MEMBRANE AND TEMPERATURE BASED METHODS FOR PROCESSING AND PURIFYING MONOCLONAL ANTIBODIES

By

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Descriptive Notes

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Abstract

Monoclonal antibodies (mAbs) as therapeutic proteins have shown great potential in treatment of various human diseases because of their highly specific nature. This has attracted worldwide attention leading to increased demand for such mAb products. To meet this demand large scale manufacturing is carried out using recombinant mammalian cell culture techniques for high yields and faster production. mAb products are worth the investment if produced in their native state. The quantity of mAb present in such cell cultures is very less and therefore special care is needed while handling them. Purifying antibody molecules from heterogeneous cell culture impurities and maintaining their native functional state is a critical task mainly because these antibodies are labile in nature. Care also need to be exercised during processing because mAbs have inherent tendancy to aggregate which is undesirable since such aggregates in antibody formulation produces immunogenic reaction when injected in humans. The other important factor in mAb purification is the processing cost involved since majority of the total production cost is utilized for purification of mAb. Protein-A chromatography is the first choice for purifying antibodies and is widely adopted. However failure in distinguishing between monomer and aggregate antibody molecules along with harsh acidic processing conditions necessitates the use of further purification steps.

In this work various techniques for mAb processing are discussed and are outlined below:

Removal of impurities from mAbs is a major challenge and this thesis discusses various processing options available to purify these mAbs. Impurities in mAb products are usually the aggregate byproducts formed due to unfolded monomer antibody molecules. These molecules are naturally hydrophobic in nature and display great differences in hydrophobicity on aggregation. Hydrophobic interaction membrane chromatography (HIMC) makes use of this hydrophobicity difference and helps in removal of aggregate impurities from monomer antibody.

Heavy chain mAbs (hcmAbs) are promising new developments in the area of biopharmaceuticals because of their unique structural composition. Similar to

conventional mAbs these hcmAbs are also rapidly finding their way into therapeutic markets. Purifying hcmAbs will be an important step in their development and for this purpose we use HIMC technique for removing impurities and obtain pure product.

Antibody molecules are almost always lost as aggregates which leads to great economic losses and the ability to disaggregate these mAb oligomers would be of significant practical and scientific interest. In this work a novel thermalcycling technique is discussed to disaggregate such mAb oligomers and potentially recover functional monomer mAb molecules.

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1. Introduction

Antibodies as therapeutic proteins have gained significant importance since the isolation of immunoglobulins (Igs) from human serum as described by Cohn's fractionation technique.[1] Reports indicate the earliest use of Igs as therapeutics was in the early 19th century for treatment of measles, hepatitis A and poliomyelitis. During World War II human serum was subjected to alcohol fractionation and antibodies were obtained to treat wounded individuals. Further in 1952, Bruton's report[2] on treatment of recurrent pneumonococcal sepsis highlighted an important finding wherein an individuals' poor innate defense mechanism, caused by agammaglobulinemia, was responsible for prolonged illness and this was successfully overcome by the administration of immune human serum globulin.

Earliest antibody production was based on selective precipitation of Igs using alcohol to fractionate individual protein components. This helped in obtaining IgG upto 99% purity along with trace amounts of Igs A, D, E and M. Major breakthrough in development was the production of monoclonal antibodies (mAbs) belonging specifically to IgG class using hybridoma technique in 1975[3]. Principle involved in this technique was to generate myeloma using spleen cells from an immunized donor mouse. This made possible to produce antibodies against a specific target antigen. mAbs for therapeutic applications generally belong to IgG class and are Y-shaped molecules with 150kDa molecular weight comprising of 2 heavy and 2 light chain units. Top part of the antibody molecule consists of 2 similar Fab (fragment antigen binding) regions and the bottom part is the Fc (fragment crystallizable) region. Generally an IgG type antibody would look like as shown in Figure.1.1. Fab sites are responsible for neutralizing the antigenic species and Fc region helps carrying out effector functions after the antibody-antigen complex is formed.

Earliest mAb produced was Campath-1M against T-cell antigens[4] present in humans. This IgM class antibody helped in fixation of the human complement and was

able to react simultaneously with all T- and B-cells.[5] Main application of this antibody was to prevent the Graft vs Host Disease (GvHD) which would normally occur during

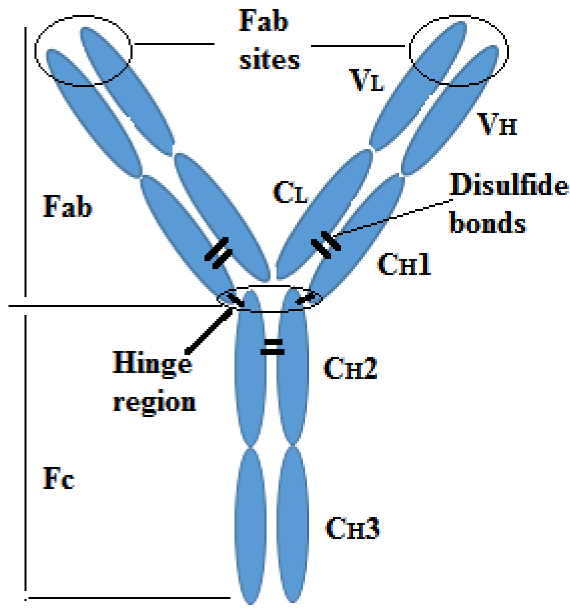

Figure 1.1. Structure of an immunoglobulin G (IgG) antibody molecule. Fab – fragment antigen binding region; Fc – fragment crystallizable region; C_H1, C_H2, C_H3 – constant heavy domains 1, 2 and 3 respectively; V_L and C_L are variable and constant light chain domains.

bone marrow transplants. Further developments led to the production of Campath-1G, an IgG type mAb. The advantage that Campath-1G had over Campath-1M was in its ability to provide prolonged lowering of lymphocytes[6] when tested in patients suffering from leukemia. However, poor interests in commercializing of Campath-1G inspired the development of Campath-1H. Use of recombinant DNA techniques made possible the production of chimeric mAb which retained mouse variable Fab regions and replaced mouse Fc region with human Fc.[7–9] Campath-1H was produced against CD52 antigen for treatment of chronic lymphocytic leukemia.[10] Immunogenic reaction in patients resulting from undesired activity of the antibody molecules was the driving force for developing fully human mAbs.[11,12]

The success in treating various life threatening diseases such as cancer has led to increased demand for these mAb products and to meet this demand, large scale production is required. Conventional method of antibody manufacturing using animal models had limitations in producing large volumes of the product. Moreover the ethical issues of animal usage as well as immunogenicity resulting from these mAbs was an added drawback. This fostered into investigating alternative methods of obtaining mAbs and led to the development of recombinant technology which revolutionized mAb production. By using mammalian cell lines and culturing the cells in controlled environment to produce antibody with desired functionality proved highly favorable and received great interests over the conventional method.[13]

Current literature mentions using high antibody yield producing cell lines which includes chinese hamster ovary (CHO), baby hamster kidney (BHK), human embryonic kidney (HEK), mouse spleen cells SP 2/0 and NS0.[14] One of the main advantages with these expression systems is that the cellular processes ensure solubility of the final mAb product. In addition to this, antibody produced contains similar glycosylation patterns when compared to those produced naturally in humans which is very essential for proper functioning of every mAb.[15,16] Conventionally produced mAbs had low expression levels and the final antibody concentration would be of the order of few milligrams/liter. However growing cells in a reactor with recombinant technique increased production volumes upto 25,000 liters with the final antibody concentration produced being 5 g/L or in some cases upto 10 g/L and this has resulted into tremendous leap in the production processes to obtain high purity antibody.

Purifying mAb from such high volume cultures is a major challenge in the biopharmaceutical industry. High concentration of antibody often results in unwanted aggregation problems. Additionally various enzymes present in culture solution are known to degrade the mAb product. For these mAb products to be effective their native functional structure should be preserved. Presence of aggregates in therapeutic drugs have previously proven harmful for human injection with varying degrees of adverse

reactions.[17–19] To solve such problems, downstream processing of these antibodies is performed for purifying monomeric mAbs from aggregates and cell culture impurities. A typical purification process is shown in Figure.1.2. The most critical step in downstream processing is the initial capture of mAbs and removal of cell culture impurities. These mAbs are usually purified using protein-A affinity chromatography because of high specificity offered by protein-A towards IgG antibody molecules.[20] However acidic condition used during purification affects mAb stability and has shown to form aggregates as well as antibody-aggregate complexes.[21]

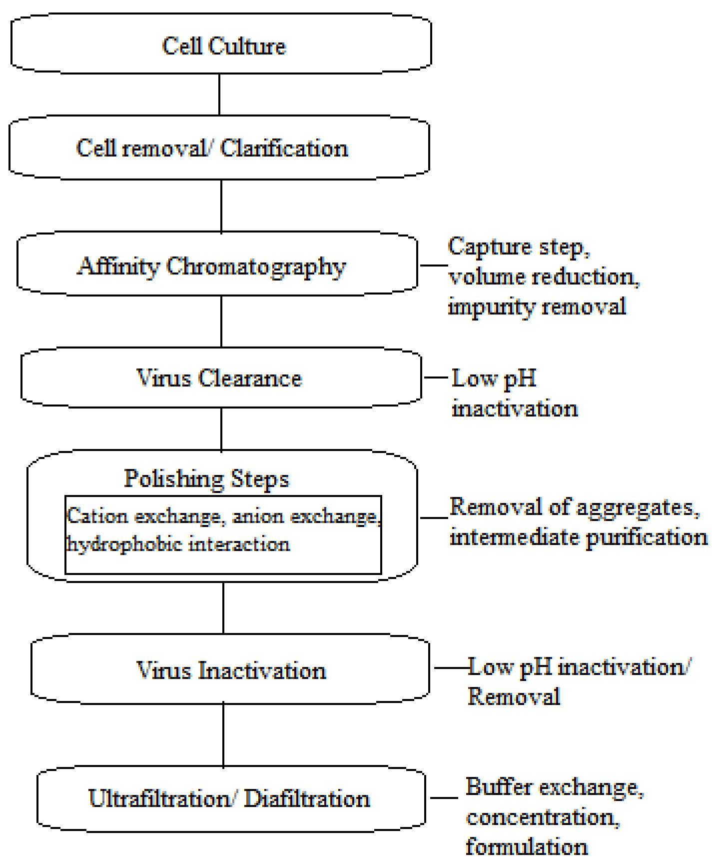

Figure 1.2. Typical process flow-chart for production of monoclonal antibody.

Even after purification, the antibody product undergoes various environmental stresses during storage which adversely affects its stability. These include factors like temperature, solution pH, salt content and physical mixing/shear forces.[22] Critical stage in aggregate formation is the initial unfolding of native protein chains which serves as nucleation site as explained by various aggregation theories[23,24] (Figure.1.3.). Additionally non-covalent interactions such as the electrostatic effects and adsorption, and covalent interactions have been responsible for reversible aggregate formation under physiological conditions.[25] Antibody aggregates formed under native conditions are often reversible and tend to dissociate upon dilution. However the most notorious species are the non-native aggregates which are irreversible in nature and are formed from completely denatured antibody molecules. It is important to characterize the nature of aggregates formed and monitor the conditions that favor aggregation.

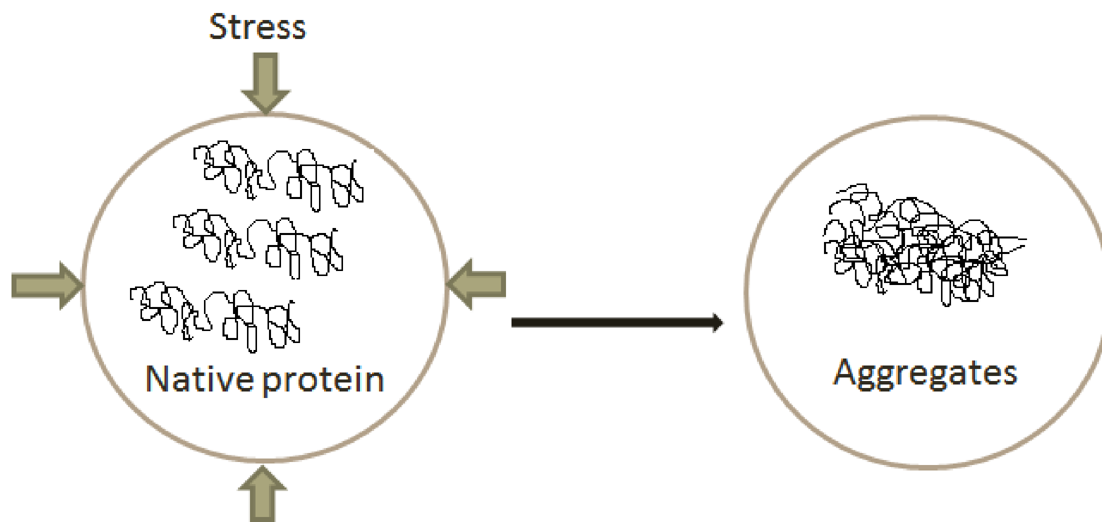

Figure 1.3. Aggregate formation with native protein molecules under stress.

Due to the diverse nature of aggregates, it is difficult to characterize them using a single known technique. One of the simplest techniques for antibody detection is the size exclusion chromatography (SEC) which is useful with non-native aggregates.[26]

However there are concerns regarding the potential interaction between aggregates and column packing which would result in incomplete estimation of the aggregate levels in sample.[27] Another important technique uses static and dynamic light scattering for characterization. The advantage in using this technique is quick and non-invasive estimation of aggregates although the requirements for high concentration samples and thorough clean-up procedures restrict its usage. Analytical ultracentrifugation (AUC) is another technique that measures the movement of proteins/aggregates under centrifugal forces which is monitored using various optical methods. Based on their relative movement, the nature of these aggregates can be studied and this technique is useful with reversible aggregates.[28] Field flow fractionation (FFF) is another such technique employing semipermeable membranes that can be used to fractionate monomer protein from aggregates.[26] Apart from this, some of the most common methods for determining aggregates involve using gel electrophoresis and turbidity measurement systems.

As mentioned earlier, aggregates present in antibody are unwanted products and need to be removed so that the monomer product can be used for injection in patients. Different processing methods are available for handling antibody aggregates. Some of the earliest known techniques involved pasteurization of the aggregate containing sample to reduce the aggregates by breaking the interactions holding them together.[29,30] Use of chemical denaturants to solubilize the non-native aggregates and then obtaining the monomer protein chains by controlled refolding is also an attractive option to reduce impurities. However, the refolding yield is never 100% and results in some antibody forming aggregates.[31] High pressure systems have shown 100% conversion of aggregates into monomer proteins[32–34] and although these processes are successful in obtaining the protein in its native form by solubilizing the aggregates, they are not suitable for large scale processing.

Final stage in purification process is polishing of the antibody molecules which helps to remove trace impurities and any aggregate components resulting in a product that is safe for developing formulations. Column chromatographic techniques have shown

good results in separating antibody components from mixture.[35,36] Traditional purification techniques were based on using column chromatography principle such as ion-exchange chromatography, hydrophobic interaction chromatography and hydroxyapatite chromatography.[37] Products obtained after processing were of high purity with minor differences in yield among different techniques. However drawbacks associated with these column techniques arising due to longer process times and a separation principle governed by diffusion mechanism affected processing of large feed volumes. Further using protein-A affinity chromatography is considered as the most important development in purifying antibodies. Using this method mAbs are captured by specific interactions between antibody and protein-A media and allows removal of impurities from target antibody.[20] However this process too generates impurities in the form of antibody aggregates and protein-A-antibody complexes.[38,39]

With the development of membrane chromatography, most drawbacks in column processes were ruled out. Membrane chromatography works on the principle of convective transport of matter instead of diffusion as in column chromatography. Use of small porous particles in column chromatography as the interaction media causes protein molecules to diffuse into dead-end pores for interaction while in membrane set-up the interaction takes place as the liquid flows through pores. Convective transport helps in faster processing of feed and pressure drop is reduced as thin membranes are used.[40] Figure.1.4 shows the comparison of mass transport between a packed bed and membrane. Reports also mention the use of affinity chemistry modifying membrane surfaces for capture of mAbs[41] in large scale purification process.

The advent of hydrophobic interaction was a critical development for use in protein separation. Other techniques like ion-exchange and affinity interaction makes use of physical characteristics of protein molecules for their processing while hydrophobic interaction chromatography uses the intrinsic property of hydrophobicity for processing.

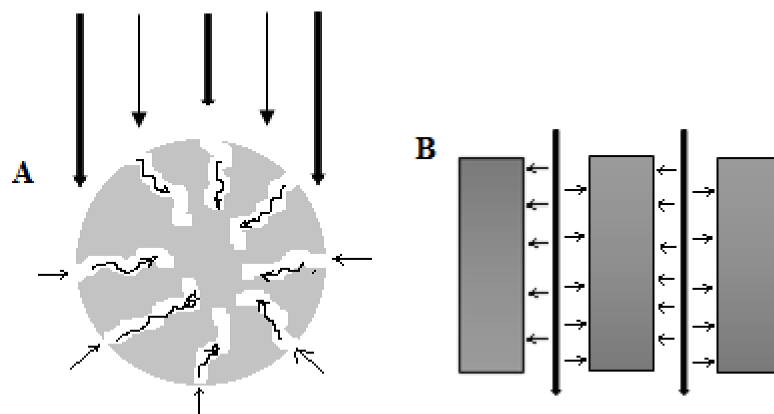

Figure.1.4. Comparison of mass transport in (A) Resin bed and (B) Membrane pore. Thick arrow-convection; Thin arrow-diffusion; Curly arrow-pore diffusion.

For the first time it was shown that monoclonal antibodies are hydrophobic in nature and bind to the membrane surfaces[42] which can then be purified from feed containing other serum proteins. The main factor contributing to this separation was the hydrophobicity difference between BSA and antibody molecules. Based on this information we use membrane chromatography to further study the binding characteristics of antibody molecules containing aggregates and ways to fractionate such aggregate species from monomer antibody molecules.

Major portion of the work discussed in this thesis deals with removal of aggregates from monomer antibody molecules. Studies with antibody aggregates is also performed using thermal methods to disaggregate the monoclonal antibody aggregates present in protein solutions. Information about the work is as provided below:

- Chapter 2 will provide information on preliminary experiments to study the binding characteristics of monoclonal antibodies in presence of anti-chaotropic salt. Aggregates present are fractionated from monomer antibody molecules using gradient elution technique. This information is then used to develop a polishing step for fractionating aggregate containing sample with the objective to obtain monomer in flow-through and dimers in the eluate.

- In Chapter 3 the main objective is to use HIMC for capturing chimeric heavy-chain mAb EG2 (cHCMAb-hFc EG2) from cell culture supernatant and purifying it from impurities. Preliminary experiments are performed with partially pt-A purified EG2 sample to study its binding. Maintaining similar experimental conditions, a scaled-up purification is shown that captures mAb EG2 from cell culture supernatant thus avoiding pt-A purification step. Antibody purity is further verified using Native PAGE analysis.
- Finally chapter 4 will focus on disaggregating monoclonal antibody aggregates using a novel thermal cycling technique. In this work, mAb samples containing aggregates are subjected to rapid heating and cooling in a thermal cycler. This helps in breaking the interactions between aggregate molecules and the aggregates are separated into individual units. The samples obtained after thermal cycling will be analyzed using HIMC, SEC and CD spectroscopy techniques to verify the results.

1.1 References

(1) Cohn, E.; Strong, L. E.; Hughes, W. L.; Mulford, D. J.; Ashworth, J. N.; Melin, M.; Taylor, H. Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *Journal of the American Chemical Society*, **1946**, *68*, 459-475.

(2) Bruton, O. Agammaglobulinemia. *Official Journal of the American Academy of Pediatrics*, **1952**, *9*, 722-728.

(3) Kohler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **1975**, *256*, 495-497.

(4) Kung, P.; Goldstein, G.; Reinherz, E.; Schlossman, S. Monoclonal antibodies defining distinctive human T cell surface antigens. *Science*, **1979**, *206*, 347-349.

(5) Hale, G.; Bright, S.; Chumbley, G.; Hoang, T.; Metcalf, D.; Munro, A.; Waldmann, H. Removal of T cells from bone marrow for transplantation: A monoclonal antilymphocyte antibody that fixes human complement. *Blood*, **1983**, *62*, 873-882.

(6) Dyer, M. J.; Hale, G.; Hayhoe, F. G.; Waldmann, H. Effects of CAMPATH-1 antibodies in-vivo in patients with lymphoid malignancies: Influence of antibody isotype. *Blood*, **1989**, *73*, 1431-1439.

(7) Morrison, S. L.; Johnson, M. J.; Herzenberg, L. A; Oi, V. T. Chimeric human antibody molecules: Mouse antigen-binding domains with human constant region domains. *Proceedings of the National Academy of Sciences*, **1984**, *81*, 6851-6855.

(8) Riechmann, L.; Clark, M.; Waldmann, H.; Winter, G. Reshaping human antibodies for therapy. *Nature*, **1988**, *332*, 323-327.

(9) Boulianne, G.; Hozumi, N.; Shulman, M. Production of functional chimaeric mouse/human antibody. *Nature*, **1984**, *312*, 643-646.

(10) Osterborg, A; Fassas, A. S.; Anagnostopoulos, A; Dyer, M. J.; Catovsky, D.; Mellstedt, H. Humanized CD52 monoclonal antibody Campath-1H as first-line treatment in chronic lymphocytic leukaemia. *British Journal of Haematology*, **1996**, *93*, 151-153.

(11) Sgro, C. Side-effects of a monoclonal antibody, muromonab CD3/orthoclone OKT3: Bibliographic review. *Toxicology*, **1995**, *105*, 23-29.

(12) Hwang, W. Y. K.; Foote, J. Immunogenicity of engineered antibodies. *Methods*, **2005**, *36*, 3-10.

(13) Shukla, A.; Thömmes, J. Recent advances in large-scale production of monoclonal antibodies and related proteins. *Trends in Biotechnology*, **2010**, *28*, 253-261.

(14) Durocher, Y.; Butler, M. Expression systems for therapeutic glycoprotein production. *Current Opinion in Biotechnology*, **2009**, *20*, 700-707.

(15) Jefferis, R. The glycosylation of antibody molecules: functional significance. *Glycoconjugate Journal*, **1993**, *10*, 358-361.

(16) Li, H.; D'Anjou, M. Pharmacological significance of glycosylation in therapeutic proteins. *Current Opinion in Biotechnology*, **2009**, *20*, 678-684.

(17) Schellekens, H. Factors influencing the immunogenicity of therapeutic proteins. *Nephrology, Dialysis, Transplantation*, **2005**, *20 Suppl 6*, vi3-vi9.

(18) De Groot, A. S; Scott, D. Immunogenicity of protein therapeutics. *Trends in Imunology*, **2007**, *28*, 482-490.

(19) Stefani, M.; Dobson, C. Protein aggregation and aggregate toxicity: New insights into protein folding, misfolding diseases and biological evolution. *Journal of Molecular Medicine*, **2003**, *81*, 678-699.

(20) Ey, P.; Prowse, S.; Jenkin, C. Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. *Immunochemistry*, **1978**, *15*, 429-436.

(21) Arakawa, T.; Philo, J.; Tsumoto, K.; Yumioka, R.; Ejima, D. Elution of antibodies from a protein-A column by aqueous arginine solutions. *Protein Expression and Purification*, **2004**, *36*, 244-248.

(22) Wang, W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *International Journal of Pharmaceutics*, **1999**, *185*, 129-188.

