ANTIBODY PURIFICATION WITH THIOPHILIC AFFINITY CHROMATOGRAPHY

TİYOFİLİK AFİNİTE KROMATOGRAFİSİ İLE ANTİBADİ SAFLAŞTIRILMASI

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This is to certify that we have read this thesis and that our opinion it is fully adequate in scope and quality, a thesis for degree of Master of Science in Chemistry.

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ABSTRACT

Antibodies are glycoproteins which are 150 kDa in molecular weight. They are becoming a dominant protein class for various human therapeutical applications and they represent the largest number of molecules in clinical trials today.

Several affinity and non-affinity chromatographic methods were employed for isolation and purification of antibodies. One popular method is Thiophilic Affinity Chromatography (TAC). TAC is based on the interaction between antibodies and sulfur containing ligand immobilized on a suitable support at a high lyotropic salt content. Elution is performed by reducing the salt concentration. The effectiveness against different classes of antibodies, the easiness of preparation, cheapness and mild conditions are the advantages of the method. The choice of matrix in cryogel form introduces several advantages especially in column studies. The low back pressure and the mass transfer by convection are due to interconnected macropores. These gel matrices of monomers or precursors of polymers which are prepared in partially frozen solvents are used in various biological applications owing to their osmotic, chemical and mechanical features.

The presented study involves the synthesis of poly(2-hydroxy ethyl methacrylate), P(HEMA) cryogel membranes and poly(2-hydroxy ethyl methacrylate-ethylene glycol dimethacrylate), P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes. The resulting membranes were activated with divinylsulfone (DVS), and the ligand, 2-mercaptoethanol was attached to these matrices. The IgG adsorption capacities of the resulting matrices were examined.

The surface area was 18.48 m^2/g for thiophilic P(HEMA) cryogel membranes, and 32.7 m^2/g for microbeads embedded P(HEMA) cryogel membranes. The inner structure and surface morphology were identified with SEM micrographs. The ligand content was 26.36 μmol/g polymer and 82.98 μmol/g polymer for P(HEMA)

and for microbeads embedded P(HEMA) cryogel membranes, respectively. The ligand attachment was also proved by FTIR spectra. The effects of parameters such as pH, initial concentration, temperature and salt type were investigated for adsorption of IgG from aqueous solutions in batch mode.

The adsorption capacity was 68.7 mg/g and 27.5 mg/g for particles embedded P(HEMA) and P(HEMA) cryogel membranes, respectively. The increase in IgG adsorption capacity with increasing Na_2SO_4salt concentration was in accordance with predictions based on Hofmeister series. No significant effect of temperature was recorded. 1M NaCl was treated as desorption agent. Following 2 h treatment, the desorption ratio was more than 95 % of the adsorbed IgG molecules. There was no remarkable decrease in adsorption capacity when adsorption-desorption cycle was repeated ten times, proving the reusability of the resulting matrices.
The best fit was obtained with Langmuir model for adsorption isotherms and pseudo-second-order model for kinetics.

The cryogel matrices were also successfully used for human IgG adsorption from plasma. The purity assayed by SDS-PAGE was 89 %. The adsorption capacities were 74.8 mg/g and 137.4 mg/g for 1:2 diluted (PBS) plasma samples for thiophilic P(HEMA), and thiophilic microparticles embedded P(HEMA) matrices.

Keywords: Cryogel, Thiophilic Affinity Chromatography, Immunoglobulin Purification, Bead Embedding.
Advisor: Prof. Dr. Serap ŞENEL, Hacettepe University, Chemistry Department.

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Monireh BAKHSHPOUR

ÖZ

Antibadiler (IgG) molekül ağırlığı 150 kDa olan glikoproteinlerdir. Tedavi amaçlı uygulamalarda yoğun olarak kullanılan bir protein türüdür ve bugün klinik denemelerde en çok kullanılan moleküllerdir.

Afinite ve afinite bazlı olmayan bir çok kromatografik yöntem antibadilerin izolasyon ve saflaştırılması için kullanılmıştır. Popüler bir yöntem Tiyofilik Afinite Kromatografisidir (TAK). TAK, yüksek liyotropik tuz varlığında uygun bir desteğe immobilize edilmiş kükürt içeren ligantla antibadiler arasındaki etkileşime dayalıdır. Elusyon tuz derişimini azaltarak gerçekleştirilir. Farklı antibadi sınıflarına karşı etkinlik, hazırlama kolaylığı, ucuzluk ve ılımlı koşullar yöntemin avantajlarıdır. Matriksin kriyojel formunda seçimi özellikle kolon çalışmalarında çeşitli avantajlar getirir. Düşük geri basınç ve konveksiyon ile kütle transferi, birbiriyle bağlantılı makro gözenekler nedeniyledir. Kısmen donmuş çözücülerde hazırlanan monomer veya polimer öncüllerinin jel matrisleri olan kriyojeller , ozmotik kimyasal ve mekanik özelliklerine bağlı olarak çeşitli biyolojik uygulamalarda kullanılırlar.

Sunulan çalışma poli(2-hidroksietilmetakrilat), P(HEMA) kriyojel ve poli(2-hidroksietilmetakrilat etilenglikoldimetakrilat), P(HEMA-EGDMA) mikro partiküller gömülü P(HEMA) kriyojel membranlarının sentezini içerir. Sentezlenen membranlar divinilsulfon (DVS) ile aktive edilmiş ve bu matrikslere 2-merkaptoetanol ligandı bağlanmıştır. Sentezlenen matrikslerin IgG adsorpsiyon kapasiteleri araştırılmıştır.

Tiyofilik P(HEMA) kriyojel membranları için yüzey alanı 18.48 m^2/g ve mikropartikül gömülü P(HEMA) kriyojel membranlar için 32.7 m^2/g dır. İç yapı ve yüzey morfolojisi SEM mikrograflarıyla aydınlatılmıştır. P(HEMA) ve mikropartikül gömülü P(HEMA) kriyojel membranları için ligand içerikleri sırasıyla 26.36 µmol/g polimer ve 32.98 µmol/g polimerdir. Ligand bağlanması, FTIR spektrumlarıyla da

kanıtlanmıştır. Sulu çözeltilerden IgG adsorpsiyonu için pH, başlangıç derişimi, sıcaklık ve tuz türü gibi parametrelerin etkisi kesikli düzende araştırılmıştır. Partikül gömülü P(HEMA) ve P(HEMA) kriyojel membranlar için adsorpsiyon kapasitesi 68.7 mg/g ve 27.5 mg/g dır. Artan Na_2SO_4 tuz derişimi ile IgG adsorpsiyon kapasitesindeki artış Hofmeister serisine dayalı öngörülerle uyum içindedir. 1M NaCl desorpsiyon ajanı olarak kullanılmıştır, 2 saatlik işlem süresini takiben adsorplanan IgG moleküllerinin % 95 den fazlası desorbe olmuştur. Adsorpsiyon-desorpsiyon çevrimi 10 kez tekrarlandığında sentezlenen matrikslerin tekrar kullanılabilirliğini kanıtlayacak şekilde adsorpsiyon kapasitesinde kayda değer bir azalma yoktur. Sıcaklığın adsorpsiyona belirgin bir etkisi kaydedilmemiştir.

Adsorpsiyon izotermleri ve kinetiği için en iyi uyum, sırasıyla Langmuir modeli ve pseudo ikinci derece mode ilel sağlanmıştır.

Kriyojel matriksleri, plazmadan, insan IgG adsorpsiyonu için başarıyla kulanılmıştır. SDS-PAGE ile belirlenen saflık 89 % dır. Tiyofilik P(HEMA) ve tiyofilik partikül gömülü P(HEMA) matrikslerin 1:2 seyreltilmiş (PBS) plazma örnekleri için IgG adsorpsiyon kapasiteleri 74.8 mg/g ve 137.4 mg/g dır.

Anahtar Kelimeler: Kriyojel, Tiyofilik Afinite Koromatografi, İmmunoglobülin Saflaştırılması, Partikül Gömme

Danışman: Prof. Dr. Serap ŞENEL, Hacettepe Üniversitesi, Kimya Bölümü.

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1. INTRODUCTION

The number of antibody-based therapeutics approved by Food and Drug Administration (FDA) has increased in recent years. The total market for therapeutic monoclonal antibodies is $ 48 billion worldwide in 2009 (Knol .google-com/k/krishan-magfon/topten-antibodies-/143-cached). Large doses of monoclonal antibodies are used in therapy-up to several grams of active pharmaceutical ingredient per patient. The main function of these therapeutic antibodies is to block cell receptor sites and the active sites of other proteins in the body. To perform these activities in a quick and effective way, a large excess of the antibody molecules is required. Therefore, homogeneous antibody preparation is needed. There is also interest in polyclonal derived therapeutics. Polyclonal antibodies are considered more efficacious as they can bind to multiple epitopes on the disease-causing agent (Subramanian, 2004). The number of steps for purification is greater for in-vivo applications than ex-vivo ones due to stringent purity standards.

Commercial monoclonal antibody productions are in vivo cultivation as ascites in mouse or rabbit and in vitro cell culture in flasks or bioreactors (Nilsang et al. 2007). The antibodies may be isolated from body fluids of immunized animal or human beings, hybridoma, or bacterial cells. The complexity of the antibody molecules and the capability of the humoral immune system to produce an unlimited number of different antibodies are difficulties for purification (Huse et al. 2002). On the other hand, they tolerate relatively harsh treatments (Aldington and Bonneriea, 2007).

The crude source is subjected to a combination of different processes such as precipitation, filtration and chromatography. Ammonium sulfate precipitation followed by size-exclusion chromatography is the least expensive option. The most widely used technique is protein A chromatography. There are several non-affinity and affinity chromatographic methods for purification. But these methods lack the selectivity of protein A. Generally, 1 mL of ascites should yield 1 to 4 mg of purified antibody and 1 mL of monoclonal antibody supernatant should yield 0.5 to 50 µg of purified product (Andrew and Titus; 2000). Among the several chromatographic methods, Thiophilic Affinity Chromatography-using sulfone-

thioether ligand - Protein A mimetic ligand - has been used for non-covalent immobilization and purification of antibodies.

In this study, immunoglobulin G (IgG) purification was investigated by 2-mercaptoethanol immobilized 2-hydroxyethylmethacrylate (HEMA)-based cryogels. Micron sized P(HEMA-EGDMA) particles were embedded into cryogels and also tried to improve adsorption characteristics.

2. GENERAL INFORMATION

The choice of matrix to which a specific ligand is covalently bound is highly important in affinity chromatography. An ideal matrix should be insoluble and hydrophilic, mechanically strong, easy for derivatization and immobilization of ligand, show minimal amount of non-specific binding properties and be macroporous for better binding kinetics (Ngo et al. 2007). Naturally occurring polysaccharide based gels such as chemically cross-linked agarose, dextran and cellulose are used as matrices. (Cutler. 2004).Inorganic supports consisting of pure SiO_2, TiO_2, Al_2O_3 or composites of them and hydroxyapatite have been used as well (Ngo. 1993). The lack of chemical stability at alkaline conditions and small pore size are the disadvantages of inorganic and dextran or cellulose-based media, respectively. Organic polymers like vinyl polymers, polyacrylamide, polyvinylstyrene, and polyvinylalcohols are also widely used as stationary phases (Ho, et al. 2003).

Porous particulate and monolith forms of these matrices have the advantages of low back-pressure and the ability to transport the solutes by convection, respectively. Several activation and coupling chemistries are used for ligand coupling. Cyanogen bromide, oxirans, carbonyldiimidazole, sulfonyl chloride, periodate, glutaraldehyde and fluoromethyl pyridinium sulfone are common activation agents (Roe, 2001). For affinity purification of antibodies, macromolecular affinity ligands such as protein A, protein G, protein L or pseudo specific affinity ligands (Protein A mimetic ligands) are coupled to the matrix.

2.1. Cryogels

Cryogels are macroporous polymeric gels, synthesized by cryotropic gelation. The basic steps in synthesis are moderate freezing, storing in a frozen state and subsequent thawing of solutions or colloidal dispersions containing monomer or polymeric precursors (Lozinsky, 2002). Cryogels can be obtained through the formation of both physically and covalently cross-linked heterogeneous polymer networks. The initial system should have the capability of forming a gel. The basic requirement is freezing of the low molecular weight liquid present in the initial

system. The polymer gel phase can be formed in one of the basic steps during cryogenic treatment.

Cryotropic gelation and concominant chemical reactions are liquid phase processes occurring in the non-frozen liquid microphase of moderately frozen (i.e. not lower than by several tens of degrees from the freezing point of the pure solvent) specimens (Lozinsky, 2008). Cryotropic gelation can take place in both aqueous and organic frozen media provided that the solvent used and the conditions of cooling ensure crystallization. The general scheme of cryotropic gelation is shown in Figure 2.1.

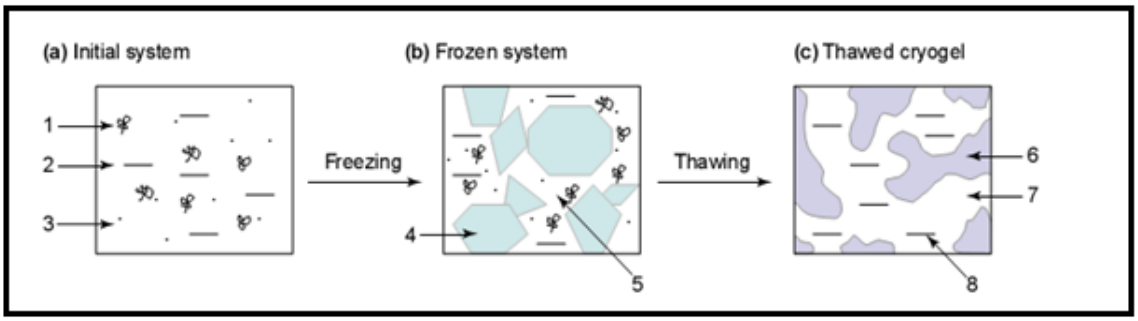


Figure. 2.1. Principal scheme for the formation of the polymeric cryogels:1, high-molecular-weight precursor; 2, solvent; 3, low-molecular-weight precursors or soluble substances; 4, polycrystals of the frozen solvent; 5, unfrozen liquid microphase (ULMP); 6, polymeric framework of the cryogel (gel walls of macropores); 7, macropores; 8, defrosted solvent (Adapted from Lozinsky, 2008).

