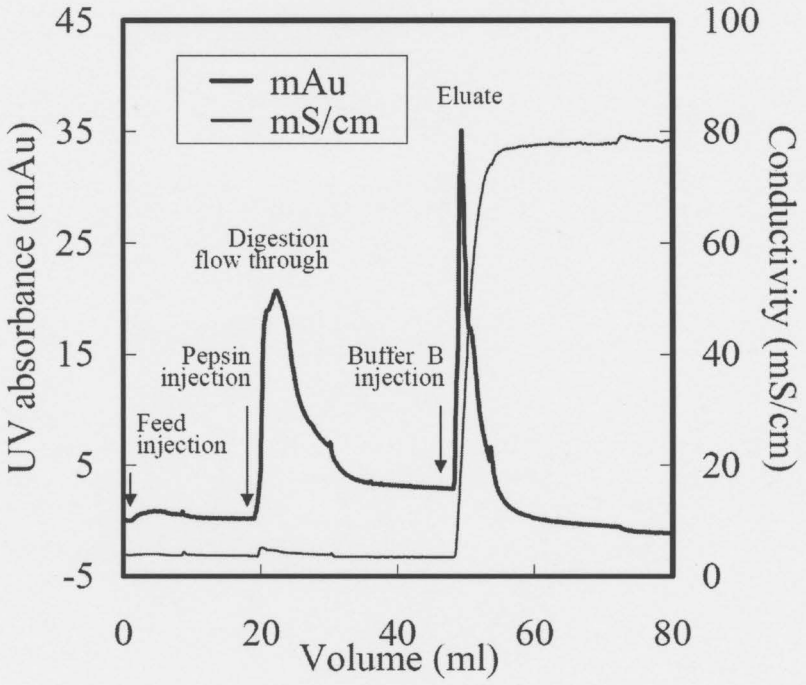


Figure 7.5 10% non-reducing SDS-PAGE for comparison of liquid phase and RAMBS based pepsin digestion of hIgG. M: marker. 1: hIgG. 2: liquid phase reaction mixture in 50 mM acetate buffer (pH 4.0). 3 and 4: Flow through and elution peak from cation-exchange based RAMBS: Binding and digestion buffer, 50 mM acetate buffer (pH 4.0); elution buffer, binding buffer + 1.0 M NaCl.

A typical batch of pepsin digestion of hIgG using cation-exchange based RAMBS was shown in Fig. 7.6A. When hIgG was fed into the cation-exchange membrane, only trace amount of hIgG flowed through while most of them bound on membrane. Papain was then injected into the membrane to digest bound hIgG. The small peptides digested from Fc flowed through while $F(ab')_2$ was still bound on membrane and then recovered by elution. SDS-PAGE analysis in Fig. 7.6B showed high conversion of hIgG and high purity of $F(ab')_2$ in eluate (Lane 2). No band shown in flow through (Lane 3) indicated high recovery of $F(ab')_2$ in eluate. Gel scanning showed the recovery of $F(ab')_2$ was 86.9%. Based on SEC analysis (Fig. 7.8), the purity of $F(ab')_2$ was 96.4% and the hIgG conversion using RAMBS was 99.5%. Using the same digestion conditions, i.e. the same buffer, hIgG and pepsin amount, liquid phase digestion only reached 81.9% conversion. The efficient digestion using RAMBS was further demonstrated by its digestion rate of 6.6×10^{-6} mmol hIgG / mg papain / min, compared with 1.8×10^{-6} mmol hIgG / mg papain / min for liquid phase digestion. Close to 100% conversion and much higher digestion rate by cation-exchange based RAMBS may reveal that IgG interacts with cation-exchange membrane through $F(ab')_2$ while exposing Fc to the environment, greatly facilitating pepsin attack of the cleavage sites on Fc. This finding indicates the potential of RAMBS as a useful tool for studying ion-exchange based IgG binding on membrane.

(A)



(B)

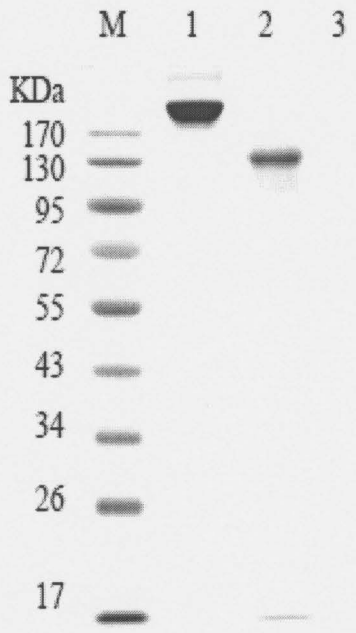
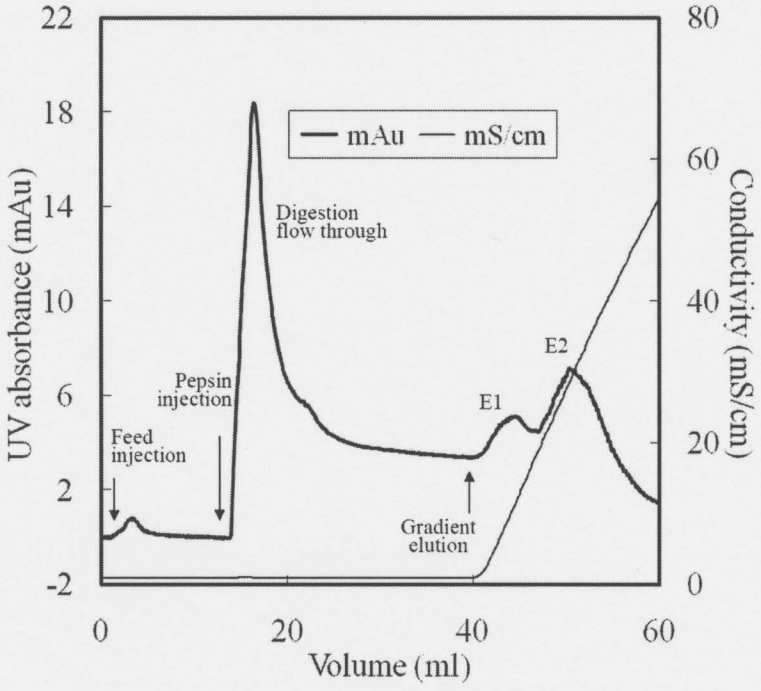


Figure 7.6 (A). UV absorbance and conductivity profiles obtained with the bioreactor effluent during pepsin digestion of hIgG using cation-exchange based RAMBS system (membrane: Sartobind S, membrane diameter: 18 mm, number of discs in stack: 15, temperature: 37°C, binding buffer: 50 mM acetate pH 4.0, eluting buffer: binding buffer + 1.0 M NaCl, flow rate: 1 mL/min for equilibration, washing and elution, 0.1 mL/min during IgG digestion, IgG loading: 1 mg; pepsin amount: 0.05 mg dissolved in 2 mL binding buffer). (B). 10% non-reducing SDS-PAGE analysis of samples from (A). M: marker; 1: hIgG; 2, 3: respectively digestion flow through and eluate in (A).

Another interesting result was found for $F(ab')_2$ production using cation-exchange based RAMBS. After pepsin digestion, when gradient elution instead of one step elution was used to elute $F(ab')_2$ from the membrane, two peaks were found (Fig. 7.7A). They were collected and analyzed by SDS-PAGE (Fig. 7.7B). The digestion flow through showed no protein bands (Lane 4), the same result as shown in Fig. 7.6B. The first peak E1 (Lane 3) and second peak E2 (Lane 4) showed $F(ab')_2$ bands with the same molecular weight. However, E1 and E2 are different in amount and the peak separation indicated they have different pI. We presumed that E1 and E2 are $F(ab')_2$ fragment respectively from hIgG2 and hIgG1. Human IgG consists of four subclasses, IgG1 and IgG2 being the two most abundant, IgG1 comprising 43-75% and IgG2 comprising 16-48% of total serum IgG (Hamilton 1987). Since IgG1 and IgG2 have no difference to pepsin susceptibility (Shakib and Stanworth 1980), the higher abundance of IgG1 than IgG2 corresponds well with the larger peak area of E2 than E1. Further evidence is provided by pI and charge analysis. The pIs of IgG1 and IgG2 were respectively 8.6 ± 0.4 and 7.4 ± 0.6 (Tracy, 1982). The four human IgG subclasses have virtually identical amino acid sequences in the constant region domains (Tan et al, 1990). Therefore, the pI difference between IgG1 and IgG2 actually represents the pI difference between their respective $F(ab')_2$ fragments. At acidic condition, IgG1 $F(ab')_2$ is more positively charged than IgG2 $F(ab')_2$, therefore during gradient elution, the latter being eluted first followed by the former. This interesting result showed that cation-exchange based RAMBS may provide a method to directly produce IgG1 $F(ab')_2$ and IgG2 $F(ab')_2$ from human serum.

(A)



(B)

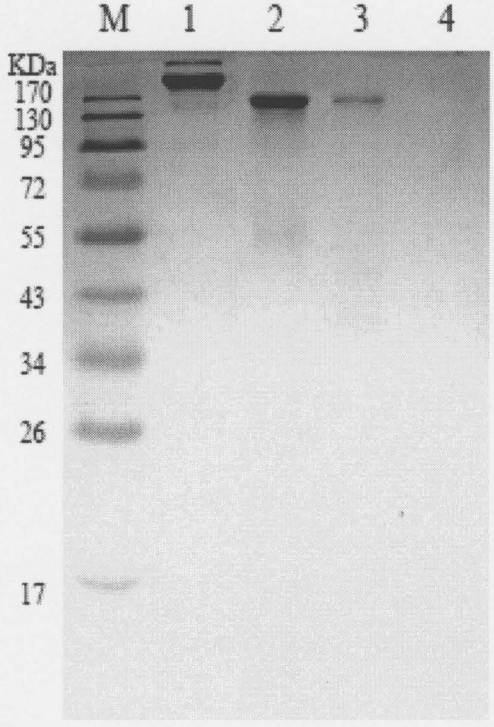


Figure 7.7 (A). UV absorbance and conductivity profiles obtained with the bioreactor effluent during pepsin digestion of hIgG and gradient elution using cation-exchange based RAMBS system (membrane: Sartobind S, membrane diameter: 18 mm, number of discs in stack: 15, temperature: 37°C, binding buffer: 50 mM acetate pH 4.0, eluting buffer: binding buffer + 1.0 M NaCl, gradient elution: 25 ml from 0-100%, flow rate: 1 mL/min for equilibration, washing and elution, 0.1 mL/min during IgG digestion, IgG loading: 1 mg; pepsin amount: 0.05 mg dissolved in 2 mL binding buffer). (B). 10% non-reducing SDS-PAGE analysis of samples from (A). M: marker; 1: hIgG; 2, 3, 4: respectively E2, E1 and digestion flow through in (A).

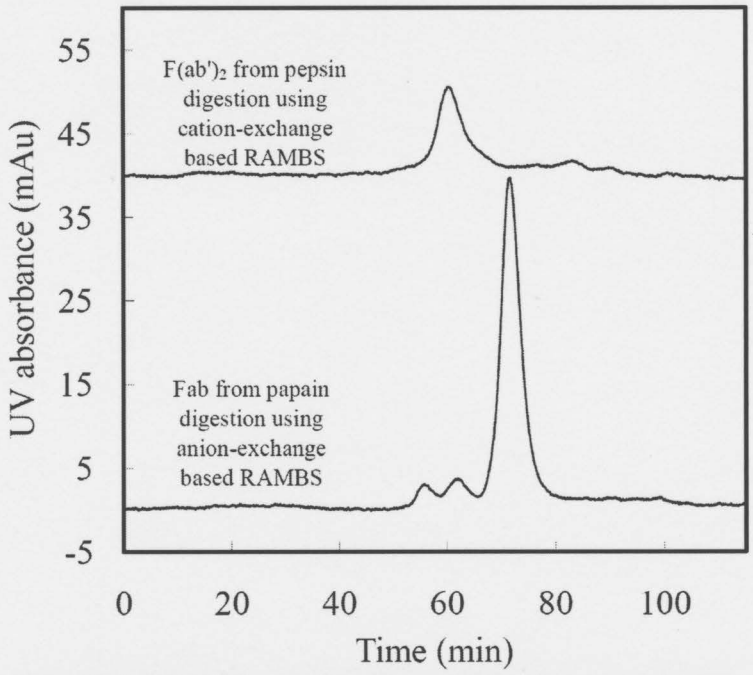


Figure 7.8 SEC analysis of $F(ab')_2$ product from pepsin digestion using cation-exchange based RAMBS and Fab product from papain digestion using anion-exchange based RAMBS (column: Superdex 200 10/300 GL, mobile phase: 20 mM sodium phosphate buffer containing 150 mM NaCl pH 7.0, flow rate: 0.2 mL/min).

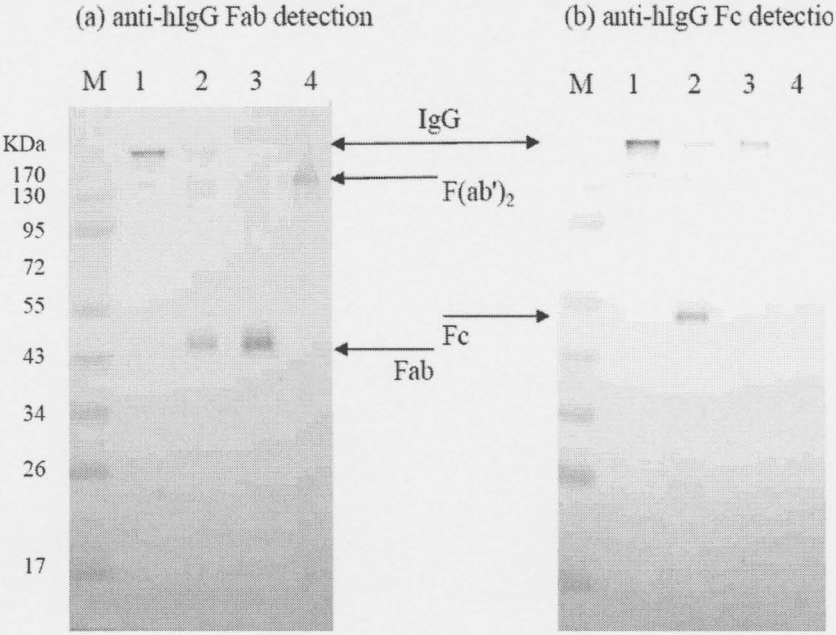


Figure 7.9 Western blot analysis of sample obtained during enzymatic digestion of hIgG using RAMBS (a) anti-hIgG-Fab detection, (b) anti-hIgG-Fc detection (M: marker, 1: pure hIgG; 2 and 3: eluate and flow through from papain digestion using anion-exchange based RAMBS; 4: eluate from pepsin digestion using cation-exchange based RAMBS).

The Fab and $F(ab')_2$ products from RAMBS processes were further characterized by western blotting (Fig. 7.9). Anti-hIgG Fab detection (Fig. 7.9A) showed the flow through from anion-exchange based RAMBS contained very pure Fab without Fc (Lane 3) while the eluate contained hIgG and small amount of Fab (Lane 2). The eluate from cation-exchange based RAMBS showed pure $F(ab')_2$ (Lane 4). Anti-hIgG Fc detection (Fig. 7.9B) showed the flow through from anion-exchange based RAMBS contained trace amount of unreacted hIgG (Lane 3) while the eluate contained hIgG and Fc (Lane 2). The eluate from cation-exchange based RAMBS did not show any bands, meaning $F(ab')_2$ product completely free from hIgG and Fc (Lane 4). Except for hIgG fragmentation introduced in this chapter, the same IEMC-RAMBS system was also used for fragmentation of monoclonal antibody hIgG1-CD4 (results not shown). This indicates the applicability of IEMC-RAMBS to a wide range of antibodies.