(23) Wetzel, R. Mutations and off-pathway aggregation of proteins. *Trends in Biotechnology*, **1994**, *12*, 193-198.

(24) Chi, E. Y.; Krishnan, S.; Randolph, T. W.; Carpenter, J. F. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. *Pharmaceutical Research*, **2003**, *20*, 1325-1336.

(25) Philo, J. S.; Arakawa, T. Mechanisms of protein aggregation. *Current Pharmaceutical Biotechnology*, **2009**, *10*, 348-351.

(26) Gabrielson, J.; Brader, M.; Pekar, A.; Mathis, K.; Winter, G.; Carpenter, J.; Randolph, T. Quantitation of aggregate levels in a recombinant humanized monoclonal antibody formulation by size-exclusion chromatography, asymmetrical flow field flow fractionation, and sedimentation velocity. *Journal of Pharmaceutical Sciences*, **2007**, *96*, 268-279.

(27) Arakawa, T.; Philo, J.; Ejima, D.; Tsumoto, K.; Arisaka, F. Aggregation analysis of therapeutic proteins, Part1. *Bioprocess Technical*, **2006**, *4*, 32-42.

(28) Laue, T.; Stafford, W. Modern applications of analytical ultracentrifugation. *Annual Review of Biophysics and Biomolecular Structure*, **1999**, *28*, 75-100.

(29) Gonzhleza, M.; Gerardo, D. Thermal stability of human immunoglobulins with sorbitol. *Vox Sanguinis*, **1995**, *68*, 1-4.

(30) Uemura, Y.; Uriyu, K.; Hirao, Y.; Takechi, K.; Ishikawa, H.; Nakajima, T.; Kagitani, Y.; Yokoyama, K.; Funakoshi, S.; Nishida, M. Inactivation and elimination of viruses during the fractionation of an intravenous immunoglobulin preparation: liquid heat treatment and polyethylene glycol fractionation. *Vox Sanguinis*, **1989**, *56*, 155-161.

(31) Clark, E. Refolding of recombinant proteins. *Current opinion in Biotechnology* **1998**, *9*, 157-163.

(32) St John, R. J.; Carpenter, J. F.; Randolph, T. W. High pressure fosters protein refolding from aggregates at high concentrations. *Proceedings of the National Academy of Science*, **1999**, *96*, 13029-13033.

(33) Gorovits, B. M.; Horowitz, P. M. High hydrostatic pressure can reverse aggregation of protein folding intermediates and facilitate acquisition of native structure. *Biochemistry*, **1998**, *37*, 6132-6135.

(34) St John, R. J.; Carpenter, J. F.; Balny, C.; Randolph, T. W. High pressure refolding of recombinant human growth hormone from insoluble aggregates. Structural transformations, kinetic barriers, and energetics. *The Journal of Biological Chemistry*, **2001**, *276*, 46856-46863.

(35) Kamp, R.; Bosserhoff, A.; Kamp, D.; Wittmann-Liebold, B. Application of high-performance liquid chromatographic techniques to the separation of ribosomal proteins of different organisms. *Journal of Chromatography A*, **1984**, *317*, 181-192.

(36) Clezardin, P.; McGregor, J.; Manach, M.; Boukerche, H.; Dechavanne, M. One-step procedure for the rapid isolation of mouse monoclonal antibodies and their antigen binding fragments by fast protein liquid chromatography on a mono Q anion-exchange column. *Journal of Chromatography A*, **1985**, *319*, 67-77.

(37) Pavlu, B.; Johansson, U.; Nyhlen, C.; Wichman, A. Rapid purification of monoclonal antibodies by high-performance liquid chromatography. *Journal of Chromatography A*, **1986**, *359*, 449-460.

(38) Giovannini, R.; Freitag, R.; Tennikova, T. B. High-performance membrane chromatography of supercoiled plasmid DNA. *Analytical Chemistry*, **1998**, *70*, 3348-3354.

(39) Carter-Franklin, J.; Victa, C.; McDonald, P.; Fahrner, R. Fragments of protein A eluted during protein A affinity chromatography. *Journal of Chromatography A*, **2007**, *1163*, 105–111.

(40) Thommes, J.; Kula, M-R. Membrane chromatography-An integrative concept in the downstream processing of products. *Biotechnology Progress*, **1995**, *11*, 357-367.

(41) Brandt, S.; Goffe, R.; Kessler, S.; O'Connor, J.; Zale, S. Membrane-based affinity technology for commercial scale purifications. *Nature Biotechnology*, **1988**, *6*, 779 -782.

(42) Ghosh, R. Separation of proteins using hydrophobic interaction membrane chromatography. *Journal of Chromatography. A*, **2001**, *923*, 59-64.

2. Removal of monoclonal antibody aggregates using hydrophobic interaction membrane chromatography

Abstract: Current monoclonal antibody (mAb) production using cell culture technique allows large scale production from a single batch operation. Higher expression levels as well as heterogeneity of culture environment drives aggregate formation. For using these mAb products as therapeutics, aggregates should be removed since their presence causes immunogenic reactions. Column based size exclusion chromatography (SEC) technique has shown good results in identifying different components present in an antibody product. But longer processing time and difficulties in scale-up of the process prevents large scale application of this technique. Hydrophobic interaction membrane chromatography (HIMC) has been effective in fractionating protein molecules based on their hydrophobicity difference. HIMC process is easily scalable and the processing time can be reduced by operating at higher flow rates because of lower pressure drop during operation. mAb molecules are hydrophobic in nature and the hydrophobicity change on aggregate formation can be used for fractionating monomer from aggregates. In this work we use HIMC to fractionate antibody sample for removing aggregate components from monomer. Preliminary fractionation experiment is performed using PVDF membranes and the result is compared with Sartobind® membrane adsorber. Results from size exclusion chromatography (SEC) are used to confirm the presence of aggregate components in antibody sample.

2.1 Introduction

Hydrophobicity as a property for protein binding was used in 1970s to study binding of proteins, exhibiting hydrophobic character, on agarose gels modified by hydrophobic alkyl substituents.[1] Important finding from this study was that binding to the

substrate increased with hydrophobicity of substituents and salt concentration. Developments into hydrophobic interaction technique led to the synthesis of customized column packings to modulate hydrophobic interactions suitable for high performance processes.[2] This further helped in using hydrophobic packings to separate proteins from a mixture based on their hydrophobicity differences.[3,4] Not only does the ligand density affect hydrophobic binding of proteins but minute changes in operating conditions like temperature and addition of chemicals such as urea also contribute towards separation efficiency.[5,6] Other factors like type of solute, column capacity and mobile phase were also shown responsible in affecting the separation process.[7] While developments in modifying the packing materials by use of substituent groups was still in progress, one study showed that columns with very high ligand densities were in reality not beneficial for analyzing some proteins as they caused proteins to denature while passing through column packings[8] due to use of stronger solvent conditions for elution.

By using hydrophobic substrate and combining it with gel filtration allowed simultaneous fractionation of antibodies from impurities present in egg yolk.[9] However first reports on fractionation of proteins proved that hydrophobic differences exist and can also be used to fractionate serum proteins.[6] Results from this work proved that serum albumin is less hydrophobic and has lower molecular weight than gamma globulins and this was achieved by use of silica packing material. This further made possible the use of different chemistries with silica-based packing materials involving alkyl substituents for separation of different proteins from mixture and the results obtained show good performance in terms of separation characteristics.[10]

Most important is the mechanism that causes hydrophobic interactions to occur. This mechanism can be divided into two main processes:

- anti-chaotropic salts added to water act as diluents as they reduce the concentration of water molecules present in solution. In addition these added salts also cause changes in protein conformation such that sufficient hydrophobic regions are exposed during binding with hydrophobic substrates, and

- water molecules act as displacing agents wherein they displace salts around the protein molecules. This disrupts hydrophobic interactions between protein molecules and membrane surfaces thus effecting elution process.[11,12]

Membrane chromatography for the first time showed separation of proteins from a mixture[13] and their use allowed modification of surface with ligands that can alter surface hydrophobicity in presence of salts. Not only was hydrophobic interaction possible but also ion-exchange and affinity based separations can be carried out by appropriate surface modifications.[14] Information on fractionating protein molecules from mixture was available but no such information existed for fractionating serum proteins using membrane chromatography. It was until when Ghosh for the first time showed that membrane chromatography can be used to separate serum proteins and immunoglobulins from a mixture.[15] Later work also showed effectiveness of using membrane chromatography to separate human plasma proteins HSA and hIgG.[16]

mAbs form stable dimeric aggregates and in this work we make use of HIMC technique to fractionate antibody samples into monomer and aggregate species. Initial testing with SEC was performed to identify the components in each mAb sample. The results obtained were compared with HIMC chromatograms. We also test this HIMC technique to fractionate mAb samples belonging to IgG1 sub-class obtained from different sources and results show some level of aggregation in all these samples. Initial fractionation experiments were performed with low binding PVDF membranes. Parameters like salt concentration and flow rate were chosen to design experiments with Sartobind® phenyl membrane adsorbers. Due to higher binding, large quantity of antibody can be loaded on these adsorbers. Experiments with sartobind adsorbers were optimized to meet the polishing requirements wherein monomer antibody molecules were obtained in flow-through and dimer molecules in eluate fraction.

2.2 Materials and Methods

2.2.1 Materials

Humanized purified mAb hIgG1-CD4 (Batch 12, 23rd March 1999) “Monomer” and hIgG1-CD4 aggregate containing mAb sample “Dimers” (Batch D6, 22nd July 1993) was kindly donated by the Therapeutic Antibody Centre, Oxford, United Kingdom. For simplicity these mAbs will be named as “Monomer” and “Dimer”. IgG1 mAbs obtained from National Research Council (NRC) Canada were cultured using HEK and CHO cell lines. Henceforth, these antibodies will be labelled as HEK-IgG and CHO-IgG. Sodium phosphate monobasic (S0751), sodium phosphate dibasic (S0876), ammonium sulphate (A4418) were purchased from Sigma-Aldrich, St.Louis, MO, USA. Sodium chloride (SOD 002.205) was purchased from Bioshop, Burlington, ON, Canada. Purified water (18.2 MΩcm) was obtained using DiamondTM NANOpure (Barnstead, Dubuque, IA, USA) water purification unit and used for preparing buffer and test solutions.

2.2.2 Size exclusion chromatography

Antibody samples were first subjected to buffer exchange with 20mM sodium phosphate buffer at pH 7.0 and concentrated using Amicon Ultracel-50 membrane filters. After concentrating these samples were analyzed on Varian Star HPLC system (Varian, Palo Alto, CA) using phosphate buffered saline (PBS) pH 7.0 as the mobile phase at flow rate of 0.3 mL/min. PBS contained 20mM sodium phosphate buffer with 250mM sodium chloride. All the antibody samples were analyzed using WTC-030S5 Wyatt Column purchased from Wyatt Technology Corporation, Santa Barbara, CA, USA.

2.2.3 Hydrophobic interaction membrane chromatography

For HIMC analysis hydrophilized PVDF (GVWP14250) 0.22µm pore size membranes purchased from Millipore, Billerica, MA, USA were used as hydrophobic interaction media. The membranes housed in a custom-designed module were integrated

with an AKTA prime plus liquid chromatography system (GE Healthcare Biosciences, QC, Canada). Figure 2.1 shows the set-up for the HIMC experiment. 0.3 mg/mL of "Monomer" sample was first analyzed. The feed sample was prepared with 1.5M ammonium sulfate salt (in 20mM sodium phosphate containing buffer, pH 7.0) to allow the antibody molecules to bind to the membrane surface. Membrane module used consisted of 5 membrane discs and the experiment was carried out at a flow rate of 2 mL/min. Bound antibody molecules were eluted by lowering the salt concentration. Other IgG mAb samples as well as "Dimer" were also analyzed under similar binding and eluting conditions using 10 membrane discs in the module and 4 mL/min flow rate.

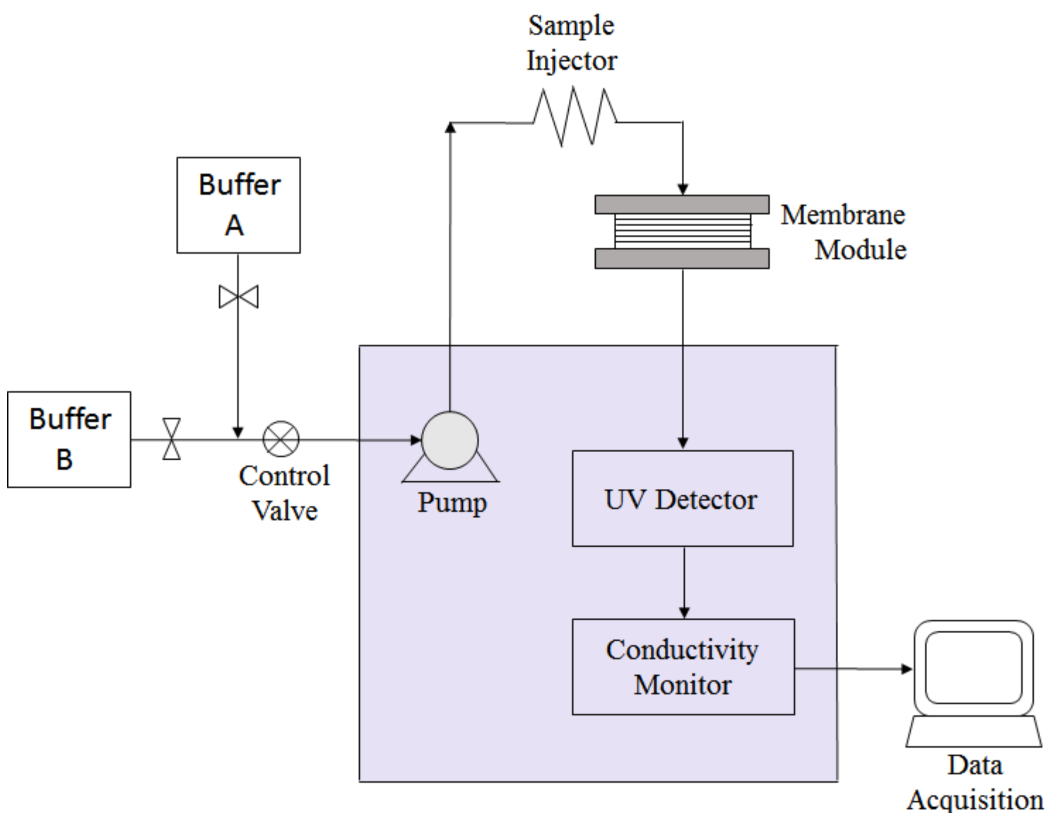

Figure 2.1 Experimental set-up for HIMC analysis. Shaded region represents the components of an AKTA system.

High binding Sartobind (Phenyl Nano 3 mL) membrane adsorber was also tested for fractionation of antibody molecules. This membrane adsorber was obtained from Sartorius Stedim Biotech, Goettingen, Germany. In the initial experiments with Sartobind adsorber "Dimer" was analyzed at flow rate of 4 mL/min. Higher binding capacity allowed use of low salt concentration in feed. These adsorbers are functionalized using phenyl ligands and therefore the membrane pore structure is not affected in presence/absence of salts, unlike with PVDF membranes which are coated with polymers, there was no significant change in system pressure and this allowed use of very high flow rates. So further fractionation experiments were performed at 10 mL/min flow rate to verify the effectiveness of these adsorbers in removing aggregates.

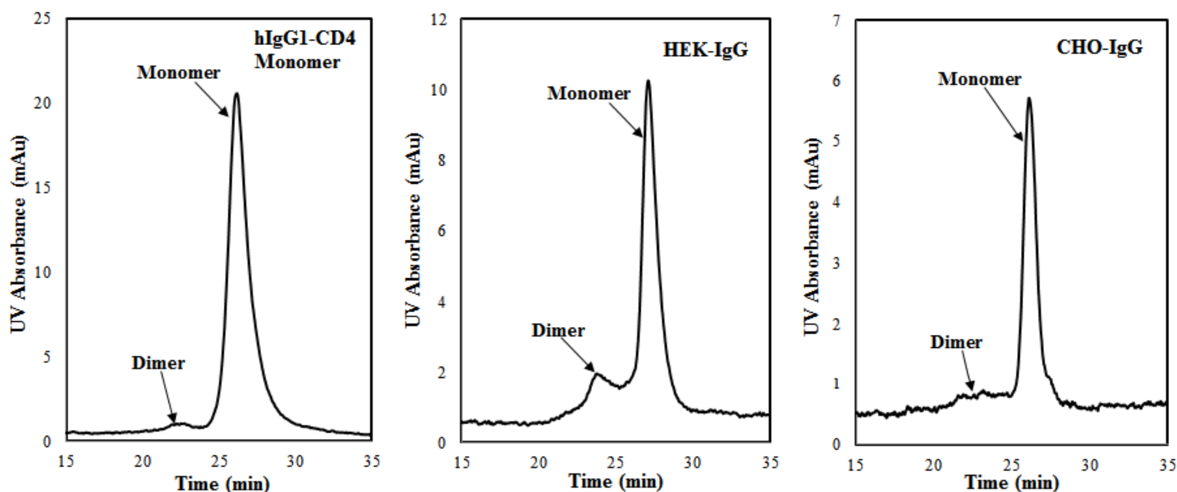

Figure 2.2 SEC chromatogram of mAb samples showing monomer and aggregates (dimer).

Hydrophilized surface of PVDF membranes are stimuli responsive in nature[17] and turn hydrophobic in presence of salts. Anti-chaotropic salt causes the grafted polymer chains to expand and results in pore blockage thus making it difficult for proteins and liquid to flow through membrane discs. These polymer chains collapse when no salt is present thus allowing easy access to membrane pores. In this situation, liquids can flow through membrane pores without much resistance. If large number of membrane discs are

used, in presence of salts high pressure is created in the system which is not suitable for use with faster flow rates. At the same time, salt is essential for proteins for binding to membranes. Hence there is a trade-off between the number of membrane discs in module and flow rate used during fractionation using PVDF membranes. When salt free buffer passes through the membrane discs, hydrophobic interactions are disrupted and membrane structure collapses allowing released proteins to elute out of the membranes. In contrast to this stimuli-responsive behaviour, sartobind adsorbers have functionalized surfaces with phenyl ligands that bind to proteins in presence of anti-chaotropic salts without affecting the porous membrane structure. As a result of this, efficient binding conditions are maintained as well as higher flow rates can be used.

2.3 Results and Discussion

SEC analysis with "Monomer", HEK-IgG and CHO-IgG was initially performed to identify the different components present in these mAb samples. The chromatograms obtained are shown in Figure.2.2. Based on the principle of size exclusion, molecules with largest hydrodynamic size move faster due to lack of diffusion in column packing while smallest particles elute in the end due to higher diffusion caused by small hydrodynamic size. This creates distribution of particles as they travel along the column length.

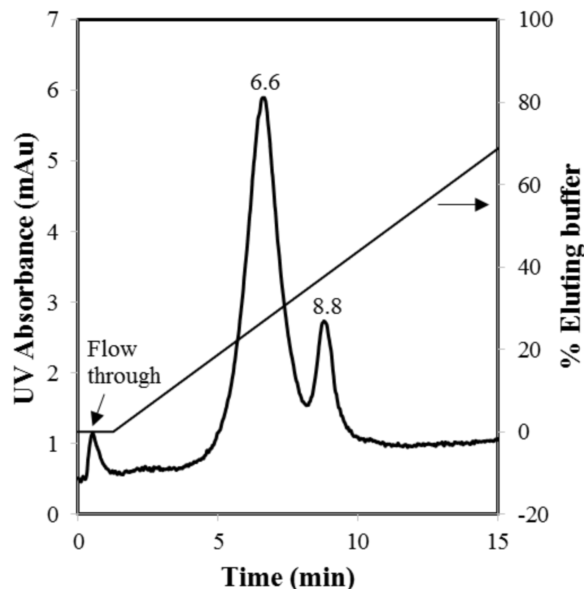

Figure 2.3 HIMC chromatogram for "Monomer" sample using PVDF membranes (5-membrane discs). 2 mL/min flow rate, 40 mL elution gradient.

The final outcome is separation of particles according to their relative molecular size. Dimer molecules exceed the hydrodynamic size when compared with monomer present in same sample so the chromatogram obtained shows a smaller peak corresponding to the dimer component and the principal peak represents monomer molecules.

Hydrophobic binding of antibodies have been studied to determine the interacting regions of antibody molecule. It is known that Fab region is more hydrophobic that Fc region[18,19] and therefore the hydrophobic interactions should predominantly involve Fab regions. In one such study it was found that the hydrophobic interaction occurs through the region between hinge and C_H2 domains.[20,21] Earlier work on studying the binding of mAbs to membrane surfaces have shown that at 1.5M anti-chaotropic salt concentration both the monomer and aggregates are strongly bound to membrane surface.[22] Based on this information the HIMC analysis for "Monomer" sample was performed and is shown in Figure.2.3.

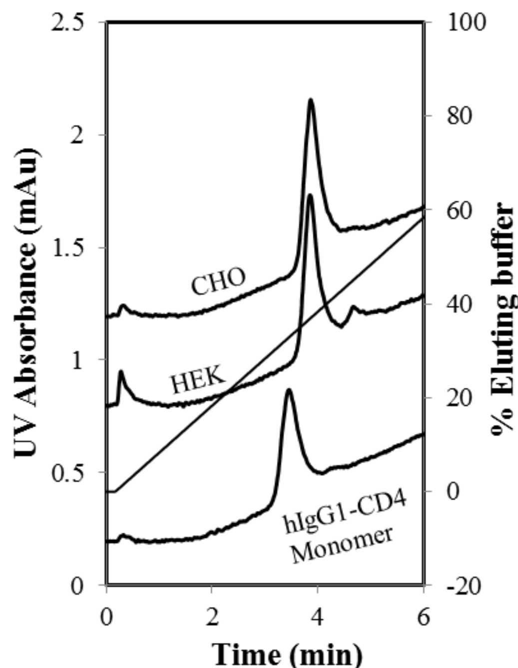

Figure 2.4 HIMC chromatogram for "Monomer", HEK-IgG and CHO-IgG samples using PVDF membranes (10-membrane discs). 4 mL/min flow rate, 40 mL elution gradient.

The chromatogram indicates presence of principal monomer peak at time 6.6 min and a small dimer peak at 8.8 min. After sample injection, binding buffer was allowed to flow through membranes for efficient binding of antibody molecules. Elution using linear gradient was carried out by applying salt free buffer 0% to 100% over 40 mL volume. Presence of ammonium sulfate in binding buffer establishes hydrophobic interaction and once the salt free buffer is applied, this interaction weakens and antibody molecules elute out in the order of increasing hydrophobicity.

When a gradient in the form of salt free buffer is applied, the environment surrounding bound protein molecules changes and is occupied by molecules from salt free buffer. This dilutes the effect of ammonium sulfate and weakens hydrophobic binding. As a result protein molecules are released immediately from the bound state once interaction is completely broken [11,12]. It is well known that monomer antibody molecules form dimer species and these are stable even in dilute solution conditions [23–25]. Aggregates produced

from monomer molecules show higher hydrophobicity than monomer. So when negative salt gradient is applied interaction between membrane surface and monomer molecules is affected thus releasing monomer molecules first followed by dimer species. Therefore on elution of the antibody sample two different eluate peaks i.e. “monomer” and “dimer” are seen in chromatogram. To further study fractionation of mAbs, experiments were performed with HEK-IgG and CHO-IgG and the results compared with “Monomer”. Figure 2.4 shows the result for HIMC analysis of these mAbs. Profile for all three mAbs is similar consisting of parent monomer peak and smaller dimer peak. These mAbs belong to same sub-class of antibody molecules and form this chromatogram it can be concluded that they follow similar mechanism of aggregate formation. The elution volume for “Monomer” sample is lower than HEK- and CHO-IgG samples indicating that these mAbs bind more strongly to membrane under similar conditions and are more hydrophobic than “Monomer”.