The properties of the cryogels are determined by several factors such as the temperature of the cryogenic process, the nature of the solvent, the type and concentration of gel-forming reagents, and the duration of storage in the frozen state. One of the principal differences between cryogels and other macroporous materials with pores of similar sizes is that the cryogels possess a tissue-like elasticity and withstand extensive deformations without being destroyed (Plieva, et al. 2008). Cryogels may be formed upon polymerization or polycondensation of suitable monomers in frozen solutions induced by chemical initiators or radiation (Lozinsky, 2002). Another possibility is the formation of cryogels in solvent-

polymer-cross-linking agent systems. If the solvent-polymer system has an upper critical solution temperature with a polymer concentration above the critical point and the rate of material solidification on cooling is high, non-covalent physical gels are produced simply by cooling (Lozinsky, 1998).

In all cases, the critical concentration of gel formation is reduced during gelation in a frozen system. It is also possible to apply cryogenic treatment for polyelectrolytes and to prepare ionic or coordination polymer materials possessing macroporous morphology (Mattiasson, et al, 2010). Only the precursors of heat-induced gels cannot be used for the preparation of cryogels. The classification of gel forming systems is given in Figure 2.2.

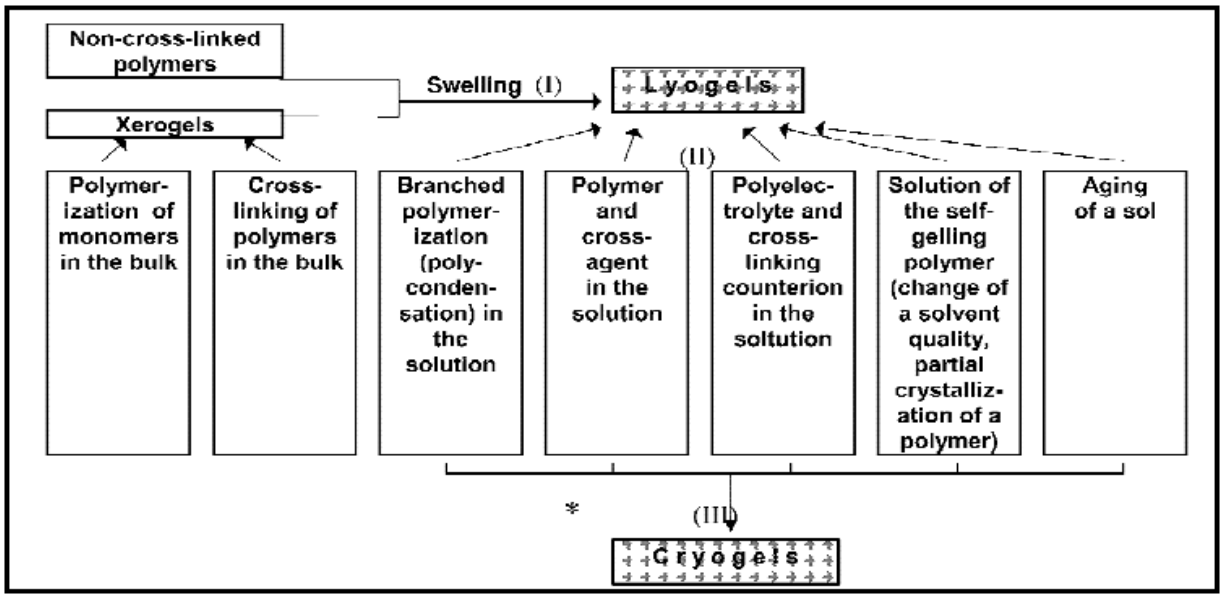


Figure. 2.2. Classification of gel forming systems (Adapted from V.I.Lozinsky, et al, 2003).

In most cases, wide pores in cryogels are interconnected, because during freezing of the initial solution of the gel-forming precursors each crystal of the solvent grows until it begins to contact with the facet of the adjacent crystal. Pore dimension changes between tenth fractions of micrometer to hundreds of micrometer. So, convective mass transfer of particles, soluble substances, and particles with a size of whole cells becomes possible.

In moderately-frozen solutions, part of the solvent is unfrozen. This unfrozen liquid microphase contains nearly all dissolved components present in the initial solution. High concentrations of dissolved substances accelerate chemical reactions. Melting of frozen solvent crystals leaves cavities in the cryogel, which become filled with liquid solvent. Solvent acts like a porogen. Surface tension at the interface of gel and the liquid causes the shape of the initially sharply angled cavities to become rounded. The lower the freezing temperature, the larger amount of small solvent crystals form and hence the smaller the pores. Most of the water present in spongy cryogels is capillary bound and can be removed mechanically by squeezing it out.

There are many potential uses of cryogels in biotechnological and biomedical applications such as the use of carriers for enzyme immobilization, microbial cell and antibody immobilization, gel bases for bioaffinity sorbents and collagen materials for medical purposes (Lozinsky, et al. 2003: Lozinsky, et al. 2002; Savina, et al. 2007).

2.2. Plasma Proteins

Blood constitutes ~8% of the total body weight, the major functions being distribution of oxygen and essential nutrients to body cells and tissues. It is also crucial for removing waste products from cells and tissues. Responsibility for clotting, immune system responses, regulation of body temperature are the other important functions. Plasma is the fluid medium of blood to which blood cells and other components are suspended. Plasma contains 0.1-0.2 % water, albumin, and globulin, hormones, clotting factors, nutrients, salts and nitrogenous waste products. When clotting factors are removed, serum is obtained. Proteins account for 7%of plasma. Hundreds of different proteins are present in plasma. The important proteins are albumin, globulins and fibrinogen. Almost all plasma proteins are glycoproteins. The main functions of plasma proteins are maintaining fluid and electrolyte balance in the blood and regulation of the body’s osmotic pressure. Albumin serves an osmotic function.

Fibrinogen leads to the formation of blood clots. Immunoglobulins mark the invading bacteria and other microorganisms for destruction by other immune cells. Globulin protein is subdivided into four categories: gamma globulin, alpha-1 globulin, alpha-2 globulin, and beta. The alpha and beta globulins primarily act as transporters for fat soluble vitamins, hormones and lipids. They are synthesized in the liver, gamma globulins are created by the lymphoid tissue.

2.3. Immunoglobulins

Antibodies or immunoglobulins are glycoproteins made up of light (L~ 25 kDa) and heavy (H~50 kDa) polypeptide chains linked by disulfide bonds, the basic role being in the functioning and regulation of the immune system of all mammals. The simplest molecule has a Y-shape and consists of four polypeptide chains. The structure is shown in Figure 2.3. L and H chains are subdivided into variable and constant regions which are composed of 3D folded, repeated segments called domains. An L-chain consists of one variable (VL) and one constant (CL) domain. Most H chains consist of one variable (VH) and three constant (CH) domains. Each domain is approximately 110 amino acids long. The variable regions are responsible for antigen binding, whereas the constant regions are responsible for various biological functions. Based on the electrophoretic migration rate, three globulins are: alpha, beta and gamma.

Five different H chains (α, δ, ε, γ, μ) and two different L chains (K and λ) determine the immunoglobulin classes. There are five classes of antibodies: IgG, IgM, IgA, IgD and IgE. There are 4 subclasses for IgG, IgG1-IgG4, based on antigenic differences in the H chains and on the number and location of disulfide bonds, (Table 2.1).The most common sources of specific antibodies are plasma, serum, ascites fluid and cell culture supernatant. The major contaminants are albumin, transferrin and non-specific endogenous antibodies. Monoclonal antibodies are identical since they were produced by one type of immune cell (B cell), all clones of a single parent cell. Polyclonal antibodies are from multiple clones of B. lymphocytes.

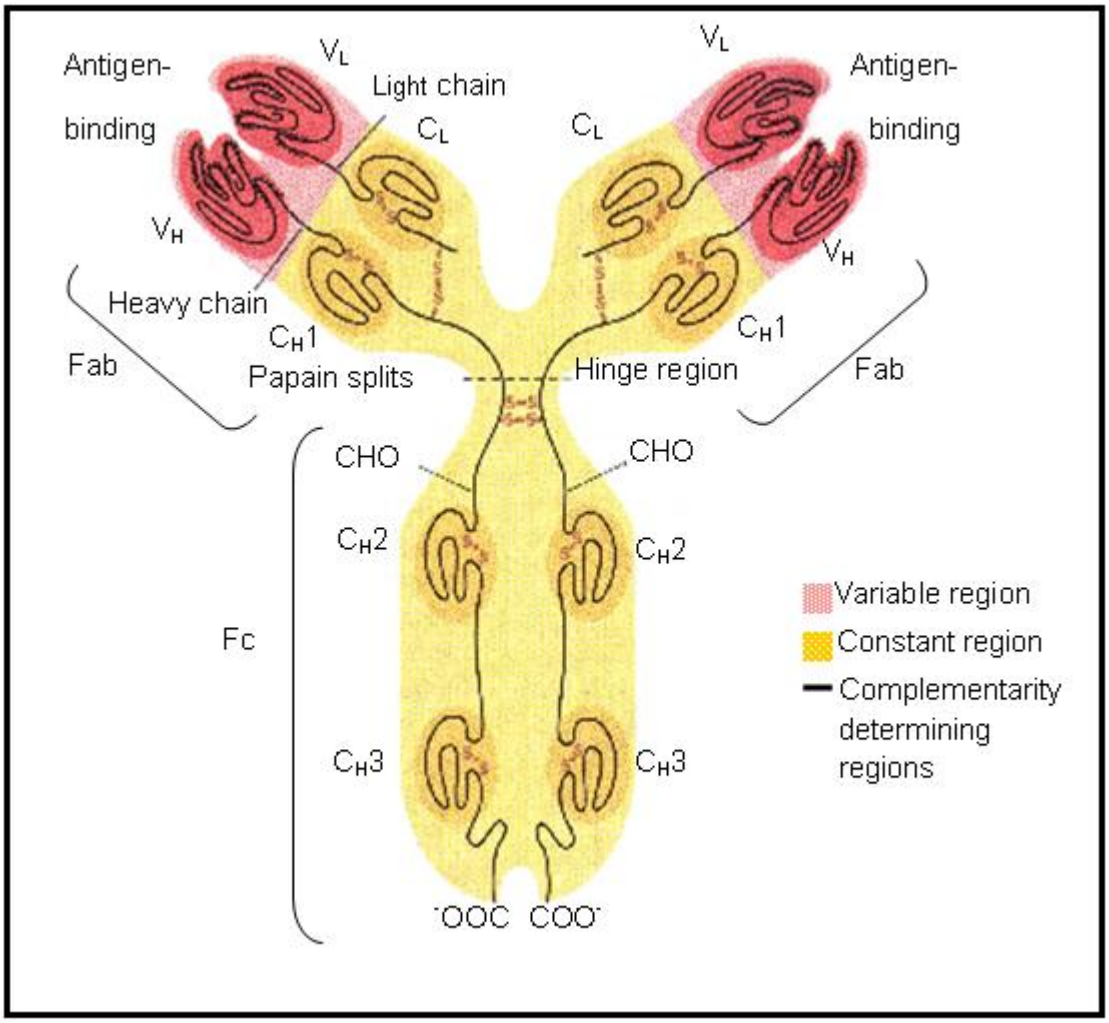


Figure. 2.3. Structure of an antibody molecule; the Y-shaped IgG consists of two light chains and two heavy chains. Each heavy chain consists of a variable region and a constant region that is divided into three domains: CH1, CH2, CH3. The CH2 domain contains the complement binding site and the CH2 domain is the attachment site of IgG to receptors on neutrophils and macrophages. The antigen binding site is formed by the variable regions of both the light and heavy chains.

Table. 2.1. Classes of Human Immunoglobulins.

Class	Heavy Chain	Light Chain	Molecular Mass (kD)
IgA	α	κ or λ	360-720
IgD	δ	κ or λ	160
IgE	ε	κ or λ	190
IgG[a]	γ	κ or λ	150
IgM	μ	κ or λ	950

a: IgG has four subclasses, IgG_1, IgG_2, IgG_3, IgG_4

Polyclonal antibodies are often raised by hyperimmunizing animals with antigens (Josic and Lim, 2001). The use of hybridoma technology allows the growth of clonal populations of cells secreting antibodies with a designed specificity: monoclonal antibodies. Fab or $F(ab)_2$ fragments and the Fc part which consists of the constant region are obtained by limited proteolytic degradation. Each kind of antibody is produced by a special clone of plasma cells after their differentiation from B-lymphocytes. After stimulation by the antigen, these clones proliferate and produce considerable amounts of the specific antibody.

In vitro diagnostic procedures, like enzyme-linked immune sorbent assay (ELISA), radioimmunoassay (RIA), or special blot techniques with radiolabeled antigens or antibodies allow the detection of minimal amounts of proteins, nucleic acids, lipids and complexes of carbohydrates in various media (Huse, et al. 2002).Therapeutically antibodies have been used in vivo for passive immunization in case of viral and bacterial infections or contamination with biological and chemical toxins or transplant rejection therapies (Beryer, et al. 2002; Phalipon, et al. 1995; Gosling, 2000; Hage, 1999). Radiolabeled monoclonal antibodies reactive with tumor-associated antigens can be targeted to malignant cells and deliver diagnostic isotopes selectively in vivo. Antibodies are also important tools for the purification of their antigens for their use as pharmaceuticals on an industrial scale. The use in purification of drugs and imaging the target are

important in clinical applications (www.authorstream.com;-/a SGuest 77764-70814-monoclonal-antibody-ppt/-Onbellek).