7.5 Conclusions

Ion exchange based RAMBS system greatly simplified as well as intensified the production processes of Fab and $F(ab')_2$ fragment by integrating the reaction and separation. Using anion-exchange based RAMBS, Fab fragment could be directly produced from hIgG with high purity of 94.2% and high recovery of 84.3%. Micro-adjustment of pH from 9.2 to 9.0 increased Fab recovery in flow through. Reaction pH significantly influenced pepsin activity, pH 4.0 providing much higher conversion of hIgG than pH 4.5. Pepsin digestion of IgG using cation-exchange based RAMBS provided $F(ab')_2$ product with high purity of 96.4% and high recovery of 86.9%.

Furthermore, cation-exchange based RAMBS using gradient elution could be a useful approach to produce IgG1 $F(ab')_2$ and IgG2 $F(ab')_2$ simultaneously. Both anion-exchange based RAMBS and cation-exchange based RAMBS demonstrated higher conversions and more than three folds higher digestion rates than respective liquid phase reaction. The catalytic efficiency was therefore higher when hIgG was immobilized on membrane and it indicated the presentation of hIgG on membrane could facilitate the enzyme catalysis.

7.6 References

Arakawa T, Philo JS, Tsumoto K, Yumioka R, Ejima D. 2004. Elution of antibodies from a Protein-A column by aqueous arginine solutions. Protein Expr Purif 36:244-248.

Bennich H, Turner MW. 1969. The release of Fc' and pFc' fragments from human immunoglobulin G following enzymic digestion: a time study. Biochim Biophys Acta 175:388-395.

Bohak Z. 1969. Purification and characterization of chicken pepsinogen and chicken pepsin. J Biol Chem 244:4638-4648.

Bommarius AS, Ribel BR. 2004. Biocatalysis, Fundamentals and Application. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA. p 109-110.

Cheung GLM, Thomas TM, Rylatt DB. 2003. Purification of antibody Fab and $F(ab')_2$ fragments using Gradiflow technology. Protein Expr Purif 32:135-140.

Cohen SA, Trikha M, Mascelli MA. 2000. Potential future clinical applications for the GPIIb/IIIa antagonist, abciximab in thrombosis, vascular and oncological indications. Pathol Oncol Res 6:163-174.

Cresswell C, Newcombe AR, Davies S, Macpherson I, Nelson P, O'Donovan K, Francis R. 2005. Optimal conditions for the papain digestion of polyclonal ovine IgG for the production of bio-therapeutic Fab fragments. Biotechnol Appl Biochem 42(Pt 2):163-168.

Gallagher S, Winston SE, Fuller SA, Hurrell JGR. 1998. Immunoblotting and immunodetection, in: Current Protocols in Immunology. New York: John Wiley & Sons. p. 8.10.8-8.10.10.

Gekas VC. 1986. Artificial membranes as carriers for the immobilization of biocatalysts. Enzyme Microb. Technol 8:450-460.

Giorno L, Drioli E. 2000. Biocatalytic membrane reactors: applications and perspectives. Trends Biotechnol 18:339-349.

Hamilton RG. 1987. Human IgG subclass measurements in the clinical laboratory. Clin Chem 33:1070-1075.

Harlow E, Lane D. 1988. Antibodies, A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press. p 626-629.

Heath C, Belfort G. 1990. Membranes and bioreactors. Int J Biochem 22:823-825.

Jones RGA, Landon L. 2002. Enhanced pepsin digestion: a novel process for purifying antibody $F(ab')_2$ fragments in high yield from serum. J Immunol Methods 263:57-74.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Ljunglöf A, Lacki KM, Mueller J, Harinarayan C, van Reis R, Fahrner R, Van Alstine JM. 2007. Ion exchange chromatography of antibody fragments. Biotechnol Bioeng 96:515-524.

Malamud D, Drysdale, JW. 1978. Isoelectric points of proteins: A table. Anal Biochem 86: 620-647.

Mateo C, Abian O, Fernandez-Lorente G, Pedroche J, Fernandez-Lafuente R, Guisan JM, Tam A, Daminati M. 2002. Epoxy sepabeads: A novel epoxy support for stabilization of industrial enzymes via very intense multipoint covalent attachment. Biotechnol Prog 18:629-634.

Morais JF, De Freitas MCW, Yamaguchi IK, Dos Santos MC, Dias da Silva W. 1994. Snake antivenoms from hyperimmunized horses: comparation of the antivenom activity and biological properties of their whole IgG and $F(ab')_2$ fragments. Toxicon 32:725-734.

Morais V, Massaldi H. 2005. Effect of pepsin digestion on the antivenom activity of equine immunoglobulins. Toxicon 46:876-882.

Parham P. 1983. On the fragmentation of monoclonal IgG1, IgG2a, and IgG2b from BALB/c mice. J Immunol 131:2895-2902.

Raweerith R, Ratanabanangkoon K. 2003. Fractionation of equine antivenom using caprylic acid precipitation in combination with cationic ion-exchange chromatography. J Immunol Methods 282:63-72.

Rea DW, Ultee ME. 1993. A novel method for controlling the pepsin digestion of antibodies. J Immunol Methods 157:165-173.

Roque AC, Lowe CE, Taipa MA. 2004. Antibodies and genetically engineered related molecules: Production and purification. Biotechnol Prog 20:639-654.

Rousseaux J, Rousseaux-Prévost R, Bazin H. 1983. Optimal conditions for the preparation of Fab and $F(ab')_2$ fragments from monoclonal IgG of different rat IgG subclasses. J Immunol Methods 64:141-146.

Shakib F, Stanworth DR. 1980. Human IgG subclasses in health and disease. (A Review) Part I. Ric Clin Lab 10:463-479.

Smith EL, Kimmel JR, Brown DM. 1954. Crystalline papain. II. Physical studies; The mercury complex. J Biol Chem 207: 533-549.

Tan LK, Shopes RJ, Oi VT, Morrison SL. 1990. Influence of the hinge region on complement activation, C1q binding, and segmental flexibility in chimeric human immunoglobulins. Proc Natl Acad Sci USA 87:162-166.

Tracy RP. 1982. Two dimensional gel electrophoresis of sera from patients with monoclonal gammopathies. Clin Chem 28:900-907.

Yamaguchi Y, Kim H, Kato K, Masuda K, Shimada I, Arata Y. 1995. Proteolytic fragmentation with high specificity of mouse immunoglobulin G. J Immunol Methods 181:259-267.

Yu D, Ghosh R. 2009. Integrated fragmentation of human IgG and purification of Fab using a reactant adsorptive membrane bioreactor separator system. Biotech Bioeng 104:152-161.

Chapter 8

Method for Studying Immunoglobulin G Binding on Hydrophobic Surfaces

This chapter was organized based on the paper in press in Langmuir (DOI: 10.1021/la902395v. 2009) by Deqiang Yu and Raja Ghosh.

8.1 Abstract

We used a reactant adsorptive membrane bioreactor separator (or RAMBS) system to examine hydrophobic interaction based binding of human immunoglobulin G (hIgG) on synthetic microporous membranes possessing tunable hydrophobicity. Membrane bound hIgG on being pulsed with papain resulted in Fab being obtained in the flowthrough with Fc remaining bound to the membrane. On the other hand when

membrane bound hIgG was pulsed with pepsin, Fc sub-fragments were obtained in the flowthrough with $F(ab')_2$ remaining bound to the membrane. These product profiles suggest that hIgG bound to the membrane through its middle region. Enzyme linked immunoadsorbent assay (ELISA), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometric analysis of eluate samples obtained from the RAMBS experiments provided evidence that the binding of hIgG took place primarily through the segment consisting of the hinge and C_H2 domain of Fc. The experimental approach described in this paper could potentially be more widely applicable for studying protein interactions with membrane and surfaces in general.

8.2 Introduction

The study of immunoglobulin G (IgG) binding on hydrophobic surfaces is of interest in areas such as membrane chromatography, immunoassays, and biomaterials development. Some researchers are of the opinion that the Fc portion of IgG interacts with hydrophobic surfaces (Bujis et al. 1996; Bujis et al. 1997; Erp et al. 1992; Nagaoka et al. 2000; Nagaoka et al. 2001), the Fab portions remaining free to interact with antigen (Hoffman et al. 1991; Solomon et al. 1993). Buijs et al. (1996) used reflectometry data to show that IgG adsorbed on hydrophobic methylated surfaces through its Fc portion. Erp et al. (1992) hypothesized that the adsorption of monoclonal IgG on polystyrene latex took place due to the increased local hydrophobicity of the Fc region. Nagaoka et al. (2000 and 2001) demonstrated that IgG bound onto a hydrophobic fluorinated polyimide surface through the Fc portion by showing that the bound molecule could interact with

anti-F(ab')$_2$. Other researchers differ, e.g. Vermeer et al. (2001) used differential scanning calorimetry and circular dichorism data to suggest that antibody binding on a hydrophobic surface took place through the Fab portion. Earlier studies by Vermeer and Norde (2000a, 2000b, 2000c) on the relative hydrophobicity of antibody fragments were used to support these observations.

Most previous studies examined IgG binding on strongly hydrophobic surfaces. The comparison of relative hydrophobicity of the different portions of the IgG molecule was therefore difficult. We felt that this problem could be overcome by using surfaces with tunable hydrophobicity such as membranes used for hydrophobic interaction membrane chromatography (or HIMC) (Ghosh 2001; Wang et al. 2006). The environment-responsive membranes used for HIMC are hydrophobic in the presence of lyotropic salts but are quite hydrophilic in their absence and can therefore be made to bind IgG in a reversible manner (Ghosh 2001; Wang et al. 2006). Such hydrophilized membranes can be prepared by grafting hydrophilic polymers on the surface of hydrophobic membranes (Hester and Mayes 2002). In the presence of lyotropic salts, these grafted polymers lose their hydration layer and collapse, resulting in a hydrophobic membrane suitable of IgG binding. When the salt is removed, the polymer chains regain their hydration layer resulting in a hydrophilic membrane no longer able to bind IgG.

We examined the binding of human IgG (hIgG) on hydrophilized polyvinylidine fluoride (PVDF) membrane in the presence of ammonium sulfate using a newly developed reactant adsorptive membrane bioreactor separator (or RAMBS) system which integrated enzymatic catalysis and membrane bioseparation into a single unit

operation (Yu and Ghosh 2009). Membrane-bound hIgG was fragmented by pulsing proteolytic enzymes pepsin and papain and by analyzing the composition of the flowthrough and material eluted from the membrane after enzymatic digestion by ELISA, SDS-PAGE and mass spectrometry, the manner in which hIgG bound to the membrane was determined. The primary goal of this study was to use the RAMBS system to study how hIgG bound on hydrophobic membranes. The results of this study are expected to help in designing new adsorptive membranes for antibody purification and immunoassays and in minimizing IgG binding in applications such as ultrafiltration, microfiltration and dialysis.

8.3 Experimental

8.3.1 Materials

Human IgG (I4506), insulin (I0516), anti hIgG (Fab-specific) alkaline phosphatase conjugated antibody (A8542), anti hIgG (Fc-specific) alkaline phosphatase conjugated antibody (A9544), BCIP®/NBT-purple liquid substrate system for membranes (B3679), SIGMA *FAST*™ pNPP tablet (N1891), TWEEN® 20 (P5927), L-cysteine (30089), disodium EDTA salt dihydrate (E4884), iodoacetamide (I6125), sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), ammonium sulfate (A4418), sodium chloride (S3014), citric acid (C0759) and sodium citrate (S4641) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium acetate trihydrate (SX0255-1) and acetic acid, glacial (AX0073-6) were purchased from EMD (Gibbstown, NJ, USA). High quality water (18.2 MΩ cm) obtained from a Barnstead

Diamond™ NANOpure water purification unit (Dubuque, IA, USA) was used to prepare all the test solutions and buffers. Hydrophilic PVDF membrane (GVWP) with 0.22 μm nominal pore diameter was purchased from Millipore Corporation (Billerica, MA, USA). Hybond™-ECL™ nitrocellulose membrane was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA).

8.3.2 Reactant adsorptive membrane bioreactor separator system

The RAMBS system used in the current work was recently developed by Yu and Ghosh (2009). The key unit is a custom-designed membrane bioreactor for holding the membrane stack. The temperature within the membrane bioreactor was maintained by a combination of hot water circulation through its jacket and pre-heating of influent using a heat exchanger. A MultiTemp™ III thermostatic circulator (GE Healthcare Bio-Sciences) was used to supply the heating water. The bioreactor was integrated with an AKTA Prime liquid chromatography system (GE Healthcare Bio-Sciences).

8.3.3 Enzymatic digestion of hIgG and fragment separation using RAMBS

Enzymatic digestion of hIgG was separately carried out using papain and pepsin. A stack of 30 hydrophilized PVDF membrane discs each having 18 mm diameter was housed within the membrane bioreactor to provide total bed volume of 0.95 mL. The eluting buffers used in the papain and pepsin digestion experiments were 20 mM sodium phosphate buffer (pH 7.5) and 50 mM sodium acetate buffer (pH 4.0) respectively. The corresponding binding buffers were prepared by adding ammonium sulfate to the eluting buffers to obtain 1.5 M salt concentration while maintaining the respective pH values.

The RAMBS system was first equilibrated using binding buffer at 1 mL/min flow rate and maintained at 37 ± 0.5 °C by hot water circulation. 4 mg hIgG prepared in 10 mL of binding buffer was then injected into the system at 1 mL/min flow rate and the membrane stack was then washed with binding buffer until a stable UV absorbance baseline was obtained. Papain or pepsin solutions (each prepared by dissolving 0.8 mg of the respective enzyme in 5 mL of corresponding binding buffer; the papain solution containing in addition 10 mM cysteine and 10 mM EDTA) was then injected into membrane module at 0.1 mL/min flow rate to digest the membrane-bound hIgG. The membranes were then washed with binding buffer until stable UV absorbance baseline was obtained. The bound material in each case was eluted using a 40 mL negative salt gradient (0% to 100% eluting buffer). Feed, flow through and eluate peaks obtained from these experiments were analyzed by SEC, SDS-PAGE and western blotting.

8.3.4 Size exclusion chromatography

Samples obtained from the RAMBS experiment were analyzed by size exclusion chromatography (SEC) using a Superdex 200 10/300 GL high performance pre-packed column (17-5175-01 GE Healthcare Bio-Sciences) fitted to a HPLC system (Varian, Lake Forest, CA, USA). The mobile phase used in the SEC experiments was 20 mM sodium phosphate buffer (pH 7.0) containing 150 mM NaCl, at a flow rate of 0.2 mL/min.

8.3.5 SDS-PAGE and Western Blotting

SDS-PAGE experiments were run according to the work of Laemmli (1970) using a Hoefer MiniVE system (GE Healthcare Bio-Sciences). After running the gels, the protein bands obtained were either directly stained by Coomassie blue or electrotransferred to a Hybond^{TM}-ECL^{TM} nitrocellulose membrane using a MiniVE blot module (GE Healthcare Bio-Sciences). The electrotransfer was carried out for 1 h in the presence of Towbin buffer at 25 V. The membrane was then blocked with Tween-TBS followed by immunoprobing with anti-HIgG (Fab-specific or Fc-specific) alkaline phosphatase conjugated antibody diluted 2000-fold with Tween-PBS followed by washing. BCIP$^{®}$/NBT-purple liquid substrate was then used to visualize the bands on the membranes (Gallagher et al. 1998).