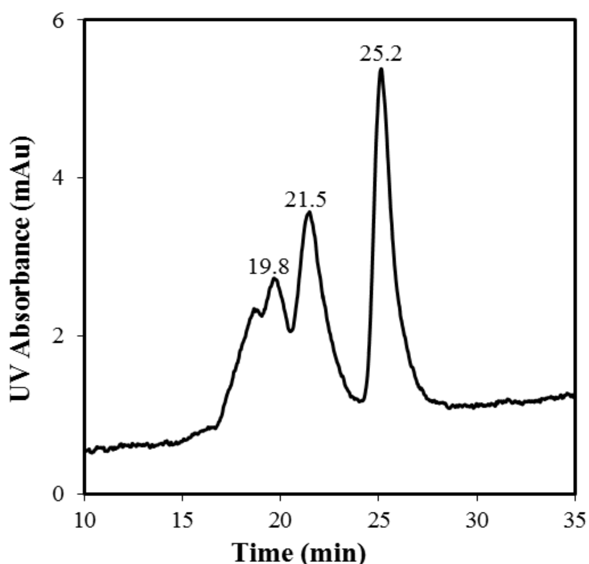

Figure 2.5 SEC chromatogram for “Dimer” containing Monomer, Dimer and Higher aggregate components. Flow rate used was 0.3 mL/min.

Prior to analysis of "Dimer" sample using HIMC technique, SEC analysis was performed. The chromatogram for "Dimer" sample in Figure.2.5 shows multiple peaks indicating different components present in sample. Monomer antibody molecules aggregate to form dimers and these dimer molecules in presence of monomer can lead to formation of higher aggregates. So the eluate peak at time 19.8 min represents higher aggregates, peak at time 21.5 min consists of dimer molecules and monomer molecules elute out at 25.2 min. For HIMC experiments, the sample was first analyzed using PVDF membranes. Sample analysis was performed at 4ml/min using a 40 mL gradient elution. Result obtained is shown in Figure.2.6. Clearly the components seen from SEC technique are also identifiable in HIMC results. Thus membrane chromatography has the potential to fractionate not only monomer and dimer molecules but the higher aggregates present in sample can be separated too.

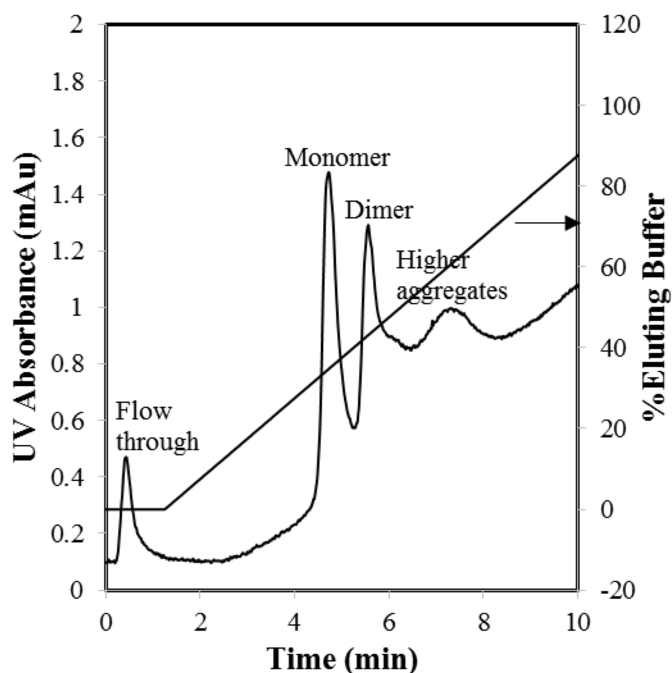

Figure 2.6 HIMC chromatogram for "Dimer" sample using PVDF membranes. 10-membrane discs, 4 mL/min flow rate, 40 mL elution gradient.

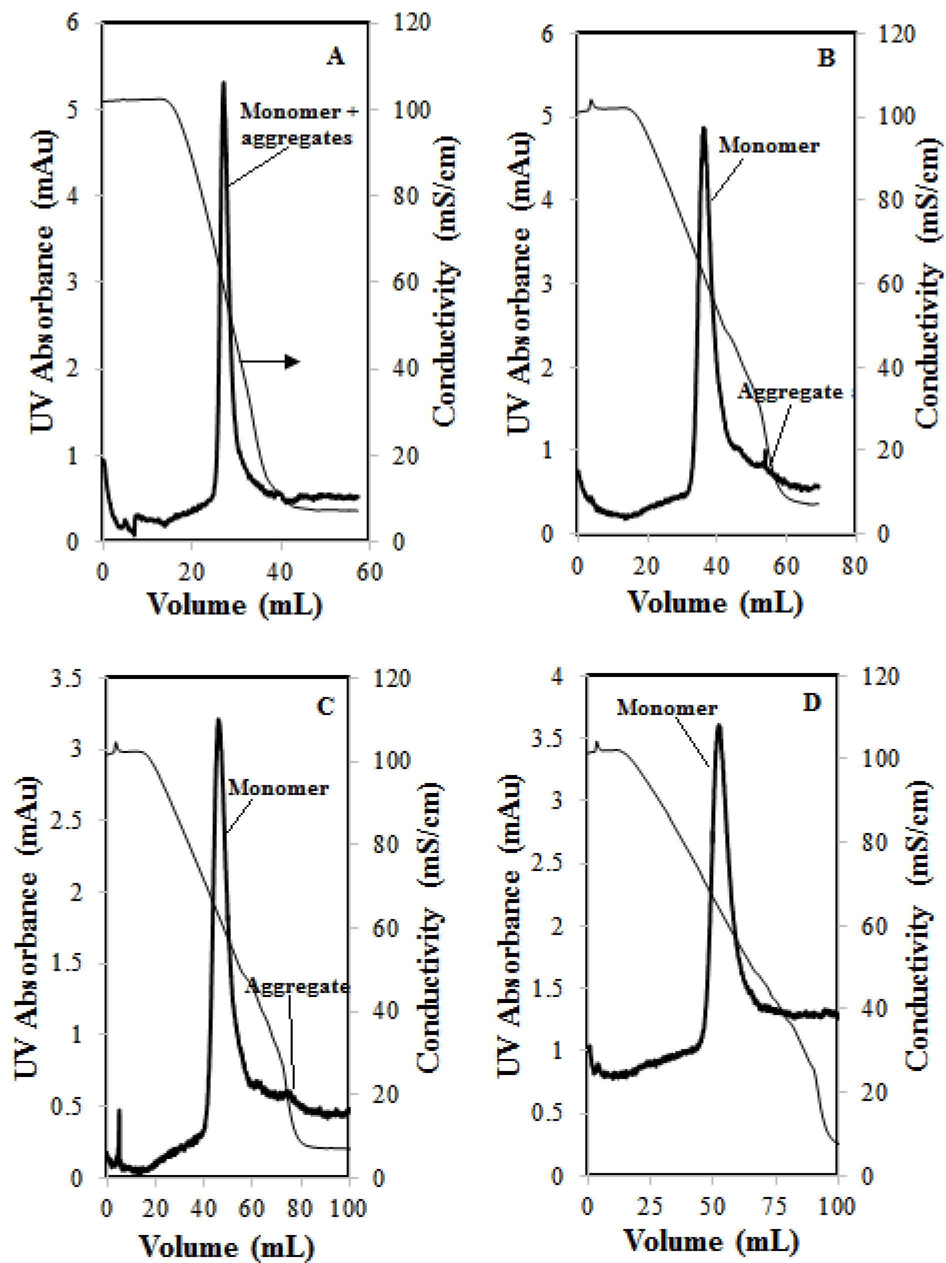

Figure 2.7. HIMC chromatogram for "Monomer" using Sartobind adsorber at different gradients. A-20 mL; B-40 mL; C-60 mL; D-80 mL. Flow rate used was 4 mL/min.

As mentioned earlier, experiments involving PVDF membranes restricts the use of high protein concentration because of low binding capacity while sartobind adsorbers have no

such limitations. Sartobind adsorbers are commercial products developed for polishing and capture of monoclonal antibodies. Specifications of the Nano 3 mL adsorber recommends use of flow rates as high as 15 mL/min. To test this module, experiments were performed initially with 100% "Monomer", "Monomer" spiked with "Dimer" and 100% "Dimer" samples at 4ml/min flow rate to determine the efficiency of separation between monomer and aggregates. Since these adsorbers have high binding capacity 0.75M salt concentration was used. The result obtained is shown in Figure.2.7. The binding strength at various gradient lengths was evaluated in this experiment. Initially at 20ml gradient elution, the monomer peak elutes after the gradient is completed indicating strong binding between antibody and membrane.

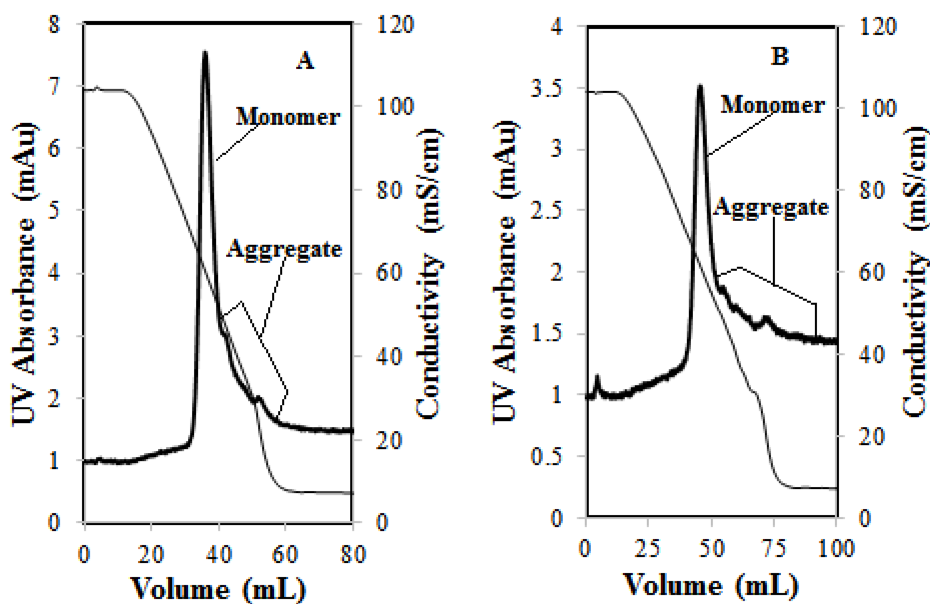

Figure 2.8. HIMC chromatogram for "Monomer" spiked with "Dimer" using Sartobind adsorber at different gradients. A-40 mL; B-60 mL. Flow rate used was 4 mL/min.

The peak obtained is sharp with no separation between monomer and dimer peaks. From the conductivity values it can be concluded that the eluate peak is seen when the buffer conductivity drops below 75 mS/cm until 20 mS/cm. In the subsequent runs, gradient

length was increased to obtain better resolution between monomer and dimer peaks. Increasing the gradient length to 40, 60 and 80 mL resulted in better resolution between monomer and dimer upto 60mL gradient length. However at 80 mL length, the dimer peak is not clearly seen. This is because the adsorber has dead volume of 3 mL which dilutes the sample after elution. Therefore, at steep gradients less dilution occurs before the effective salt concentration for elution is reached resulting in sharp peaks with better resolution while at longer gradient there is considerable dilution to detect the peak.

Since the level of aggregates in “Monomer” sample was low it resulted in poor resolution between monomer and dimer peaks. So to test the separation between monomer and dimer with better resolution, “Monomer” was spiked with 40% “Dimer”. During “Monomer” analysis the separation between monomer and dimer can be clearly seen only with 40 mL and 60 mL gradient and therefore the analysis for “Monomer” spiked with “Dimer” was performed only for 40 mL and 60 mL elution gradients. The result obtained is shown in Figure.2.8.

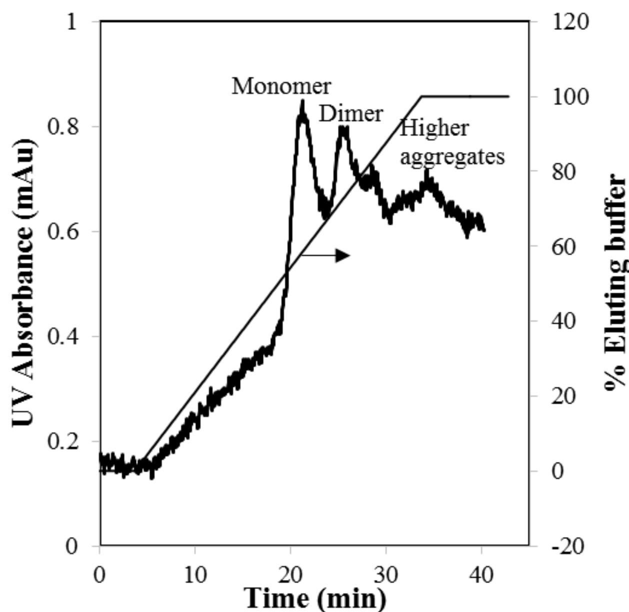

Figure 2.9. HIMC chromatogram for “Dimer” sample using Sartobind adsorber. Experiment was performed using 4 mL/min flow rate and 60 mL elution gradient.

Comparing the result for 40 mL and 60 mL gradient length, the separation between monomer and aggregate components is better with 60 mL with peaks clearly seen for dimer and higher aggregates which is slightly better with better than with 40 mL gradient. Therefore the experiment with 100% "Dimer" was performed at 60 mL gradient and result obtained is shown in Figure.2.9.

The result shows 2 distinct peaks and a third peak not clearly resolved is merged with dimer peak. When compared with the result from PVDF experiments sartobind chromatograms show broad peaks with poor resolution. This is because of dead volume in the module which dilutes sample after elution. The other contributing factor for poor peak resolution that the hydrophobic binding mechanism with sartobind adsorbers is different from PVDF membranes i.e. there is no changes to the membrane pore structure in presence or absence of anti-chaotropic salts. From the results above it is clear that with Sartobind adsorbers it is possible to fractionate aggregate components from monomer antibody molecules.

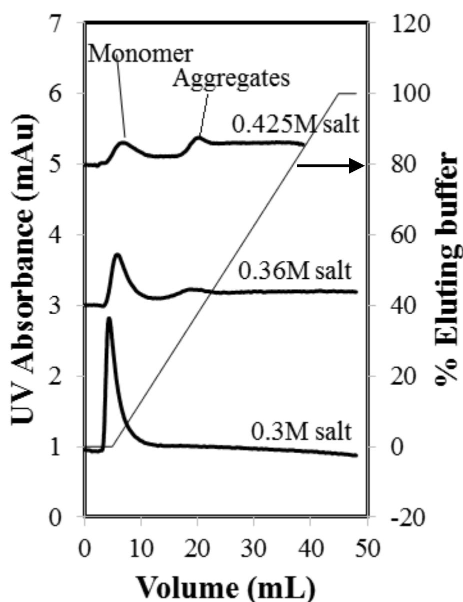

Figure 2.10. HIMC chromatogram for "Dimer" sample using Sartobind adsorber obtained under different salt concentrations. Flow rate used was 10 mL/min with 40 mL elution gradient.

During downstream purification processes, presence of aggregates in the final product is undesirable and often additional polishing steps are included which can effectively separate monomer antibody product from aggregates. From earlier experiments it is clear that Sartobind adsorbers can be tuned to show differential binding capacity towards monomer and aggregate components by controlling the salt gradients. In order to test the performance of Sartobind adsorber for polishing application, experiments were performed with an aim to obtain monomer in the flow through and aggregates in eluate fraction using “Dimer” as the feed. Since the adsorber allows use of higher liquid flow rates, analysis was performed at 10 mL/min for faster analysis and using 40mL elution gradient. The earlier experiments performed at 0.75M salt concentration bound both the monomer and aggregate components, so for this set of experiments the concentration was reduced considerably. Binding conditions were tested starting with 0.3M salt concentration and gradually increased thereafter until 0.425M. Result obtained is shown in Figure.2.10. Initially at 0.3M salt concentration only a single flow-through peak is seen indicating that nothing was bound to the membrane. So for binding to occur the salt concentration was increased and at 0.36M two different peaks are clearly seen corresponding to monomer and aggregates. Since the two peaks are not properly separated from each other, salt concentration was further increased to 0.425M. Under this condition both the monomer and aggregate peaks are separated from each other with monomer in the flow-through and aggregates in the eluate fraction.

2.4 Conclusion

Hydrophilized PVDF membranes on account of stimuli-responsive behaviour provide good results in terms of peak resolution and peak separation. The number of discs used in the module doesn’t affect the separation of individual components. So these membranes can be useful in preparative separation studies. With Sartobind membrane adsorbers, both peak resolution and peak separation is poor and even with lower flow

rates and considerable peak spreading is observed. Since these adsorbers can bind proteins differentially depending on their hydrophobicity even at higher liquid flow rates under low salt concentration conditions, they can be used in large scale processing where aggregate removal is of prime concern. Results from SEC and HIMC analysis suggests presence of aggregates in antibody samples and their successful fractionation into separate peaks. HIMC chromatograms for "Monomer", HEK-IgG and CHO-IgG shows that IgG1 molecules obtained from HEK and CHO have higher hydrophobicity than "Monomer". The presence of aggregates in these samples also indicate that IgG1 molecules obtained from different sources undergo similar aggregation mechanisms.

2.5 References

(1) Rosengren, J.; Pahlman, S.; Glad, M.; Hjerten, S. Hydrophobic interaction chromatography on non-charged sepharose derivatives. *Biochimica Et Biophysica Acta*, **1975**, *412*, 51-61.

(2) Fausnaugh, J.; Gupta, P.; Regnier, F. High-performance hydrophobic interaction of proteins. *Analytical Biochemistry*, **1984**, *137*, 464-472.

(3) Kato, Y.; Kitamura, T.; Hashimoto, T. High-performance hydrophobic interaction chromatography of proteins. *Journal of Chromatography A*, **1983**, *266*, 49-54.

(4) Gooding, D.; Schmuck, M.; Gooding, K. Analysis of proteins with new, mildly hydrophobic high-performance liquid chromatography packing materials. *Journal of Chromatography A*, **1984**, *296*, 107-114.

(5) Gooding, D.; Schmuck, M.; Nowlan, M.; Gooding, K. Optimization of preparative hydrophobic interaction chromatographic purification methods. *Journal of Chromatography A*, **1986**, *359*, 331-337.

(6) Goheen, S.; Matson, R. Purification of human serum gamma globulins by hydrophobic interaction high-performance liquid chromatography. *Journal of Chromatography A*, **1985**, *326*, 235-241.

(7) Fausnaugh, J.; Regnier, F. Solute and mobile phase contributions to retention in hydrophobic interaction chromatography of proteins. *Journal of Chromatography A*, **1986**, *359*, 131-146.

(8) Ingraham, R.; Lau, S.; Taneja, A.; Hodges, R. Denaturation and the effects of temperature on hydrophobic interaction and reversed-phase high performance liquid chromatography of proteins. *Journal of Chromatography A*, **1985**, *327*, 77-92.

(9) Hassl, A.; Aspock, H. Purification of egg yolk immunoglobulins - A two-step procedure using hydrophobic interaction chromatography and gel filtration. *Journal of Immunological Methods*, **1988**, *110*, 225-228.

(10) Alpert, A. High-performance hydrophobic-interaction chromatography of proteins on a series of poly(alkyl aspartamide)-silicas. *Journal of Chromatography A*, **1986**, *359*, 85-97.

(11) Geng, X.; Guo, L.; Chang, J. Study of the retention mechanism of proteins in hydrophobic interaction chromatography. *Journal of Chromatography A*, **1990**, *507*, 1-23.

(12) Queiroz, J.; Tomaz, C.; Cabral, J. Hydrophobic interaction chromatography of proteins. *Journal of Biotechnology*, **2001**, *87*, 143-159.

(13) Tennikova, T.; Bleha, M.; Svec, F.; Almazova, T.; Belenkii, B. High-performance membrane chromatography of proteins: A novel method of protein separation. *Journal of Chromatography A*, **1991**, *555*, 97-107.

(14) Tennikova, T.; Svec, F. High-performance membrane chromatography: Highly efficient separation method for proteins in ion-exchange, hydrophobic interaction and reversed-phase modes. *Journal of Chromatography A*, **1993**, *646*, 279-288.

(15) Ghosh, R. Separation of proteins using hydrophobic interaction membrane chromatography. *Journal of Chromatography A*, **2001**, *923*, 59-64.

(16) Ghosh, R. Fractionation of human plasma proteins by hydrophobic interaction membrane chromatography. *Journal of Membrane Science*, **2005**, *260*, 112-118.

(17) Wandera, D.; Wickramasinghe, S.; Husson, S. Stimuli-responsive membranes. *Journal of Membrane Science*, **2010**, *357*, 6-35.

(18) Vermeer, A.; Giacomelli, C.; Norde, W. Adsorption of IgG onto hydrophobic teflon. Differences between the F(ab) and F(c) domains. *Biochimica Et Biophysica Acta*, **2001**, *1526*, 61-69.

(19) Vermeer, A.; Bremer, M.; Norde, W. Structural changes of IgG induced by heat treatment and by adsorption onto a hydrophobic Teflon surface studied by circular dichroism spectroscopy. *Biochimica Et Biophysica Acta*, **1998**, *1425*, 1-12.

(20) Sun, X.; Yu, D.; Ghosh, R. Study of hydrophobic interaction based binding of immunoglobulin G on synthetic membranes. *Journal of Membrane Science*, **2009**, *344*, 165-171.

(21) Yu, D.; Ghosh, R. Method for studying immunoglobulin G binding on hydrophobic surfaces. *Langmuir*, **2010**, *26*, 924-929.

(22) Wang, L.; Hale, G.; Ghosh, R. Non-size-based membrane chromatographic separation and analysis of monoclonal antibody aggregates. *Analytical Chemistry*, **2006**, *78*, 6863-6867.

(23) Phillips, J.; Drumm, A; Harrison, P.; Bird, P.; Bhamra, K.; Berrie, E.; Hale, G. Manufacture and quality control of CAMPATH-1 antibodies for clinical trials. *Cytotherapy*, **2001**, *3*, 233-242.

(24) Tankersley, D.; Preston, M.; Finlayson, J. Immunoglobulin G dimer: An idiotype-anti-idiotype complex. *Molecular Immunology*, **1988**, *25*, 41-48.

(25) Wan, Y.; Vasan, S.; Ghosh, R.; Hale, G.; Cui, Z. Separation of monoclonal antibody alemtuzumab monomer and dimers using ultrafiltration. *Biotechnology and Bioengineering*, **2005**, *90*, 422-432.