2.4. Future of the Antibody Purification

Methods of synthesing monoclonal antibodies are heading towards expanding genetical applications. Nowadays, monoclonal antibodies are synthesing through producing antibodies in animals and then imposing these exchangeable parts of immunoglobulines into the order of IgG genes. At the same time, human antibodies can be produced as bacterial antigens (Vaughan et al., 1996). For some applications it is enough to produce only parts of monoclonal antibody and it can be even more preferable. In these cases the application of genetic arrangements are preferred (Zhu et al., 1996).

For instance using $F(ab)_2$ fragment in ELISA can reduce non-specific reactions (Yurov et al., 1994). Radioisotopes of Fab and $F(ab)_2$ fragments are used in curing tumores. Because, in comparison with a complete molecule of immunoglobulin, it can diffuse into the tumors and be removed more easily (Proudfoot et al., 1992).Furthermore, biospecific monoclonal antibodies make a bridge between sitotoxic hole and tumor hole and destroy the tumor (De Jonge et al., 1995).

2.5. Purification of Antibodies

Monoclonal antibodies have long development time, are expensive to produce and often have a more limited range of uses. Polyclonal antibodies are faster and cheaper to develop, often give higher sensitivity by recognizing multiple epitopes on the antigen and can be more versatile. But, the multiple specificity results in increased risk of cross-reactivity with irrelevant proteins. In plasma fractionation precipitation is still the method of choice. Precipitation agents may be salts (e.g. ammonium sulfate), organic solvents (e.g. acetone or ethanol) or an organic polymer (e.g. polyethylene glycol).

Water molecules grouped around the hydrophobic patch of protein molecules form a hydration shell. This shell is disrupted by salts or displaced by organic solvent molecules. Aggregation and precipitation occur due to hydrophobic interaction in

the case of salts. Electrostatic and van der Waals forces are responsible for precipitation in the case of organic polymers and solvents. Cohn fractionation (Kistler and Nitschmann, 1962), involves fractional precipitation with ethanol at temperatures near or below 0°C, with careful control of pH and ionic strength and additions of certain bivalent metal ions, especially Zn^{2+}. Fraction 1 contains fibrinogen; 2, gama-globulins; 3, beta-globulins; 4, alfa-globulin; 5, albumin; 6, glycoproteins. Affinity precipitation is performed by smart polymers or affinity macroligands. The requirement to customize the macroligand with each target antigen is a limiting factor. A more convenient alternative is to use cationic or anionic polyelectrolytes (Dainiak, et al., 1999). The formation of protein-polyelectrolyte complexes is managed by changing the solution and polyelectrolyte. Non-chromatographic techniques such as aqueous two-phase systems, magnetic separation, and preparative electrophoresis are other options for purification (Denizli, 2001; Matejtschuk, 1997).

2.5.1. Non-Affinity Chromatographic Methods

Size Exculusion Chromatography (SEC), Ion-exchange Chromatography (IEC), Hydroxyapatite Chromatography (HAC), Hydrophobic Interaction Chromatography (HIC), and Reversed Phase (RP) Chromatography are well-known options.

2.5.1.1. Size Exclusion Chromatography (SEC)

It is widely used for laboratory scale purification of antibodies. Separation is based on the size of the molecules. The medium is typically porous beads with the size of the pore determining the exclusion limit of the column. The elution mode is isocratic. Low capacity, low resolution are major limitations. It is usually combined with other chromatographic steps following precipitation. The method is useful in purifying antibodies of the IgM isotype (Josic and Lim, 2001).

2.5.1.2. Ion Exchange Chromatography (IEC)

The method is based on the electrostatic attraction between opposite charges on the support and the antibody molecules. Two charge modes (anion and cation exchangers) with several ionic forms (strong and weak) are available for resins. The columns are loaded in low ionic strength buffer. The elution is performed with either step or linear gradients with buffers of higher ionic strength. Since antibody molecules have a more basic isoelectric point than the majority of other serum or contaminating proteins, IEC is useful in purifying antibodies regardless of isotype (Deschamps, et al. 1985). But, IEC resin needs to be explored and determined experimentally on an individual basis due to the change in isoelectric points of antibodies.

2.5.1.3. Hydroxyapatite Chromatography (HAC)

The interaction of antibodies with hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$ is based on attraction of the amino and carboxyl groups of the molecules to the phosphate and calcium sites of matrix (Josic and Lim.2001). Phosphate sites provide ionic interactions and calcium sites provide affinity. HAC is useful for fractionating antibodies regardless of species, class or subclass. The samples are usually applied in 5-50 mM phosphate buffer at pH=6.8 and the antibodies are eluted by a gradient elution of phosphate buffer up to 0.5 M at pH=6.8. The crystalline structure degrades rapidly below pH=5.

2.5.1.4. Hydrophobic Interaction Chromatography (HIC)

HIC involves retention on the basis of hydrophobic interaction. HIC is water-eluted hydrophobic interaction chromatography. Reversed phase (RP) is solvent-eluted form. RP is usually chosen for purification of peptides and small proteins. HIC is used for large proteins and proteins with subunits, such as IgG.

Loading in HIC is done in a high concentration of a cosmotropic salt and elution is achieved with a gradient down to lower ionic strength buffer (http=//www.pall.com/laboratory-4561.asp). Molecules are adsorbed on

hydrophobic groups (i.e. phenyl, butyl), covalently bonded to matrix and the adsorption is enhanced by the presence of high salt concentration. Since albumin and transferrin are usually unretained under antibody binding conditions, HIC becomes a suitable first purification step. In combination with IEC, complete removal of the non-specific antibodies can be achieved.

2.5.2. Affinity Chromatographic Methods

Affinity chromatographic techniques separate molecules on their differing affinities for a ligand. This may be achieved by a number of traditional techniques such as gel permeation chromatography, high performance liquid chromatography, chromatofocusing, electrophoresis, centrifugation, etc., in that the process relies on the differences in the physical properties (e.g., size, charge and hydrophobicity) of molecules to be treated. This is due to the condition of surface geometry and special arrangement of the ligand and the binding site of the biomolecule . Affinity chromatography enables the separation of almost any biomolecule on the basis of its biological function or individual chemical structure. Examples can be found from all areas of structural and physiological biochemistry, such as in multimolecular assemblies, effector-receptor interactions, DNA-protein interactions, and antigen-antibody binding. Schematic representation of bioaffinity is shown in Figure 2.4. Affinity chromatography demonstrated in this figure is based on the simple principle that every biomolecule usually recognizes another natural or artificial molecule. A wide variety of ligands may be covalently attached to an inert support matrix, and subsequently packed into a chromatographic column.

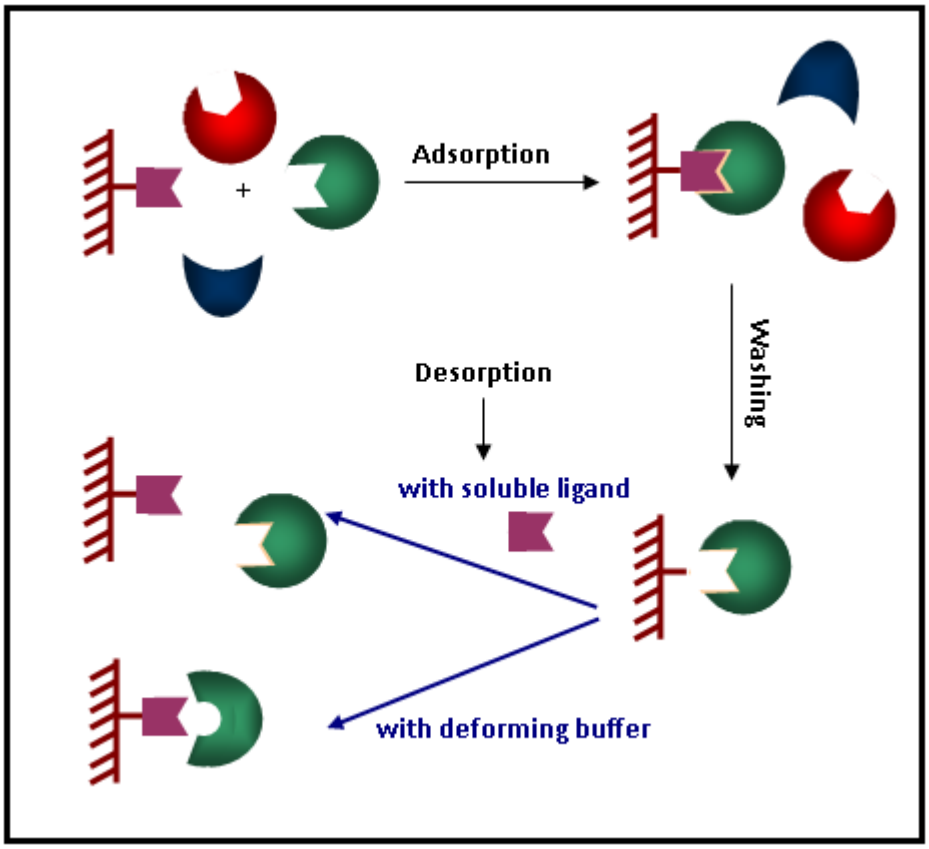


Figure. 2.4. Schematic representation of the main steps in affinity chromatography.

2.5.2.1. Immobilized Antigen Affinity Chromatography

The biospecific interaction between antigen and antibody molecules is the basis of the method (Figure 2.5). Purification of monospecific antibodies from a mixture of polyclonal antibodies is achieved (Josic and Lim, 2001). The need of large quantities of pure antigen and elution conditions are disadvantages of the method.

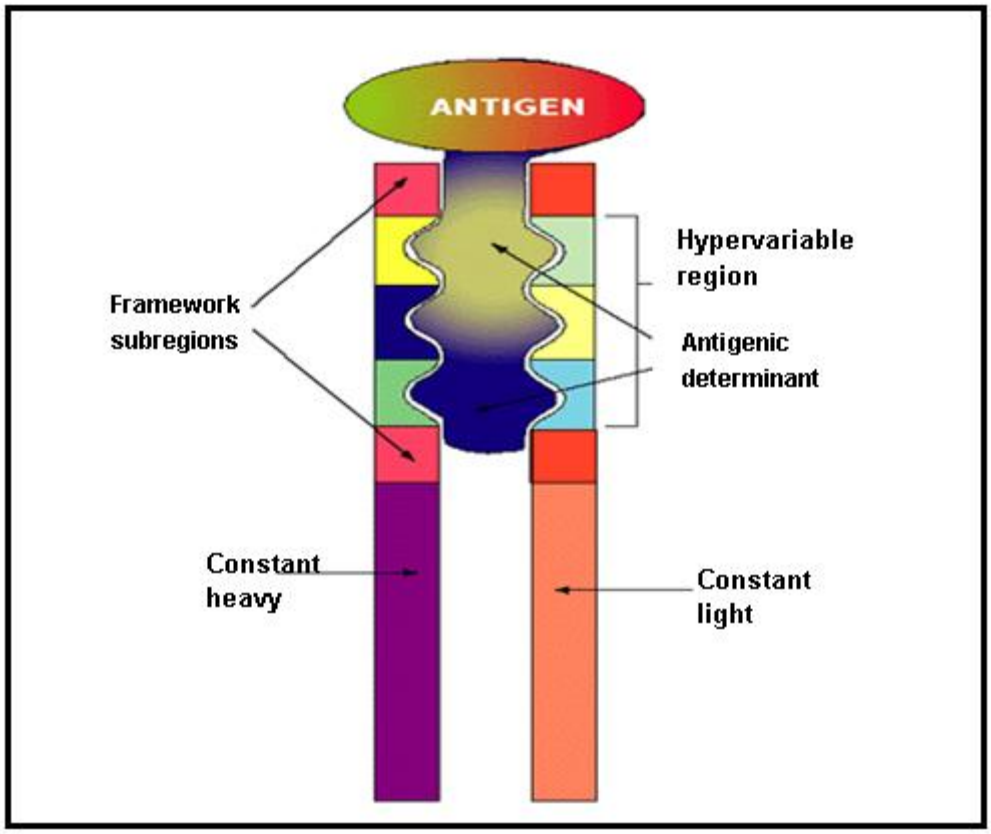


Figure. 2.5. Immobilized Antigen.

2.5.2.2. Immobilized Metal Affinity Chromatoghraphy

Interaction of specific amino acid side chains, particularly those of histidine, cystein and tryptophan, with transition metal ions that are bound to the chelating groups of a chromatographic resin result in retention of immunoglobulins. Immobilized Cu^{2+}, Ni^{2+} and Zn^{2+} ions have been shown to be effective in retaining the His-tagged antibody fragments with up to 90% purity on elution. The most common ligand is iminodiacetic acid (IDA) which is tridentated. The method is widely used for purification of native and recombinant proteins (Martins, et al. 2006).

2.5.2.3. Histidine Ligand Affinity Chromatography

In immobilized form, histidine can interact with antibodies through its amino, carboxyl or imidazole groups by hydrophobic and charge-induced interactions. The efficiency of the method was also proven for the purification of human IgG from

plasma (Roque, et al.2007). Peptide and DNA-degrading activities within variable region of natural antibodies are relevant to some autoimmune disorders. Using this type of chromatography, intact antibodies with peptidase activities with a recovery of high-purity IgG directly from the sera of patients are managed (Huse, et al. 2002).

2.5.2.4. Lectin Affinity Chromatography

Lectins are glycoproteins of non-immune origin, isolated from plants and animals, capable of binding carbohydrates. Jacalin is a galactose-binding lectin that can be used to purify human IgA1 (Wilson and Walker, 2000). Mannan-binding protein is mannose and N-acetyl glucose-amine- binding lectin found in mammalian sera. When coupled to an inert support, it can be used to purify mouse IgM.