8.3.6 ELISA

Samples S8 and S9 were analyzed on BRAND 96-Well ELISA plates (80087-049, VWR, Mississauga, ON, Canada). The samples were first diluted by phosphate buffered saline (PBS) to ca. 40 μg/mL protein concentration to obtain the stock samples. These were further diluted by PBS to ca. 20, 10, 5, 2.5 and 1.25 μg/mL. 50 μL of each sample was loaded on the ELISA plate and incubated overnight at 4° C. The wells were then washed five times using 150 μL PBS-Tween. The detection antibodies i.e. anti hIgG (Fab-specific) or anti hIgG (Fc-specific) alkaline phosphatase conjugated were serially diluted using PBS-Tween, the optimized dilution factors for detection being 1600 and 2000 respectively. 50 μL of detection antibody was loaded to the PBS-Tween washed

wells and incubated for 2 hours at room temperature followed by five washes with 150 μL PBS-Tween. 150 μL of substrate solution freshly prepared from SIGMA *FAST*™ pNPP tablets was then added to each well followed by 30 minutes incubation. The absorbance was measured immediately after the incubation period at 405 nm using a 680 XR microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Ovalbumin solution and PBS were used as negative control while hIgG solution was used as positive control, the volume loaded in each case being 50 μL. Ovalbumin and hIgG solutions were diluted by PBS to obtain the concentrations similar to those for S8 and S9, i.e. 20, 10, 5, 2.5 and 1.25 μg/mL.

8.3.7 Mass spectrometry (MS)

The S8 and S9 samples were analyzed by TOF MS and MSMS. 0.015 μg of trypsin in 30 μl of 50 mM ammonium bicarbonate solution containing 10 % acetonitrile was added to samples containing ca. 5 μg protein and these were incubated at 37° C overnight. The digested proteins samples were desalted and concentrated using Millipore C18 ZipTip with the peptides being eluted in 8 μl of 50% aqueous acetonitrile containing 0.2% formic acid. The samples were then analyzed using a Micromass Q-TOF Ultima Global mass spectrometer (Waters, USA) with a nanoES source. The capillary voltage was in the 1.2-1.6 kV range while the cone voltage was in the 50-100 V range. Mass spectra in TOF MS and MSMS mode were in the 50-1800 m/e range with a resolution of 8000 FWHM. Argon was used as collision gas.

8.4 Results

8.4.1 Digestion of hIgG using papain and separation of fragments

Fig. 8.1 shows the UV absorbance profile of the effluent obtained from the RAMBS system during the digestion of membrane-bound hIgG using papain followed by gradient elution of bound material. The results of SEC, SDS-PAGE and western blot analysis of samples obtained during the above reaction-separation experiment are shown in Fig. 8.2a, 8.2b and 8.2c respectively. Samples 2–9 were collected as shown in Fig. 8.1 where arrows are used to indicate start and end of fraction collection; that is, the entire amount of eluate between two arrows was collected, desalted, and concentrated prior to the electrophoretic and SEC analysis. Sample 1 was standard hIgG which showed a retention time (RT) of 56.6 min on the SEC chromatogram (Fig. 8.2a) and was obtained as a band close to the 170 kDa molecular weight marker on the gel (Fig. 8.2b). Samples 2 and 3 (the reaction flow through peak and the tail respectively) contained primarily Fab (RT of 73.8 min and band close to the 43 kDa marker) and trace amounts of Fc and Fc sub-fragments. Sample 4 contained primarily Fc (RT of 72.0 min and band close to the 50 kDa marker) and a small amount of hIgG while sample 5 consisted mainly of hIgG. Samples 6 and 7 consisted primarily of half Fc (RT of 85.9 min and molecular weight close to the 26 kDa marker) and small amounts of hIgG. The presence of these different entities in the above samples was verified by western blotting (Fig. 8.2c). Samples 8 (S8) and 9 (S9) contained proteins having similar molecular weights (close to the 17 kDa marker) but different hydrophobicity.

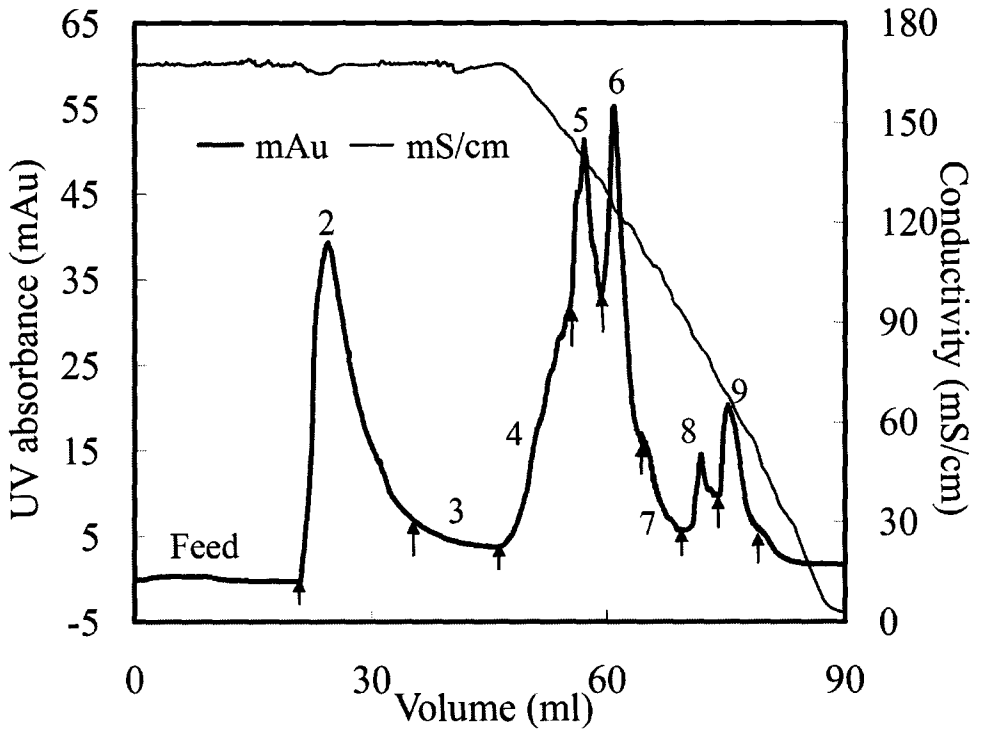


Figure 8.1 UV absorbance and conductivity profiles of the bioreactor effluent obtained during hIgG digestion using papain followed by gradient elution of bound material (membrane: 0.22 micron hydrophilized PVDF, membrane diameter: 18 mm, number of discs in stack: 30, temperature: 37°C, eluting buffer: 20 mM sodium phosphate pH 7.5, binding buffer: eluting buffer + 1.5 M ammonium sulfate, flow rate: 1 mL/min for equilibration, washing and elution, 0.1 mL/min during hIgG digestion, gradient elution: 40 ml from 0 to 100% eluting buffer, hIgG loading: 4 mg; papain amount: 0.8 mg dissolved in 5 mL binding buffer containing in addition 10 mM cysteine and 10 mM EDTA).

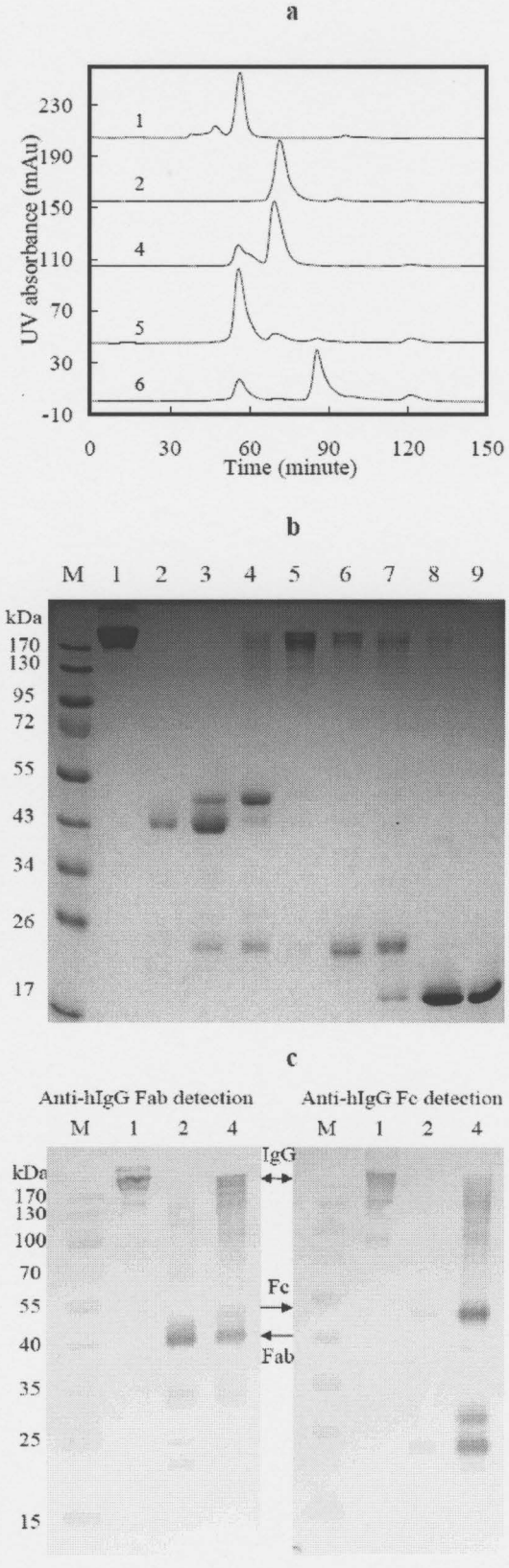


Figure 8.2 The numbered samples from Figure 1 and feed hIgG (sample 1) were analyzed by different methods. (a) SEC analysis of samples (column: Superdex 200 10/300 GL, mobile phase: 20 mM sodium phosphate buffer containing 150 mM NaCl, pH 7.0, flow rate: 0.2 mL/min). (b) Coomassie blue stained 10% non-reducing SDS-PAGE (M, marker). (c) Western-blot analysis using anti hIgG Fab and anti hIgG Fc detection (M, marker).

8.4.2 Digestion of hIgG using pepsin and separation of fragments

Fig. 8.3 shows the UV absorbance profile of the effluent obtained from the RAMBS system during the digestion of membrane-bound hIgG using pepsin followed by gradient elution of bound material. The results of SEC, SDS-PAGE and western blot analysis of samples obtained during the above reaction-separation scheme are shown in Fig. 8.4a, 8.4b and 8.4c respectively. Samples 2–6 were collected as shown in Fig. 8.3 where arrows are used to indicate start and end of fraction collection; that is, the entire amount of eluate between two adjacent arrows was collected, desalted, and concentrated prior to the electrophoretic and SEC analysis. The reaction flow through (sample 2) contained small peptide fragments and possibly amino acids and these were individually non-detectible on either SEC or SDS-PAGE (Fig. 8.4b). Samples 3 and 4 contained primarily $F(ab')_2$ (RT of 62.4 min and band between the 130 kDa and 95 kDa markers) while sample 5 containing both $F(ab')_2$ and hIgG. Sample 6 contained in addition to $F(ab')_2$ and hIgG a small fragment (RT of 78.9 min), probably pFc', which has a molecular weight of 26 kDa (Turner and Bennich 1968). SDS-PAGE of the same sample revealed the presence of a small fragment just above 10 kDa marker. This small fragment was half pFc', the pFc' fragment being broken up into two by SDS treatment (Turner and Bennich 1968; Bennich and Turner 1969).

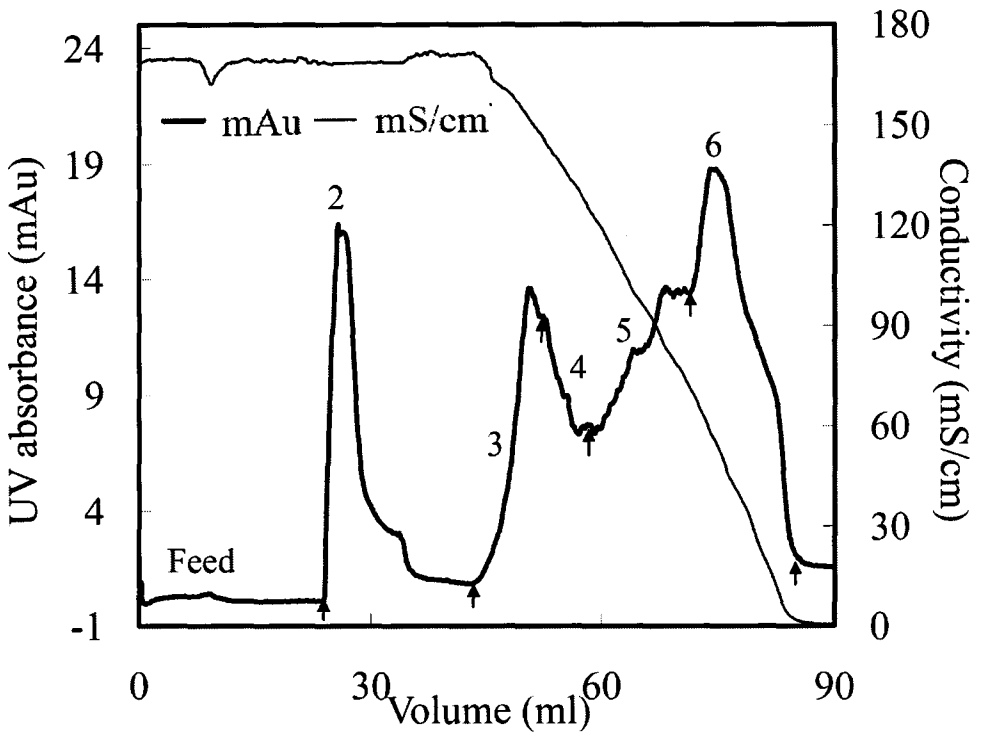


Figure 8.3 UV absorbance and conductivity profiles of the bioreactor effluent obtained during hIgG digestion using pepsin followed by gradient elution of bound material (membrane: 0.22 micron hydrophilized PVDF, membrane diameter: 18 mm, number of discs in stack: 30, temperature: 37°C, eluting buffer: 20 mM sodium acetate buffer pH 4.0, binding buffer: eluting buffer + 1.5 M ammonium sulfate, flow rate: 1 mL/min for equilibration, washing and elution, 0.1 mL/min during hIgG digestion, gradient elution: 40 ml from 0 to 100% eluting buffer, hIgG loading: 4 mg; pepsin amount: 0.8 mg dissolved in 5 mL binding buffer).