3. Single step purification of chimeric heavy chain monoclonal antibody-EG2 using hydrophobic interaction membrane chromatography

Abstract: Monoclonal antibodies (mAbs) produced in a cell culture form aggregates due to their high concentration in the heterogeneous cell culture mixture. Protein-A (pt-A) chromatography is the preferred step for capturing monoclonal antibody (mAb) from such heterogeneous mixture because of specific interactions with Fc region of mAb. Acidic condition used for antibody elution can also leach pt-A molecules which in turn can form pt-A-mAb complex. Acidic environment also impacts mAb stability and is known to cause antibody aggregation. The complex formed as well as the antibody aggregates are unwanted products in antibody formulation and should be removed. Hydrophobic interaction membrane chromatography (HIMC) is an important polishing step in the downstream purification of antibody monomer from aggregates. This work demonstrates the potential use of HIMC for capturing heavy-chain mAb EG2 molecules by replacing conventional pt-A chromatography and simultaneously purifying mAb from aggregate impurities. Binding and eluting conditions were determined from preliminary experiment performed with partially pt-A purified EG2. On increasing the binding capacity and maintaining similar binding-eluting conditions, mAb EG2 was successfully captured from cell culture supernatant feed ensuring efficient removal of impurities. Applying gradient elution resulted in multiple eluate peaks which on analysis with Native PAGE confirmed the purification of monomeric EG2 from aggregates. The effectiveness of HIMC for capturing mAb EG2 was further verified from similar glycan profiles of pt-A and HIMC purified antibody samples.

3.1 Introduction

Monoclonal antibody (mAb) provides passive defense in patients against invading pathogens by forming antibody-antigen complexes. These mAbs were earlier produced according to the method developed by Kohler and Milstein.[1] Difficulties involved in this method included: ethical issues in using animals for producing antibodies, low titer volumes and immunogenic reaction in humans. To overcome these problems recombinant technology has enabled mAb production with significant transformation, though gradually, from being of the mouse origin to completely human derived.[2] With this technique it is possible to introduce favorable genes while undesirable genes were knocked out from expression systems.[3] This has made recombinant technique one of the most important development in biopharmaceutical industry. Currently, the therapeutic market is dominated by mAbs belonging to IgG class obtained from such recombinant techniques. According to the recent market reports the number of mAbs launched and the ones in clinical and preclinical stages as well as increasing demand for these antibodies make them important candidates in biopharmaceutical industry.[4]

Conventional IgG mAbs are multidomain molecules composed of 2 heavy and 2 light chain units with 150kDa molecular weight. Disulfide bonds link together heavy and light chains forming a Y-shaped structure. Top part of the molecule forms Fab region and lower part forms Fc region. Interaction between antigen and antibody occurs through Fab region while Fc region is responsible in effector functions for clearance of antibody-antigen complexes as well as binding to the cell surface receptors to increase its serum half-life.[5] Inspite of these characteristics antibodies are not always required in their intact form. For neutralizing toxins or tumor imaging purposes antibodies have shown poor performance.[6] Problems such as tissue penetration and high specificity towards antigen prevents these antibodies from reaching the interior of such tissues.[7]

Use of recombinant technology has also made available wide range of immunoglobulin (Ig) fragments many of which have already been approved by US FDA and few others are in the clinical and preclinical stages.[8] Such fragments carry complementarity determining regions (CDR) of intact antibody molecule with potential antigen binding capabilities. Recombinant scFv fragments were efficient with tissue penetration compared to intact IgG[7] which enabled their use in radioimmunoassays (RIAs) for imaging the target antigenic sites.[9,10] Diabodies which are equivalents of Fab in terms of specificity and valency have proved important molecules in targeting antigens[11]. Fab fragments obtained traditionally from enzymatic digestion process are not always reliable but recombinant Fab has shown good results with antigen-binding characteristics.[12] Even though these antibody fragments were good for targeting antigens and tissue penetration, lack of an Fc region caused their rapid clearance. Fc region of an antibody molecule is multifunctional in nature. It helps in binding to pt-A molecules during purification of mAbs and also participates in cellular recognition. It also interacts with FcRn and help mAbs to extend their half-life.[13] The half-life of these antibody fragments was improved using molecular conjugation technique by covalently attaching small molecules.[14,15]

Though all these antibody fragments were useful for diagnostic applications their small size had shortcomings due to their structure. Hydrophobic residues in these fragments remain exposed to solvent molecules causing poor solubility and eventually leading to aggregation. Search for small molecules with functionality similar to IgG antibodies helped researchers to identify antibodies containing only heavy chain units. Such heavy chain antibodies (hcAbs) were naturally present in the serum of camelid species (camel, llama and dromedary) forming 75% of total serum proteins. Similar to naturally occurring IgG antibodies, these heavy chain antibodies also exist as different isotypes and play important role in providing passive immunity in new born camelids.[16]

hcAbs were also naturally found in humans as a result of pathological disorder and were non-functional since the light chains and C_H1 domain were absent.[17] On the contrary, naturally occurring hcAbs in camelid are fully functional and have shown good antigen binding properties.[18] But similar to murine IgG antibodies, hcAbs could also present immunogenicity problems due to their camelid origin. To solve immunogenicity problem camelid antibodies have been successfully produced with recombinant technique by adding human Fc region.[19] Unlike the stability concerns with antibody fragments and conjugate molecules, hcAbs possess good thermal stability similar to conventional IgG antibodies. Reversibility of thermal denaturation also shows the inherent stability of these antibodies. The robust structure is unaffected even by strong denaturants like Urea or GnHCl.[18]

Numerous affinity techniques are available for capturing IgG type antibodies[20] but pt-A chromatography by far is most widely accepted because of specific interaction through the Fc region.[21] When a feed containing IgG is passed through pt-A column, impurities flow without binding and the bound IgG is recovered by reducing the buffer pH. hcmAb EG2 used in this work is recombinantly produced, containing human Fc region, and therefore can be purified using pt-A chromatography. This way EG2 can be captured and separated from impurities present in a cell culture. However IgG purification studies under strong acidic conditions for elution have created problems with purity of the product as well as damage to the native structure leading to aggregate formation.[22] In some cases pt-A-antibody complexes have also been found. These byproducts are non-desirable and at the same time leaching of pt-A under acidic conditions can potentially threaten the use of such contaminated mAb product.[23] To find an alternative purification method, we make use of hydrophobic interaction membrane chromatography (HIMC).

The main objective in this work is to capture and purify mAb EG2 using HIMC technique. Our hypothesis is based on the result obtained in a study which showed that

IgG antibodies bind to hydrophobic membranes in presence of anti-chaotropic salt through the region between hinge and C_H2 domain.[24,25] Since mAb EG2 also contains similar hinge and C_H2 domain it is expected to bind in presence of anti-chaotropic salt. To verify this hypothesis preliminary analysis was initially performed with partially pt-A purified EG2 with binding conditions being similar to that used in studying binding of IgG1 antibody. These conditions were then carried over for a scaled-up purification experiment for capturing EG2 directly from cell culture supernatant. Applying gradient elution simultaneously fractionated bound EG2 into multiple components. These individual fractions were then analyzed with Native PAGE to confirm the presence of aggregates and purity of monomer EG2 captured.

3.2 Materials and Methods

3.2.1 Materials

Humanized purified mAb hIgG1-CD4 (Batch 12, 23rd March 1999) monomer was kindly donated by the Therapeutic Antibody Centre, Oxford, United Kingdom. Day 4 samples: pt-A purified mAb EG2 as well as the cell culture supernatant was obtained from Dr. Michael Butler (Department of Microbiology, University of Manitoba, MB, Canada). Sodium phosphate monobasic (S0751), Sodium phosphate dibasic (S0876), Ammonium sulphate (A4418), Sodium citrate (S4641), Citric acid (C0759) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Sodium chloride (SOD 002.205) for preparing SEC mobile phase was purchased from Bioshop, Burlington, ON, Canada. Purified water (18.2 MΩ cm) was obtained using DiamondTM NANOpure (Barnstead, Dubuque, IA, USA) water purification unit and used for preparing buffer and test solutions. Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-30 membrane (Millipore, Billerica, MA, USA) were used for diafiltration and buffer exchange.

3.2.2 Harvesting and separation of mAb EG2 from cell culture

EG2 was cultured according to the procedure mentioned elsewhere[26] using CHO cells under optimized conditions. To separate mAb EG2, culture was harvested and centrifuged at 1500 rpm forming supernatant (top layer) and pellet (precipitate) in centrifuge tube. Supernatant containing antibody was then sterile filtered using 0.2µm membrane filter to remove impurities. Filtrate media was then concentrated 10X using 30K MWCO Amicon filters and frozen at -20°C. Prior to pt-A purification, the frozen media was thawed and subjected to buffer exchange with PBS using 30K Amicon membrane filters. Day-3 and day-4 harvested and filtered supernatant samples were provided for analysis. Cell count in day-3 sample was $2x10^6$ cells/mL and in day-4 sample was $3.3x10^6$ cells/mL. Since day-4 sample had maximum mAb content, only the results with this sample are presented here.

3.2.3 Protein-A purification of cell supernatant

Purification was performed on GE pt-A column (HiTrap pt-A HP) as per the column instructions. Supernatant containing antibody was loaded and elution was carried out using 0.1M sodium citrate buffer pH 3.6. Eluted sample was then neutralized with 1 mL of 0.5M Tris-HCl, pH 9.0. Purified sample was kept frozen at -20°C prior to shipment. This product is the pt-A purified sample.

3.2.4 Hydrophobic interaction membrane chromatography

To develop a method for simultaneously capturing and polishing mAb EG2 and eliminating non-mAb components from cell culture supernatant (feed) was the main objective in this work. For HIMC experiments, a custom-designed module was fabricated that housed hydrophobic interaction media in the form of membrane discs. Hydrophilized polyvinylidene fluoride (PVDF) membranes can be tuned for hydrophilic/hydrophobic properties in absence/presence of anti-chaotropic salts and were employed in these experiments. Chromatographic separation was performed after integrating the membrane

module with AKTA prime plus liquid chromatography system (GE Healthcare Biosciences, QC, Canada). Preliminary binding experiments with pt-A purified EG2 were performed using 10 membrane discs. Eluting buffer consisted of 20mM sodium phosphate, pH 7.0 and binding buffer was prepared by adding 1.5M ammonium sulfate to eluting buffer (pH 7.0). 0.1 mg/mL sample was injected in the form of a pulse by using a sample loop attached directly to AKTA system. Hydrophobic binding of mAb EG2 took place in presence of anti-chaotropic salt, ammonium sulfate. Bound antibody was eluted using gradient technique by allowing eluting buffer (from 0% to 100%) to flow over the membrane discs for 40ml eluting volume. Throughout the HIMC experiments 4 mL/min flow rate was used.

PVDF membranes have lower binding capacity compared to commercially designed membrane adsorbers used for polishing and removal of antibody aggregates. However the peak resolution obtained with these membranes makes it attractive for use in HIMC. To maximize the capture of mAb EG2 directly from cell culture supernatant, binding capacity was increased by increasing the number of membrane discs in the module to 20. Feed sample for injection was prepared by mixing 1:1 ratio of buffer and supernatant containing EG2 by adjusting the salt concentration to 1.5M for binding. Sample loop of higher capacity was used for injecting large volume of supernatant. Binding and eluting conditions used were the same as mentioned above. Sample measurements were recorded at 280nm using an in-line UV detector integrated with AKTA system.

3.2.5 Gel electrophoresis

3.2.5.1 Reducing and non-reducing SDS-PAGE

Pt-A purified mAb EG2 sample was analyzed using 10% gels, with (reducing) and without (non-reducing) β-mercaptoethanol in the sample buffer. Addition of surfactant sodium docecyl sulfate (SDS) to the sample buffer imparts uniform negative charge to the

antibody molecules. Electrophoresis was performed at 140V in Hoefer MiniVE system (GE Healthcare Bio-Sciences, Canada) using Tris-glycine running buffer, pH 8.3. Discontinuous buffer system consisting of stacking gel (pH 6.8) and separating gel (pH 8.8) prepared using Tris-HCl buffering system was used. After completion of electrophoresis, gels were stained using Coomassie Brilliant Blue dye for 15 minutes. Destaining was performed using the solution containing methanol, acetic acid and water. This analysis was carried out according to the method developed by Laemmli.[27]

3.2.5.2 Native PAGE

Eluate samples obtained from scaled-up HIMC purification were subjected to acidic Native PAGE analysis performed using 10% gel without adding SDS in sample buffer. This allows proteins to separate according to their inherent charge and size. Sample buffer consisted of acetic acid, Tris-base, glycerol and pyroninY. Addition of sample buffer to antibody imparts positive charge, so electrophoresis was performed with reverse polarity i.e. cathode (positively charged) and anode (negatively charged). Stacking gel (pH 6.0) and separating gel (pH 4.8) were prepared using Tris-HCl buffer. Electrophoresis was performed at 200V in Hoefer MiniVE (GE Healthcare BioSciences, Canada) with the running buffer containing a mixture of acetic acid and β-alanine. Gels were stained using Coomassie Brilliant Blue dye solution for 15 minutes and destained in solution containing methanol, acetic acid and water.

3.2.6 Glycosylation profiling

Pt-A purified as well as HIMC purified mAb EG2 samples were analyzed for the presence of different types of oligosaccharide (sugar) molecules. mAb samples were first analyzed using SDS-PAGE under reduced conditions to obtain sharp bands. These gel bands were carefully removed and subjected to PNGase digestion to extract the oligosaccharides. These oligosaccharides were labeled using 2-AB (2-aminobenzamide) and analyzed using normal phase HILIC system.

3.3 Results and Discussion

mAbs in a cell culture are recombinantly produced by expressing the genes for each fragment of an antibody which then combines covalently forming the whole molecule.[28] While this happens, post-translational processes are also activated which modify the mAb molecule and imparts appropriate functionality.[29–31] Since the growth medium contains heterogeneous mixture of nutrients and modifying elements it becomes difficult to obtain mAb as desired. Therefore once the required cell density is attained, cells are harvested and antibodies are separated to avoid major changes to their structure. In this work mAb EG2 producing cells were cultured and harvested on day-4 from which the antibody molecules were separated by centrifugation. Prior to further processing the supernatant containing mAb EG2 was pt-A purified as mentioned in "materials and methods" section. The purified EG2 obtained was immediately analyzed for the presence of antibody and its molecular weight as well as testing its purity using SDS-PAGE under reducing condition. Following samples were analyzed: (i) supernatant and (ii) pellet precipitated after centrifugation. This analysis was done at the University of Manitoba using 4-15% gradient gels. The supernatant was concentrated 48X using 30K Amicon Ultracel membranes before analysis. Result obtained is shown in Figure.3.1. Under reducing condition both the supernatant and the pellet shows presence of a single dark band corresponding to 40kDa molecular weight which is the expected weight for EG2 since it exists as homodimer (80kDa) molecule. The pellet remaining after centrifugation contains impurities mainly cell debris and therefore multiple faint bands corresponding to such impurities are seen.

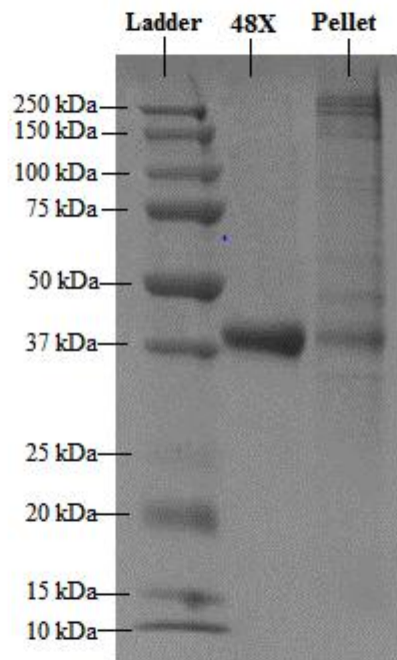

Figure 3.1. SDS-PAGE (reducing) analysis of EG2 containing sample (48X) and pellet from cell culture supernatant.

To determine the binding characteristics of mAb EG2 under hydrophobic conditions, it was analyzed using HIMC technique. Supernatant containing mAb EG2 after pt-A purification was used as the feed. mAb EG2 is still under development and not characterized thoroughly. So the HIMC data was compared with mAb IgG1 which has been studied extensively. The characteristic feature of these mAbs is that even though they have different Fab region but they carry similar Fc region qualifying them for HIMC analysis.

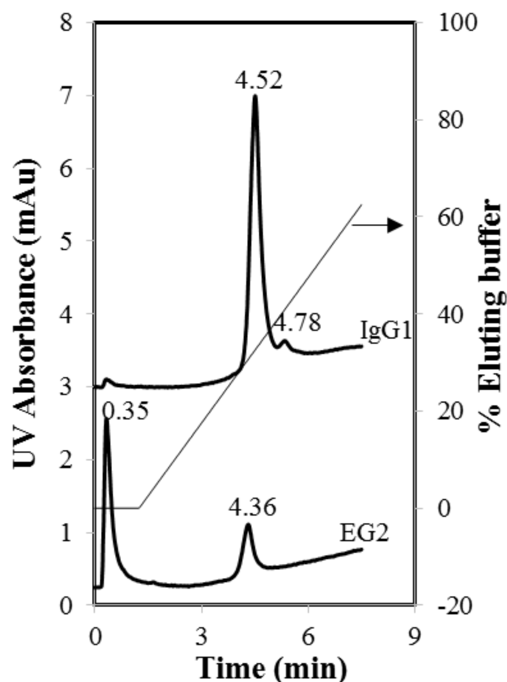

Figure 3.2. Preliminary HIMC chromatogram for mAbs IgG1 showing Monomer - (4.52 min) and Dimer - (4.78 min), and EG2 with flow-through - (0.35 min) and Monomer EG2 - (4.36 min) obtained using 10 membrane discs at 4 mL/min and 40 mL elution gradient.

Antibody molecules are hydrophobic in nature and their binding characteristics have been thoroughly studied through hydrophobic interactions. Earlier work on binding between IgG1 mAb and PVDF membrane resulted in strong binding of monomer and dimer at 1.5M salt concentration.[32] Based on this information, analysis of mAb IgG1 was performed first as a reference for comparing the result obtained with mAb EG2. Chromatogram obtained is as shown in Figure.3.1. With mAb IgG1, a single eluate peak at 4.52 min confirms the presence of monomeric antibody molecule while dimer elutes at 4.78 min. Analyzing EG2 under similar conditions also shows a single eluate peak at 4.36 min which is very close to the retention time for IgG1 indicating that both antibody molecules undergo hydrophobic interaction with membrane and the strength of binding is similar in both cases. However the chromatogram for EG2 also shows a peak at 0.35 min.

Since the components in this peak do not bind to the membrane surface so these must be essentially non-hydrophobic in nature.

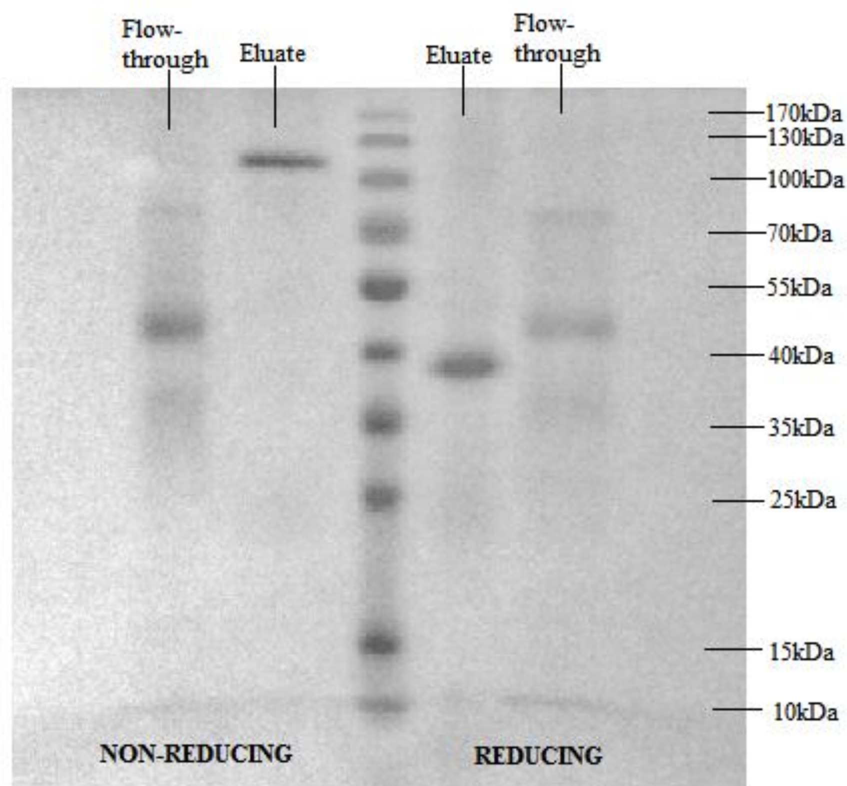

Figure 3.3. SDS-PAGE gel (10%) analysis of fractionated EG2 sample under reducing and non-reducing conditions. Eluate and flow-through were collected from preliminary HIMC fractionation of EG2 (Figure 3.1)

Since a single eluate peak was obtained for HIMC analysis of mAb EG2 as shown in Figure.3.2, its purity and molecular weight was analyzed using SDS-PAGE analysis. Individual peak fractions were collected from multiple HIMC runs and pooled together. Gel image obtained is shown in Figure.3.3. EG2 is a small molecule with approximately 80kDa molecular weight. Non-reducing gel analysis shows the molecule is over 100kDa which contradicts the actual molecular weight of EG2. Presence of a single band however

confirms purity of the sample. Under reducing conditions the added β-mercaptoethanol in sample buffer reduces disulfide bonds of EG2 into free thiol groups. Thus the heavy chains are separated into 2 individual fragments having same molecular weight. Under reduced conditions the band obtained corresponds to molecular weight of approximately 40kDa which is true given that it is the weight of a single heavy chain and this result confirms EG2 being 80kDa since it exists as heavy-chain homodimer. Result for the flow-through component shows multiple bands at 36kDa, 46kDa and 80kDa. These three bands are unaffected under reducing and non-reducing conditions which confirms that these are non-mAb impurities.

Having proved that EG2 antibody binds to membrane surfaces under hydrophobic conditions and is obtained in highly pure state, a scaled-up purification was carried out. The cell culture is a heterogeneous mixture of antibody producing cells, nutrients, enzymes and media. Obtaining antibody from such a mixture involves capture of mAb EG2 directly from cell culture supernatant and further purifying it. Antibody concentration in supernatant is usually low and large feed volumes need to be processed which would require high capacity membranes. Therefore the binding capacity was increased by adding 20 membrane discs in the module. So the scaled-up experiment was performed using 2 mL sample loop for feed injection and 20 membrane discs for higher binding capacity.

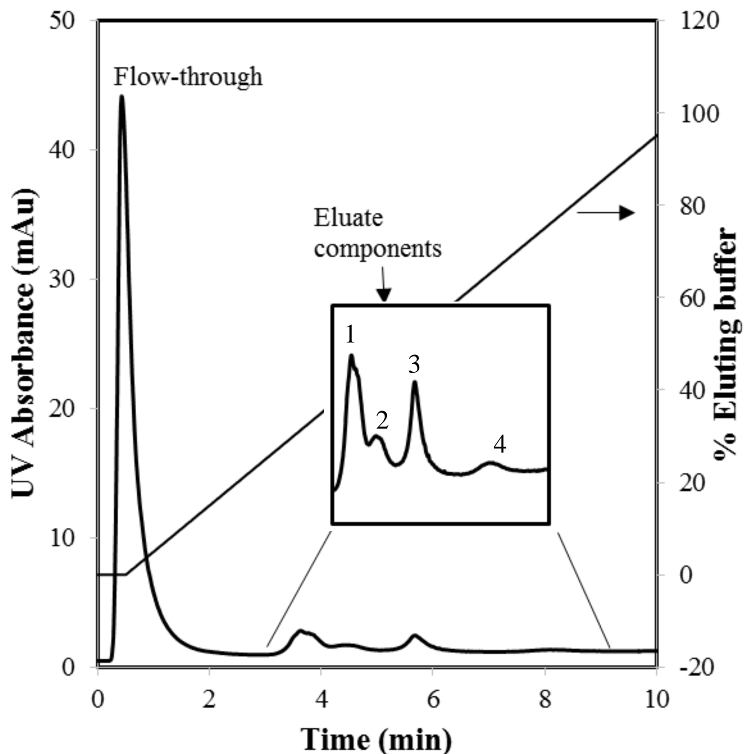

Figure 3.4. HIMC chromatogram for cell culture supernatant used as feed. 20 membrane discs were used in module. Flow rate of 4 mL/min and 40 mL elution gradient was used.