Glycoproteins bind to columns at pH values close to neutrality. Elution is performed by inclusion of a competing ligand or changing the pH of the eluting buffer (Walsh, et al. 2007).

2.5.2.5. Anti-Antibodies As Affinity Ligands

Proteins can also be used as ligands targeting the constant domains of both heavy and light chains of antibodies. Especially, for the isolation of rat monoclonal antibodies, resistant to Protein A and Protein G purification, anti-rat-IgG light chain antibodies bonded to agarose has been used (Huse, et al. 2002).

2.5.2.6. Immunoaffinity Chromatography

Adsorbents also can be defined as materials which are capable of binding substances in biological fluids particularly from blood or plasma. The base of immune adsorption is based on affinity adsorption and chromatographic methods (Figure 2.6). A ligate, e.g., a pathogenic antibody or immune complex is selectively and reversibly adsorbed to an immobilized ligand.

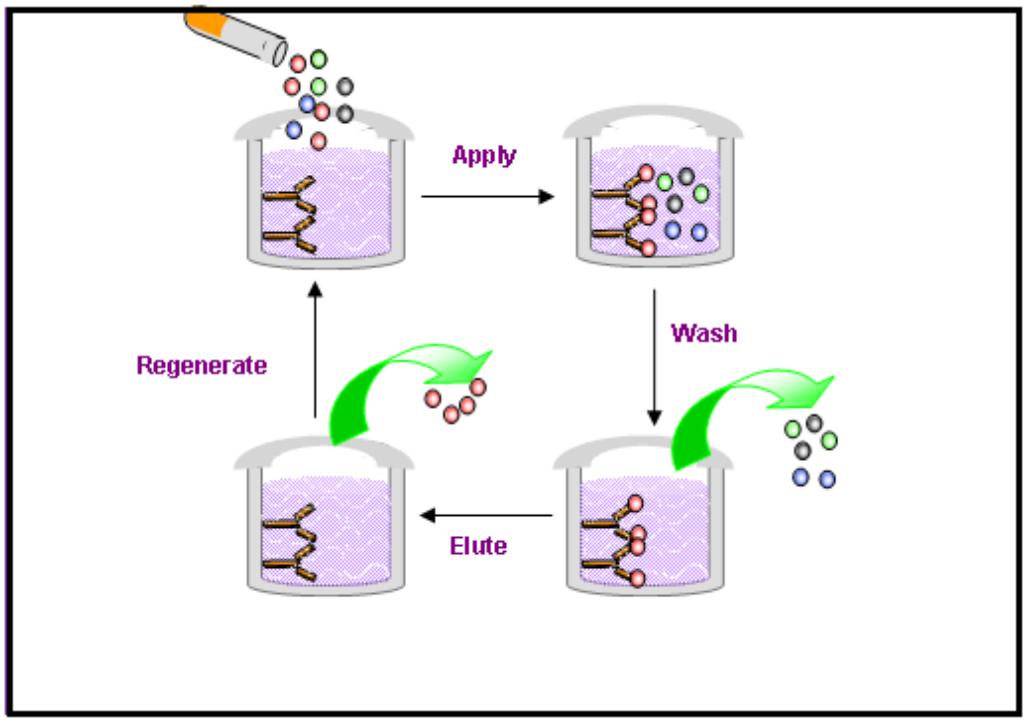


Figure. 2.6. Operating scheme for immunoaffinity chromatography.

In order to apply immunosorption techniques more practically, three fields may be distinguished (Kirstiansen, 1978):

1. Removing of unwanted antigens from sample solutions by batch method,
2. Enriching or isolating antigen and antibody populations, in relatively abundant supply, by batch or column procedures,
3. High recoveries of the fully active, precious antigens and antibodies on adsorbents of high stability which can be regenerated many times without losing their activity.

In addition to this, immunoaffinity chromatography represents a sensitive tool for detection of immunoactive substances.

2.5.2.7. Protein A Affinity Chromatography

Protein A (Cowan strain I) is a wall component of *staphylococcus oueveus* that binds the Fc portion of IgG specifically. Most mammalian and mouse/rat immunoglobulins bind well to Protein A (Hober, et al. 2007). Protein A consists of a single polypeptide chain containing five highly homologous antibody-binding domains. It is stable over a wide range of pH (pH; 2-11). Total IgG purity of 95 %

can be achieved in a single step but non-specific antibodies co-elute with the target IgG. Contamination can be reduced with linear gradient pH elution but it cannot be eliminated completely.

α_2-macroglobulin and kinogen also bind to Protein A causing contamination. Uncharged and hydrophobic histidine residues at alkaline pH strength the interaction between the target and matrix. Harsh elution conditions causing the loss in antibody activity, leaching and cost of ligand, the possibility of degradation of ligand by proteases in the feedstocks are problems.

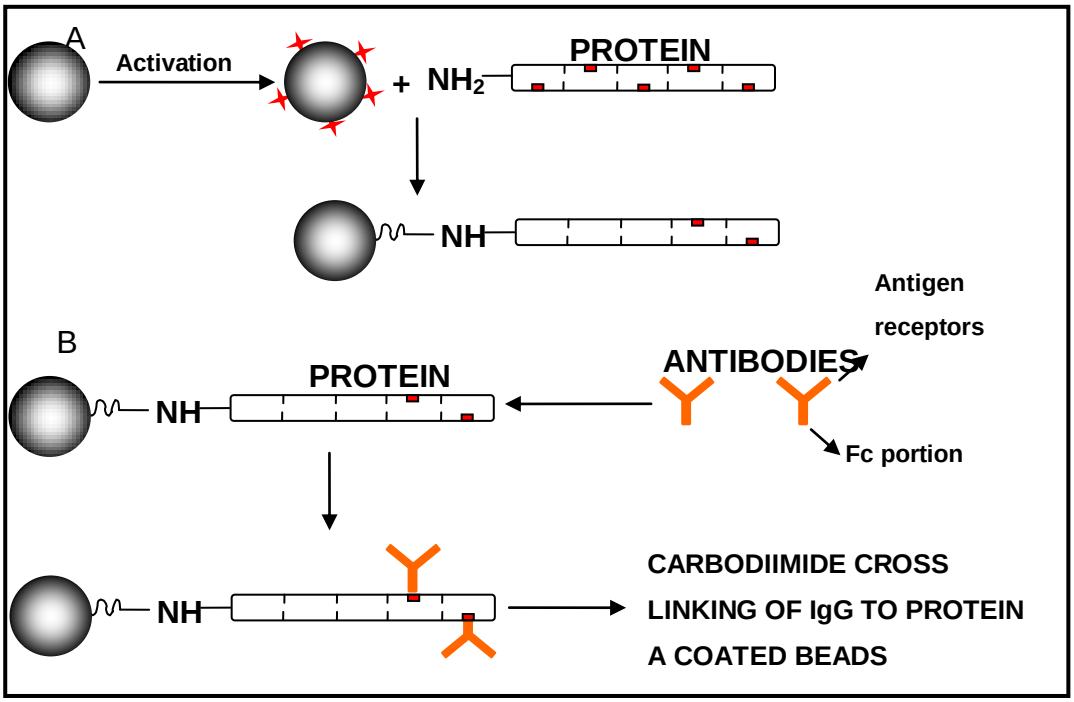


Figure. 2.7. Diagrammatic representation of the process for binding antibodies to Protein A-coated beads. (A) Illustration of the chemistry used to couple Protein A to the activated/derivatized beads. (B) Mechanism of antibody binding to the Fc receptors on the Protein A coated beads.

2.5.2.8. Other Bacterial Ligands

Protein G is derived from group C and G *streptococci*. For monoclonal IgG_3 and mouse IgG_1 and immunoglobulins for goat, chicken and rat, protein G is preferred. Again, non-specific antibody contamination and ligand leaching are important

restrictions (Huse, et al, 2002). Another microbial protein, Protein L, isolated from *peptostreptococcus magnus* strains, interacts with the Fab portion of antibodies, specifically with K light chains without interfering with the antigen-binding site (Roque, et al, 2004). This protein allows a wider range of antibody classes and subclasses than Protein A or Protein G. It is also useful for purifying single chain variable fragments.

2.5.2.9. Thiophilic Adsorption Chromatography (TAC)

TAC is a group-specific purification method having an adsorption affinity towards immunoglobulins and α_2-macroglobulins and promoted by lyotropic salts. Desorption is achieved by lowering or removing the salt. The method was orijinally derived by Porath et al. (Hutchens and Porath, 1987). SH-containing ligands, originally 2-mercaptoethanol, are immobilized on divinylsulfone-activated matrices. The resulting matrix is given the name T-gel. The term thiophilic refers to an affinity for sulfone groups that lie in close proximity to thioether groups. The general formula for a T-gel matrix is as follows: (Matrix)-O-CH_2-CH_2-SO_2-CH_2-CH_2-S-CH_2-CH_2-OH.

Polyclonal as well as monoclonal antibodies have been purified by this technique. Other types of proteins such as horseradish peroxidase, allergens, glutathione peroxidase, can also be purified. (www.gbiosciences.com/../(Thiophilic. Adsorption)Kit.aspx). Thiophilic purification of antibody single-chain variable region (ScFv) molecules is also possible. Binding is almost independent of the antibody source and class. Aliphatic, aromatic and heteroaromatic ligands have been studied (Knudsen, et al, 1992). Binding occurs at high concentration of lyotropic salts. Sulfates and phosphates are mostly used in TAC. Interaction of the non-ionic sulfone-thioether ligand with appropriated acceptor sites on the protein surface is quite specific and results in the formation of pseudo S-S bonds.

Lyotropic salt allows the protein molecules to accumulate at the phase boundary, the protein into close proximity with the sulfone-thioether group where short-range forces are effective. An electron donor-acceptor or proton transfer mechanism may

be involved (Hutchens; Porath, 1987). Permanent sulfone dipole and the free electron pair of the thioether potentially act in concert as electron acceptor and donor site, respectively. A possible mechanism for interaction is given in Figure 2.8.

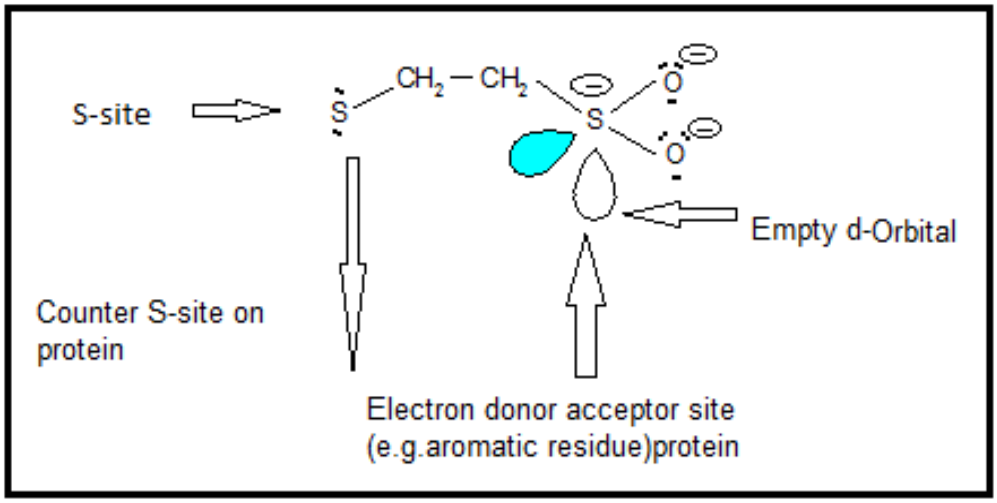


Figure. 2.8. Possible Interaction Mechanism (Hutchens; Porath,1987).

The sulfone grup and thioether sulfur (or other electron donor) cooperate to form some kind of ring structure by transfer of electrons or protons between the ligand site and the protein principally phenylalanine and trypthophan residues (Cutler, 2004). Mild conditions for elution at neutral pH, the low cost and stability of adsorbent, high recovery rate, selectivity for IgG, IgM, IgE and IgY are the main advantages of the method. The need for using a high concentration of salt is a disadvantage, since it tends to aggregate the immunoglobulins. Recently, a new technique, Hydrophobic Charge Induction Chromatography, does not require the high salt loadings by means of a new mercaptoheterocyclic ligand, (4-mercaptoethylpyridineligand) attached affinity matrix (Guerrier, et al, 2000).

The requirement for a thiophilic adsorbent is that it has a nucleophilic atom spaced three away from the sulfone group. The most effective is sulfur, but unprotonated nitrogen and weakly oxygen also show thiophilic effects (Hutchens, Porath, 1987). The inability to bind albumin under high salt conditions, the enhancement of adsorption capacity by lyotropic salts (i.e. $(NH_4)_2$ SO_4, not with NaCl), the

weakness of thiophilic interaction at higher temperatures are the distinctive properties of TAC, from HIC (Roque, et al, 2007).

2.6. Synthetic Mimic Ligands

These ligands are generally designed to improve the performance of natural binding ligands (i.e. Protein A). Several peptide-based ligands were synthesized screening of multimeric combinatorial peptide libraries. Non-peptidyl ligands were also treated for purification of antibodies. The multimeric peptide-TG 19318 binds IgG from different sources. It was also used for one-step purification of IgM, IgA, IgE and IgY (Roque, et al, 2004). A cyclic dimeric peptide-peptide H-was used for IgG purification from rat and mouse (Fassina, et al.2001).

Biomimetic dyes, triazine-based ligands, mixed-mode ligands were successfully used in applications (Agostino, et al, 2008). The ligand 22/8 (an artificial Protein A ligand) consists of two organic aromatic amines linked to a scaffold of cyanuric chloride and tried for human IgG purification from plasma (Kabir, 2002). Another triazine ligand, ligand 8/7(an artificial Protein L ligand) has the ability to bind to the Fab portion of antibodies (Roque, et al, 2004).