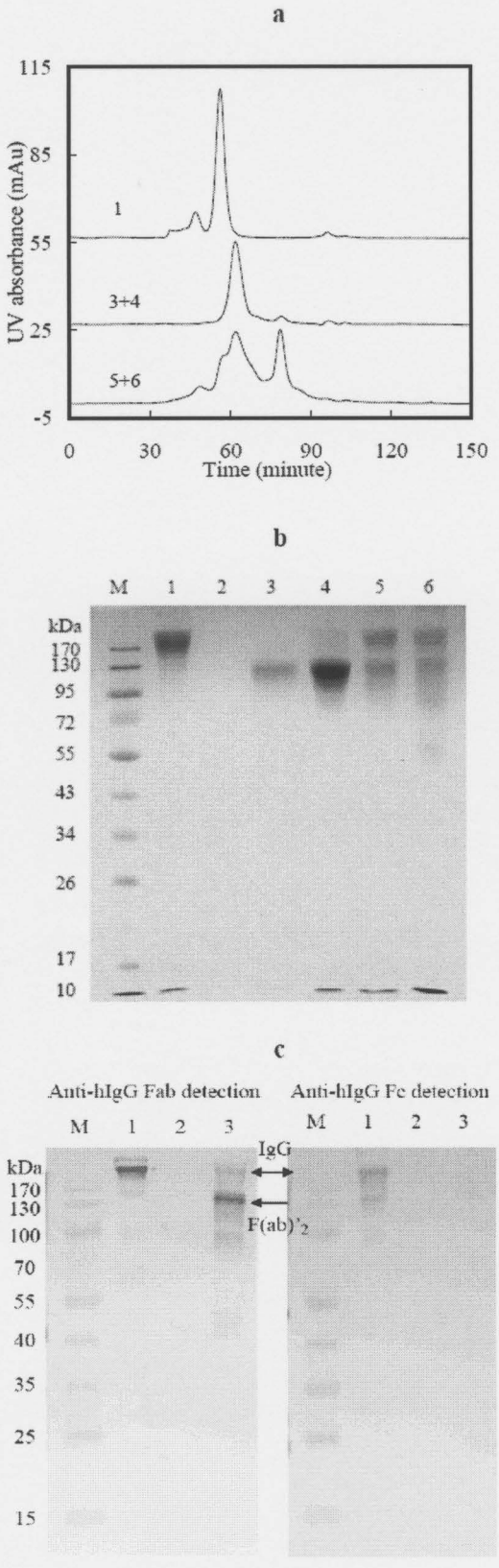


Figure 8.4 The numbered samples from Figure 3 and feed hIgG (sample 1) were analyzed by different methods. (a) SEC analysis of samples (column: Superdex 200 10/300 GL, mobile phase: 20 mM sodium phosphate buffer containing 150 mM NaCl pH 7.0, flow rate: 0.2 mL/min). (b) Coomassie blue stained 10% non-reducing SDS-PAGE (M, marker). (c) Western-blot analysis using anti hIgG Fab and anti hIgG Fc detection (M, marker).

8.4.3 SDS-PAGE analysis of S8 and S9

Samples S8 and S9 were analyzed using 20% SDS-PAGE under both reducing and non-reducing conditions (Fig. 8.5). Under non-reducing condition, S8 and S9 bands were obtained just above the 15 kDa marker, S8 being slightly larger than S9. Under reducing condition both S8 and S9 bands were located higher than those obtained under non-reducing condition.

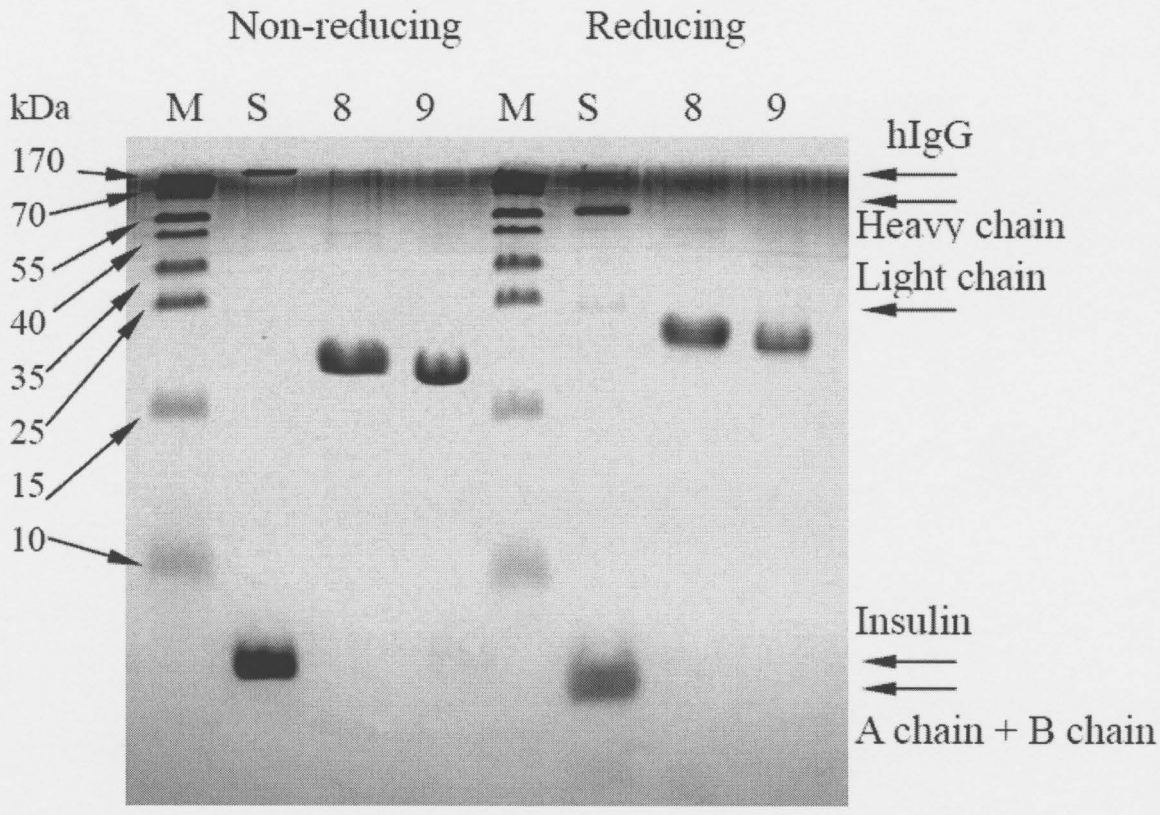


Figure 8.5 SDS-PAGE analysis (20%, reducing and non-reducing) of S8 and S9 obtained by digestion of hIgG with papain using the RAMBS system (M: Marker; S: standard hIgG and insulin; 8: S8; and 9: S9).

8.4.4 Mass spectrometry analysis of S8 and S9

Table 8.1 summarizes the sequencing results obtained with samples S8 and S9 (Fig. 8.1). The sixteen-amino acid peptide K.FNWYVDGVEVHNAK.T from human IgG1 chain C region was identified at two different ionization forms in S8. The peptides K.NSWGTGWGENGYIR.I and SAVVTIEGIIK from papain were identified in S9.

Table 8.1 Peptide ion identification of protein samples S8 and S8 by mass spectrometry.

Sample	Protein description	Database / accession code	Peptide sequence	M_r (exp)	M_r (calc)	MOWSE score*
S8	Human IgG1 chain C region	MSDB / GHHU	K.FNWYVDGVEVHNAK.T	1676.79	1676.79	38 (34)
		MSDB / GHHU	K.FNWYVDGVEVHNAK.T	1676.68	1676.79	63 (34)
S9	Papain (EC 3.4.22.2) precursor – papaya	MSDB / PPPA	K.NSWGTGWGENGYIR.I	1595.67	1595.71	55 (47)
		MSDB / PPPA	SAVVTIEGIIK	1128.65	1128.68	47 (62)

* Figures in parenthesis indicate MOWSE score required for identity or extensive homology ($P<0.05$).
M_r (exp) = experimentally determined molecular mass.
M_r (calc) = calculated molecular mass for the peptide sequence.

8.5 Discussion

When membrane-bound hIgG was digested using papain, Fab was obtained in the flow through while Fc, undigested hIgG and small fragments remained membrane-bound and could be eluted using salt-free buffer. Therefore, Fab clearly did not play any important role in the binding of hIgG on the membrane. Gradient elution showed that the order of relative hydrophobicity was: Fab < Fc < hIgG < S8 < S9. Presumably therefore if S8 and/or S9 originated from IgG, they played key roles in membrane binding. When

membrane-bound hIgG was pulsed with pepsin, Fc was cleaved off followed by its further digestion to small peptide fragments. $F(ab')_2$ remained bound on membrane and could be subsequently eluted by lowering the salt concentration. By combining information from the two RAMBS experiments, an idealized representation of the two enzymatic reaction-separation schemes can be obtained (Fig. 8.6). The product profiles obtained in the two experiments can only be explained if hIgG bound to the membrane through the overlapping region of Fc and $F(ab')_2$, i.e. the middle region consisting of the hinge and the portion of C_H2 domain of Fc adjacent to it.

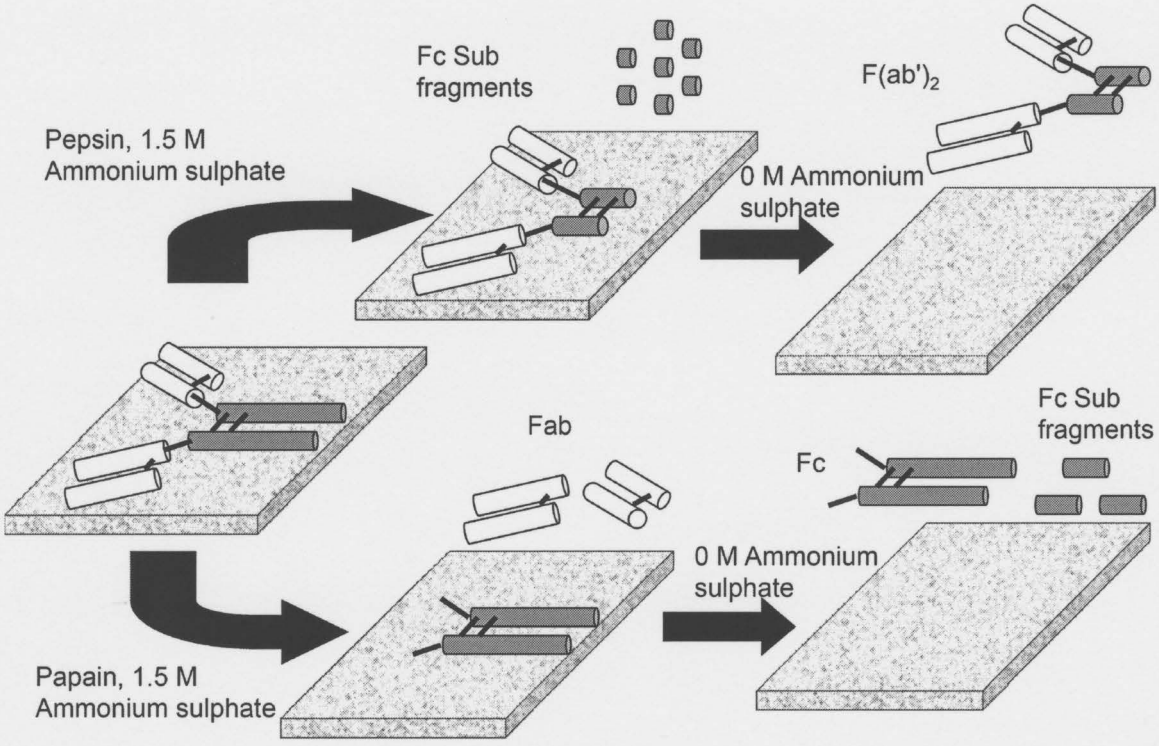


Figure 8.6 Idealized diagram showing hIgG digestion with pepsin and papain using the RAMBS system.

Based on the above information, we presumed that fragments S8 and S9 obtained in the papain digestion experiments were from the middle region of hIgG. Analysis of S8 and S9 using ELISA (data not shown) revealed that S8 could be strongly detected by both anti-hIgG Fab and anti-hIgG Fc while signals obtained with S9 were weak. S8 possibly contained the hinge region since it serves as the bridge between Fab and Fc and is most likely to be recognized by both anti-Fab and anti-Fc. In an earlier paper (Yu and Ghosh 2009) we reported that two bands were obtained with papain on SDS-PAGE, the lower molecular weight band having a molecular weight of ca. 17 kDa. It is therefore highly likely that S9 contained papain. Analysis of S8 and S9 using 20% SDS-PAGE (see Fig. 8.5) showed that S8 had a slightly higher molecular weight than S9 at both reducing and non-reducing conditions. Interestingly, under reducing condition both S8 and S9 bands obtained were higher than corresponding bands obtained under non-reducing condition. This is counter-intuitive since most proteins maintain the same mobility or move faster under reducing condition due to separation of chains linked by disulfide bonds, as demonstrated using hIgG and insulin (Fig. 8.5). The SDS-PAGE results obtained with S8 and S9 could be explained in terms of the breakage of intra-chain disulfide bonds which would stretch out the chains resulting in lower mobility. A similar phenomenon has been described without explanation by other researchers (Shaunak et al. 2006) who reported that interferon under reducing condition had lower mobility than at non-reducing condition. Papain is a single chain protein with three intra-chain disulfide bonds (Mitchel et al. 1970). Intra-chain disulfide bonds are present both in Fab and Fc. However S8 was clearly derived from Fc and not from Fab since the latter

is not further degraded by papain into smaller fragments. On the other hand earlier studies have shown that sub-Fc fragments could be produced by secondary cleavage by papain in the presence of cysteine (Bennich and Turner 1969; Poulik and Shuster 1965; Poulik 1966). The main cleavage site for papain on hIgG is located just above the hinge region while the two secondary cleavage sites lie within Fc (Turner and Bennich 1968; Grey and Abel 1967; Nardella and Teller 1985). Cleavage at the secondary sites, i.e. at amino acid residues 14 and 105 (numbered from the C-terminal end) would split Fc into three groups of peptide fragments: short C-terminus fragments (ca. 3 kDa), the Fc′ fragments containing two C_H3 domains (ca. 21 kDa), and those containing two C_H2 domain and hinge region (ca. 28 kDa). Since each C_H2 domain is glycosylated by an oligosaccharide chain which is close to 3 kDa (Ghirlando et al. 1999), the two C_H2 domains and the hinge region add up to ca. 34 kDa. On an SDS-PAGE the two larger fragments would show molecular weights corresponding to their respective single chain, i.e. ca. 10 kDa for the Fc′ fragment and ca. 17 kDa for the C_H2 domain-hinge fragment. Based on the SDS-PAGE results shown on Fig. 8.5 combined with the fact that S8 was recognized both by anti-hIgG Fab and anti-hIgG Fc in ELISA experiments, it may be concluded that S8 consisted of the hinge and the C_H2 domains. The further evidence on the origin of S8 was obtained by mass spectrometry which confirmed that S8 contained peptide sequence from the C chain of hIgG1, the main hIgG subclass. The identified peptide K.FNWYVDGVEVHNAK.T is present in the C_H2 domain of the hIgG1 C chain (Ellison et al. 1982). The hinge region sequence was not found in S8. Presumably the

sample pretreatment methods for mass spectrometry disrupted the hinge into small pieces making detection difficult.

The RAMBS experiments discussed above provided conclusive evidence that the binding of hIgG on a hydrophobic membrane primarily took place through its middle region consisting of the C_H2 domain of Fc and the hinge. The results from this study in addition to helping understand how hIgG interacts with hydrophobic surfaces is likely to be useful in designing and developing materials and processes for antibody bioseparation and immunoassays. Moreover, the RAMBS based experimental approach described in this paper could find wider application for studying protein interactions with membranes and surfaces and in proteomic analysis. Proteolytic enzymes with specific cleavage sites are commonly used in proteomic analysis to generate fragments which are further analyzed for molecular mass and amino acid sequence. Such fragmentation is typically carried out as a liquid phase reaction, thus generating a complex pool of fragments. Carrying out such enzymatic reactions using surface bound target proteins would potentially allow for fragments to be released in a controllable fashion, and this could in turn simplify the task of isolating and identifying fragments.

8.6 Conclusions

The use of a membrane with tunable hydrophobicity facilitated the study of subtle hydrophobicity differences between IgG and its fragments, which in turn revealed information on the nature of IgG binding on a hydrophobic surface. The reactant adsorptive membrane bioreactor separator or RAMBS system used in our study

generated IgG fragments *in situ* and these could be obtained in the reactor effluent in order of increasing hydrophobicity. The product profiles obtained from the two RAMBS experiments using papain and pepsin respectively could only be possible if the IgG molecule bound to the membrane through the overlapping region of $F(ab')_2$ and Fc, i.e. the middle region of IgG consisting of the hinge and C_H2 domain. This hypothesis was conclusively verified by ELISA, electrophoresis and mass spectrometry of S8, one of the most hydrophobic components eluted from the bioreactor.