The chromatogram obtained is shown in Figure.3.4. At 40 mL gradient elution, the sample is fractionated into multiple eluate peaks along with large flow-through which contains non-hydrophobic mAb impurities. For a mixture containing multiple protein components the least hydrophobic protein elutes first followed by the most hydrophobic. Therefore the chromatogram indicates presence of multiple components in (supernatant) feed with peak 1 being least hydrophobic and peak 4, most hydrophobic. Hydrophobic studies carried out on antibody molecules containing aggregates suggest that antibody aggregates are more hydrophobic than parent antibody. So the peak 1 shown in chromatogram is the parent antibody monomer EG2 while peak 2 (dimer), peak 3 (trimer) and peak 4 (tetramer) are aggregates of mAb EG2. The individual fractions collected from HIMC fractionation of supernatant were analyzed using SDS-PAGE under reducing

conditions before performing the glycol-analysis. Result is shown in Figure.3.5. Lanes 2-5 are for eluate fractions while lane 1 is for the flow-through fraction. Lanes 2-5 show single band with molecular weight close to 40 kDa verifying the presence of EG2 antibody. Lane 2 also shows faint band close to 100 kDa which could be due to error in collecting the fraction from HIMC experiment. While lane1 corresponding to the flow-through does not show presence of antibody.

Antibody molecules are always purified from cell culture supernatant using pt-A chromatography. Presence of an Fc region in mAb allows binding with pt-A molecules while the impurities are washed away. However in this fractionation no such pt-A step was involved.

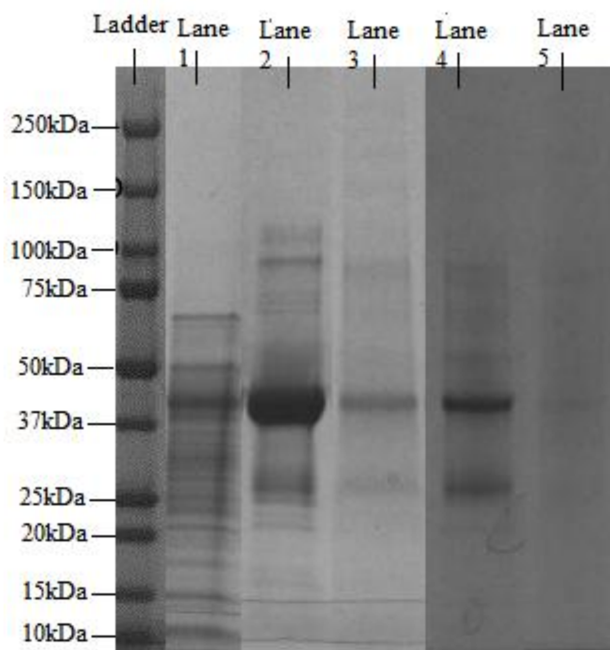

Figure 3.5. SDS-PAGE analysis of individual fractions collected from HIMC fractionation of cell culture supernatant. Lane-2- Eluate peak 1; Lane-3 – Eluate peak 2; Lane-4 – Eluate peak 3; Lane-5 – Eluate peak 4; Lane-1 – Flow-through (peak numbers correspond to eluate fractions in Figure 3.4. inset)

To ensure all the antibody molecules are bound to membrane during HIMC analysis and no trace quantities of mAb were present in flow-through fraction, pt-A chromatographic analysis was carried out with the flow-through fraction as feed. Antibody sample prepared in 20mM sodium phosphate buffer, pH 7.0 which also served as binding buffer and 0.1M sodium citrate buffer, pH 3.0 was used for elution.

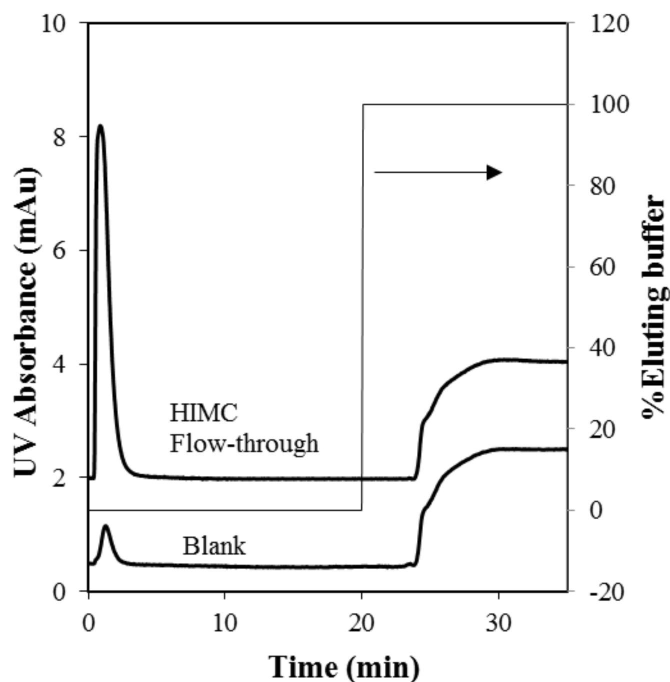

Figure 3.6. Chromatogram showing protein-A result for HIMC flow-through (feed) compared with a blank injection of 20mM sodium phosphate buffer, pH 7.0.

A blank sample injection containing 20mM sodium phosphate buffer was also carried out for comparison. Result obtained is shown in Figure 3.6. As seen from chromatogram the injected sample elutes immediately even before the elution gradient was applied. When compared with the profile for blank injection, the flow-through fraction from HIMC does not show presence of antibody and this result verifies that the

flow-through from HIMC experiment contains non-mAb impurities. Individual fractions collected from HIMC fractionation experiment (mentioned above) were analyzed for the presence of aggregates using Native PAGE. This technique uses inherent charge and size of the protein molecules to separate them without affecting their native state. The image obtained is shown in Figure.3.7. Lanes 1, 2, 3 and 4 are the corresponding eluate components 1, 2, 3 and 4 from HIMC fractionation. When a protein mixture containing monomer and aggregates is analyzed using Native PAGE, monomer molecules owing to their small hydrodynamic size travel greater distances than their aggregate counterparts. Lane 1 and 2 consists of components which are least hydrophobic and migrate to greater extent in gel analysis. Since a single dark band is obtained for sample in lane 1, it indicates presence of pure monomer EG2. Due to lower concentration for the sample loaded in lane 2, a faint band is seen corresponding to the monomer component which indicates presence of some monomer molecules. But lane 2 also contains aggregate molecules which is not seen due to lower concentration. On the other hand components present in lanes 3 and 4 show two different bands which are well separated. These components show higher hydrophobicity in HIMC analysis and are aggregated forms of EG2 mostly the trimer and tetramer alongwith some monomer EG2 molecules. Presence of dark bands for aggregates and faint bands for monomer signifies that these components are essentially rich in aggregates.

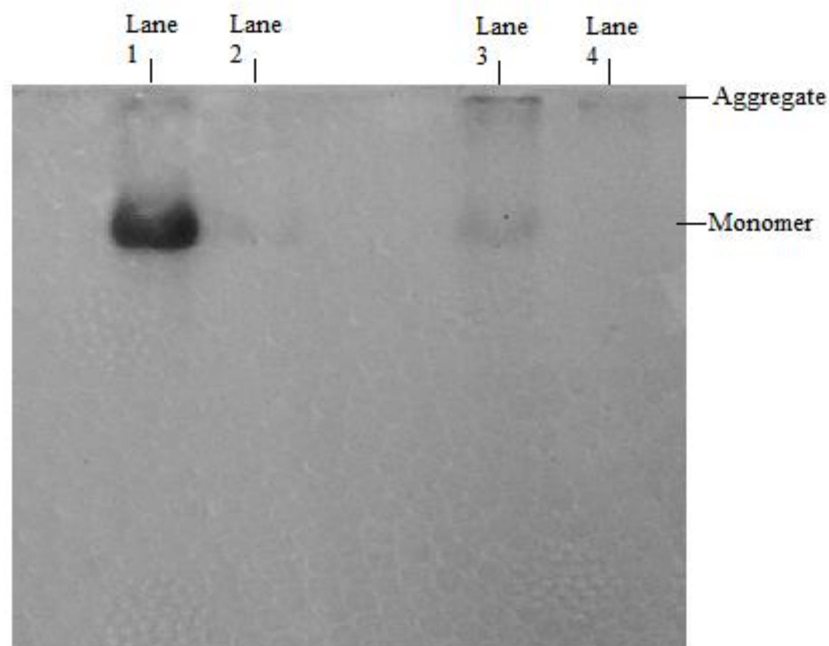

Figure 3.7. Native PAGE analysis for EG2 fractionated using HIMC showing monomer and aggregate components. Lane1-Eluate peak 1 (figure 3.5); Lane2-Eluate peak 2; Lane3-Eluate peak 3; Lane4-Eluate peak 4.

Antibodies are manufactured with an aim to target specific antigens. As discussed earlier the functional parts of an IgG antibody involves Fab and Fc regions to greater extent. Each of these region has multiple functions to carry out. Fab region is primarily the antigenic binding site and Fc region is used for cell recognition and increasing the serum half-life of antibody. Fc region also undergoes post translational modification during the manufacturing stages which involves attaching polysaccharides that will be recognized by cell receptors to perform the desired function of the antibody.

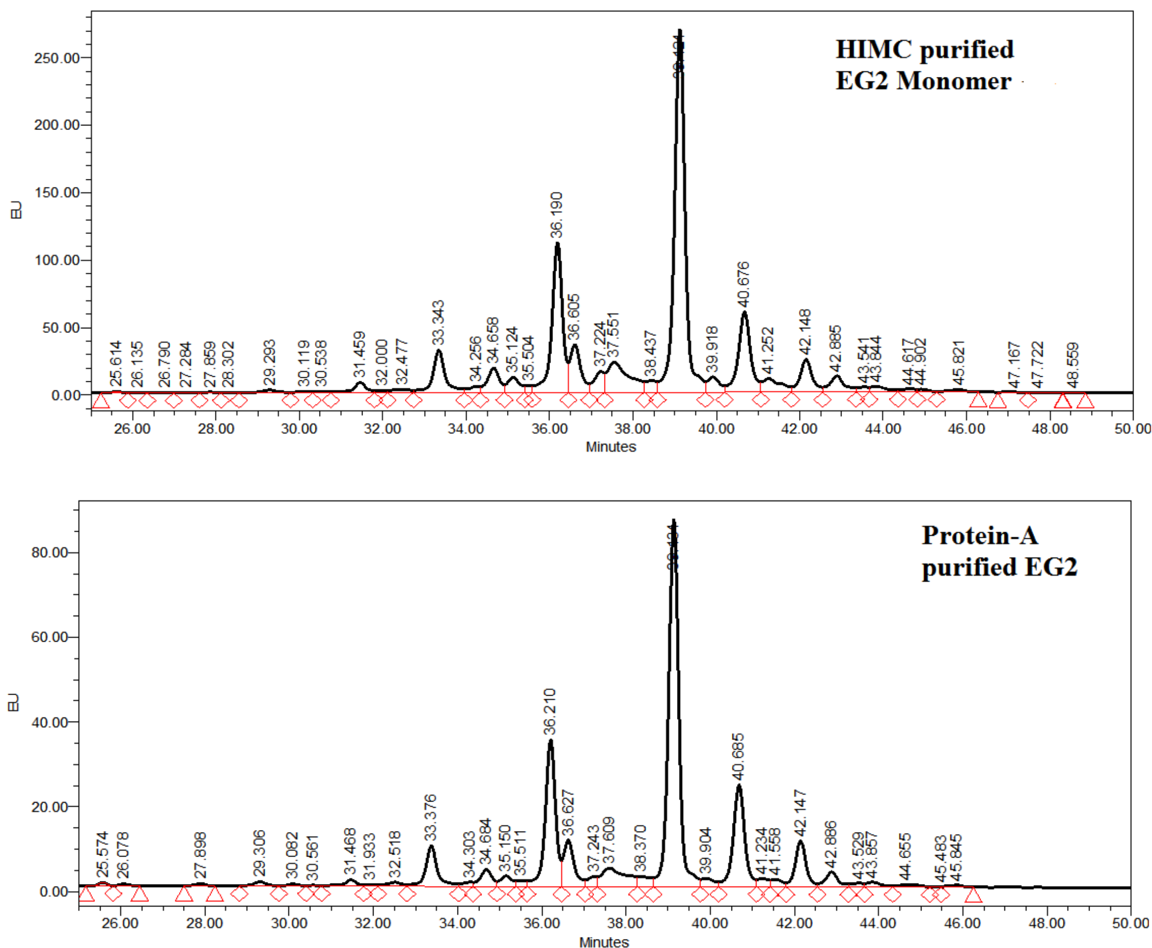

Figure 3.8. Glycoanalysis of EG2 sample purified using HIMC and protein-A techniques. HIMC purified EG2 (monomer from HIMC fractionation).

The heterogeneity in cell culture media doesn't allow single population of sugar residues to attach to the Fc region and the product always contains mixture of multiple sugar residues differing slightly in their composition. Since mAb EG2 was successfully fractionated into monomer and aggregate components, these fractions were tested for their oligosaccharide content. For comparison partially pt-A purified mAb EG2 was also analyzed for its sugar content. The result obtained is shown in Figure.3.8. It can be seen from the results that the glycosylation profile for protein-A as well as HIMC purified EG2

are almost similar containing same type of oligosaccharides indicating the efficiency of mAb capture using HIMC technique.

3.4 Conclusion

Antibody sample from cell culture supernatant contains significant amount of non-mAb impurities. As a result the conventional pt-A purification cannot be successfully used since these impurities can block the active sites and also react with pt-A molecules and affect the mAb purification. Inefficiency in purifying antibody was evident from the impurities present in pt-A purified mAb sample. HIMC provides an efficient way to remove these impurities and capture mAb EG2 in a single step. Antibody molecules were completely captured from supernatant sample as verified from subsequent pt-A analysis. Using gradient elution, the bound antibody was successfully fractionated into monomer and aggregates. Monomer antibody molecule collected from HIMC eluate fraction showed similar glycan composition as obtained with pt-A purified mAb. These results indicate that HIMC technique can be successfully used to capture monoclonal antibody molecules thereby replacing conventional pt-A chromatography and by increasing the binding capacity of membrane, the process can be scaled to purify large sample volumes.

3.5 References

(1) Kohler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **1975**, *256*, 495-497.

(2) Sandhu, J. S. Protein engineering of antibodies. *Critical Reviews in Biotechnology*, **1992**, *12*, 437-462.

(3) Birch, J. R.; Racher, A. J. Antibody production. *Advanced Drug Delivery Reviews*, **2006**, *58*, 671-685.

(4) Nelson, A. L.; Dhimolea, E.; Reichert, J. M. Development trends for human monoclonal antibody therapeutics. *Nature Reviews*, **2010**, *9*, 767-774.

(5) Edelman, G.; Cunningham, B.; Gall, W.; Gottlieb, P.; Rutishauser, U.; Waxdal, M. The Covalent Structure of an entire IgG molecule. *The Journal of Immunology*, **1969**, *63*, 78-85.

(6) Jain, M.; Kamal, N.; Batra, S. Engineering antibodies for clinical applications. *Trends in Biotechnology*, **2007**, *25*, 307-316.

(7) Yokota, T.; Milenic, D. E.; Whitlow, M.; Schlom, J. Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin. *Cancer Research*, **1992**, *52*, 3402-3408.

(8) Holliger, P.; Hudson, P. Engineered antibody fragments and the rise of single domains. *Nature Biotechnology*, **2005**, *23*, 1126-1136.

(9) Raag, B.; Whitlow, M. Single-chain Fvs. *The FASEB Journal*, **1995**, *9*, 73-80.

(10) Adams, G. P.; Tai, M.-S.; McCartney, J. E.; Marks, J. D.; Stafford, W. F.; Houston, L. L.; Huston, J. S.; Weiner, L. M. Avidity-mediated enhancement of in vivo tumor targeting by single-chain Fv dimers. *Clinical Cancer Research*, **2006**, *12*, 1599-1605.

(11) Holliger, P.; Prospero, T.; Winter, G. Diabodies: small bivalent and bispecific antibody fragments. *Proceedings of the National Academy of Sciences*, **1993**, *90*, 6444-6448.

(12) Venturi, M.; Seifert, C.; Hunte, C. High level production of functional antibody Fab fragments in an oxidizing bacterial cytoplasm. *Journal of Molecular Biology*, **2002**, *315*, 1-8.

(13) Ravetch, J.; Kinet, J. Fc Receptors. *Annual Reviews in Immunology*, **1991**, *9*, 457-492.

(14) Chapman, a P.; Antoniw, P.; Spitali, M.; West, S.; Stephens, S.; King, D. J. Therapeutic antibody fragments with prolonged in vivo half-lives. *Nature Biotechnology*, **1999**, *17*, 780-783.

(15) Trüssel, S.; Dumelin, C.; Frey, K.; Villa, A.; Buller, F.; Neri, D. New strategy for the extension of the serum half-life of antibody fragments. *Bioconjugate Chemistry*, **2009**, *20*, 2286-2292.

(16) Wernery, U. Camelid Immunoglobulins and Their Importance for the new-born - A review. *Journal of Veterinary Medicine B*, **2001**, *48*, 561-568.

(17) Seligmann, M.; Mihaesco, E.; Preud'homme, J.; Danon, F.; Brouet, J. Heavy chain diseases: current findings and concepts. *Immunological Reviews*, **1979**, *48*, 145-167.

(18) Spinelli, S.; Frenken, L. G.; Hermans, P.; Verrips, T.; Brown, K.; Tegoni, M.; Cambillau, C. Camelid heavy-chain variable domains provide efficient combining sites to haptens. *Biochemistry*, **2000**, *39*, 1217-1222.

(19) Li, S.; Liang, S.; Guo, N.; Wu, A.; Fujita-Yamagachi, Y. Single-chain antibodies against human insulin-like growth factor I receptor : expression, purification, and effect on tumor growth. *Cancer Immunology Immunotherapy*, **2000**, *49*, 243-252.

(20) Huse, K.; Bohme, H.; Scholz, G. Purification of antibodies using affinity chromatography. *Journal of Biochemical and Biophysical Methods*, **2002**, *51*, 217-231.

(21) Ey, P.; Prowse, S.; Jenkin, C. Isolation of pure IgG1, IgG2a and IgG2b immuneglobulins from mouse serum using protein A-sepharose. *Immunochemistry*, **1978**, *15*, 429-436.

(22) Arakawa, T.; Philo, J.; Tsumoto, K.; Yumioka, R.; Ejima, D. Elution of antibodies from a protein-A column by aqueous arginine solutions. *Protein Expression and Purification*, **2004**, *36*, 244-248.

(23) Carter-Franklin, J.; Victa, C.; McDonald, P.; Fahrner, R. Fragments of protein A eluted during protein A affinity chromatography. *Journal of Chromatography A* **2007**, *1163*, 105-111.

(24) Sun, X.; Yu, D.; Ghosh, R. Study of hydrophobic interaction based binding of immunoglobulin G on synthetic membranes. *Journal of Membrane Science*, **2009**, *344*, 165-171.

(25) Yu, D.; Ghosh, R. Method for studying immunoglobulin G binding on hydrophobic surfaces. *Langmuir*, **2010**, *26*, 924-929.

(26) Zhang, J.; Liu, X.; Bell, A.; To, R.; Baral, T. N.; Azizi, A.; Li, J.; Cass, B.; Durocher, Y. Transient expression and purification of chimeric heavy chain antibodies. *Protein Expression and Purification*, **2009**, *65*, 77-82.

(27) Laemmli, U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **1970**, *227*, 680-685.

(28) Schlatter, S.; Stansfield, S.; Dinnis, D.; Racher, A.; Birch, J.; James, D. On the optimal ratio of heavy to light chain genes for efficient recombinant antibody production by CHO cells. *Biotechnology Progress*, **2005**, *21*, 122-133.

(29) Lodish, H. Post-translational modification of proteins. *Enzyme and Microbial Technology*, **1981**, *3*, 178-188.

(30) Han, K.; Martinage, A. Post-translational chemical modifications of proteins. *International Journal of Biochemistry*, **1992**, *24*, 19-28.

(31) Dwek, R.; Butters, T.; Platt, F.; Zitzmann, N. Targeting glycosylation as a therapeutic approach. *Nature Reviews*, **2002**, *1*, 65-75.

(32) Wang, L.; Hale, G.; Ghosh, R. Non-size based membrane chromatographic separation and analysis of monoclonal antibody aggregates. *Analytical Chemistry*, **2006**, *78*, 6863-6867.

4. A thermal cycling based method for disaggregating non-native monoclonal antibody aggregate-complexes

Abstract: Proteins are known to form aggregates by various pathways. Presence of such aggregates in therapeutic protein formulations results in problems such as toxicity and immunogenic reactions in patients and therefore these should not be present in the finished product. Protein aggregation adds to the production cost, both in terms of material loss and additional processing. In this paper we examine a novel thermal cycling technique for disaggregating non-native aggregate-complexes of monoclonal antibody. Molecules belonging to the immunoglobulin G (or IgG) class are known to form stable non-native aggregates through Fab-Fab interactions. By exposing the aggregate containing samples to rapid temperature changes in a thermal cycler, interactions that hold molecules in aggregate-complexes are broken. During this process, the unfolding of the native proteins is likely to result in some fresh aggregate formation i.e. disaggregation and aggregation may occur simultaneously. However, the net result of controlled thermal cycling was found to be disaggregation accompanied by protein renaturation as observed using different analytical techniques such as hydrophobic interaction membrane chromatography (HIMC), size exclusion chromatography (SEC) and circular dichroism (CD) spectroscopy. Constant temperature heating experiments were also performed for comparison with thermal cycling results. While some disaggregation was also observed in constant temperature heated samples, this was accompanied with protein denaturation and prolonged heating even led to protein fragmentation.