Lowe et al.designed a ligand, mimicking the key dipeptide Phe-132; Tyr-133 motif of Protein A to purify IgG by a triazine scaffold. The IgG adsorption capacity of the gel was 51.9 mg IgG/g-moist weight gel (Hober, et al. 2007).

Table. 2.2. Comparison of Natural Affinity Ligands with Synthetic Affinity Ligands.

	Natural affinity ligand	Synthetic peptidic affinity ligands	Synthetic non-peptidicaffinity ligands
Preparation	Produced by recombinant bacterial system; difficult purification	Chemically defined and characterized products; easily synthesized	Chemically defined and characterized products; easily synthesized
Specificity, elution	High, harsh (typically glycine, HCl pH 2-3)	Moderate to high mild	Moderate to high mild
Product contamination	Leaking; high toxicity; high immunogenicity	Potentially leaking; low toxicity; low immunogenicity	Non-leaking; low/non-toxic; low/non-immunogenecity
Capacity, reusability	Low (0.01-1%ligand utilization) poor biological and chemical stability	High (>10%ligand utilization) peptidic bonds subject to degradation	High (>10%ligand utilization) high stability; no fissile bonds
Economy	High cost	Low cost	Low cost

(Adapted from Roque, et al. 2004).

2.7. Adsorption IgG Capacities for Various Thiophilic Carriers

The adsorption capacities changing over a wide range were reported in literature for several matrix-ligand pairs.

Table. 2.3. IgG adsorption capacities for various thiophilic carriers. (Adapted from, Denizli, 2011).

Carrier	Ligand	Qmax	[R]
Agarose	Divinylsulphone	6.3 mg/mL	H. Qian, et al, 2009.
	2-Mercapto ethanol	6.9 mg/mL	
Sepharose CL-6B	4-(1H-imidazol-1-yl)aniline	59.0 mg/mL	J. Noel, et al, 1996.
Cellulose beads	2-Mercapto-5-benzimidazolesulfonic acid	30.0 mg/mL	P. Girot, et al, 2004.
Poly(ethylene vinyl alcohol)	Mercapto methyl imidazole	16.0 mg/mL	Y. Coffinier, et al, 2004.
	Mercapto purine	14.9 mg/mL	
	Mercapto nicotinic acid	10.3 mg/mL	
	Mercapto methyl pyrimidine	4.9 mg/mL	
Poly(ethylene vinyl alcohol)	DVA/2-Mercaptoethanol	3.2 mg/mL	Y. Coffinier, et al, 2002.
Cellulose beads	Mercapto ethyl pyridine	30.0 mg/mL	O. Pitiot, et al, 2002.
Sepharose	2-Mercapto ethanol	4.0 mg/mL	J. Hardouin, et al, 2007.
P(styrene-vinyl acetate)	2-Mercapto nicotinic acid	14.0 mg/mL	H. Qian, et al, 2009.
Agarose	Divinylsulphone	28.1 mg/mL	B. Nopper, et al, 1985.
Agarose	Divinylsulphone	150.0 mg/g	A. Lihme, et al, 1995.
Agarose	DVS/2-Amino pyridine	30-60 mg/mL	K.L. Knudsen, et al, 1992.
Silica	Mercapto thiazoline	25.0 mg/mL	A. Schwarz, et al, 1995.
Agarose	Mercapto thiazoline	30.0 mg/mL	
Sepharose	Sulfanyl ethylamine	55.6 mg/mL	S.J. Mountford, et al, 2011.

3. EXPERIMENTAL

3.1. Materials

Human immunoglobulin G (HIgG), divinylsulfone (DVS), 2-mercaptoethanol were obtained from Sigma (St Louis, USA). Hydroxyethylmethacrylate (HEMA) and ethyleneglycoldimethacrylate (EGDMA) were obtained from Fluka A.G. (Buchs, Switzerland).4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was supplied from Sigma (St Louis, USA). Poly(vinylalcohol) (PVAL; MW: 140.000, 98% hydrolyzed) was obtained from Aldrich Chem. Co. (USA). N,N,N',N'-tetra-methyl-ethylendiamine (TEMED), N,N'-methylene-bisacrylamide (MBAAm), ammonium persulfate (APS) and benzoyl peroxide (BPO) were obtained from Fluka A. G. (Switzerland). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system.

3.2. Synthesis of P(HEMA-EGDMA) Microbeads

Poly(2-hydroxy ethyl methacrylate-ethylene glycoldimethacrylate), [P(HEMA-EGDMA)] microbeads were prepared by a radical suspension polymerization technique (Denizli et.al., 1995). Toluene and EGDMA were included in the recipe as the diluent (as a pore former) and cross-linker, respectively. The monomer phase containing HEMA, EGDMA and benzoyl peroxide (BPO-initiator) was added to the dispersion medium within a laboratory type reactor (i.e., a two neck flask with a volume of 500 mL) provided with a blade type stirrer (Figure 3.1).

In order to produce beads of about < 63 μm in diameter and with a narrow size distribution, the HEMA/EGDMA ratio, the monomer phase/dispersion phase ratio, the amounts of EGDMA, BPO, and the agitation speed were optimized and the optimum polymerization conditions and the recipe were given in Table 3.1.

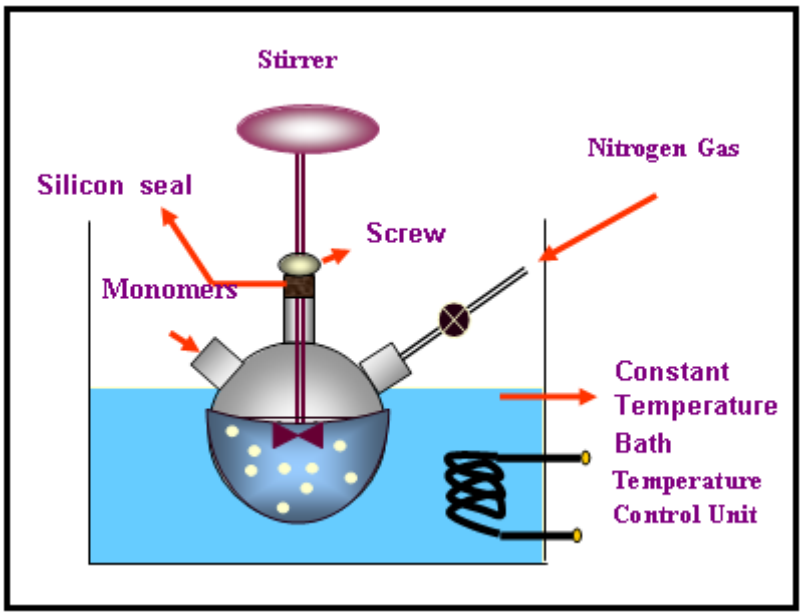


Figure. 3.1. Schematic representation of polymerization system used in the preparation of P(HEMA-EGDMA) microbeads.

Table. 3.1. Recipe and the polymerization conditions for the preparation of P(HEMA-EGDMA) microbeads.

Aqueous Dispersion Phase	Organic Phase
Distilled water: 50 mL	HEMA: 4.0 mL
PVAL: 0.2 g	EGDMA: 8.0 mL
Toluene: 12 mL	
BPO: 0.06 g	
Polymerization Conditions	
Reactor volume: 500 mL	
Stirring Rate: 600 rpm	
Temperature and Time: first at 65°C for 4 h, and then at 90°C for 2 h	

3.3. Preparation of P(HEMA) Cryogel Membranes

Preparation of P(HEMA) cryogel membranes is described below. Briefly, monomers (2.6 mL HEMA and 0.56 g MBAAm) were dissolved in deionised water (5 mL) and the mixture was degassed under vacuum for 5 min to eliminate soluble oxygen. The cryogel was produced by free radical polymerization initiated by

APS/TEMED. After adding APS (40 mg, 1% (w/v) of the total monomers), the solution was cooled in an ice bath for 2–3 min. TEMED (50 µL, 1% (w/v) of the total monomers) was added and the reaction mixture was stirred for 1min. Then, the reaction mixture was poured between two glass sheets with closed outlet at the bottom. The polymerization solution was frozen at −16°C for 24 h and then thawed at room temperature. After washing with 200 mL of water, the cryogel was cut into circular disks (0.5 cm in diameter) with a perforator.

3.4. Activation with Divinylsulfone (DVS) and 2-Mercaptoethanol Attachment

This activation reaction is at the basis of a large number of affinity chromatography resins, where the ligand is attached after DVS activation. Activation of the matrix with DVS and attachment of 2-mercaptoethanol are described below.

First P(HEMA) cryogel membranes and P(HEMA- EGDMA) microbeads were washed with water one by one in a beaker. Then they were suspended in 100 mL of 0.5 M sodium carbonate. DVS (10 mL) was added in a dropwise manner into these solutions with constant stirring over a 15 min period. When addition was completed, each one of the beaker suspension was stirred for 1 h at room temperature at 100 rpm. Next step was adding of 5 mL of 2-mercaptoethanol into each one of the beaker and stirring them for 24 h at room temperature at 100 rpm. The ligand attached polymers were extensively washed with water until the filtrate was no longer acidic.

3.5. Synthesis of Thiophilic P(HEMA-EGDMA) Microbeads Embedded P(HEMA) Cryogel Membranes

The thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes were prepared using the same polymerization recipe for P(HEMA) cryogel membranes differing only by the inclusion of 100 mg of P(HEMA-EGDMA) microbeads into the monomer mixture. The monomer mixture consisted of 2 mL HEMA and 0.56 MBAAm. Then radical polymerization with vigorous stirring was

initiated by TEMED (50 µL) and APS (40 mg).The resulting membranes were cut into circular disks (0.5 cm diameter) with a perforator.

3.6. Characterization of P(HEMA) Cryogels

3.6.1. Swelling Test

Water uptake ratios of the thiophilic P(HEMA-EGDMA) microbeads and thiophilic P(HEMA) cryogel membranes were determined in distilled water. The experiment was conducted as follows: initially dry cryogels were carefully weighed before being placed in a 50 mL vial containing distilled water. The vial was put into an isothermal water bath with a fixed temperature (25 ±0.5°C) for 2 h. The bead sample was taken out from the water, wiped using a filter paper, and weighed. The weights of dry and wet samples were recorded. The water content of the cryogels was calculated by using the following expression:

$$\text{Water uptake ratio, \%} = [(W_s - W_o)/ W_o] \times 100 \quad (3.1)$$

where W_o and W_s are the weights of cryogels before and after uptake of water, respectively.

3.6.2. Surface Morphology

The surface morphology of the P(HEMA) cryogel membranes, thiophilic P(HEMA-EGDMA) microbeads and thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes were examined using scanning electron microscope (SEM). The samples were initially dried in air at 25°C for seven days before being analyzed. A fragment of the dried cryogels was mounted on a SEM sample mount and was sputter coated for 2 minutes. The sample was then mounted in a scanning electron microscope (Model: Raster Electronen Microscopy, Leitz-AMR-

1000, Germany). The surface of the sample was then scanned at the desired magnification to study the morphology of the cryogels.

3.6.3. Surface Area Measurements

The specific surface area of the P(HEMA-EGDMA)) microbeads, thiophilic P(HEMA) cryogel membranes and thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes were measured according to the Brunauer-Emmett-Teller (BET) model using multi point analysis and a Flowsorb II2300 from Micromeritics Instrument Corporation, Norcross, GA.

3.6.4. Elemental Analysis

To evaluate the degree of DVS activation and 2-Mercaptoethanolattachment,the synthesized P(HEMA) basedcryogels were subjected to elemental analysis using a Leco Elemental Analyzer (Model CHNS-932).

3.6.5. FTIR Studies

FTIR spectra of the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes and P(HEMA) cryogel membranes were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry cryogels (about 0.1 g) was thoroughly mixed with KBr (0.1 g, IR Grade, Merck, Germany), and pressed into a pellet and the FTIR spectrum was then recorded.

3.6.6. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In this method, human serum was ran on the poly(acrylamide) gel in the presence of SDS (sodium dodecyl sulphate). Observing each protein and also subunits of proteins are feasible via SDS-PAGE. SDS-PAGE was performed to observe IgG purified from human blood plasma by thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes and to monitor purity of IgG. SDS-PAGE was performed by using 12% acrylamide gel (1.0 mm). SDS-PAGE were loaded

with plasma solutions diluted in different ratios for initial, final and desorption samples (10 µl) mixed with buffer having the same ratio. Each sample and standard were mixed with 20% glycerol, 20% mercaptoethanol, 4% sodium dodecyl sulphate (SDS), 0.02% bromophenol blue (BPB) buffer and protein concentrations for each sample were fixed to 20 µg/mL. System was run in the presence of Tris-glycine (pH=8.3) running buffer including 0.1% SDS for 8 h and 150 V. As a next step, Commassie Brilliant Blue G-250 was added to gel ejected from system and waited for a day. $–SO_3H$ active group of Commassie Brilliant Blue G-250 is bound to amino groups of proteins. 20% methanol and 10% acetic acid were applied to final mixture for every 20 min for 2 h. The prestained wide spectrum protein marker (10-170 kDa) was used as protein standard for SDS-PAGE application.

3.7. Adsorption and Desorption Studies

3.7.1. IgG Adsorption Studies from Aqueous Solutions

The adsorption capacity of the thiophilic cryogel membranes for IgG was determined in batch mode. 40 mg [weight of the two thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes] of the adsorbent prepared as described above was washed with deionized water. Then, the thiophilic cryogel membranes were incubated with 5 mL of IgG solution for 120 min (i.e., equilibrium time), agitating magnetically at 100 rpm. IgG concentration was determined by absorbance measurements at 280 nm of a UV-Visible spectrophotometer.

pH effect was examined for 0.5 mg/mL IgG concentration at a fixed temperature of 25°C. Phosphate (pH: 6.0-8.0), acetate (pH: 4.0-5.5), and HEPES (pH: 7.0-8.0) buffers were empolyed.