8.7 References

Buijs J, Berg P, Lichtenbelt J, Norde W, Lyklema J. 1996. Adsorption dynamics of IgG and its $F(ab')_2$ and Fc fragments studied by reflectometry. Coll Interf Sci 178:594-605.

Buijs J, White DD, Norde W. 1997. The effect of adsorption on the antigen binding by IgG and its $F(ab')_2$ fragments. Colloids Surf B 8:239-249.

Erp R, Linders YEM, Sommeren APG, Gribnau TCJ. 1992. Characterization of monoclonal antibodies physically adsorbed onto polystyrene latex particles. J Immunol Methods 152:191-199.

Nagaoka S, Kanno M, Kawakami HJ. 2001. Evaluation of blood compatibility of fluorinated polyimide by immunolabeling assay. Artif Organs 4:107-112.

Nagaoka S, Ashiba K, Kawakami H. 2000. Interaction between biocomponents and surface modified fluorinated polyimide. Mat Sci Eng C 20:181-185.

Hoffman WL, Jump AA, Kelly PJ, Ruggles AO. 1991. Binding of antibodies and other proteins to nitrocellulose in acidic, basic, and chaotropic buffers. Anal Biochem 198:112-118.

Solomon B, Schmitt S, Schwartz F, Levi A, Fleminger G. 1993. Eupergit C-coated membranes as solid support for a sensitive immunoassay of human albumin. J Immunol Methods 157:209-215.

Vermeer AWP, Giacomelli CE, Norde W. 2001. Adsorption of IgG onto hydrophobic teflon. Differences between the Fab and Fc domains. Biochemica et Biophysica Acta 1526:61-69.

Vermeer AWP, Norde W. 2000a. The thermal stability of immunoglobulin: Unfolding and aggregation of a multi-domain protein. Biophys J 78:394-404.

Vermeer AWP, Norde W. 2000b. The influence of the binding of low molecular weight surfactants on the thermal stability and secondary structure of IgG. Colloids Surf A: Physicochem Eng Asp 161:139-150.

Vermeer AWP, Norde W. 2000c. The unfolding/denaturation of immunogammaglobulin of isotype 2b and its Fab and Fc fragments. Biophys J 79:2150-2154.

Ghosh R. 2001. Separation of proteins using hydrophobic interaction membrane chromatography. J Chromatogr A 923:59-64.

Wang L, Hale G, Ghosh R. 2006. Non-size-based membrane chromatographic separation and analysis of monoclonal antibody aggregates. Anal Chem. 78:6863-6867.

Hester JF, Mayes AM. 2002. Design and performance of foul-resistant poly(vinylidene fluoride) membranes prepared in a single-step by surface segregation. J Membr Sci 202:119-135.

Yu D, Ghosh R. 2009. Integrated fragmentation of human IgG and purification of Fab using a reactant adsorptive membrane bioreactor separator system. Biotech Bioeng 104:152-161.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Gallagher S, Winston SE, Fuller SA, Hurrell JGR. 1998. Current Protocols in Immunology. New York: John Wiley & Sons. p 8.10.8-8.10.10.

Turner MW, Bennich H. 1968. Subfragments from the Fc fragment of human immunoglobulin G. Isolation and physicochemical charaterization. Biochem J 107: 171-178.

Bennich H, Turner MW. 1969. The release of Fe' and pFc' fragments from human immunoglobulin G following enzymic digestion: a time study. Biochim. Biophys. Acta 175:388-395.

Shaunak S, Godwin A, Choi JW, Balan S, Pedone E, Vijayarangam D, Heidelberger S, Teo I, Zloh M, Brocchini S. 2006. Site-specific PEGylation of native disulfide bonds in therapeutic proteins. Nat Chem Biol 2:312-313.

Mitchel RE, Chaiken IM, Smith EL. 1970. The complete amino acid sequence of papain. Additions and corrections. J Biol Chem 245:3485-3492.

Poulik MD, Shuster J. 1965. Role of cysteine in the production of Fc and F'c sub-components of gamma-G myeloma globulins. Nature 207:1092-1093.

Poulik MD. 1966. F'c fragment of immunoglobulins. Nature 210:133-134.

Grey HM, Abel CA. 1967. Structural characteristics of the Fc' fragment of human gamma-G-globulin. Immunochemistry 4:315-317.

Nardella FA, Teller DC. 1985. Fc intermediate (Fci), a papain-generated fragment of human IgG, intermediate in charge, molecular weight and cleavage between the Fc and Fc' fragments of IgG. Mol Immunol 22:705-713.

Ghirlando R, Lund J, Goodall M, Jefferis R. 1999. Glycosylation of human IgG-Fc: influences on structure revealed by differential scanning micro-calorimetry. Immunol Lett 68:47-52.

Ellison JW, Berson BJ, Hood LE. 1982. The nucleotide sequence of a human immunoglobulin C gamma1 gene. Nucleic Acids Res 10:4071-4079.

Chapter 9

Paper-PEG Based Membranes for Hydrophobic Interaction Chromatography: Purification of Monoclonal Antibody

The chapter was organized based on the published paper in Biotechnology and Bioengineering 99: 1434-1442 (2008) by Deqiang Yu, Xiaonong Chen, Robert Pelton and Raja Ghosh.

9.1 Abstract

This paper discusses the preparation of novel Paper-PEG interpenetrating polymer network based membranes as inexpensive alternative to currently available adsorptive membranes. The Paper-PEG membranes were developed for carrying out hydrophobic interaction membrane chromatographic (HIMC). PEG is normally very hydrophilic but can undergo phase separation and become hydrophobic in the presence of high

antichaotropic salt concentrations. Two variants of the Paper-PEG membranes, Paper-PEG 1 and Paper-PEG 2 were prepared by grafting different amounts of the polymer on filter paper and these were tested for their hydraulic properties and antibody binding capacity. The better of the two membranes (Paper-PEG 1) was then used for purifying the monoclonal antibody hIgG1-CD4 from simulated mammalian cell culture supernatant. The processing conditions required for purification were systematically optimized. The dynamic antibody binding capacity of the Paper-PEG 1 membrane was about 9 mg per ml of bed volume. A single step membrane chromatographic process using Paper-PEG 1 membrane gave high monoclonal antibody purity and recovery. The hydraulic permeability of the paper based membrane was high and was maintained even after many runs, indicating that membrane fouling was negligible and the membrane was largely incompressible.

9.2 Introduction

Protein A based column chromatography is widely used for purifying monoclonal antibodies (mAbs) from simulated mammalian cell culture supernatant. The major limitations with column chromatography particularly those employing soft gel based media are: high back pressure, low product throughput and scale-up problems. The main problem associated with using protein A based media is the leakage of this ligand which is immunotoxic (Carlson 2005). Therefore, additional steps for removal of protein A and stringent quality control measures increase the processing cost. Another problem associated with protein A affinity chromatography is the acidic condition used for elution,

one that could result in formation of antibody aggregates (Arakawa et al. 2004). Membrane chromatography is a fast growing bioseparation technique which combines the advantages of membrane technology and chromatography, thereby leading to the possibility of developing high-throughput, high-resolution purification processes (Ghosh 2002). Membrane chromatography using non-protein A approaches such as ion exchange and hydrophobic interaction have been examined for monoclonal antibody purification (e.g. Knudsen et al. 2001; Philips et al. 2004; Ghosh 2001; Ghosh and Wang 2006). However, the cost of commercial adsorptive membranes is quite high and some of these membranes show fouling tendencies. The competitive edge of membrane chromatography could be further enhanced by addressing these issues, i.e. cost and membrane fouling. The current work attempts to do this by using novel membranes prepared by interpenetrating polymer network (IPN) formation of polyethylene glycol (PEG) and filter paper (i.e. cellulose). These membranes can be manufactured economically and due to their open structure have less fouling tendency.

Filter paper was selected as base material for making the hydrophobic interaction membranes due to its high hydraulic permeability and low cost. Also, cellulose is highly compatible with biological macromolecules such as monoclonal antibodies and would have very low tendency to foul. Yang and Chen (2002) have discussed the use of chitosan coated filter paper modified with spacer arm linked to protein A for IgG separation. The use of hydrophobic interaction membrane chromatography (HIMC) with polyvinylidine fluoride (PVDF) membranes for purification of monoclonal antibody from simulated mammalian cell culture supernatant has recently been reported by Ghosh and Wang

(2006). To the best of our knowledge the use of filter paper based hydrophobic interaction media has not yet been reported in the literature.

PEG is a hydrophilic polymer that can undergo phase separation in the presence of antichaotropic salts such as sodium sulphate and ammonium sulphate (Ananthapadmanabhan and Goddard 1987). Under such conditions PEG displays a mildly hydrophobic behaviour. This reversible change from hydrophilic to hydrophobic could be used for hydrophobic interaction based protein separation. We postulated that a membrane prepared from PEG and cellulose would bind hydrophobic molecules such as antibodies in the presence of high antichaotropic salt concentration. However, on account of the extremely hydrophilic nature of PEG at low salt concentrations, the bound protein would be completely released. This would be better than using traditional hydrophobic interaction ligands such as phenyl, butyl and octyl which are hydrophobic even at eluting condition and consequently give very poor release of bound material. Poor recovery of protein happens to be one of the major limitations with currently available hydrophobic interaction matrices and hence our approach is designed to overcome this. Interaction of proteins with PEG does not affect their biological activity (Wang et al. 2006). PEG modified beads for column chromatography has been reported by Ling and Mattiasson (1983). Protein interaction with PEG is also utilized in PEG-salt aqueous two phase extraction (Ananthapadmanabhan and Goddard 1987; Andrews et al. 1996; Huddleston et al. 1996). The current work examines two types of Paper-PEG membranes. The better of the two is then used for purifying monoclonal antibody hIgG1-CD4 from simulated CHO cell culture supernatant. hIgG1-CD4 is an investigative drug which has been shown to be

quite promising in the treatment of refractory psoriasis and rheumatoid arthritis (Isaacs et al. 1997).

9.3 Materials and methods

9.3.1 Materials

Humanized monoclonal antibody hIgG1-CD4 (batch 12) was kindly donated by the Therapeutic Antibody Center, University of Oxford, UK and was used as received. Cellulose filter paper (Grade 5) was purchased from Whatman. Serum-free CHO cell culture media (Cat. No. C1707) and bovine albumin (Cat. No. A7906) were purchased from Sigma-Aldrich. Other chemical reagents, including poly (ethylene glycol) methyl ether methacrylate solution (macro-monomer, 50 wt% in water), methylenebisacrylamide (>99%), potassium persulfate (99.99%), sodium phosphate (mono- and di-basic), ammonium sulphate, sodium citrate, citric acid and sodium chloride were purchased from Sigma-Aldrich. Isopropylacrylamide (99%) was purchased from Acros Organics and re-crystallized before use. All buffers and protein solutions were prepared using ultra-pure water (18.2MΩ-cm) obtained from a Diamond Nanopure water purification unit (Barnstead).

9.3.2 Preparation of PEG grafted filter papers and SEM characterization

Paper-PEG membranes were prepared by interpenetrating polymer network (IPN) technique (Sabaa and Mokhtar 2002; Wang and Xu 2006). Monomer/ initiator solution was prepared by adding 4.3 mg of methylenebisacrylamide, 3.3 mg of potassium

persulfate, 0.5 g of poly (ethylene glycol) methyl ether methacrylate solution, 68 mg of isopropylacrylamide and 1.5 ml of ultra-pure water into a 500 ml beaker. A piece of dry filter paper (0.568 g) was conditioned and weighed in a constant temperature and humidity (CTH) room (23 degrees Celsius, 50% humidity) and then put into the beaker to absorb the solution under N_2 atmosphere maintained by bubbling the gas through it. After absorption, the excess monomer/initiator solution was removed and the beaker was sealed for reaction at 60 degrees Celsius for 2 hrs and 70 degrees Celsius for 2 hrs. This was followed by the introduction of 2 ml water to the system and the reaction was continued at 70 Celsius for an additional 20 hrs. The resulting membrane was washed with ultra-pure water three times and submerged in fresh water overnight. The membrane was then dried in the CTH room (23 degrees Celsius, 50% humidity) to constant weight and mass increase of the paper was weighed. The original filter paper and the Paper-PEG membranes were scanned by SEM (Scanning Electron Microscopy) to observe the change in morphology caused by Paper-PEG interpenetrating network formation.

9.3.3 Membrane module and chromatography equipment

The Paper-PEG membrane samples were cut into discs and housed in a custom-designed membrane module (Ghosh and Wong 2006) which provided excellent flow distribution to the membrane and collection from it. The effective diameter of the membrane within the module was 18 mm. This module was then integrated with an AKTA prime liquid chromatography system (GE Healthcare Biosciences). The effluent from the membrane module was continuously monitored for UV absorbance at 280 nm, pH and conductivity, the data being logged into a computer using Prime View software

(GE Healthcare Biosciences). The system pressure was also continuously monitored and recorded using the built-in pressure sensor of the AKTA prime system.

9.3.4 Monoclonal antibody binding experiments

The monoclonal antibody binding capacities of the Paper-PEG membrane samples at different experimental conditions were determined using the AKTA prime system. The binding buffer consisted of ammonium sulfate solutions of different concentrations prepared using the 20 mM sodium phosphate buffer (pH 7.0) while 20 mM sodium phosphate buffer (pH 7.0) on its own was used as the eluting buffer. The monoclonal antibody samples were prepared in the corresponding binding buffer. All binding experiments were carried out at 1.5 ml/min flow. The module was first equilibrated with binding buffer followed by injection of appropriate volume of the mAb sample. The module was then washed with binding buffer until the UV absorbance returned to the baseline. The eluting buffer was then used to recover the bound monoclonal antibody and the amount of antibody bound was determined from the eluted peak area, based on appropriate calibration. The binding experiments were performed in duplicate and found to be reproducible. Averaged numerical data is reported.

9.3.5 Hydraulic characterization of Paper-PEG membranes

The hydraulic properties of membranes were characterized at the eluting and binding solution conditions: with 20 mM sodium phosphate buffer (pH 7) and with a 1.75 M ammonium sulphate solution prepared using the same buffer. In each test the solution was passed through the membrane bed housed within a module at different flow rates and

the corresponding transmembrane pressure was recorded. The flow rate was increased and then decreased in a stepwise fashion. The pressure change caused by change in salt concentration was also examined by switching back and forth several times between high salt binding solution and ammonium sulfate free eluting solution.

9.3.6 Monoclonal antibody purification and sample analysis

The mAb was purified from serum-free CHO cell culture medium spiked with the appropriate amount of mAb. The purification experiments were similar to those used for determining binding capacity. The feed solutions were prepared using appropriate ammonium sulphate containing binding buffer and these were injected into the membrane module using sample loops. The feed, flow through samples and eluted peak samples were analyzed for mAb content and purity. The purification experiments were performed in duplicate and found to be reproducible. Averaged numerical data is reported.

A HiTrap Protein-A FF affinity column (GE Healthcare Biosciences) was used for analyzing mAb purity utilizing the selective binding of antibody by this column. The affinity chromatography experiments were run at 1.0 ml/min flow rate with 20 mM sodium phosphate buffer (pH 7.0) as binding buffer and 100 mM sodium citrate buffer (pH 3.0) as eluting buffer. The feed, flow through and eluted peak samples were also analyzed by SDS-PAGE (Laemmli, 1970). Both reducing (7.5% gel) and non-reducing (12.5% gel) SDS-PAGE experiments were used for samples analysis (Laemmli, 1970). The electrophoresis experiments were carried out using a Hoefer MiniVE device (GE Healthcare Biosciences).