4.1 Introduction

Monoclonal antibodies (mAbs) were first developed in 1975[1] and over the many years that have followed, continued efforts have resulted in the successful development of fully human monoclonal antibodies for biopharmaceutical application. The therapeutic

protein market is dominated by mAbs and these account for billions of dollars' worth of sale[2] and profits, clearly highlighting their importance. More than 15 mAbs have been successfully launched[3] with a large number of mAb products in the development pipeline[4]. mAbs used for therapeutic applications are usually delivered as high concentration doses as injections to patients.[5] Stability of mAbs is highly susceptible to changes in solution conditions due to combination of physical and chemical factors.[6,7] Instability often lead to the formation of native and non-native aggregates which typically results in loss of protein activity[8] and causes toxic and immunogenic reactions.[9]

Aggregation of immune globulins (Ig) was first reported with intravenous immunoglobulin (IVIg) infusions[10] which when injected resulted in rapid activation of anti-complement activity. Protein aggregation depends primarily on factors that affect protein folding. Under physiological conditions, a protein molecule is in an equilibrium state with folding and unfolding mechanisms occurring simultaneously.[11] Any disturbance in the form of external energy disturbs this equilibrium leading to the formation of large unfolded intermediates. Presence of such intermediates in large quantities could trigger the formation of fibril-like amyloid structures or inclusion bodies[12–14] that are the commonly observed aggregated forms of proteins. Such aggregation is reported to be the cause of diseases like Alzheimer's, Parkinson's and Huntington's diseases in humans.[15,16] mAbs produced for therapeutic application also are prone to aggregation.[17–19] Campath-1H which is a humanized CD52 mAb of the IgG1 type binds to human blood lymphocytes including the B- and T-cells and is effective in the treatment of chronic lymphocytic leukaemia.[20] Studies with Campath-1H indicate presence of stable dimeric species[21] which are known to form by Fab-Fab interactions between antibody molecules.[22] In serum IgG-based treatment, large doses of concentrated protein are injected either intravenously, intramuscularly or through subcutaneous routes. Serum IgG is fractionated from plasma obtained from multiple donors and analysis of purified IgG samples have indicated the presence of stable dimeric species.[23]

Investigations have shown that the formation of dimer involve Fab-Fab interactions between the involved IgG molecules.[24]

From a thermodynamic angle a protein molecule represents a macromolecular system in which each domain contributes independently towards overall stability.[25] The native state of a protein is therefore determined by complex events that tend to minimize the free energy of individual polypeptide chains while the denatured state is highly disordered and possesses relatively high free energy. Various mechanisms for native and non-native aggregate formation have been explained for proteins in solutions.[26–29] Electrostatic interaction is a common mechanism for native aggregation.[28] The presence of complementary surface charges among multiple chains frequently lead to the formation of oligomers, particularly in concentrated protein solutions. Studies with insulin show formation of such reversible aggregates at neutral pH and high concentration, which tends to dissociate on dilution as well as during SEC analysis due to interactions with chromatographic media.[30] These reversible aggregates are soluble until a specific size is attained beyond which they turn into insoluble and frequently irreversibly aggregated species.[28]

Non-native aggregation which is accompanied by protein denaturation is a major challenge with protein based therapeutics as they can cause adverse effects in human beings. Apart from being irreversible in nature, non-native aggregates also contain highly ordered β-structures[31] indicative of protein denaturation. Under ordinary physiological conditions, formation of non-native aggregates occurs slowly.[32] Techniques to obtain native protein from non-native aggregates by refolding have been extensively studied.[33,34] The general approach is to completely denature the protein using chemical agents and then subject it to controlled refolding and renaturation by removal of the denaturing agent. Traditional refolding methods makes use of high concentration of chaotropes[35] like Urea or Guanidine HCl for denaturation. Sometimes ionic detergents are also used to denature the proteins by simultaneously maintaining their secondary structures for ease in

refolding. Different conditions are described[36,37] to remove these denaturants and the mechanism to obtain protein chains in their native state. However the resulting chains do re-aggregate again due to difficulty in controlling the dilution rates and the refolding is never complete. Occasionally, these techniques also make use of co-solutes as additives to ensure proper refolding of the denatured protein chains[38–40] and improve the refolding yield. Alternatively, molecular chaperone pathway is employed[41,42] for proper folding of the denatured protein chains. Chaperones are the protein molecules that assist in folding of denatured protein chains by providing isolated compartments and shielding from unwanted hydrophobic interactions thus protecting them from aggregation. Another category of renaturing/refolding technique employs very high pressures, typically around 2000 atmospheres, in presence of chaotropes[43] to obtain the native protein chains. One such study carried out with recombinant human growth hormone (rhGH) involved denaturation of aggregated chains with chaotropes and the refolding was achieved[44] with very high pressures maintained for 24 hours. In this work, the rhGH aggregates were broken down into individual chains by applying high pressures. Under such conditions hydrophobic residues were exposed to solution environment and complete hydration of these residues was possible in presence of high concentration of chaotropes. Use of high pressures prevented the intermolecular hydrophobic interactions thereby avoiding potential re-aggregation and at the same time allowed the unfolded protein chains to refold into native structure. The refolding achieved was almost complete but longer process times were involved and the technique was highly energy intensive.

Apart from the chemical and high pressure methods for obtaining monomer protein chains from aggregates, use of higher temperatures have also shown good results in reducing the aggregate content. IVIg formulations prepared using alcohol fractionation contains polymers/aggregates, dimer and monomer components. A method for reducing the aggregate content from protein formulations is by heating such formulations.[45] In one such study pasteurization was performed at 60°C of the aggregate containing

formulations and analysis after pasteurization confirmed only trace amounts of aggregates with improved immunological properties and very little effect on secondary structure of IgG molecules.[46]

We hypothesize a novel thermal cycling technique to achieve disaggregation with simultaneous renaturation of aggregate containing mAb samples. At room temperature mAb molecules present in a solution are in a random state and with increase in temperature the molecular collisions between mAb molecules also increases. This tends to destructure the surrounding hydration layer causing disruption of the solvent molecules. According to thermodynamics of protein molecules in solution, higher temperatures favor protein-protein interactions because of the overall reduction of the free energy[47]. Two different heating techniques were tested: (i) thermal cycling between two fixed temperatures and (ii) constant temperature heating.

When a mAb sample containing aggregates is heated at a temperature which is close to denaturation temperature of the Fab domain (involved in aggregate formation), net aggregation is seen because of increased collisions between unfolded chains. On the contrary, during thermal cycling these aggregate samples are rapidly heated and cooled and in this case too the heating temperature is chosen very close to unfolding of Fab domain of mAb. Due to rapid heating and cooling of aggregate containing samples Fab-Fab interactions in aggregate molecules are disrupted and individual chains are released. However at the same time collisions between unfolded chains at higher temperature causes can cause some of these unfolded chains to aggregate. But the pronounced effect of disaggregation is seen on thermal cycling. In addition to disaggregation the cooling cycle allows protein chains to restructure themselves and return to more stable configuration i.e. effecting renaturation of the protein chains.

Initial heating experiments were performed with antibody sample containing only monomer and dimer species. To compare the results, similar experiments were performed

with antibody sample containing monomer, dimer and higher aggregate species. Antibody sample that was not subjected to any heat treatment was used as “control” for comparison. Changes to the aggregate content of both the antibody samples on heat treatment was verified using hydrophobic interaction membrane chromatography (HIMC) and size exclusion chromatography (SEC) techniques. Changes in the secondary structure on heat treatment was confirmed by circular dichroism (CD) spectroscopy in terms of denaturation/renaturation of the overall antibody structure.

4.2 Materials and Methods

4.2.1 Materials

Humanized purified mAb hIgG1-CD4 (Batch 12, 23rd March 1999) monomer and hIgG1-CD4 aggregate containing mAb sample “Dimers” (Batch D6, 22nd July 1993) was kindly donated by the Therapeutic Antibody Centre, Oxford, United Kingdom. The purified monomer on storage led to the formation of small amounts of aggregates and this sample is referred to as the “Monomer-rich” sample. There was some vial to vial variation and the “Monomer-rich” sample contained on an average of 94% monomer and 6% aggregates (as measured by size exclusion chromatography or SEC). The “Dimers” which is referred to as the “Aggregate-rich” sample contained on an average about 37% monomer, 28% dimer and 35% higher aggregates (trimer and tetramer). Sodium phosphate monobasic (S0751), Sodium phosphate dibasic (S0876), Ammonium sulphate (A4418) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Sodium chloride (SOD 002.205) was purchased from Bioshop, Burlington, ON, Canada. Purified water (18.2 MΩcm) was obtained using DiamondTM NANOpure (Barnstead, Dubuque, IA, USA) water purification unit and used for preparing buffer and test solutions. Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-50 membrane (Millipore, Billerica, MA, USA) were used for buffer exchange and desalting. Genuine Axygen Quality PCR tubes

(PCR-05-C; 0.5mL thin wall, clear, flat cap) for carrying out thermal cycling were purchased from Axygen Inc, CA, USA.

4.2.2 Heating and thermal cycling of monoclonal antibody

Antibody samples were subjected to buffer exchange with 20mM sodium phosphate buffer at pH 7.0 and concentrated using Amicon Ultracel-50 membrane filters. Thermal cycling with antibody samples was performed using Thermal cycler (Eppendorf Mastercycler® personal, Applied Biosystems, CA, USA). For thermal cycling, mAb sample (concentration was adjusted according to requirements of SEC and CD experiments) was pipetted in Eppendorf tubes and placed into the wells of thermal cycler. To test the behavior of aggregate containing samples under different heating conditions "Monomer-rich" and "Aggregate-rich" samples were examined as follows: (i) sample subjected to thermal cycling (10°C to 60°C) and (ii) sample heated at 60°C. The results obtained were then compared with "control" sample at room temperature. Henceforth the "Monomer-rich" and "Aggregate-rich" samples that were treated as "control" will be termed as "untreated monomer sample" and "untreated aggregate sample" respectively. Preliminary thermal cycling experiments with aggregate samples were performed with various combination of low-end and high-end temperatures (e.g. 10°C-30°C, 30°C-40°C, 10°C-40°C, 10°C-60°C, 20°C-60°C) and the combination that significantly affected the aggregate species was selected. Accordingly, the lower-end and higher-end of temperature cycling was chosen as 10°C and 60°C respectively. To avoid long exposure time at high temperatures and cause minimal damage to antibody samples during thermal cycling, heating rate of 3°C/sec was chosen. For comparing thermal cycling results, aggregate samples were also subjected to constant temperature heating in a constant water heating unit maintained at 60°C. Unlike with constant temperature heating at 60°C, during thermal cycling heat was supplied in the form of a pulse as the temperatures varied between 10°C and 60°C. The heated samples from thermal cycler as well as from

constant temperature water unit were equilibrated at room temperature (22±1°C) and diluted to appropriate concentration before further analysis.

4.2.3 Size exclusion chromatography

After concentration and buffer exchange using Amicon Ultracel-50 membrane filters samples were analyzed on Varian Star HPLC system (Varian, Palo Alto, CA, USA) using phosphate buffered saline (PBS) as the mobile phase at flow rate of 0.3ml/min. PBS contained 20mM sodium phosphate buffer with 0.25M sodium chloride, pH 7.0. "Monomer-rich" and "Aggregate-rich" samples were analyzed on WTC-030S5 Wyatt SEC column (Wyatt Technology Corporation, Santa Barbara, USA).

4.2.4 Hydrophobic interaction membrane chromatography

Usually protein-A capture of antibody is the first step in purifying monoclonal antibody from cell culture supernatant. However protein-A technique fails to distinguish between monomer and dimer antibody molecules as both the species have intact Fc region. Furthermore, acidic processing conditions and leaching of protein-A molecule calls for additional purification steps. Earlier work carried out on antibody aggregates using HIMC demonstrated the fractionation of monomer and aggregate components present in humanized monoclonal antibody hIgG1-CD4.[48] This was possible because monomer antibody molecules are less hydrophobic compared to aggregate species and in presence of anti-chaotropic salts antibody aggregates bind to the membrane surface stronger than monomer molecules. Sun et.al., investigated the monoclonal antibody region that was responsible for binding on membrane surfaces[49] in presence of anti-chaotropic salts. For this study antigens specific to Fab and Fc regions were used. This work proved that though the antibody was able to bind through both Fab and Fc regions to the respective antigens, binding on the membrane surface occurred via the region near the hinge. So for an intact IgG molecule, binding on a hydrophobic surface will occur

from the region near the hinge and this was the main motivation in using HIMC technique for analyzing our aggregate containing samples.

"Monomer-rich" sample was analyzed by HIMC technique using 5 hydrophilized polyvinylidene fluoride (PVDF) membrane discs housed in custom-designed membrane module integrated with AKTA primeplus liquid chromatography system (GE Healthcare Biosciences, QC, Canada). Two different buffers were used: binding buffer (1.5M ammonium sulphate solution prepared in eluting buffer, pH 7.0) and eluting buffer (20mM sodium phosphate buffer, pH 7.0). 0.3 mg/mL of "Monomer-rich" sample was prepared (from stock concentration 10mg/2.1mL) by appropriate dilution in buffer. Sample was injected into module in the form of a pulse and subsequent binding onto PVDF membranes was accomplished by the presence of anti-chaotropic salt (ammonium sulphate) with elution carried out by applying negative salt gradient using salt-free buffer from 0% till 100% over gradient length of 40ml. For this experiment, flow rate of 2 mL/min was used. Eluting conditions were optimized so as to get good resolution between monomer and dimer components present in the "Monomer-rich" sample. All the components were analyzed by UV measurements at 280 nm wavelength using a detector present in-line with the AKTA Primeplus system.

Similarly, the "Aggregate-rich" sample was analyzed using HIMC performed with 10 hydrophilized PVDF membrane discs, flow rate of 4 mL/min at sample concentration of 0.1 mg/mL prepared (from concentration of 4mg/3.1mL) by appropriate dilution in buffer. Other conditions were maintained same as with "Monomer-rich" sample.

4.2.5 Circular dichroism

CD measurements were carried out to analyze the changes in secondary structure and study the potential aggregation tendency of "Monomer-rich" and "Aggregate-rich" samples after different heat treatments. Analysis was performed using CD spectrometer Model No.416 (AVIV Biomedical Inc. Lakewood, New Jersey, USA). Quartz cuvettes

having path lengths 1 mm were used for the far-UV (190 nm-260 nm) measurements. Samples were scanned at the rate of 1 nm every 3 seconds. Concentration of samples used was 0.25mg/mL. Experiment was performed with constant nitrogen purging at 25°C and data obtained was recorded in terms of mean residual ellipticity measured in deg.cm^2.dmol^{-1}.

4.3 Results and Discussion

Composition of untreated “Monomer- and Aggregate-rich” samples is presented in Figure.4.1 and 4.2. “Monomer-rich” sample contains monomer and dimer components as verified from SEC and HIMC chromatograms. mAb formulations must contain only the monomeric form of the protein. However, information on mAb aggregates suggests that strong interactions between Fab-Fab regions leads to dimer formation.[21-24] So the dimer peak seen in “Monomer-rich” sample is due to Fab-Fab interactions between monomer components.

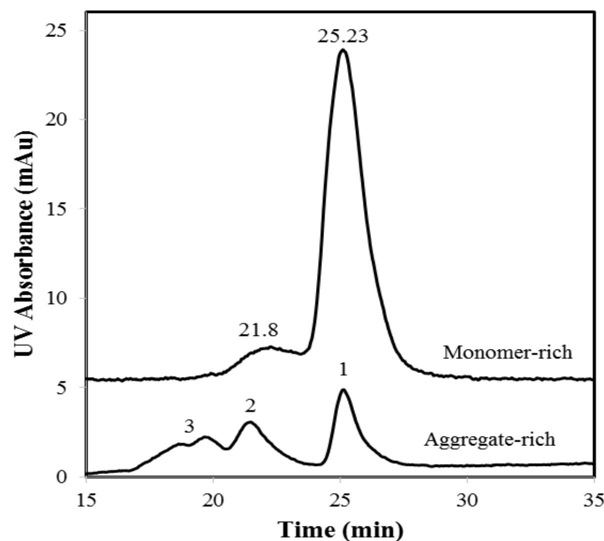

Figure 4.1. SEC chromatogram for untreated “Monomer-rich” and “Aggregate-rich” samples. 1-Monomer; 2-Dimer; 3-Higher aggregates.

These dimer species eventually turn more hydrophobic compared to monomer. Further during processing of protein formulations, often sample equilibration at room temperature is needed. Depending on the solution conditions and the surrounding temperature, increase in molecular collisions disrupts some of the bonds holding protein molecules in their native structure thus denaturing them which is also thermodynamically favorable.[47]

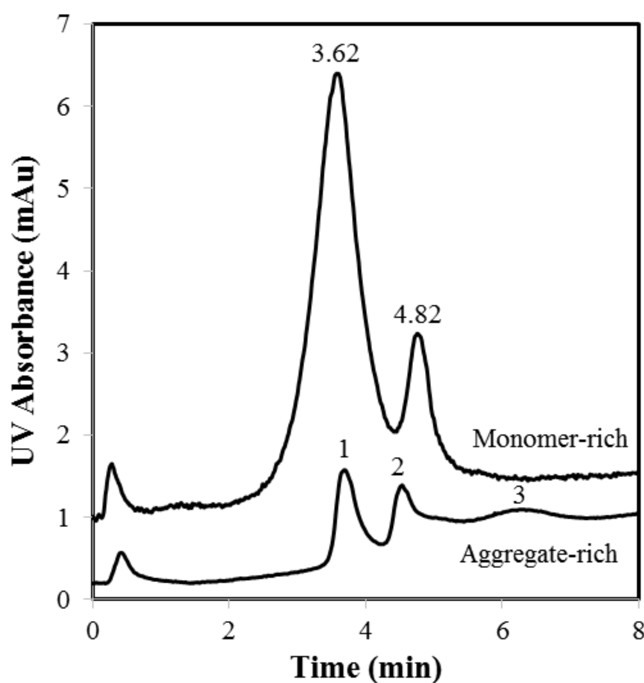

Figure 4.2. HIMC chromatogram for untreated "Monomer-rich" and "Aggregate-rich" samples. 1-Monomer; 2-Dimer; 3-Higher aggregates.

Denatured molecules are unstable and rapidly combine with other denatured monomer molecules to form dimers. This process of aggregation continues with temperature increase forming large number of such dimers and to certain extent also forms higher

aggregates. Therefore the “Aggregate-rich” sample used in this work contains monomer, dimer and higher aggregate species confirmed using SEC and HIMC results. Increase in hydrodynamic radius on aggregation causes poor diffusion in the column packing during SEC which results in their faster elution compared to parent monomer species.

Accordingly the “Monomer-rich” sample shows monomer peak at 25.23 min and dimer peak at 21.8 min while the “Aggregate-rich” sample contains parent monomer peak at 25.23 min along with a dimer and higher aggregate species eluting at 21.6 min and between 19-20 min respectively. Additionally as described in the HIMC section, aggregates and monomer species can be separated based on their hydrophobicity in presence of ammonium salts.[48] Therefore from the HIMC chromatograms we see the monomer species elute first (3.62 min) followed by dimer (4.82 min) and higher aggregates. With SEC and HIMC techniques quantitative information on samples and changes in composition on heat treatment is obtained. However qualitative information regarding the protein structure and information about the stability of these antibody molecules is still missing. So, for analysis of the secondary structure of these antibody samples CD spectroscopy was performed.

Protein molecules present in solution have a characteristic globular structure which exists due to various stabilizing forces like hydrogen bonds, electrostatic attractions, hydrophobic interactions and van der Waals forces. Proteins are multidomain molecules with each domain contributing independently in effecting overall structural change. A CD spectra provides information on the overall secondary structure of protein molecules with no details on the local structural changes. In case of such multidomain proteins, a CD signal is the summation of effects of α-helix, β-sheets, β-turns and randomly coiled conformations. Intramolecular hydrogen bonds between peptide linkages are mainly responsible for maintaining the secondary structure of native proteins.

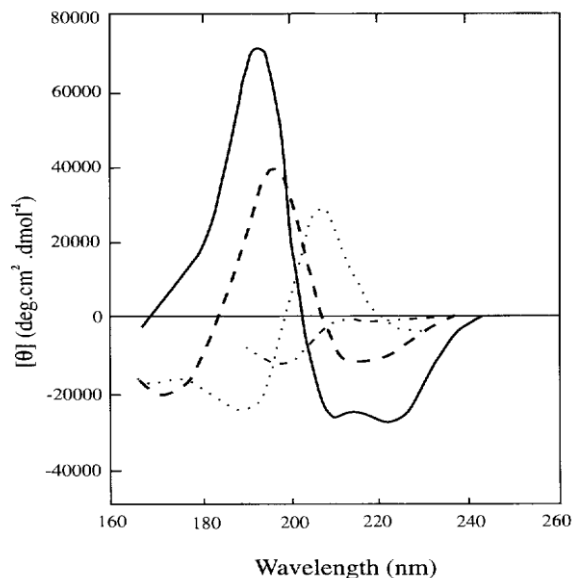

Figure 4.3. Far-UV CD spectra for different types of secondary structures. Solid line – α-helix; Dashed line – anti-parallel β-sheets; Dotted line – β-turns; Dots and short dashes – irregular structure[50].

When a protein folds/unfolds major changes occur at the secondary structure and these are typically recorded in the far-UV region (240 nm to 190 nm) of the visible spectrum. Proteins show one of the two dominant secondary structures: α-helix or β-structures (sheets and turns or random coils).[50] According to the available data,[50,51] α-helices in proteins show two negative peaks at 222 and 210 nm and single positive peak at 193 nm while the presence of β-sheets is confirmed from negative and positive peaks at 218 nm and 198 nm respectively as shown in Figure.4.3. The far-UV CD spectra for untreated monomer and untreated aggregate samples is provided in Figure.4.4 with β-sheet structure present in both samples since negative peak at 218 nm and positive maximum peak at 198 nm is characteristically displayed.[52,53] Also seen in figure is the ellipticity difference between "Monomer- and Aggregate-rich" samples. From SEC and HIMC profiles it is known that "Aggregate-rich" sample contains greater proportion of

aggregates compared to "Monomer-rich" sample. In case of protein aggregates the unfolding of native chains is a prerequisite for non-native interactions.

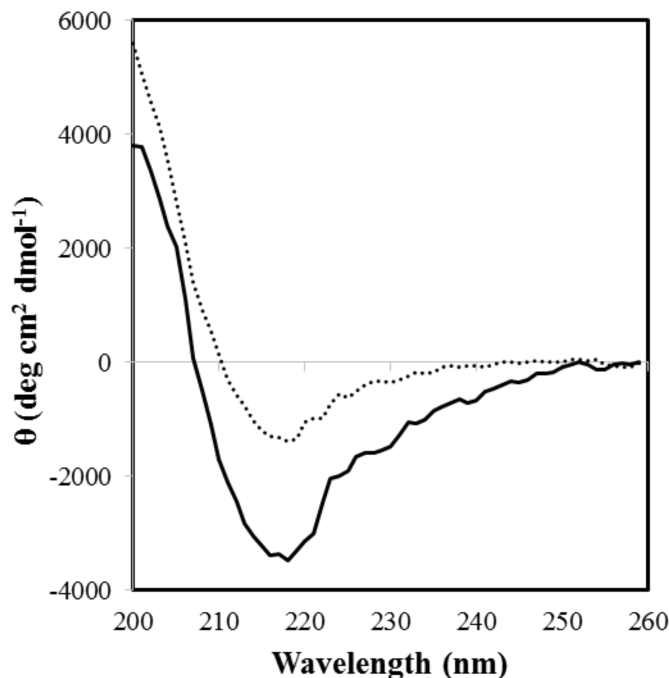

Figure 4.4. Far-UV CD spectra for untreated samples. "Monomer-rich"-Solid line; "Aggregate-rich"-Dotted line.

At higher temperatures, large number of such unfolded chains are present which are in random diffusion state. The secondary structure of such protein chains when analyzed shows disordered configuration. Since the higher aggregates are formed from such random collisions between protein chains, the higher ellipticity magnitude for "Aggregate-rich" sample is expected to result into such disordered structure.

To study the effect of heat treatment, thermal cycling of "Monomer-rich" sample was performed for 2 hours and result for the same is shown in Figure.4.5. Comparing the size exclusion (SE) chromatogram for thermal cycled sample and untreated monomer clearly indicates reduction in dimer content (Table-4.1). The chromatogram for thermal cycled

sample also shows increased monomer peak area compared to untreated sample. During thermal cycling sample was heated momentarily at 60°C and so constant temperature heating at 60°C for 2 hours was also performed to check for changes in the sample at this temperature. Analysis was done using HIMC technique and results were compared with the untreated monomer sample (Figure.4.6). For the three chromatograms, only the parent monomer and dimer peak is seen indicating that heating at 60°C does not cause the antibody sample to form higher aggregates or result into antibody fragmentation.