To study the concentration effect, the initial concentration of IgG was changed between 0.1 mg/mL and 1.0 mg/mL at 25°C, while keeping the pH constant at 8.0. The salt effect was examined for two different salts. The concentrations of salts were changed from 0.001 M to 0.1 M for NaCl and from 0.001 M to 0.40 M for Na_2SO_4, respectively.

The temperature effect was examined for 4-37°C range for IgG concentration of 0.5 mg/mL at a constant pH of 8.0. The amount of adsorbed IgG was calculated as:

$$q= [(C_o-C)V]/m \quad (3.2)$$

Here, q is the amount of IgG adsorbed onto unit mass of thiophilic cryogel membranes (mg/g); C_o and C are the concentrations of IgG in the initial solution and in the aqueous phase after treatment for certain period of time, respectively (mg/mL); V is the volume of the aqueous phase (mL); and m is the mass of the cryogel membranes used (g). Each experiment was performed in triplicate for quality control and statistical purposes.

3.7.2. Desorption and Repeated Use

In all cases adsorbed IgG molecules were desorbed by using 1 M NaCl. In a typical desorption experiment, the thiophilic cryogel sample containing IgG molecules was in contact with 50 mL of the desorption agent for 2 h at 100 rpm constant stirring rate. IgG concentration in the desorption medium was determined by measuring the absorbance at 280 nm. The desorption ratio was calculated from the amount of IgG adsorbed on the beads and the final IgG concentration in the desorption medium.

$$\text{Desorption ratio} = \frac{\text{Amount of human-IgG to the elution medium}}{\text{Amount of human-IgG adsorbed to the cryogel}} \quad (3.3)$$

In order to test the reusability of the thiophilic cryogel membranes, IgG adsorption-desorption procedure was repeated ten times by using the same affinity matrix.

When desorption was achieved, the affinity matrix was cleaned with 50 mM NaOH solution and then re-equilibrated with the starting buffer.

3.7.3. Adsorption of HIgG from Human Plasma

Adsorption of HIgG from human plasma was studied using the thiophilic cryogel membranes. The blood samples were obtained from the blood-bank of university hospital. The plasma was separated by centrifugation at 4000 g for 30 min at room temperature, then filtered (3 μm Sartorius filter) and frozen at -20°C. Before use, the plasma was thawed for 1 h at 37°C. In a typical continuous column system, 50 mL of the plasma sample was stirred for 2 min. The amount of HIgG adsorbed on the thiophilic cryogel membrane was determined by a solid-phase-enzyme-linked immunosorbent assay method (ELISA). Human anti-IgG (Sigma, I-9384) diluted in a ratio of 1/1000 in 50 mM $NaHCO_3$, pH 9.6, was adsorbed onto PVC microtitre plates at 4°C for 12 h. The plates were washed with phosphate buffered saline (PBS) containing 0.05% Tween 20 (washing buffer) and blocked with PBS containing 0.05% Tween 20, 1.5% bovine serum albumin *(*BSA), and 0.1% sodium azide (microorganism blocking buffer). Samples (2.5mL, neutralized with 0.5mL of 1.0 M trisodium citrate) or controls containing known amounts of HIgG were added and incubated at 37°C for 1 h. Bound HIgG was detected with the anti-IgG labeled with biotin (Sigma, B-3773) followed by peroxidase-conjugated streptavidin (Sigma) and o-phenylenediamine. The absorbance was measured at 492 nm.

The purity of HIgG was assayed by sodium dodecylsulfate-polyacrylamide gel electrophoresis using 10% separating gel 16x14 cm stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid-methanol-water (1:5:5, v/v/v) and destained in ethanol-acetic acid-water (1:4:6, v/v/v). Electrophoresis was run for 5 h with a voltage of 120 V.

4. RESULTS AND DISCUSSION

The objective of this thesis is to prepare a thiophilic affinity matrix, poly(hydroxyethylmethacrylate-ethyleneglycoldimethacrylate) [P(HEMA-EGDMA)] microbeads embedded poly(hydroxyethylmethacrylate) P(HEMA) cryogel membranes for the separation of IgG from human plasma and aqueous IgG solution. The main criteria of the selection of HEMA as the basic component are due to its physiological acceptability, mechanical strength, chemical and biological stability. The matrix was activated with divinylsulfone (DVS) and 2-mercaptoethanol was attached. Firstly, P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes were characterized by swelling studies, SEM analysis, surface area measurements and FTIR studies. Then the effects of buffer type, salt type and concentration, initial concentration of immunoglobulin G and temperature on adsorption capacity were investigated in batch mode. The adsorption isotherms and adsorption kinetics were reported. Then, purification of IgG from human plasma by P(HEMA-EGDMA) microbeads embedded P(HEMA) based cryogels was also performed. The purity was assayed by SDS-PAGE. The IgG desorption studies and the reusability of the P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel were also tested.

4.1. Characterization of Thiophilic P(HEMA-EGDMA) Microbeads Embedded P(HEMA) Cryogel Membranes

4.1.1. Swelling Properties

P(HEMA-EGDMA) microbeads and P(HEMA) cryogel membranes prepared in this study have hydrophilic structures, i.e., hydrogel properties. Hydrogels are water swollen; therefore, they do not dissolve in aqueous media, but do swell, depending on the degree of cross-linking and on the hydrophilicity of the matrix. The equilibrium swelling ratio (SR) of the P(HEMA) cryogel membranes was 7.79 g$H_2$0/g polymer and of the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes was 9.01 g$H_2$0/g polymer. Compared with thiophilic P(HEMA) cryogel (8.41 g$H_2$0/g polymer), the water uptake ratio of the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes increased. Increasing of surface area may affect the swelling ratio of the matrix.

Several possible factors may contribute to this result. First, incorporating EGDMA actually introduces more hydrophilic functional groups into the polymer chain which can attract more water molecules into polymer matrices. Second, reaction of EGDMA with HEMA could effectively decrease the molecular weight of the resulting polymer and reduce the crystallinity of the structure. Therefore, the water molecules penetrate into the entangled polymer chains more easily, resulting in an improvement of polymer's water uptake behavior in aqueous solutions. It should be also noted that these hydrophilic microbeads are quite rigid, and strong enough due to highly cross-linked structure.

Porosities were found as 78.75 % for thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes,73.26 % for thiophilic P(HEMA) cryogel membranes and 69.45 % for P(HEMA) cryogel membranes.

4.1.2. Surface Area Measurements

The cross-linked [P(HEMA-EGDMA)] microbeads in the spherical form were mostly in the size range of 50-63 μm. Specific surface area were found to be 47.86 m^2/g of the P(HEMA-EGDMA)) microbeads, 18.48 m^2/g for thiophilic P(HEMA) cryogel membranes, 32.7m^2/g for thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes.

4.1.3. Surface Morphology

The surface morphology and internal structure of P(HEMA) cryogel membranes, thiophilic P(HEMA-EGDMA) microbeads, and microbeads embedded cryogel membranes are exemplified by the scanning electron micrographs in Figure 4.1. As clearly seen here (Figure 4.1 B), the polymeric microbeads have a spherical form and rough surface due to the pores which formed during the polymerization procedure. The micrographs in Figure 4.1.C and D are for thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes.

P(HEMA-EGDMA) microbeads increased the surface area of P(HEMA) cryogel membranes, increased surface area increased adsorption capacity. Experimental results confirm this prediction.

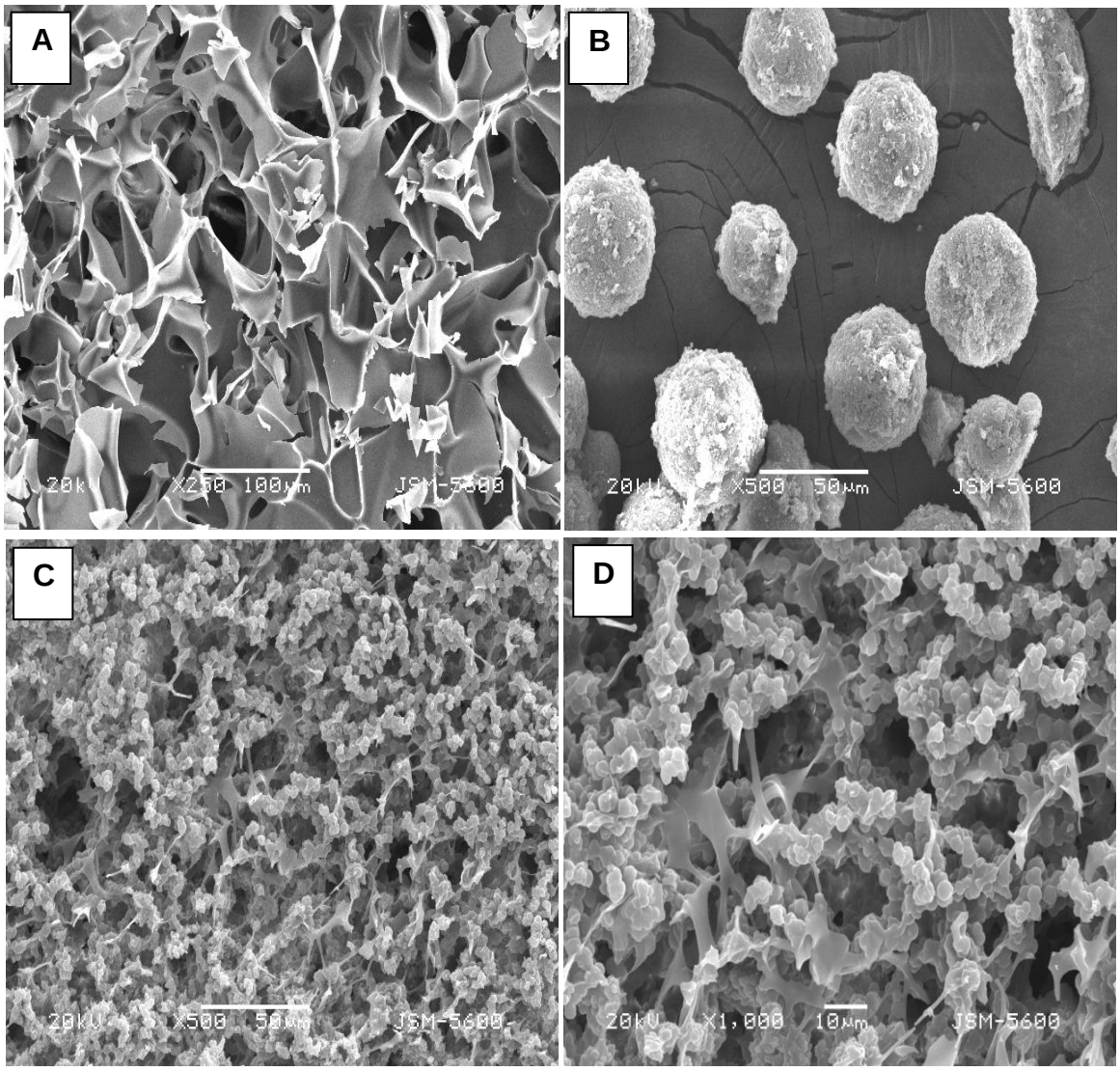


Figure. 4.1. SEM micrographs for P(HEMA) cryogel membranes (A), thiophilic P(HEMA-EGDMA) microbeads (B), thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes (C and D).

4.1.4. Elemental Analysis

Thiophilic P(HEMA) cryogel membranes, thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes and thiophilic P(HEMA-EGDMA) microbeads were analyzed. The sulfur content is expected to exist in the matrix

activated with DVS and attached with 2-mercaptoethanol. Data from the elemental analysis are given in Table 4.1.

Table. 4.1. Elemental analysis for 2-mercaptoethanol.

	μmol ligand/g polymer
Thiophilic P(HEMA) cryogel membranes	26.36
Thiophilic P(HEMA-EGDMA) microbeads	43.22
Thiophilic P(HEMA-EGDMA) microbeads-embedded P(HEMA) cryogel membranes	82.98

4.1.5. FTIR Studies

The molecular formula of synthesized HEMA-EGDMA copolymer was shown in Figure 4.2.

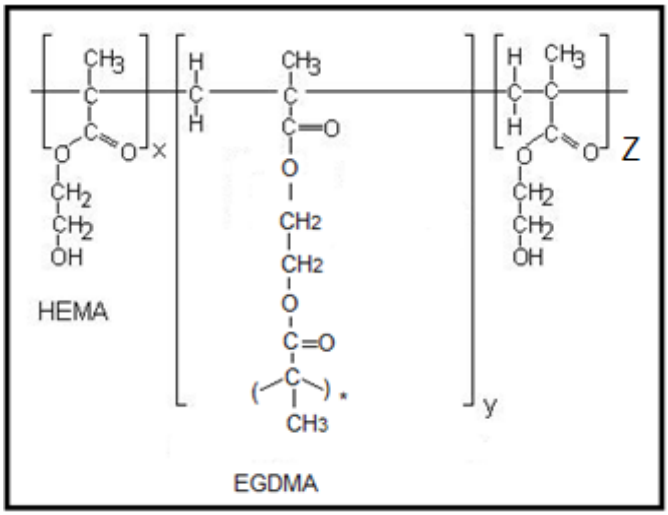


Figure. 4.2. Molecular formula of poly(HEMA-EGDMA).