9.4 Results and discussion

A cartoon image of the Paper-PEG membrane is shown in Figure 9.1. Cellulose which forms the fibre web is represented by the thick line grid structure. The PEG chains penetrate the fiber web and are also covalently bound to the cellulose. The amount of PEG grafted can be calculated based on the mass gain of the treated paper. In this study membranes prepared by 3 wt% PEG (Paper-PEG 1) and 17 wt% PEG (Paper-PEG 2) were examined for their hydraulic properties and mAb binding. SEM images of the original filter paper and the two Paper-PEG membranes are shown in Figure 9.2. These images show that that PEG covered and penetrated the cellulose fibers.

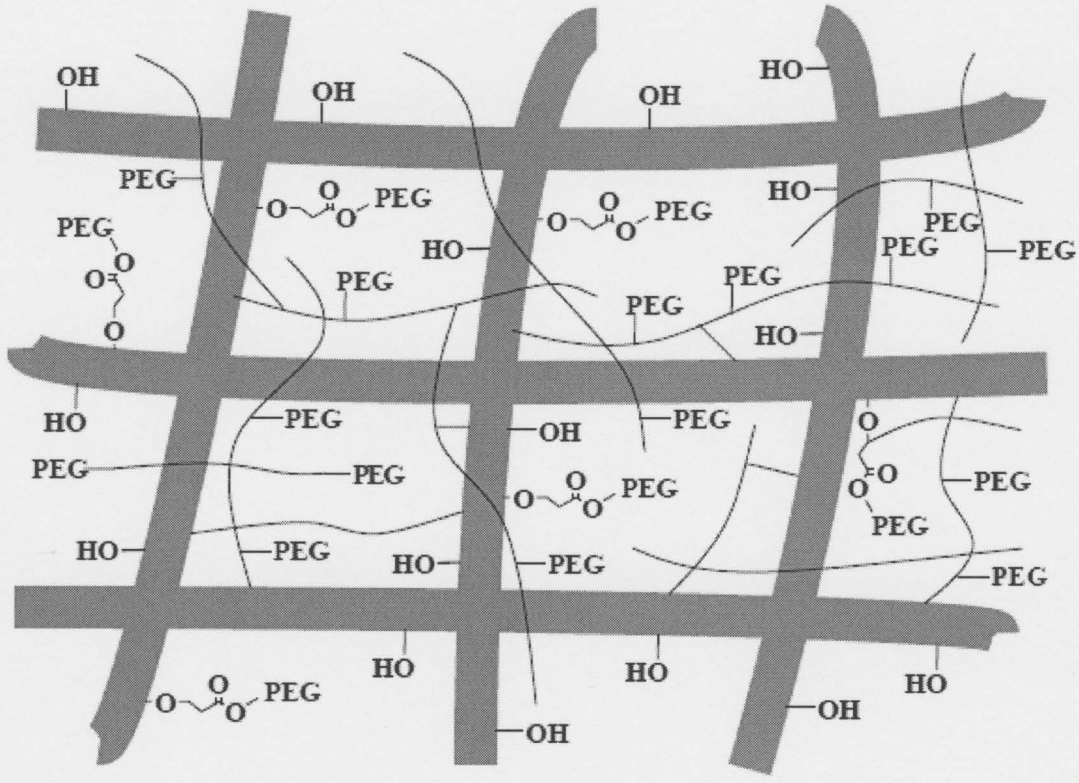


Figure 9.1 Cartoon image of PEG grafted filter paper.

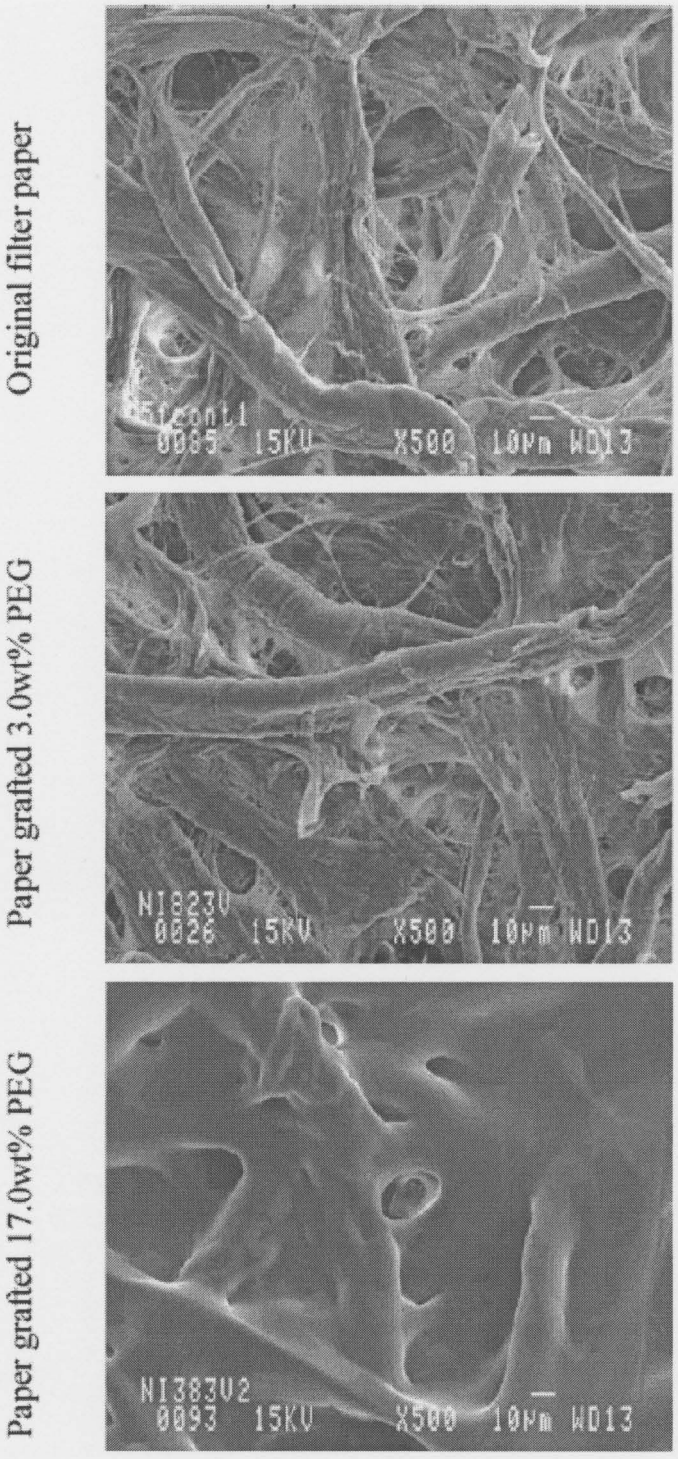


Figure 9.2 Scanning Electron Micrograph (SEM) of Whatman no. 5 filter paper and filter paper grafted with 3% and 17% PEG (% based on mass increase on grafting).

The mAb binding capacities of Paper-PEG 1 and Paper-PEG 2 were compared at 1.60 M and 1.80 M ammonium sulfate concentrations in the feed. These salt concentrations were chosen since preliminary studies showed that below 1.60 M concentration mAb binding was negligible, while at concentrations higher than 1.80 M, significant amounts of mAb were precipitated. The membrane bed volume used in these experiments was 0.159 ml which was obtained by using 5 discs each having thickness of 125 μm. The concentration of mAb in the feed solution was 0.1 mg/ml and 2 ml of this was injected. The amount of bound antibody was determined from the area of the eluted peak, the binding results being shown in Table 9.1. The binding capacity has been expressed in terms of amount of mAb bound per unit membrane bed volume. Based on the significantly higher binding capacity of Paper-PEG 1, this type was selected for carrying out the mAb purification experiments.

Table 9.1 Comparison of monoclonal antibody binding on Paper-PEG 1 and Paper-PEG 2 at different ammonium sulphate concentrations.

Membrane Type	Percentage of mAb bound at 1.6M salt concentration	Percentage of mAb bound at 1.8M salt concentration
Paper-PEG 1	35.4%	97.7%
Paper-PEG 2	6.8%	11.8%

The reason for the lower mAb binding on Paper-PEG 2 can be explained based on the micrographs shown in Figure 9.2. Paper-PEG 1 has a clearer pore network and hence the ligands would be expected to be more accessible to the mAb molecules. In contrast, the higher PEG grafting on Paper-PEG 2 resulted in severe pore occlusion, leading to

poor ligand accessibility. In other word, even though Paper-PEG 2 had a higher PEG loading, less of this was actually able to bind mAb.

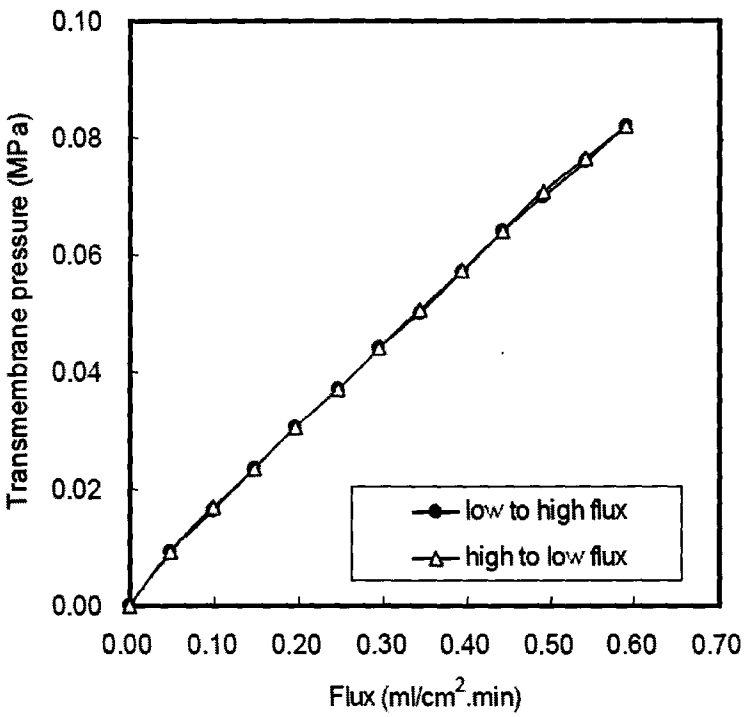


Figure 9.3 Flux versus transmembrane pressure profiles (pressure stepped up and then down) for Paper-PEG 1 obtained with 1.75 M ammonium sulfate solution prepared in 20 mM phosphate buffer (pH 7.0).

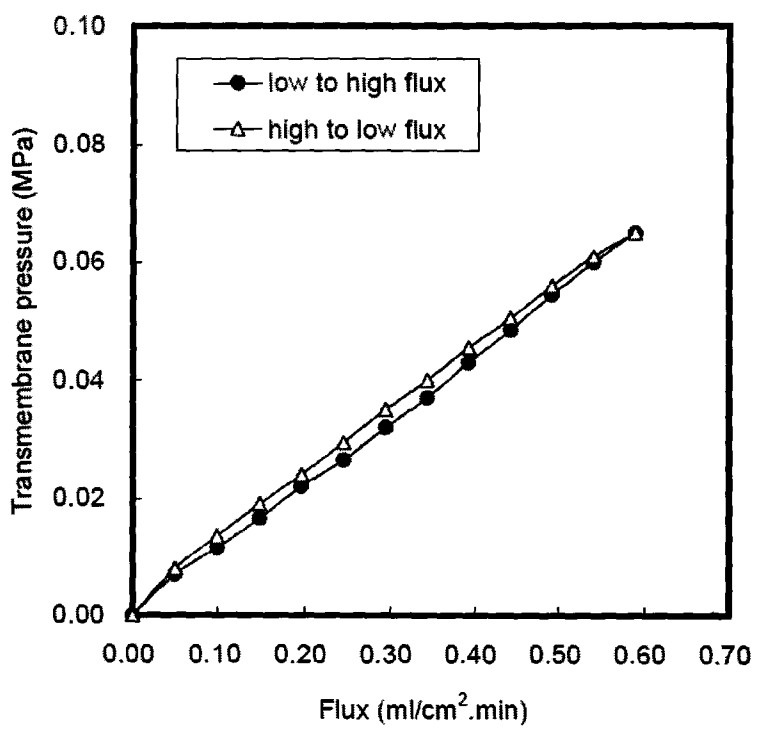


Figure 9.4 Flux versus transmembrane pressure profiles (pressure stepped up and then down) for Paper-PEG 1 obtained with 20 mM phosphate buffer (pH 7.0).

The hydraulic properties of membranes can be characterized by obtaining flux-pressure profiles. Figures 9.3 and 9.4 shows such profiles obtained using the Paper-PEG 1 membrane at two solution conditions: in 20 mM sodium phosphate buffer pH 7.0 and in 1.75 M ammonium sulphate solution prepared using the same buffer. The cross-sectional area of the membrane bed was 2.54 cm^2 while the bed height was 0.625 mm. At both solution conditions the transmembrane pressure increased linearly with permeate flux and also decrease in a linear fashion when the flux was decreased. These results indicate that the Paper-PEG 1 membrane was largely incompressible in the flux-pressure range examined. The permeability of Paper-PEG 1 was higher with the ammonium sulphate free buffer. One of the reasons for this is the higher viscosity of the 1.75 M ammonium sulphate solution. However, viscosity difference alone cannot account for this difference in permeability. At low salt concentration, PEG exists in an extensively hydrated form and hence the degree of interaction between adjacent PEG chains is minimal. However, at a high ammonium sulphate concentration PEG shows hydrophobic properties and hence the PEG chains are expected to form a tighter network. This accounts for the higher pressure observed at higher salt concentrations. Figure 9.5 shows the effect of cyclic change in ammonium sulfate concentration on transmembrane pressure across the Paper-PEG 1 bed. When the ammonium sulfate concentration was increased, the pressure increased due the combination of factors already discussed, i.e. increase liquid viscosity and shrinkage of the PEG network due to salt induced dehydration. When the salt concentration was decreased, the pressure decreased to its original value. When this sequence of salt concentration change was repeated, the same pressure pattern was

observed. These results clearly indicate that the change in property of the Paper-PEG 1 membrane caused by change in salt concentration was reversible in nature.

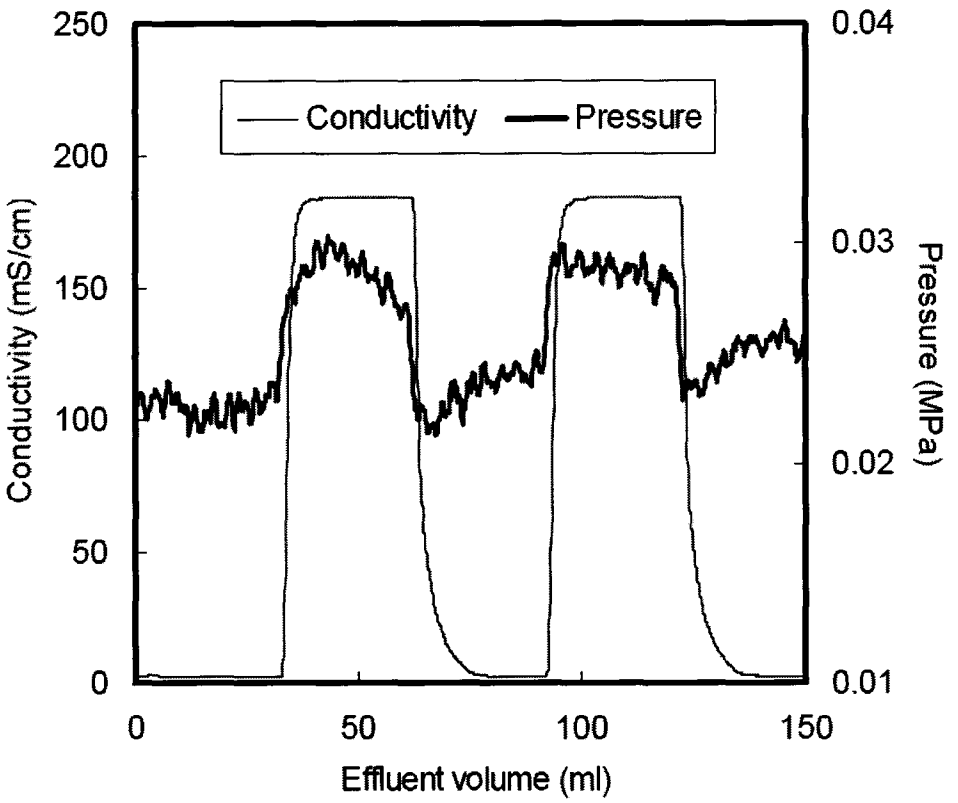


Figure 9.5 Effect of cyclic change in ammonium sulfate concentration on the pressure across a Paper-PEG 1 membrane bed. The ammonium sulfate concentration was changed from zero to 1.75 M using 20 mM phosphate buffer (pH 7.0) as base buffer. The flow rate used in this experiment was 1.5 ml/min.