Table 4.1. Comparison of composition of "Monomer-rich" control sample and that subjected to thermal cycling. Composition was determined based on SEC data.

SEC peak	**Peak area (%)**	
	Untreated "Monomer" sample	**Sample after thermal cycling**
Second (Monomer)	94.12 ± (0.23)	97.22 ± (0.30)
First (Dimer)	5.88 ± (0.13)	2.78 ± (0.15)

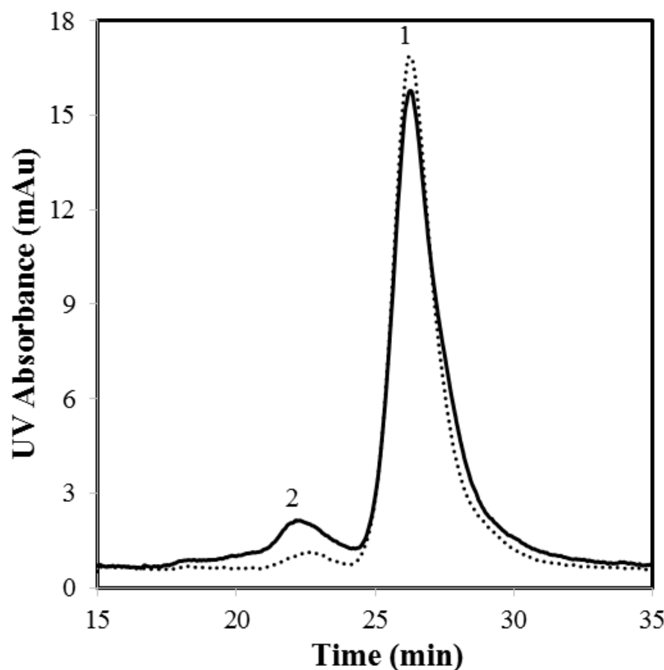

Figure 4.5. SEC chromatogram for "Monomer-rich" sample. Solid line-Untreated sample; Dotted line-Thermal cycled sample. 1-Monomer; 2-Dimer

Data from Table-4.2 suggests that thermal cycling results in an increase of monomer species with proportional reduction in the dimer species when compared with untreated monomer sample. For the sample heated at 60°C, the peak areas suggest decrease in monomer species and increase in dimer species. Thus subjecting an antibody sample containing only monomer and higher aggregates to heat, different results are observed on thermal cycling and constant temperature heating. Analysis using isothermal calorimetric study shows irreversible denaturation of Fab region for an intact antibody molecule around 61°C.[52] Hence when IgG1 type molecules are exposed to temperatures as high as 60°C complete unfolding of Fab region occurs. While rapid changes in temperature on thermal cycling (10°C-60°C) causes momentary denaturation of Fab region of the antibody molecule, rapid cooling helps nullify the effect and the molecule undergoes a relaxation phase returning to a more stable structure. When this process of rapid heating

and cooling is continued for longer duration, the interactions that hold Fab-Fab together are broken gradually.

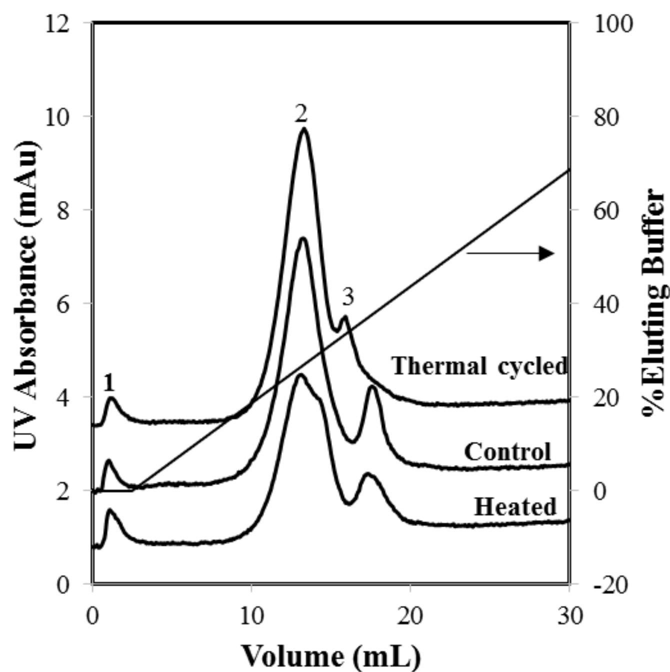

Figure 4.6. HIMC chromatogram for "Monomer-rich" sample. 1-Flow through; 2-Monomer; 3-Dimer.

Thus we see decrease in dimer component and increase in monomer component upon thermal cycling. During this process, the momentarily denatured Fab molecules are still in turbulent state due to rapid temperature changes. This results in an increased molecular collision and the denatured antibody molecules can potentially combine with other denatured/monomer antibody molecules to stabilize the denatured structure forming aggregates. However the results indicate reduction in aggregate species and increase of monomer and so we see net disaggregation of the antibody sample containing aggregate species.

Table 4.2. Comparison of composition of "Monomer-rich" control sample and those subjected to thermal cycling and constant heating. Composition was determined based on hydrophobic interaction membrane chromatography

HIMC peak	Peak area (%)		
	Untreated "Monomer" sample	Sample after thermal cycling	Sample heated at 60°C
First (Monomer)	86.68 ± (0.25)	95.8 ± (0.10)	82.72 ± (0.26)
Second (Dimer)	13.32 ± (0.22)	4.2 ± (0.18)	17.28 ± (0.14)

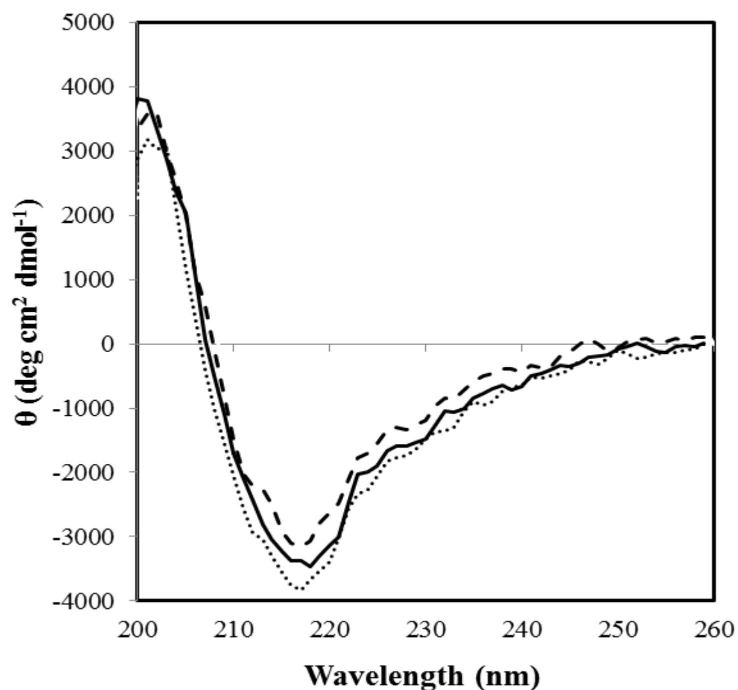

Figure 4.7. Far-UV CD spectra for "Monomer-rich" sample (0.25mg/ml) in PBS buffer. Solid line-Untreated sample; Dashed line-Heated; Dotted line-Thermal cycled.

To check for structural changes on different heat treatments, "Monomer-rich" sample was analyzed using circular dichroism (CD) spectroscopy. Result obtained for different heat treatments is shown in Figure.4.7. Analysis of the untreated, thermal cycled and constant temperature heated sample shows a single negative peak at 218 nm which indicates no significant changes to the secondary structure. With all three samples only minor changes in the ellipticity magnitude is seen. These changes are not very significant to draw strong conclusion regarding aggregation/disaggregation behavior of antibody molecules upon heating/thermal cycling and so to get a clear explanation about the actual process occurring, experiments were also performed with "Aggregate-rich" sample and CD results for the same will be discussed later.

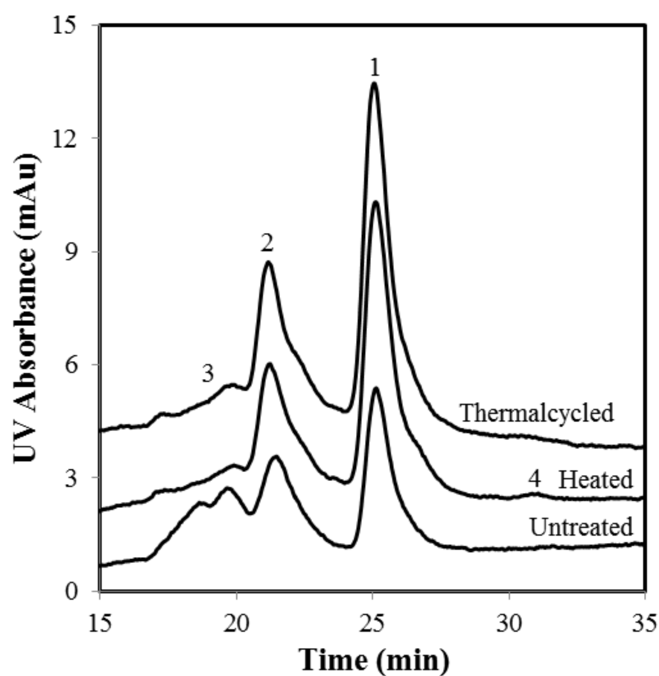

Figure 4.8. SEC chromatogram for "Aggregate-rich" sample. 1-Monomer; 2-Dimer; 3-Higher aggregates; 4-Degradation products. No degradation products are seen with untreated and thermal cycled samples.

To further investigate the effect of heat on samples containing monomer, dimer and higher aggregate species, the “Aggregate-rich” sample was also subjected to thermal cycling and constant temperature heating. Thermal cycling was carried out for 4 hours. SE chromatogram for the thermal cycled sample is shown in Figure.4.8. Result obtained after thermal cycling and constant temperature heating experiments show significant decrease in the higher aggregate species. From the chromatogram and Table-4.3 the data suggests that breakdown of higher aggregates results in increased monomer and dimer peak areas. Analysis after 4 hours of constant temperature heating shows similar trend with increase in monomer but decrease in dimer peak area.

Table 4.3. Comparison of composition of “Aggregate-rich” control sample and those subjected to thermal cycling and constant heating. Composition was determined based on size exclusion chromatography.

SEC peak	Peak area (%)		
	Untreated “Aggregate” sample	Sample after thermal cycling	Sample heated at 60°C
Monomer	36.71 ± (0.23)	47.38 ± (0.27)	41.8 ± (0.21)
Dimer	27.88 ± (0.10)	29.28 ± (0.15)	26.6 ± (0.13)
Higher Aggregates	35.41 ± (0.17)	23.34 ± (0.32)	27.01 ± (0.15)
Degradation	NA	NA	4.59 ± (0.24)

However, an additional peak is seen eluting at 31 min. Since the “Aggregate-rich” sample is free from any low molecular weight contaminants (when compared with the untreated sample) so this low molecular weight species is the degraded product resulting from prolonged heating at 60°C. HIMC analysis of the untreated and thermal cycled

“Aggregate-rich” sample is shown in Figure.4.9. Quite clearly the chromatograms indicate significant reduction of the higher aggregates. Data from Table-4.4 confirms reduction of higher aggregates and proportional increase of the monomer and dimer species.

Table 4.4. Comparison of composition of “Aggregate-rich” control sample and that subjected to thermal cycling. Composition was determined based on size exclusion chromatography.

HIMC peak	Peak area (%)	
	Untreated “Aggregate” sample	Sample after thermal cycling
Monomer	50.12 ± (0.12)	61.87 ± (0.19)
Dimer	35 ± (0.25)	36.67 ± (0.14)
Higher Aggregates	14.88 ± (0.18)	1.46 ± (0.22)

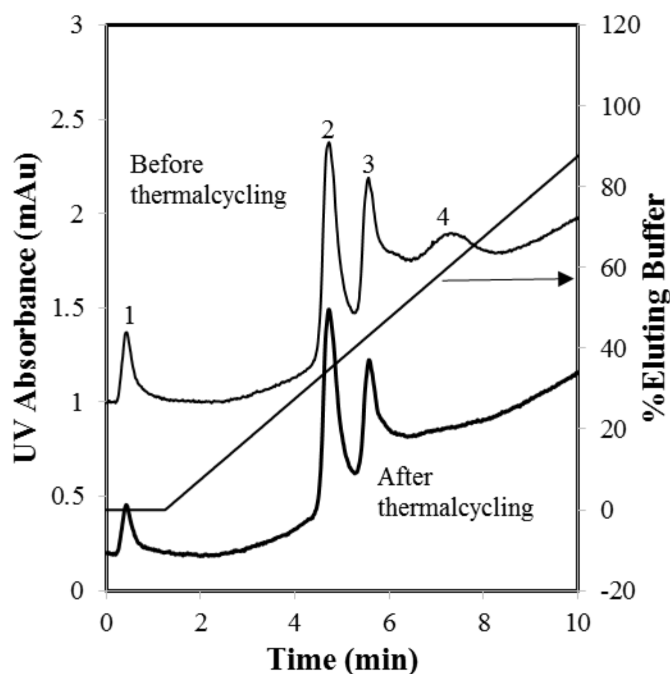

Figure 4.9. UV Absorbance profile for “Aggregate-rich” sample. 1-Flow through; 2-Monomer; 3-Dimer; 4-Higher aggregates.

With "Aggregate-rich" sample, thermal cycling causes breakdown of higher aggregates forming monomer and dimer species. However as explained earlier, the disaggregated molecules can further combine with other molecules forming new aggregates. The mechanism followed is similar to what is seen with "Monomer-rich" samples. Once the dimer species are formed they remain stable. However, the monomer antibody molecules can further combine in monomer-monomer or monomer-dimer combinations forming new dimer or higher aggregates respectively. These higher aggregates are mainly the entangled products of denatured monomer molecules with dimer molecules. So with thermal cycling the higher aggregates are affected due to the temperature fluctuations and the entangled products are released resulting in increase of monomer and dimer species.

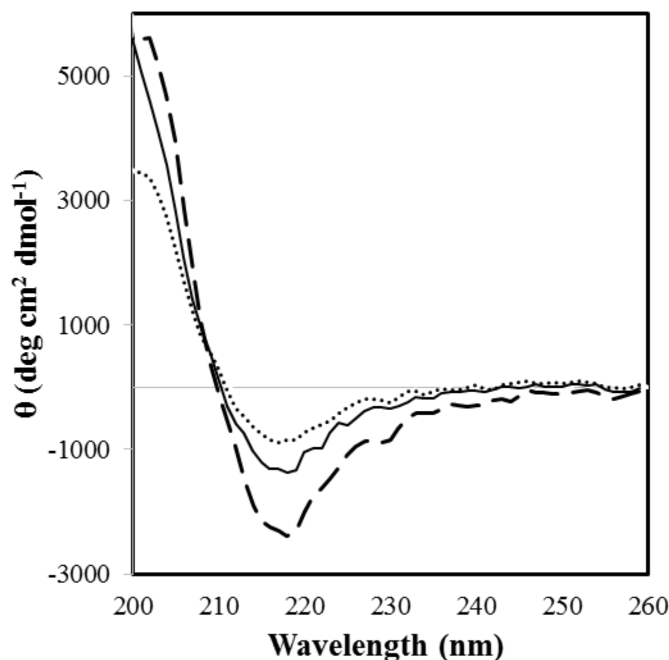

Figure 4.10. Far-UV CD spectra for "Aggregate-rich" sample (0.25mg/ml) in PBS buffer. Solid line-Untreated sample; Dashed line-Thermal cycled; Dotted line-Heated.

At the same time, the dimer molecules are also broken down into individual monomer molecules and new aggregates are formed from monomer-monomer and monomer-dimer interactions. But the net result seen is disaggregation. Heating at constant temperature does seem to produce similar effect with additional formation of degradation products. These degradation products are low molecular weight species characterized from SE chromatograms.

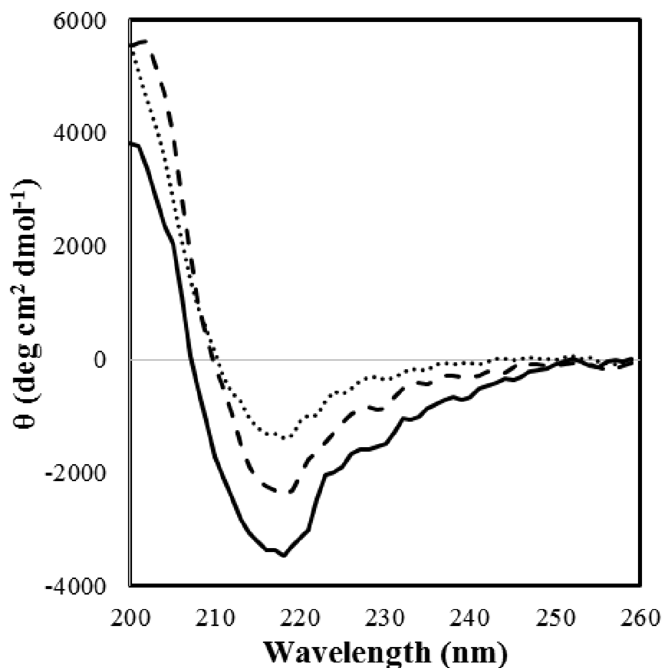

Figure 4.11. Far UV-CD spectra for "Monomer and Aggregate-rich" samples. Solid line-Untreated "Monomer-rich" sample; Dashed line-Thermal cycled "Aggregate-rich" sample; Dotted line-Untreated "Aggregate-rich" sample.

Many studies have been performed on polypeptides and proteins to analyze the secondary structural changes when subjected to thermal stress. In one such study involving collagen protein (β-sheet structure) thermal denaturation caused the native structure to convert to disordered state with significant increase in ellipticity.[54] In case of some polypeptides it is explained that the increase in ellipticity on heating is due to the

higher flexibility at elevated temperatures as opposed to rigid structure at low temperature.[55] However no evidence is available from these studies about the aggregation behavior on thermal denaturation. In case of denaturation studies performed with IgG molecules, no direct conclusion regarding the aggregation tendancy of IgG molecules can be drawn based on the direction of shift of CD profile. In fact many studies have reported the structural changes based on supporting evidence from DSC, SEC, FTIR or fluorescence intensity measurements.[15,56,57] To determine the changes occurring in the antibody molecule hydrophobic binding studies were performed and it was found that Fab region being more hydrophobic was involved in hydrophobic interactions. From DSC measurement the denaturation temperature of Fab is known to be around 61°C.[52] Therefore when an antibody molecule is heated at denaturation temperature, the Fab hydrophobic regions are affected to greater extent leading to unfolding of the hydrophobic protein chains. Comparing the changes in secondary structure on aggregation the Fab fragments shows no deviation in ellipticity signal from 218 nm while Fc fragments shift in the direction of lower (208 nm) wavelengths which is also seen with intact IgG antibody indicating the formation of α-helix structures.[53]

CD result from "Monomer- and Aggregate-rich" samples show no deviation in the ellipticity signal from 218 nm which confirms that the structural change is due to unfolding of Fab region only. From HIMC and SEC results for "Aggregate-rich" sample as well as the CD data it can be concluded that disaggregation and aggregation causes the shift in CD profile. Higher ellipticity signal on aggregation can be attributed to the unfolded antibody molecules because denaturation at 60°C causes unfolding of the Fab region. One possible reason for the lower ellipticity profile on thermal cycling could be due to low temperature (10°C) encountered during thermal cycling. But since all samples were equilibrated at room temperature before CD analysis, this possibility is ruled out. The only other explanation for lower ellipticity could be that the monomer and dimer species released after thermal cycling adopt to a new configuration that is rich in β-sheet structure and this validates the fact that IgG antibodies are predominantly rich in β-sheet

structures. The claim can also be supported by comparing the CD profile for untreated "Monomer- and Aggregate-rich" as well as thermal cycled profile for "Aggregate-rich" sample as shown in Figure.4.11. It is evident from this figure that on thermal cycling the increase in monomer and dimer while simultaneously reducing the higher aggregates shifts the profile in the direction towards "Monomer-rich" sample. Reduction in higher aggregates decreases the contribution of coiled structure to the CD signal and therefore major contribution is from β-sheet structures only. The shift in profile towards "Monomer-rich" is indicative of the fact that thermal cycling helps the aggregated molecules return to their native structure which in this case the "Aggregate-rich" sample tends to achieve the profile for "Monomer-rich". Therefore the disaggregation process can also be viewed similar to renaturation process of obtaining native monomer from denatured state.

Since the thermal cycling technique is effective in reducing aggregates from mAb sample containing varying levels of aggregates so thermal cycling experiments with another mAb sample was also performed. The mAb used was belonged to IgG1 sub-class produced using HEK293 cell lines. Chromatogram for the untreated and thermal cycled sample is shown in figure 4.12. With 2 hours of thermal cycling, the result shows significant drop in aggregate content and increase in the monomer content which is similar to the result obtained with "Monomer-rich" sample. This proves that the aggregation in IgG1 type antibody molecules generally occurs through Fab-Fab interactions. This result also shows the reproducibility of disaggregation by using thermal cycling technique. Following the thermal cycling experiments, prolonged heating of "Monomer-rich" samples was carried out to monitor the changes in sample. SEC chromatograms for this experiment are shown in figure 4.13. Heating of "Monomer-rich" sample was performed by thermal cycling and also at constant temperature. Results show degradation of the sample along with formation of low molecular weight products. Reduction in the aggregate content is also evident from the chromatograms. The shoulder component and low molecular degradation product are evident from chromatograms.

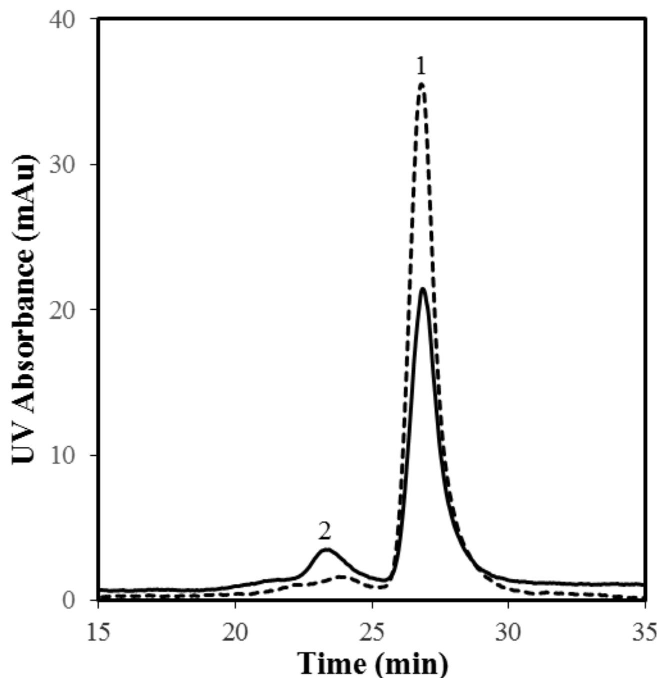

Figure 4.12. SEC chromatogram for IgG1 produced using HEK293 cell line. Solid line-Untreated sample; Dotted line-Thermal cycled. 1-Monomer; 2-Dimer.