Similar absorption bands were observed as CH stretching (alkyl, 2978 cm^{-1}), broad band for OH group of carboxylic acid (3400 cm^{-1}), stretching of C=O of acid (1729 cm^{-1}), C=C stretching (1657 cm^{-1}), and C=O bending of acid (1387cm^{-1}) in both spectra of P(HEMA) and microbeads embedded P(HEMA) cryogel membranes. (Figure 4.3. A,B). Different bands, -SO_2 stretching asymmetric (1274 cm^{-1}), -SO_2 stretching symmetric (1155 cm^{-1}), -SO stretching (S-O double bond, 1074 cm^{-1}),

-CS stretching (broad, 700-685 cm^{-1}) showed the occurrence of sulfone and 2-mercaptoethanol in structure of thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes.

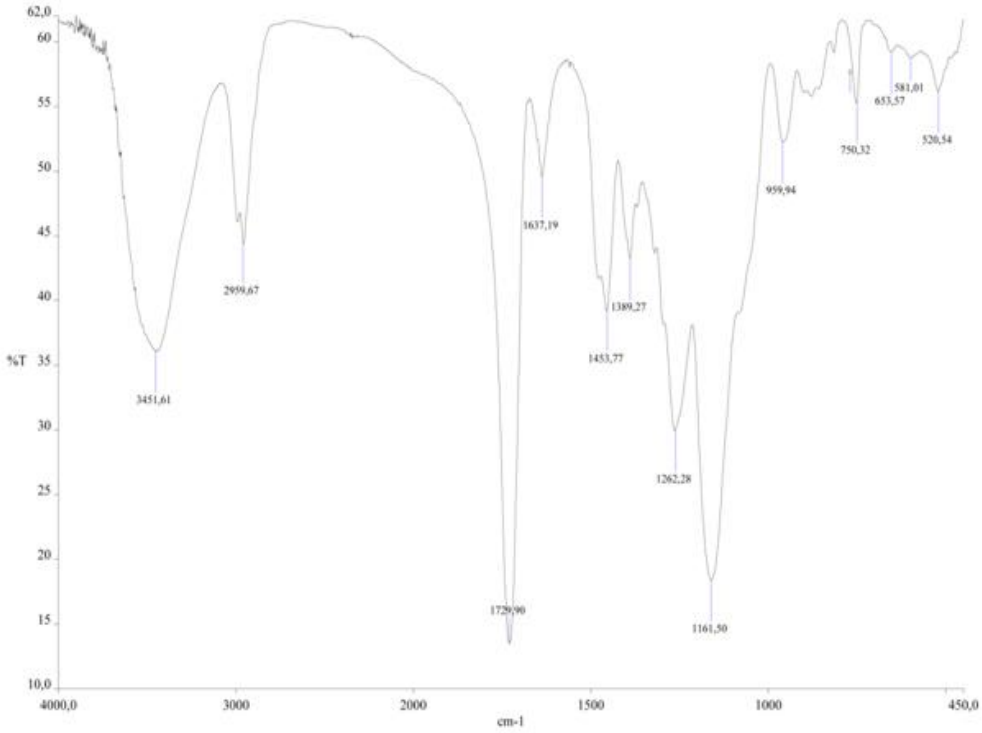


Figure. 4.3.A. FTIR spectrum of P(HEMA) cryogel membranes.

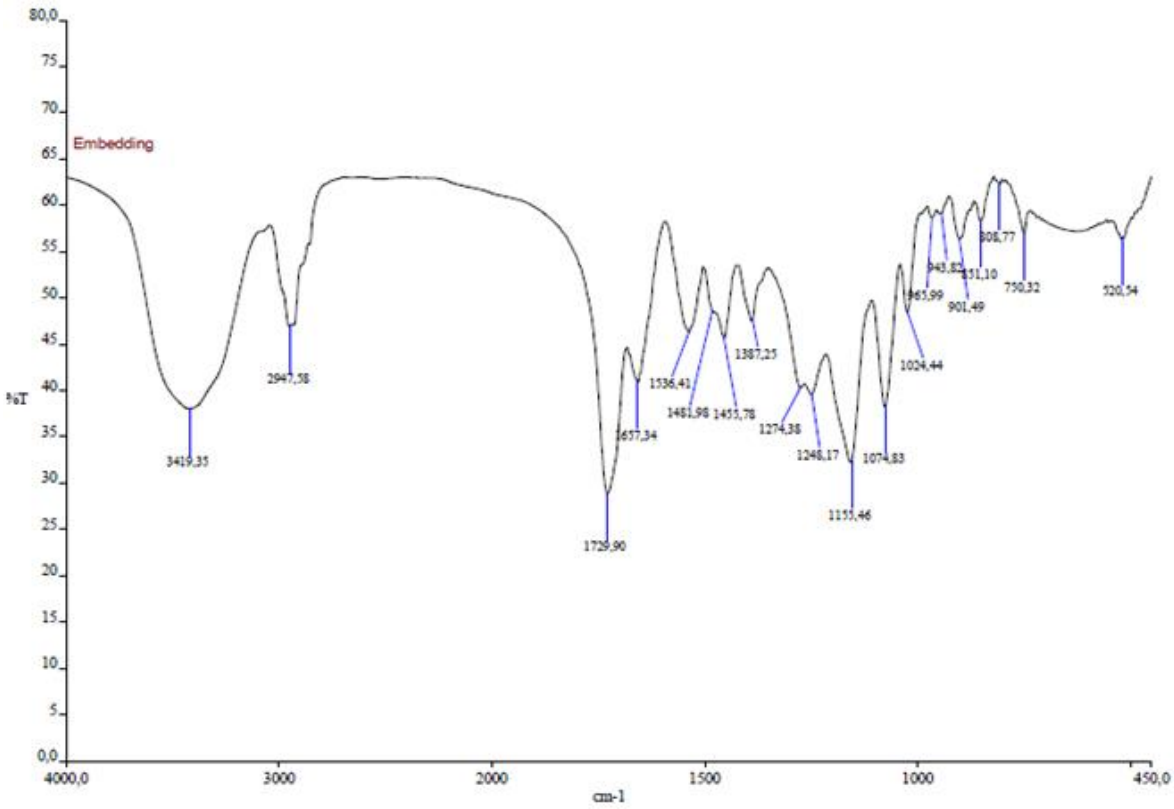


Figure. 4.3.B. FTIR spectrum of thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes.

4.2. Adsorption of IgG From Aqueous Solutions

4.2.1. Effect of pH and Buffer Type

Adsorption studies were carried out using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic (HEPES) and phosphate-acetate buffers within their respective ranges. Figure 4.4 shows IgG adsorption capacity values for these buffer systems at different pH values. Buffer ranges are 6.0-8.0 for phosphate, 4.0-5.5 for acetate and 7.0-8.0 for HEPES. Maximum adsorption capacity was observed at pH 8.0 for HEPES (51.0 mg/g). Below and over the maximum adsorption pH, adsorption capacity decreased significantly. In HEPES buffer at pH 8.0, an IgG conformational structure enhances the interaction of the biomolecule with the matrix.

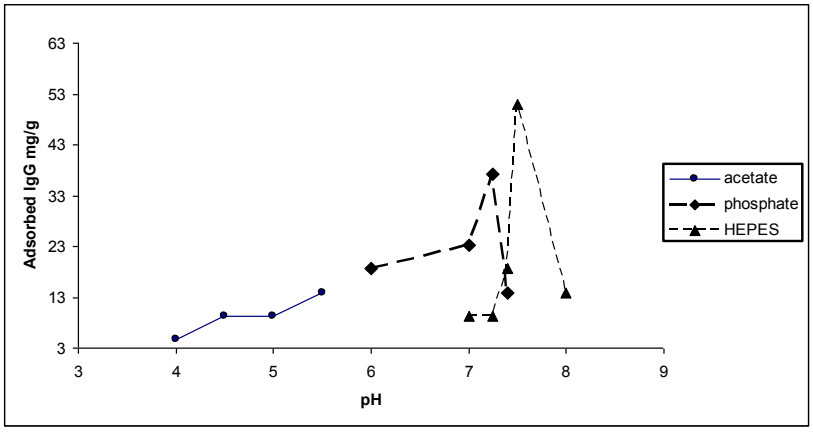


Figure. 4.4. Effect of buffer type on IgG adsorption; IgG concentration: 0.5 mg/mL; 0.250 M Na_2SO_4; time: 120 min; T: 25°C.

4.2.2. Effect of Concentration of Immunoglobulin

Effect of initial IgG concentration on adsorption is shown in Figure 4.5. When thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes were used, IgG adsorption capacity significantly increased up to 68.7 mg/g and IgG adsorption capacity for thiophilic P(HEMA) cryogel membranes was 27.5 mg/g from IgG solution. As the initial concentration of IgG increased, the amount of adsorbed IgG increased. The steep slope of the initial part of the adsorption isotherm represented a high affinity between IgG and thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes. When all the available and accessible sites of polymeric structure were occupied by IgG, adsorption value reached a plateau which represents saturation of the active adsorption sites on the cryogel membranes.

The results showed thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes have higher binding capacity for IgG than that of the thiophilic P(HEMA) cryogel membranes.

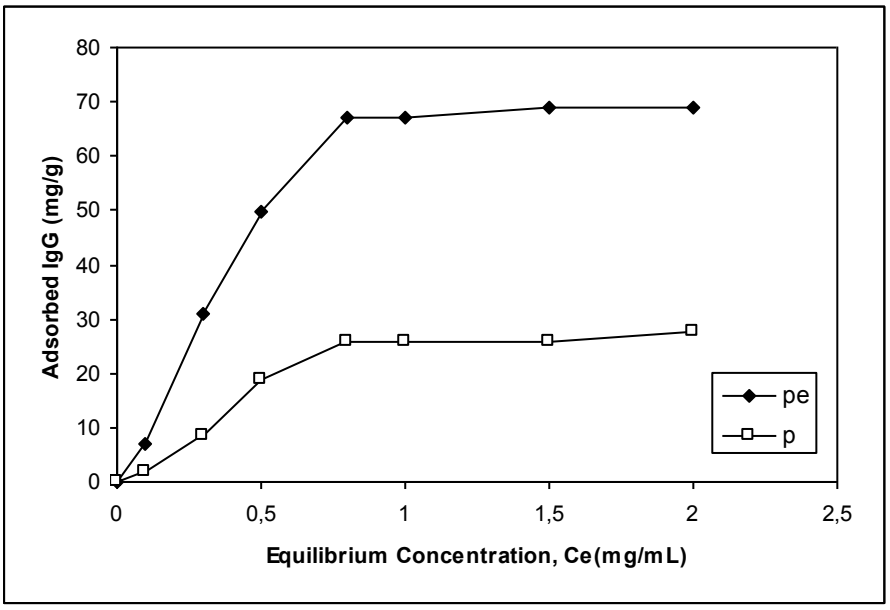


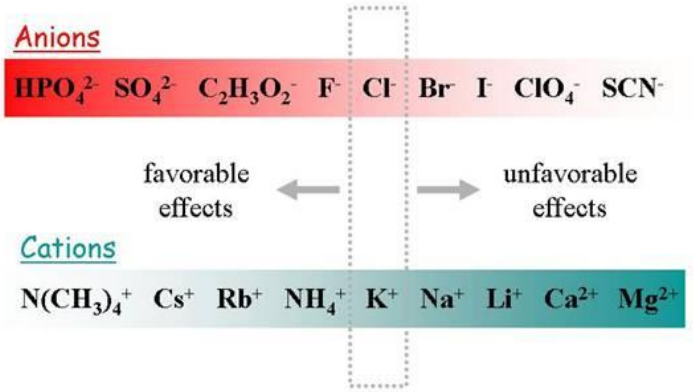
Figure. 4.5. Effect of equilibrium IgG concentration on adsorption capacity; in 20 mM HEPES buffer pH 8.0 containing 0.250 M Na_2SO_4; time: 120 min ;T: 25°C P: thiophilic P(HEMA) cryogel membranes, Pe: thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes.

4.2.3. Effect of Salt Type and Concentration

The effect of salt concentration and type on the adsorption capacity of thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes was investigated. Figure 4.6 shows the effect of salt type on the adsorption capacity of the cryogel. NaCl and Na_2SO_4 were used to investigate the effect of salt type on the adsorption capacity. The concentration of the salt varied between 0.001-0.1 M for NaCl and 0.001 - 0.400 M for Na_2SO_4. The amount of IgG adsorbed were 2.7 mg/g and 51.5 mg/g for NaCl and Na_2SO_4, respectively. As shown in Figure 4.7, IgG adsorption capacity decreased with increasing NaCl concentration in contrast with sodium sulfate. The decrease in the adsorption capacity as the salt concentration increases can be attributed to the repulsive electrostatic forces between the P(HEMA-EGDMA) microbeads and protein molecules. The distortion of existing salt bridges in the presence of salt also contributed to this low protein adsorption at high salt concentration. Maximum adsoption capacity was observed with Na_2SO_4.This result followed Hofmeister seriesor lyotropic series given in

Table 4.2.The non-ionic sulfone-thioether ligand (with appropriated acceptor sites) with appropriated acceptor sites on the protein surface appears to be quite specific, and is promoted in the presence of water structure-forming salts.

Table. 4.2. Hofmeister series.



The mechanism of the Hofmeister series is not entirely clear, but does not seem to result from changes in general water structure, instead more specific interactions between ions and proteins and ions and the water molecules directly contacting the proteins may be more important. Early members of the series increase solvent surface tension and decrease the solubility of nonpolar molecules (“salting out”) in effect, they strengthen the hydrophobic effect. By contrast, later salts in the series increase the solubility of nonpolar molecules (“salting in”) and decrease the order in water; in effect, they *weaken* the hydrophobic effect. The salting out effect is commonly exploited in protein purification through the use of ammonium sulfate precipitation.