The average permeability of the Paper-PEG 1 membrane was 2.2×10^{-6} m Pa^{-1} s^{-1} per m of bed height. This was slightly lower than that observed with the original filter paper at the same operating conditions (1.74×10^{-6} m Pa^{-1} s^{-1} per m of bed height) but significantly higher than that observed with Paper-PEG 2 (3.76×10^{-8} m Pa^{-1} s^{-1} per m of bed height). With most commercial adsorptive membranes, the permeability is in the range of 1.6×10^{-7} to 3.3×10^{-7} m Pa^{-1} s^{-1} per m of bed height. The permeability of the

Paper-PEG 1 was therefore an order of magnitude higher than commercial membranes. Hence, one of the main reasons for choosing a paper based adsorptive media i.e. obtaining high permeability is amply justified.

The mAb binding of Paper-PEG 1 was re-examined at the following ammonium sulphate concentrations: 1.55, 1.65, 1.7, 1.75 and 1.8 M. The concentration of mAb in the feed solutions used in these experiments was 0.1 mg/ml and 2 ml of this solution was injected into the module. The Paper-PEG 1 bed volume was 0.159 ml. The adsorption of CHO cell culture media proteins on Paper-PEG 1 was also examined at these salt concentrations. The total media protein concentration in the feed solution was 0.04 mg/ml and 2 ml of this was injected. The results of these salt concentration optimization experiments are shown in Figure 9.6 where the percentage of mAb and media protein bound to the Paper-PEG 1 bed at the different testing conditions is reported. When the ammonium sulphate concentration in the feed was increased in the range of 1.55 M to 1.75 M, the mAb binding increased from 23% to 98%. Increasing the salt concentration to 1.8 M did not result in higher mAb binding. The percentage of media proteins bound was in about 0.5% in the salt concentration range of 1.55 M to 1.75 M but increased to 0.9% when salt concentration was increased to 1.8 M. It further increased to 2.5% at 1.85 M concentration but mAb binding was not tested at this condition since its solubility in the feed solution was very low. Based on these results, 1.75 M ammonium sulphate concentration was chosen as the binding condition for mAb purification.

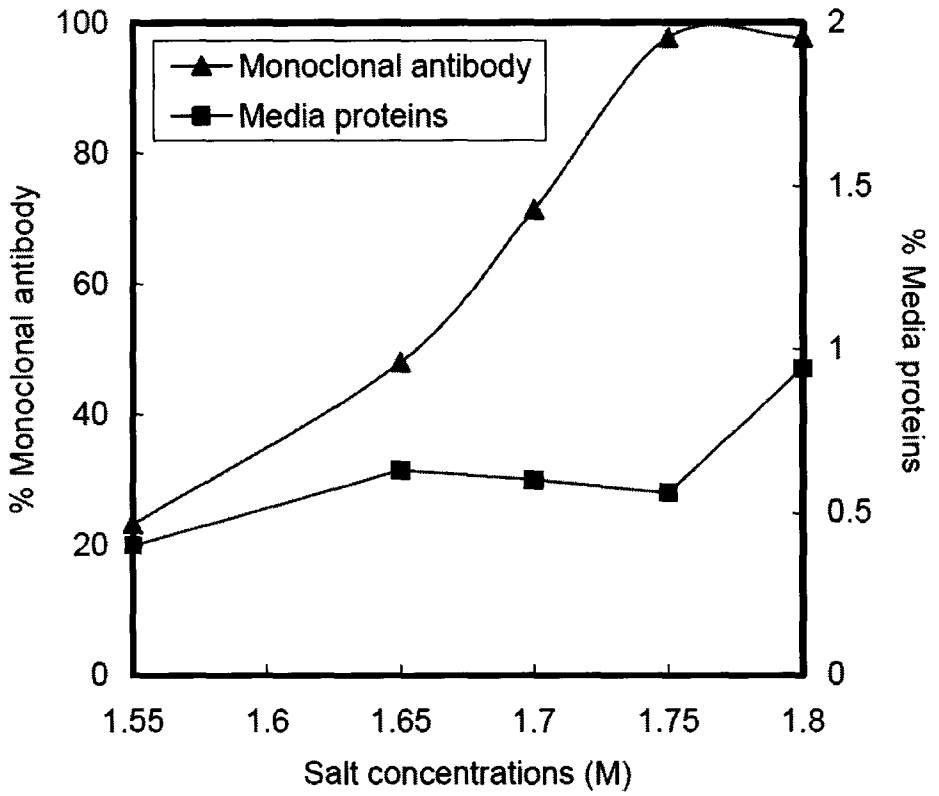


Figure 9.6 Binding of monoclonal antibody and CHO cell culture media proteins on Paper-PEG 1 membrane at different ammonium sulphate concentrations (mAb concentration in feed: 0.1 mg/ml mAb, feed volume: 2 ml; flow rate: 1.5 ml/min; membrane bed volume: 0.159 ml).

The dynamic mAb binding capacity of Paper-PEG 1 was examined at different feed concentrations and feed loading volumes. The membrane bed volume used in these experiments was 0.286 ml and the flow rate used was 1.5 ml/min. The different feed concentration-volume combinations were: 15, 30 and 45 ml of 0.1 mg/ml mAb and 90 ml of 0.05 mg/ml mAb. In these experiments the feed solutions were injected using a superloop. The membrane bed volume used was 0.286 ml and this as obtained by using 9 discs each having thickness of 125 μm. The feed flow rate used was 1.5 ml/min and the feed ammonium sulphate concentration was 1.75 M. The amount of mAb bound was determined from the area of the eluted peak. The dynamic binding capacities at different

concentration-volume combinations are shown in Table 9.2. The combination of 45 ml of 0.1 mg/ml mAb feed solution gave the highest binding capacity i.e. 9 mg/ml. When the same amount of mAb was loaded on the membrane in the form of 90 ml of 0.05 mg/ml mAb, the binding capacity was lower, i.e. 6.2 mg/ml. This can be ascribed to the higher binding of mAb at a higher feed concentration as might be expected from an adsorption isotherm. Material balance for hIgG1-CD4 was performed for the above experiments and it was observed that the antibody present in the feed could be accounted for by the chromatographic peaks.

Table 9.2 Monoclonal antibody binding capacity of Paper-PEG 1 at different mAb concentrations and loading volumes.

Feed concentration (mg/ml)	Feed volume (ml)	Binding capacity (mg/ml bed volume)
0.1	15	3.3
0.1	30	6.3
0.1	45	9.0
0.05	90	6.2

The purification of mAb from simulated CHO cell culture supernatant was carried out at two mAb feed concentrations: 0.05 mg/ml and 0.1 mg/ml. Typical defined proteins present in serum free cell culture medium include bovine albumin, insulin, ferritin and transferrin. In these experiments the media protein concentrations were kept the same. Figure 9.7 shows the chromatogram from the separation experiment carried out using 0.1 mg/ml mAb. The membrane bed volume used in the purification experiments was 0.286 ml and 5 ml of feed solution adjusted to 1.75 M ammonium sulphate concentration and

having the appropriate mAb concentration was injected in each case. The impurities are expected to be present in the flow through peak (i.e. the first peak) while the purified mAb is expected in the eluted (i.e. second) peak. Figure 9.8 shows the Protein A affinity chromatogram obtained with the purified mAb sample. The results of the purification experiments are summarized in Table 9.3. When using a mAb concentration of 0.1 mg/ml in the feed the purity of purified mAb was 89% and the recovery was 71%. When the feed mAb concentration was lowered to 0.05 mg/ml, the purity of purified mAb changed to 85% while the recovery increased to 97%. The lower purity obtained at 0.05 mg/ml feed concentration reflects the fact that the media protein to mAb concentration increased in this experiment. The higher recovery at the lower feed mAb concentration means that the mAb loading was significantly lower than the binding capacity.

Table 9.3 Purity and recovery of purified mAb obtained from CHO cell culture medium.

mAb concentration in CHO cell culture medium	Feed volume	mAb purity	mAb recovery
0.1 mg/ml	5 ml	89%	71%
0.05 mg/ml	5 ml	85%	97%

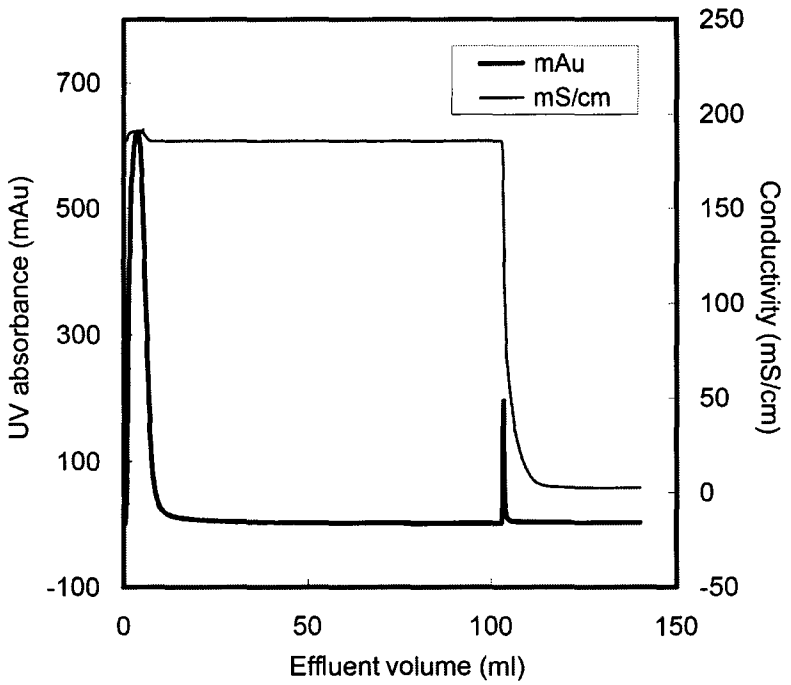


Figure 9.7 Purification of mAb from CHO cell culture medium by hydrophobic interaction membrane chromatography using Paper-PEG 1(feed: CHO cell culture supernatant spiked with 0.1 mg/ml mAb, feed volume: 5 ml; flow rate: 1.5 ml/min; membrane bed volume: 0.286 ml).

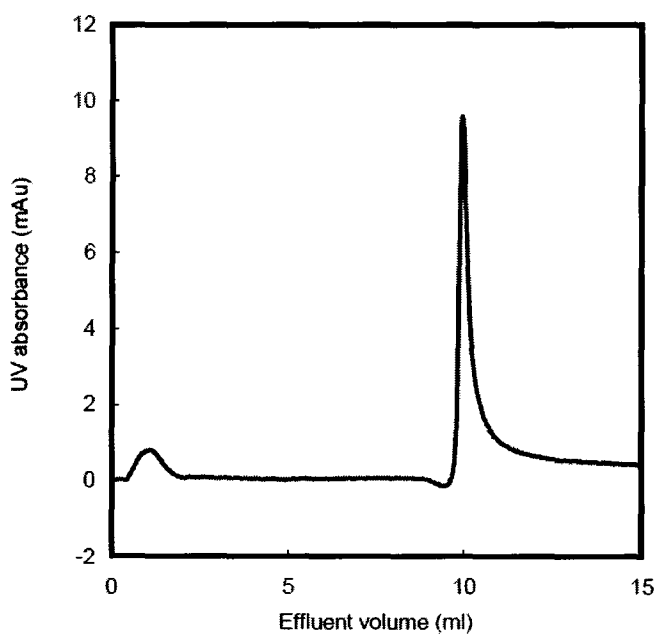


Figure 9.8 Protein A affinity chromatogram obtained with mAb purified from CHO medium (first peak represents impurities and second peak represents mAb).

The feed, flow through and purified mAb samples were analysed by SDS-PAGE. Figure 9.9 shows the 12.5% reducing gel while Figure 9.10 shows the 7.5% non-reducing gel. The purified mAb showed two bands on the reducing gel, one corresponding to the heavy chain (50 kDa) and the other corresponding to the light chain (25 kDa) while of the non-reducing gel, the mAb showed one band corresponding to 150 kDa. The two gels show that the purified mAb had higher purity than that indicated by the protein A affinity chromatogram. Once reason for this could be the presence of low molecular weight UV absorbing impurities in the sample which would show up on the chromatographic analysis but would not be picked up on an SDS-PAGE. Moreover if there are several impurities each in very small amounts and each having different molecular weights, these would be hard to detect using SDS-PAGE. In contrast, these would appear together in the unbound protein A chromatographic peak and would hence be collectively detectable.

The pressure drop required to drive a solution through a membrane module can be used to keep track of its health, i.e. whether it is getting fouled or clogged up. Figure 9.11 shows the pressure drop versus run number for several experiments carried out using the same membrane module. The membrane bed volume used in these experiments was 0.159 ml and these were carried out at 1.5 ml/min flow rate. The membrane module was not cleaned between runs but was simply rinsed out with eluting buffer. Even after 32 runs the pressure was lower than 0.025 MPa. This clearly indicated that the Paper-PEG 1 membrane had very low fouling tendency and had a long process life.

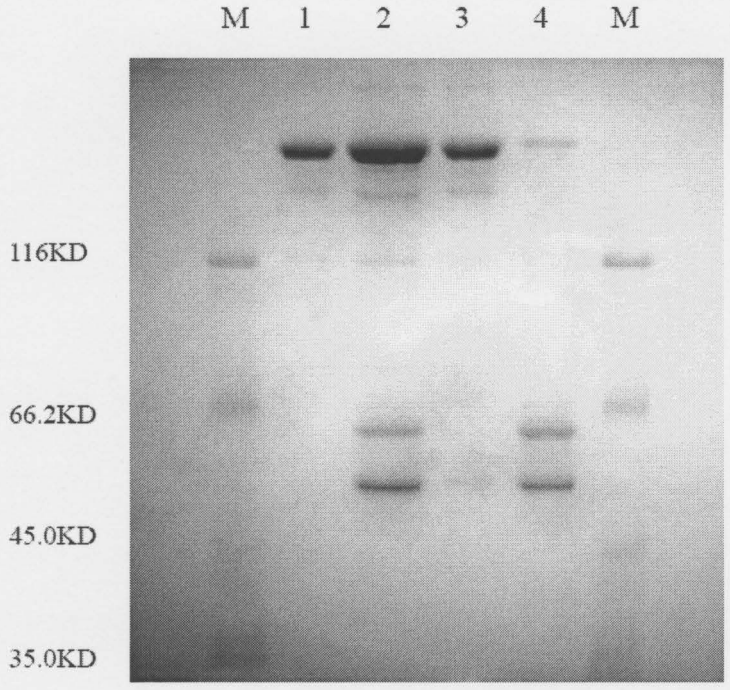


Figure 9.9 Non-reducing SDS-PAGE (7.5%): lane 1: standard mAb; lane 2: feed; lane 3: purified mAb; lane 4: flow through sample.