Similar work has been performed using recombinant IgG1 antibodies produced using NS0 cells. The degradation products obtained on prolonged heating have been studied and characterised thoroughly[58]. These results prove that longer heating times (greater than 4 hours) causes irreversible damage to the antibody sample.

The overall aggregation/disaggregation phenomena can be described as follows:

Monomer molecules from “Monomer-rich” samples can combine with similar monomer molecules through Fab-Fab interactions at elevated temperatures to form dimer molecules. When the monomer molecules approach towards each other and start interacting, they tend to disturb the solvent layer surrounding them. This causes the free energy of the system to reduce and the Fab-Fab interactions are more favorable for system to stabilize. One the dimer molecules are formed, they remain stable in the

solution. During this time the monomer molecules then entangle with the already formed dimers to produce higher aggregates as seen with "Aggregate-rich" sample.

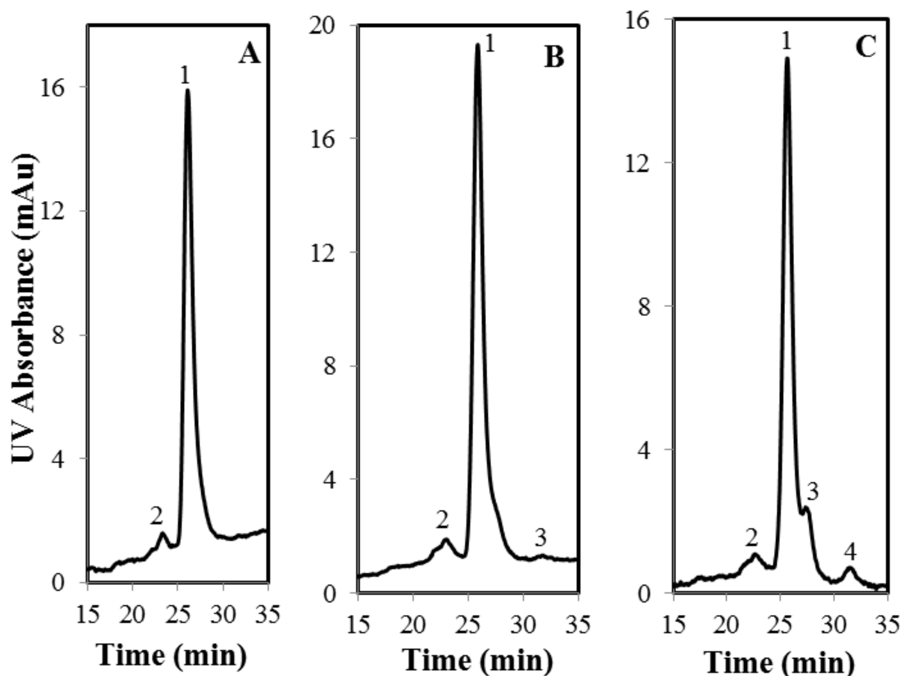

Figure 4.13. SEC chromatogram for "Monomer-rich" sample. A-Untreated sample; B-8hrs thermal cycling; C-8hrs heating. A: 1-Monomer, 2-Dimer; B: 1-Monomer, 2-Dimer, 3-Degradation product; C: 1-Monomer, 2-Dimer, 3-Shoulder degradation, 4-Degradation product.

Simultaneously monomer-monomer interactions can also produce more dimer molecules. During thermal cycling energy is supplied to the system at the melting temperature of Fab region. This tends to denature the Fab region for very short duration affecting the Fab-Fab interaction. The entropy of the system is also on the rise at high temperature which creates more impact and affecting the Fab interactions to a greater extent. Rapid cooling allows the denatured chains to align themselves into more stable configuration and according to thermodynamics, molecular collisions are reduced with lowering of temperature. On repeating the same process of heating and cooling for long time, Fab interactions break thus releasing the individual monomer molecules.

4.4 Conclusion

This work shows the disaggregation of mAbs containing aggregates using thermal cycling technique. With "Monomer-rich" sample thermal cycling leads to the formation of aggregates to some extent but the overall result as confirmed from SEC and HIMC data is disaggregation of dimer species into individual monomer molecules. However this is mainly due to the disaggregation of higher aggregates which are formed from "dimer-dimer" or "dimer-monomer" interactions. Breakdown of higher aggregates cause increase in dimer and monomer components. In this process some of the dimer also disaggregates into monomer molecules. The monomer and dimer species in "Aggregate-rich" formed on thermal cycling or already present in the sample do aggregate to smaller extent. Result with constant temperature heating is opposite to the thermal cycling result with overall aggregation as confirmed from chromatographic and spectroscopic analysis. This is because of increased molecular collisions at higher temperature. Since the aggregates formed with IgG1 type antibody molecules are known to follow the same mechanism of Fab-Fab interactions, they are also expected to show similar results when subjected to same temperature conditions. So when IgG1 type mAb samples containing aggregates produced using HEK293 cell lines are subjected to thermal cycling technique, we see net disaggregation similar to the results with "Monomer-rich" and "Aggregate-rich" sample. Constant temperature heating with "Monomer-rich" sample was further studied with longer heating times and the results show fragmentation of the sample into low molecular weight components.

4.5 References

(1) Kohler, G.; Milstein, C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **1975**, *256*, 495-497.

(2) Pavlou, A. K.; Belsey, M. J. The therapeutic antibodies market to 2008. *European Journal of Pharmaceutics and Biopharmaceutics*, **2005**, *59*, 389-396.

(3) Reichert, J.; Pavolu, A. Monoclonal antibodies market. *Nature Reviews*, **2004**, *3*, 383-384.

(4) Reichert, J. M.; Rosensweig, C. J.; Faden, L. B.; Dewitz, M. C. Monoclonal antibody successes in the clinic. *Nature Biotechnology*, **2005**, *23*, 1073-1078.

(5) Shire, S. J.; Shahrokh, Z.; Liu, J. Challenges in the development of high protein concentration formulations. *Journal of Pharmaceutical Sciences*, **2004**, *93*, 1390-1402.

(6) Wang, W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *International Journal of Pharmaceuticals*, **1999**, *185*, 129-188.

(7) Manning, M. C.; Patel, K.; Borchardt, R. T. Stability of protein pharmaceuticals. *Pharmaceutical Research*, **1989**, *6*, 903-918.

(8) Castellanos, I. J.; Cruz, G.; Crespo, R.; Griebenow, K. Encapsulation-induced aggregation and loss in activity of gamma-chymotrypsin and their prevention. *Journal of Controlled Release : Official Journal of the Controlled Release Society* **2002**, *81*, 307–19.

(9) Schellekens, H. Factors influencing the immunogenicity of therapeutic proteins. *Nephrology, Dialysis, Transplantation*, **2005**, *20*, 3-9.

(10) Barandun, S.; Kistler, P.; Jeunet, F.; Isliker, H. Intravenous administration of human gamma-globulin. *Vox Sanguinis*, **1962**, *7*, 157-174.

(11) Kim, P. S.; Baldwin, R. L. Specific intermediates in the folding reactions of small proteins and the mechanism of protein refolding. *Annual Review of Biochemistry*, **1982**, *51*, 459-489.

(12) Jaenicke, R. Folding and association versus misfolding and aggregation of proteins. *Philosophical Transactions of the Royal Society of London*, **1995**, *348*, 97-105.

(13) Kiefhaber, T.; Rudolph, R.; Kohler, H-H.; Buchner, J. Protein aggregation invitro and invivo: A quantitative model of the kinetic competition between folding and aggregation. *Nature Biotechnology*, **1991**, *9*, 825-829.

(14) Speed M. A.; Wang. D. I. C.; King, J. Specific aggregation of partially folded polypeptide chains: The molecular basis of inclusion body composition. *Nature Biotechnology*, **1996**, *14*, 1283-1287.

(15) Fink, A. L. Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Folding & Design*, **1998**, *3*, 9-23.

(16) Ross, C. A; Poirier, M. A. Protein aggregation and neurodegenerative disease. *Nature Medicine*, **2004**, *10*, 10-17.

(17) Joubert, M. K.; Luo, Q.; Nashed-Samuel, Y.; Wypych, J.; Narhi, L. O. Classification and characterization of therapeutic antibody aggregates. *The Journal of Biological Chemistry*, **2011**, *286*, 25118-25133.

(18) Ejima, D.; Tsumoto, K.; Fukada, H.; Yumioka, R.; Nagase, K.; Arakawa, T.; Philo, J. S. Effects of acid exposure on the conformation, stability, and aggregation of monoclonal antibodies. *Protein: Structure, Function, and Bioinformatics*, **2007**, *66*, 954–962.

(19) Vázquez-Rey, M.; Lang, D. A. Aggregates in monoclonal antibody manufacturing processes. *Biotechnology and Bioengineering*, **2011**, *108*, 1494-1508.

(20) Osterborg, A; Fassas, A. S.; Anagnostopoulos, A; Dyer, M. J.; Catovsky, D.; Mellstedt, H. Humanized CD52 monoclonal antibody Campath-1H as first-line treatment in chronic lymphocytic leukaemia. *British Journal of Haematology*, **1996**, *93*, 151-153.

(21) Phillips, J.; Drumm, A; Harrison, P.; Bird, P.; Bhamra, K.; Berrie, E.; Hale, G. Manufacture and quality control of CAMPATH-1 antibodies for clinical trials. *Cytotherapy*, **2001**, *3*, 233-242.

(22) Wan, Y.; Vasan, S.; Ghosh, R.; Hale, G.; Cui, Z. Separation of monoclonal antibody alemtuzumab monomer and dimers using ultrafiltration. *Biotechnology and Bioengineering*, **2005**, *90*, 422-432.

(23) Tankersley, D.; Preston, M.; Finlayson, J. Immunoglobulin G Dimer : An Idiotype-Anti-Idiotype Complex. *Molecular Immunology*, **1988**, *25*, 41-48.

(24) Remmele, R. L.; Callahan, W. J.; Krishnan, S.; Zhou, L.; Bondarenko, P. V; Nichols, A. C.; Kleemann, G. R.; Pipes, G. D.; Park, S.; Fodor, S.; Kras, E.; Brems, D. N. Active dimer of Epratuzumab provides insight into the complex nature of an antibody aggregate. *Journal of Pharmaceutical Sciences*, **2006**, *95*, 126-145.

(25) Privalov, P. L. Thermodynamic problems of protein structure. *Annual Review of Biophysics and Biophysical Chemistry*, **1989**, *18*, 47-69.

(26) Wang, W. Protein aggregation and its inhibition in biopharmaceutics. *International Journal of Pharmaceutics*, **2005**, *289*, 1–30.

(27) Chi, E. Y.; Krishnan, S.; Randolph, T. W.; Carpenter, J. F. Physical stability of proteins in aqueous solution: mechanism and driving forces in non-native protein aggregation. *Pharmaceutical Research*, **2003**, *20*, 1325-1336.

(28) Philo, J. S.; Arakawa, T. Mechanisms of protein aggregation. *Current Pharmaceutical Biotechnology*, **2009**, *10*, 348-351.

(29) Arakawa, T.; Prestrelski, S. J.; Kenney, W. C.; Carpenter, J. F. Factors affecting short-term and long-term stabilities of proteins. *Advanced Drug Delivery Reviews*, **2001**, *46*, 307-326.

(30) Yu, C.-M.; Mun, S.; Wang, N.-H. L. Phenomena of insulin peak fronting in size exclusion chromatography and strategies to reduce fronting. *Journal of Chromatography A*, **2008**, *1192*, 121-129.

(31) Dong, A; Prestrelski, S. J.; Allison, S. D.; Carpenter, J. F. Infrared spectroscopic studies of lyophilization- and temperature-induced protein aggregation. *Journal of Pharmaceutical Sciences*, **1995**, *84*, 415-424.

(32) Krishnan, S.; Chi, E. Y.; Webb, J. N.; Chang, B. S.; Shan, D.; Goldenberg, M.; Manning, M. C.; Randolph, T. W.; Carpenter, J. F. Aggregation of granulocyte colony stimulating factor under physiological conditions: characterization and thermodynamic inhibition. *Biochemistry*, **2002**, *41*, 6422-6431.

(33) Gorovits, B. M.; Horowitz, P. M. High hydrostatic pressure can reverse aggregation of protein folding intermediates and facilitate acquisition of native structure. *Biochemistry*, **1998**, *37*, 6132-6135.

(34) Clark, E. D. Protein refolding for industrial processes. *Current Opinion in Biotechnology*, **2001**, *12*, 202-207.

(35) Clark, E. Refolding of recombinant proteins. *Current Opinion in Biotechnology*, **1998**, *9*, 157-163.

(36) Tsumoto, K.; Ejima, D.; Kumagai, I.; Arakawa, T. Practical considerations in refolding proteins from inclusion bodies. *Protein Expression and Purification*, **2003**, *28*, 1-8.

(37) Lilie, H.; Schwarz, E.; Rudolph, R. Advances in refolding of proteins produced in E. coli. *Current Opinion in Biotechnology*, **1998**, *9*, 497-501.

(38) Yasuda, M.; Murakami, Y.; Sowa, A; Ogino, H.; Ishikawa, H. Effect of additives on refolding of a denatured protein. *Biotechnology Progress*, **1998**, *14*, 601-606.

(39) Arakawa, T.; Ejima, D.; Tsumoto, K.; Obeyama, N.; Tanaka, Y.; Kita, Y.; Timasheff, S. N. Suppression of protein interactions by arginine: a proposed mechanism of the arginine effects. *Biophysical Chemistry*, **2007**, *127*, 1-8.

(40) Samuel, D.; Kumar, T. K.; Ganesh, G.; Jayaraman, G.; Yang, P. W.; Chang, M. M.; Trivedi, V. D.; Wang, S. L.; Hwang, K. C.; Chang, D. K.; Yu, C. Proline inhibits aggregation during protein refolding. *Protein Science*, **2000**, *9*, 344-352.

(41) Hartl, F. U.; Hlodan, R.; Langer, T. Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends in Biochemical Sciences*, **1994**, *19*, 20-25.

(42) Hartl, F. U.; Bracher, A.; Hayer-Hartl, M. Molecular chaperones in protein folding and proteostasis. *Nature*, **2011**, *475*, 324-332.

(43) St John, R. J.; Carpenter, J. F.; Randolph, T. W. High pressure fosters protein refolding from aggregates at high concentrations. *Proceedings of the National Academy of Sciences*, **1999**, *96*, 13029-13033.

(44) St John, R. J.; Carpenter, J. F.; Balny, C.; Randolph, T. W. High pressure refolding of recombinant human growth hormone from insoluble aggregates. Structural transformations, kinetic barriers, and energetics. *The Journal of Biological Chemistry*, **2001**, *276*, 46856-46863.

(45) Uemura, Y.; Uriyu, K.; Hirao, Y.; Takechi, K.; Ishikawa, H.; Nakajima, T.; Kagitani, Y.; Yokoyama, K.; Funakoshi, S.; Nishida, M. Inactivation and elimination of viruses during the fractionation of an intravenous immunoglobulin preparation: liquid heat treatment and polyethylene glycol fractionation. *Vox Sanguinis*, **1989**, *56*, 155-161.

(46) Bridonneau, P.; Marcilly, H.; Vernois-Martin, M.; Goigoux, P.; Bourdel, V.; Laulan, A.; Deramoudt, F. X.; Desmadril, M.; Sitbon, M.; Basuyaux, B.; Steinbuch, M.; Schmitthaeusler, R. Liquid pasteurization of an immunoglobulin preparation without stabilizer: effects on its biological and biochemical properties. *Vox Sanguinis*, **1996**, *70*, 203-209.

(47) Ross, P. D.; Subramanian, S. Thermodynamics of protein association reactions: forces contributing to stability. *Biochemistry*, **1981**, *20*, 3096-3102.

(48) Wang, L.; Ghosh, R. Fractionation of monoclonal antibody aggregates using membrane chromatography. *Journal of Membrane Science*, **2008**, *318*, 311-316.

(49) Sun, X.; Yu, D.; Ghosh, R. Study of hydrophobic interaction based binding of immunoglobulin G on synthetic membranes. *Journal of Membrane Science*, **2009**, *344*, 165-171.

(50) Kelly, S.; Jess, T.; Price, N. How to study proteins by circular dichroism. *Biochimica Et Biophysica Acta*, **2005**, *1751*, 119-139.

(51) Chang, C. T.; Wu, C. S.; Yang, J. T. Circular dichroic analysis of protein conformation: inclusion of the beta-turns. *Analytical Biochemistry*, **1978**, *91*, 13-31.

(52) Vermeer, A. W.; Norde, W. The thermal stability of immunoglobulin: unfolding and aggregation of a multi-domain protein. *Biophysical Journal*, **2000**, *78*, 394-404.

(53) Vermeer, A.; Giacomelli, C.; Norde, W. Adsorption of IgG onto hydrophobic teflon. Differences between the F(ab) and F(c) domains. *Biochimica Et Biophysica Acta*, **2001**, *1526*, 61-69.

(54) Shanmugam, G.; Polavarapu, P. Structural transition during thermal denaturation of collagen in the solution and film states. *Chirality*, **2009**, *21*, 152-159.

(55) Tiffany, M.; Krimm, S. Effect of temperature on the circular dichroism spectra of polypeptides in the extended state. *Biopolymers*, **1972**, *11*, 2309-2316.

(56) Mason, B.; Schöneich, C.; Kerwin, B. Effect of pH and light on aggregation and conformation of an IgG1 mAb. *Molecular Pharmaceutics*, **2012**, *9*, 774-790.

(57) Andya, J.; Hsu, C.; Shire, S. Mechanisms of aggregate formation and carbohydrate excipient stabilization of lyophilized humanized monoclonal antibody formulationns. *AAPS Pharmaceutical Science*, **2003**, *5*, 1-11.

(58) Mazur, M. T.; Seipert, R. S.; Mahon, D.; Zhou, Q.; Liu, T. A platform for characterizing therapeutic monoclonal antibody breakdown products by 2D chromatography and top-down mass spectrometry. *The AAPS Journal*, **2012**, *14*, 530-541.

5. Conclusions

This work dealt with different aspects of processing antibody molecules but ultimately used membrane chromatography for characterization and purification, thus making it an inevitable component in downstream processing. Preliminary binding studies performed with antibody samples were inspired from earlier work focussing on binding of antibody molecules to membrane surface. Some of the highlights of this work were:

1. Antibody samples of the same isotype characterized using HIMC technique showed certain degree of aggregation. It is known for Campath-1H and several other antibody molecules that these aggregates are formed by Fab-Fab interactions. The newly developed antibody molecules HEK-IgG and CHO-IgG also showed aggregate formation indicating the universality of aggregation mechanism for antibodies of this isotype even though these antibodies were produced using different mammalian cell lines.

2. A single step antibody capture and purification technique was successfully demonstrated using hydrophobic interaction membrane chromatography. HIMC is operated at the pI of antibody sample and the anti-chaotropic salts used for hydrophobic binding helps stabilize the antibody structure as opposed to the acidic buffer conditions employed in protein-A chromatography which is not suitable for the stability of antibody molecules. Antibodies captured using protein-A technique also contains impurities in the form of antibody aggregates and antibody-protein-A complexes which needs further polishing either with ion-exchange or HIMC techniques. The advantage offered by HIMC technique is that both capture and purification operations can be performed simultaneously. In addition to this, column based purification technique including protein-A chromatography are not efficient in processing large volumes of cell culture supernatant feed mainly because the impurities in feed can cause blockage of separating media packed

inside the column. In addition to this longer processing time is a major drawback. However with membrane chromatography very little fouling was seen even with multiple runs and by increasing the binding capacity large sample can be processed within short time. Along with this the glycosylation profile also showed presence of similar glycans for the sample purified using protein-A and HIMC purification techniques.

3. A thermal cycling technique was demonstrated to reduce the aggregate content in antibody sample. Many techniques are available to obtain the native protein from its aggregated form. These techniques first denature the protein and then in a slow controlled manner try to regain the native form. Time required for the whole process is too long to obtain maximum yield of the native proteins. The objective in this study was to show that native form of antibody can be recovered from aggregates. The process was not completely successful in breaking down all aggregate molecules but has certainly shown significant reduction in the aggregate content upon thermal cycling. Advantage of this technique is that since no additives are added, unlike other denaturation/renaturation processes, the product maintains its purity. By proper control of temperature and duration of thermal cycling product deterioration can also be controlled. This process has also shown good performance compared to constant temperature heating of antibody which poses significant aggregation and degradation issues.

Recommendations

A single step capture and purification process has been successfully shown for EG2 antibody however there were certain aspects that need to be addressed for improving the performance of this process.

1. The cell culture supernatant in mAb EG2 processing contained significant amount of impurities and very less antibody product. Using PVDF membranes has shown good results in capturing antibody while removing the impurities. The peak resolution obtained is also good and helps to distinguish impurities and antibody molecules. However after several sample injections pressure in the system increased rapidly indicating blockage of membrane pores due to impurity deposits. Since the initial objective in this work was to capture antibody molecules and removal of impurities, high binding Sartobind® (Phenyl Nano, 3mL) membrane adsorbers can be efficiently utilized. These adsorbers are designed for handling large process volumes and will prove very effective in removing impurities. Unlike using 1.5M salt for binding EG2 to PVDF membranes, with these membrane adsorbers the salt needed can be further reduced. This initial capture will concentrate the antibody molecules (including aggregates) and the concentrated product can then be fractionated using PVDF membranes for analyzing the aggregates individually.
2. Since mAb EG2 contains significant amount of dimer, trimer and tetramers there is a good opportunity to study their characteristics. The resolution offered for EG2 aggregates by PVDF membranes was sufficient enough to fractionate and collect each aggregate component individually which can prove helpful for further processing of these individual fractions. EG2 from camelid has shown good thermal and chemical stability. So the mAb EG2 used in this work can also be tested for its stability. Conventional methods of unfolding/refolding performed using strong denaturants can be used with each of the aggregate fractions to solubilize and renature them. This will help in understanding the stability limits for these aggregates. Differential Scanning Calorimetry (DSC) studies are often performed to study the thermal stability and investigate the denaturation temperature for mAbs. Similar study can also be performed with these EG2 aggregates to find out the effect on each aggregate component. mAb aggregation

leads to changes in the secondary structure. There are numerous ways to monitor these changes including techniques like Circular Dichroism (CD) and fluorescence measurements which can also be used to study structural changes in mAb EG2 and aggregates. Thermal cycling technique was shown to effectively reduce aggregate contents in IgG1 type antibody molecules. This technique can be used to study the extent of disaggregation for each aggregate fraction.

The thermal cycling technique can potentially be used for the following studies:

1. Inclusion bodies expressed in prokaryotic cells are known to form aggregates due to overexpression of the protein. These inclusion bodies are formed due to the presence of non-native monomers. Our thermal cycling technique can potentially be used to disaggregate and obtain the individual protein chain in its native state since thermal cycling results into breaking aggregates to release individual protein chains and also causes renaturation.

2. The effect of rapid heating/cooling can also be monitored by ANS binding studies. ANS molecules bind to the hydrophobic regions of a protein molecule. During thermal cycling changes occur in the secondary structure of the protein chains. The fluorescence signals obtained from ANS on protein unfolding/refolding due to rapid heating/cooling can be closely mmonitored by using this technique.

3. Antigen binding studies using ELISA technique can also be carried out with thermal cycling. Antigen can be allowed to bind specific mAb and the complex can then be subjected to thermal cycling to monitor the strength of antigen-antibody bond and also to monitor whether the Fab region undergoes denaturation even when bound to antigen.