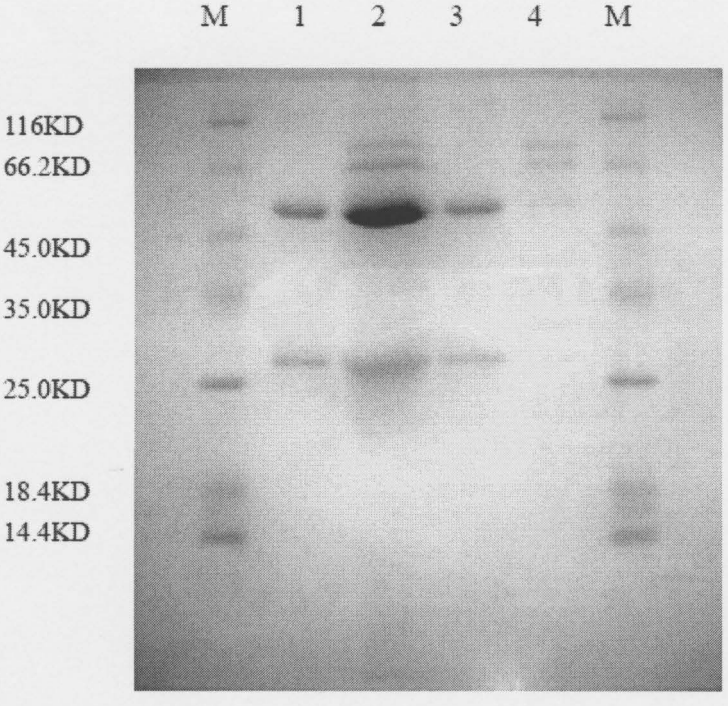


Figure 9.10 Reducing SDS-PAGE (12%): lane 1: standard mAb; lane 2: feed; lane 3: purified mAb; lane 4: flow through.

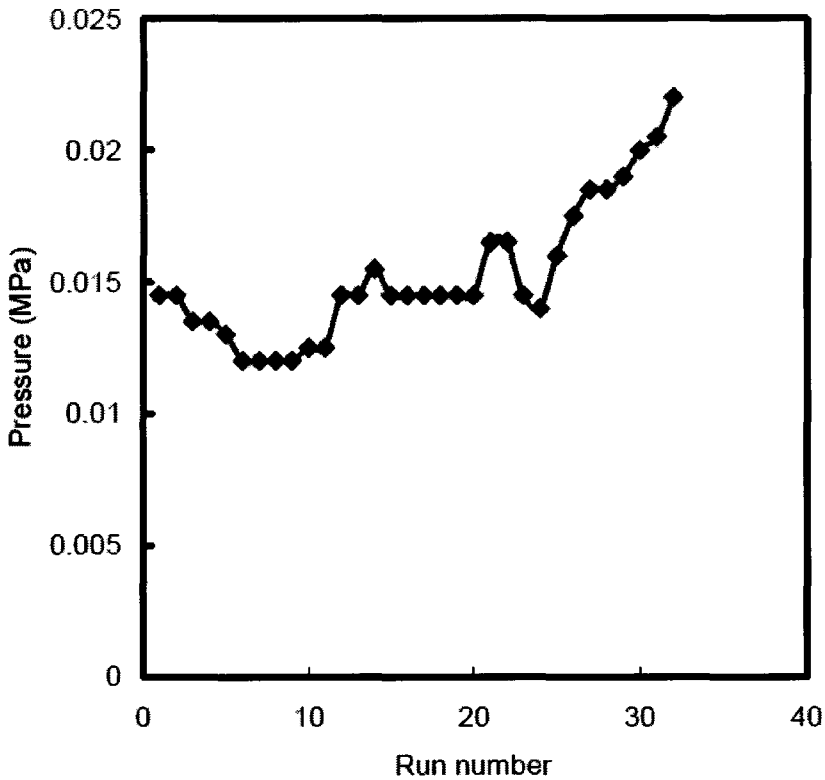


Figure 9.11 Change in pressure with run number (number of discs: 5; flow rate: 1.5 ml/min; binding buffer: 1.80 M ammonium sulfate prepared using 20 mM pH 7.0 sodium phosphate buffer; eluting buffer: 20 mM pH 7.0 sodium phosphate buffer).

Several aspects could be considered to further improve the Paper-PEG membrane. The mAb binding capacity could be improved by optimizing the PEG loading on the paper. The current work used a randomly chosen filter paper with two levels of PEG loading i.e. 3% and 17% mass gains, the purpose being to provide proof-of-concept for our approach. Since this does seem to work, the next logical step would be to try out other types of filter paper with different levels of PEG. The hydrophobicity and indeed mAb binding capacity of the grafted PEG in the presence of high antichaotropic salt concentration would also depend on the molecular weight of the PEG. A polymer with

higher molecular weight than the currently used PEG 2000 may provide better binding capacity.

9.5 Conclusions

The Paper-PEG membranes could be prepared in a controllable manner using the IPN technique. These membranes are likely to be inexpensive due to the use of paper as the fibre web material. The membrane with 3 wt% PEG showed much higher mAb binding than that with 17% PEG due to greater accessibility of the ligands. The Paper-PEG 1 membrane showed excellent hydraulic properties in terms of high permeability and practically no compressibility in the pressure range examined. The mAb binding on this membrane was shown to be selective in nature: at 1.75 ammonium sulphate concentration in the binding buffer the mAb binding was in the range of 9 mg/ml bed volume while media protein binding was very low. In a single step purification process using the Paper-PEG 1 membrane the mAbs purity obtainable was in the range of 85 to 89% (as determined by analytical protein A affinity chromatography) and the recovery obtainable was in the range of 71 to 97%, these being dependent on the mAb concentration in the starting material. The reducing and non-reducing SDS-PAGE showed higher mAb purity values than indicated by the protein A chromatograms. The permeability of the Paper-PEG 1 membrane was high even after 32 separation runs, clearly indicating it reusable nature and low susceptibility to membrane fouling. Quite clearly the Paper-PEG membrane showed significant promise in carrying out hydrophobic interaction chromatographic separations.

9.6 References

Ananthapadmanabhan KP, Goddard ED. 1987. Aqueous biphase formation in polyethylene oxide-inorganic salt systems. Langmuir 3:25-31.

Andrews BA, Nielsen S, Asenjo JA. 1996. Partitioning and purification of monoclonal antibodies in aqueous two-phase systems. Bioseparation 6:303-313.

Arakawa T, Philo JS, Tsumoto K, Yumioka R, Ejima D. 2004. Elution of antibodies from a Protein-A column by aqueous arginine solutions. Protein Expression Purif 36:244-248.

Carlson KL. 2005. Flexibility—the guiding principle for antibody manufacturing. Nat Biotechnol 23:1054-1058.

chromatographic separation processes. J Membr Sci 281:532-540.

Ghosh R, Wang L. 2006. Purification of monoclonal antibody by hydrophobic interaction membrane chromatography. J Chromatogr A 1107:104-109.

Ghosh R, Wong T. 2006. Effect of module design on the efficiency of membrane

Ghosh R. 2001. Separation of proteins using hydrophobic interaction membrane chromatography. J Chromatogr A 923:59-64.

Ghosh R. 2002. Protein separation using membrane chromatography: opportunities and challenges. J Chromatogr A 952:13–27.

Huddleston J, Abelaira JC, Wang R, Lyddiatt A. 1996. Protein partition between the different phases comprising poly(ethylene glycol)-salt aqueous two-phase systems, hydrophobic interaction chromatography and precipitation: a generic description in terms of salting-out effects. J Chromatogr B Biomed Appl 680:31-41.

Isaacs JD, Burrows N, Wing M, Keogan MT, Rebello PR, Watts RA, Pye RJ, Norris P, Hazelman BL, Hale G, Waldmann H. 1997. Humanized anti-CD4 monoclonal antibody therapy of autoimmune and inflammatory disease. Clin Exp Immunol 110:158-166.

Knudsen HL, Fahrner RL, Xu Y, Norling LA, Blank GS. 2001. Membrane ion-exchange chromatography for process-scale antibody purification. J Chromatogr A 907:145-54.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Ling TGI, Mattiasson B. 1983. Poly(ethylene glycol)- and poly(vinyl alcohol)-substituted carbohydrate gels for "mild" hydrophobic chromatography. J Chromatogr A 254:83-89.

Michel R, Pasche S, Toxtor M, Castner DG. 2005. Influence of PEG architecture on protein adsorption and conformation. Langmuir 21:12327-12332.

Phillips M, Cormier J, Ferrence J, Dowd C, Kiss R, Lutz H, Carter J. 2004. Performance of a membrane adsorber for trace impurity removal in biotechnology manufacturing. J Chromatogr A 1078:74-82.

Sabaa MW, Mokhtar SM. 2002. Material characterisation chemically induced graft copolymerization of itaconic acid onto cellulose fibers. Polym Test 21:337-343.

Wang L, Xu Y. 2006. Preparation and characterization of graft copolymerization of ethyl acrylate onto hydroxypropyl methylcellulose in aqueous medium. Cellulose 13:191-200.

Wang RW, Zhang Y, Ma GH, Su ZG. 2006. Modification of poly(glycidyl methacrylate-divinylbenzene) porous microspheres with polyethylene glycol and their adsorption property of protein. Colloids and Surf B: Biointerfaces 51:93-99.

Yang L, Chen P. 2002. Chitosan/coarse filter paper composite membrane for fast purification of IgG from human serum. J Membr Sci 205:141-153.

Chapter 10

Summary and Future Work

10.1 Summary and major contributions

The following was achieved through the research work described in this thesis.

10.1.1 Novel application in mAb purification from transgenic tobacco

For the first time, membrane chromatography based bioprocesses were established for mAb purification from transgenic tobacco, primarily addressing the challenge of low mAb abundance. The bioprocess based on IEMC and PAMC successfully purified hIgG1-CD4 from spiked tobacco juice and anti-*P. aeruginosa* from transgenic tobacco. The high mAb purity and recovery with low pressure drops demonstrated a flexible, reproducible and scalable bioprocess (Chapter 3). The bioprocess using IEMC followed by HIMC and ultrafiltration was established for mAb purification from tobacco extract (Chapter 4). It

overcome the limitations of Protein A based methods and demonstrated the feasibility of using gradient elution in membrane chromatography for high-resolution separation.

10.1.2 Novel application in purification of PEGylated proteins

This thesis first reports the application of membrane chromatography in purification of PEGylated proteins (Chapter 5). Optimized conditions were achieved for N-terminus lysozyme PEGylation. Single-step purification using IEMC highly purified mono-PEGylated lysozyme from reaction mixture with high flow rate and low pressure. The binding capacity and resolution in IEMC separation were found to be enhanced by flow rate increase. This study demonstrated membrane chromatography as a robust, high-resolution and high-throughput method for purification of PEGylated proteins.

10.1.3 Novel application in integrated bioreaction and bioseparation

Reactant adsorptive membrane bioreactor separator was developed for integrated enzymatic fragmentation of hIgG and purification of IgG fragments. HIMC based RAMBS was developed for integrated separation-reaction-separation to produce Fab fragment directly from human serum (Chapter 6). Cation-exchange based RAMBS was utilized for papain digestion of hIgG and purification of Fab (Chapter 7). Anion-exchange based RAMBS was utilized for pepsin digestion of hIgG and purification of $F(ab')_2$. The RAMBS system achieved the process intensification of reaction and separation and apparently higher catalytic efficiency than that in liquid phase reaction. Highly purified Fab and $F(ab')_2$ were produced with high recovery using a single unit operation within a single device. This system would be attractive in any application where relatively expensive macromolecular reactants are to be digested using inexpensive enzymes.

10.1.4 Mechanism of IgG binding

RAMBS system was utilized to examine hydrophobic interaction based hIgG binding on synthetic microporous membranes (Chapter 8). The membrane in RAMBS was used as a *molecular chopping board.* On site cleavage of membrane bound hIgG by enzymes followed by separation of fragments using gradient elution provided conclusive evidences that hIgG bound on membrane through its middle region, i.e. the combination of the hinge and C_H2 domain of Fc. The results from this study are useful for developing membrane chromatography, immunoassay and blood contact biomedical devices. The approach developed here could be potentially applicable for studying other protein interaction with solid support.

10.1.5 Development of novel membrane

PEG-filter paper based membrane was developed for hydrophobic interaction membrane chromatography (Chapter 9). This novel membrane addressed the limitations of high cost and fouling tendency of commercial membrane adsorbers. Single-step hydrophobic interaction membrane chromatography using this membrane could produce mAb from simulated CHO cell culture with high purity and high recovery. This inexpensive membrane showed significant promise in carrying out HIMC separations due to its selecting power, high hydraulic permeability and good reusability.

10.2 Recommendations for future work

10.2.1 Application in bioprocessing of protein therapeutics

The research reported in this thesis opens up new application areas of membrane chromatography that could be further developed based on the significant progresses achieved here. The high-resolution membrane chromatography based process was successfully developed for purification of mAb from transgenic tobacco. While this process focused on purification, the pretreatment process could be further optimized for high processing capacity. Furthermore, the scale up of current process has been started but not reported. Further work in process scale up and improvements of mAb expression could lead to the potential commercialization of transgenic tobacco based mAb.

Purification of PEGylated proteins using IEMC demonstrated a high-throughput and high-resolution process. In addition, purification of PEGylated proteins using HIMC was found very promising (not reported here). The PEGylated proteins can be bound at relatively low salt concentration and high purity can be achieved by one-step HIMC. More interestingly, this method may be widely suitable for purification of various PEGylated proteins. Further development of this method may lead to a patent.

RAMBS system was successfully developed for integrated hIgG fragmentation and purification of hIgG fragments. This method could be extended to produce antibody fragments from pure monoclonal antibody or directly from mAb in cell culture supernatant. The approach of using RAMBS to study IgG binding on membrane could be more widely applicable for studying protein interaction with membrane or other surfaces.

The development of PEG-filter paper led to the new membrane adsorber with low cost and high permeability. Other membrane adsorber based on filter paper could be further developed. Protein G-CBD-filter paper is an interesting project. Some of the work has been started. The fusion protein Protein G-CBD (cellulose binding domain) was expressed by engineered *Pichia Pastoris* provided by Dr. J.C. Hall (Department of Environmental Biology in University of Guelph). This fusion protein could be simply immobilized on filter paper by affinity interaction of CBD to cellulose and Protein G ligand is then used for antibody capture. With further understanding of CBD interaction with cellulose and optimizing the preparation, Protein G-CBD-filter paper could be a useful affinity membrane adsorber for membrane chromatography or immunoassay.

10.2.2 Application in bioprocessing of DNA, virus and cell based therapy

The researches described in this thesis focus on the application of membrane chromatography in bioprocessing of monoclonal antibodies and PEGylated proteins. Next big area for membrane chromatography would be the bioprocessing of DNA, virus and cell for new vaccine, gene therapy and stem cell therapy. These large biomolecular assemblies are especially suitable for bioprocessing using membrane chromatography which provides higher binding capacity and throughput than column chromatography due to the large pore size and convective mass transport of membrane adsorbers. Although the DNA, virus and cell based therapies are in very early stages, they are very promising solutions to currently untreatable diseases. Membrane chromatography will play significant role in developing these new biotherapies.