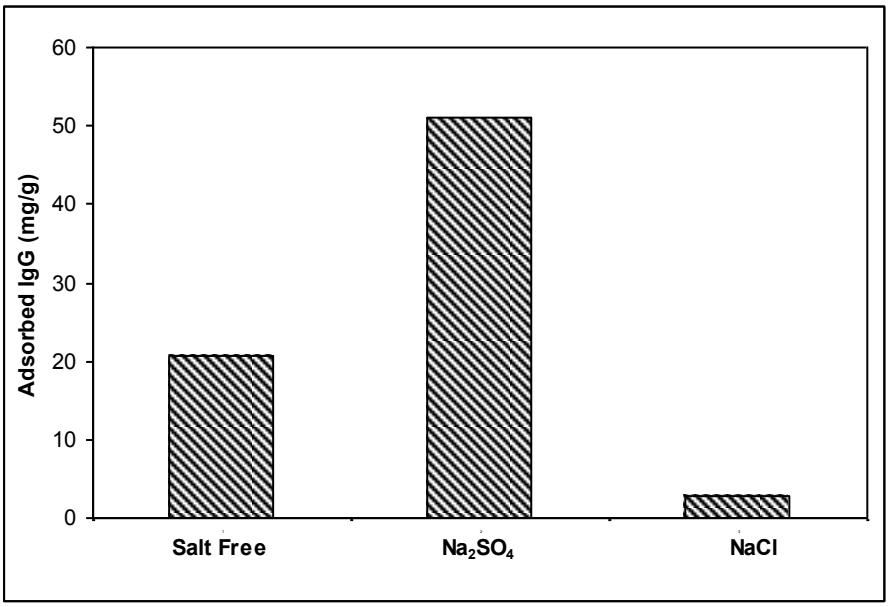


Figure. 4.6. Effect of salt type on IgG adsorption; IgG concentration: 0.5 mg/mL in pH 8.0 HEPES containing 0.250 M salt; time: 120 min; T: 25°C.

High salt concentrations in the separation medium have several important advantages in addition to increasing adsorption capacity. Proteins are frequently stabilized in solutions of water-structure-stabilizing salts. Bacterial growth is largely inhibited. Nonspecific electrostatic interactions are eliminated during salt-promoted adsorption (Hutchens and Porath, 1987).

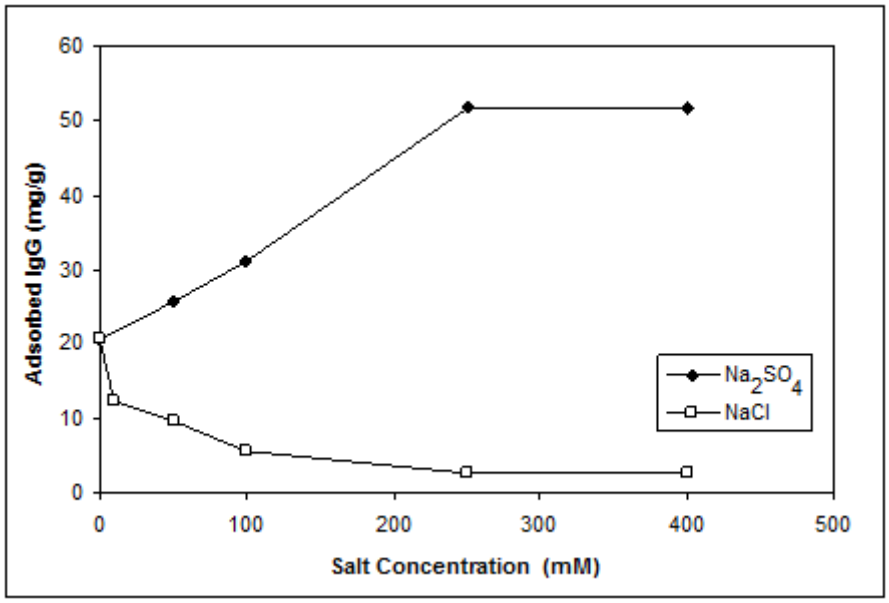


Figure. 4.7. Effect of salt concentration on IgG adsorption; IgG concentration 0.5 mg/mL; adsorbing buffer HEPES (pH: 8.0); time: 120 min; T: 25°C.

4.2.4. Effect of Temperature

The effect of temperature on IgG adsorption capacity of the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes is given in Figure 4.8. The equilibrium adsorption of IgG onto thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes decreased with increasing temperature and the maximum IgG adsorption was achieved as 54.7 mg/g at 4°C. Because the selective recognition between the thiophilic ligands and IgG is attributed to electron donor-acceptor interaction, there is no significant effect of temperature on the adsorption. TAC is not an entropy-driven process.

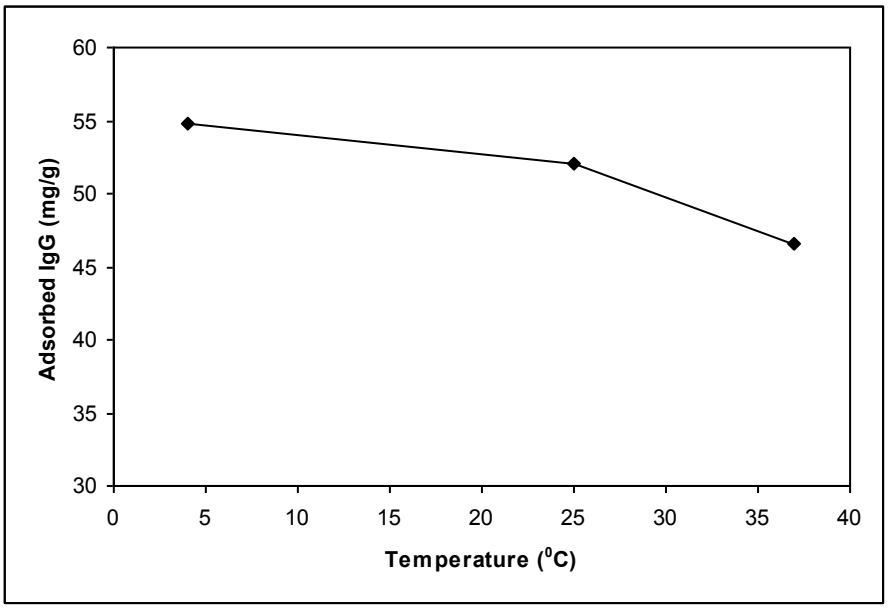


Figure. 4.8. Effect of temperature on IgG adsorption: IgG concentration: 0.5 mg/mL in 20 mM pH 8.0 HEPES buffer containing 0.250 M Na_2SO_4; time: 120 min.

The enthalpy change is always small and the entropy change is the main driving force to determine the Gibbs free energy for the hydrophobic interaction chromatography. Temperature has important role for determining the change of Gibbs free energy. Therefore, the adsorption should be greatly influenced by temperature since the interaction between the proteins and ligands is hydrophobic interaction (Altıntas et al., 2007). On the contrary, the adsorption that changes very little with temperature is believed to be electron donor-acceptor (EDA) interaction since it is an enthalpy-driven process (Porath et al., 1985). So that the driving force was mainly influenced by the EDA interaction between the thiophilic ligand and IgG. There was no remarkable effect of the entropy change of proteins on the specific recognition.

4.2.5. Adsorption Isotherms

The Langmuir adsorption model assumes that the molecules are adsorbed at a fixed number of well-defined sites, each of which is capable of holding only one molecule. These sites are also assumed to be energetically equivalent and distant from each other so that there are no interactions between molecules adsorbed on adjacent sites.

During the batch experiments, adsorption isotherms were used to evaluate adsorption properties. The Langmuir adsorption isotherm is expressed by Equation 4.1. The corresponding transformations of the equilibrium data for IgG gave rise to a linear plot, indicating that Langmuir model could be applied in these systems and described by the equation:

$$Q = Q_{max}.\ b.\ C_{eq} / (1 + b\ C_{eq}) \quad (4.1)$$

where Q is the concentration of bound IgG in the adsorbent (mg/g), $C_{eq}$is the equilibrium IgG concentration in solution (mg/mL), b is the Langmuir constant (mL/mg) and, $Q_{max}$is the maximum adsorption capacity (mg/g). This equation can be linearized so that

$$C_{eq} / Q = 1/ (Q_{max}.\ b) + C_{eq}/ Q_{max} \quad (4.2)$$

The plot of C_{eq}/ Q versus C_{eq} employed to generate the intercept of $1/ (Q_{max}.\ b)$ and the slope of $1/ Q_{max}$

The maximum adsorption capacity (Q_{max}) for the adsorption of IgG was obtained from the experimental data.

The Langmuir correlation coefficient (R^2) was high for thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes. The Langmuir adsorption model can be applied in this affinity adsorbent system.

The other well known isotherm, which is frequently used to describe adsorption behaviour, is the Freundlich isotherm. This isotherm is another form of the Langmuir approach for adsorption on a heterogeneous surface. The amount of adsorbed protein is the summation of adsorption on all binding sites. The Freundlich isotherm describes reversible adsorption and is not restricted to the formation of the monolayer. This empirical equation takes the form:

$$Q_{eq} = K_F (C_{eq})^n \quad (4.3)$$

where, K_F and n are the Freundlich constants.

The plot of $\ln Q_{eq}$ versus $\ln C_{eq}$ was employed to generate the intercept of $\ln K_F$ and the slope of n.

The adsorption isotherms of IgG were found to be linear over the whole concentration range and the correlation coefficients were high. According to the correlation coefficients of isotherms, Langmuir adsorption model is favorable.

Table 4.3 shows the Freundlich adsorption isotherm constants, n and K_F and the correlation coefficients. Regression coefficient value for Langmuir isotherm is higher than Freundlich isotherm and Langmuir Q_{max} value is in agreement with experimental data.

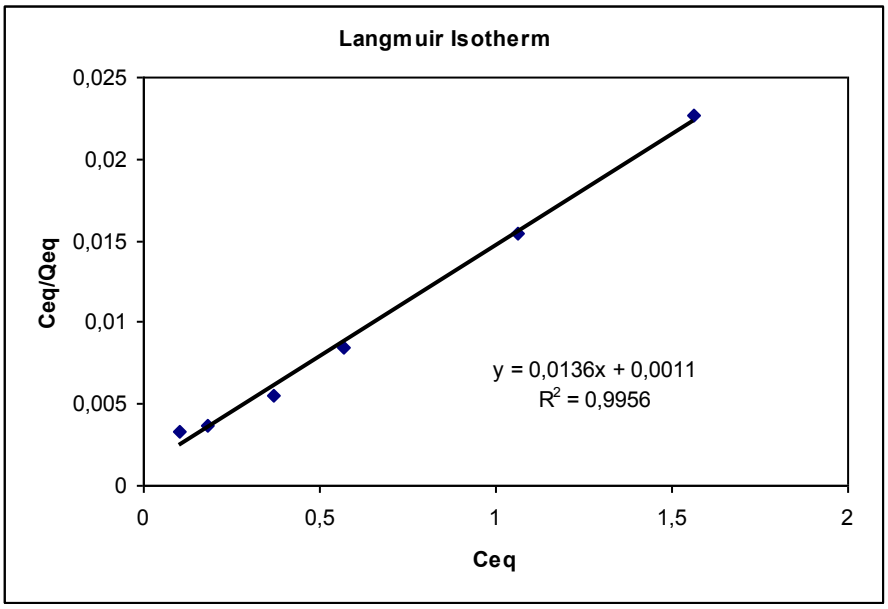


Figure. 4.9. Langmuir adsorption isotherm of the P(HEMA-EGDMA) microbeads embedded P(HEMA) based cryogels, pH: 8 HEPES; T: 25°C.

Table. 4.3. Langmuir and Freundlich adsorption constants and correlation coefficients.

	Experimental	Langmuir constants			Freundlich constants		
	q_{ex} (mg/g)	q_{max} (mg /g)	b (mL/mg)	R^2	K_F	n	R^2
Composite Cryogel	68.76	73.52	12.37	0.996	70.66	0.265	0.736

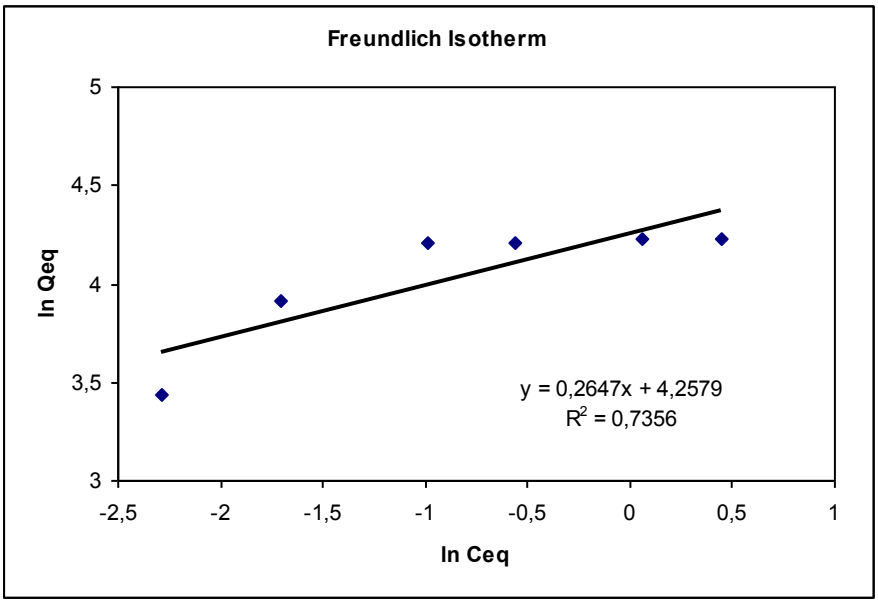


Figure. 4.10. Freundlich adsorption isotherm of the P(HEMA-EGDMA) microbeads embedded P(HEMA) based cryogels, pH: 8 HEPES, T: 25°C.

4.2.6. Effect of Time on IgG Adsorption

The effect of time on IgG adsorption capacity of the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes is given in Figure 4.11. At the start adsorption was observed relatively fast and equilibrium is achieved in nearly 60 min.

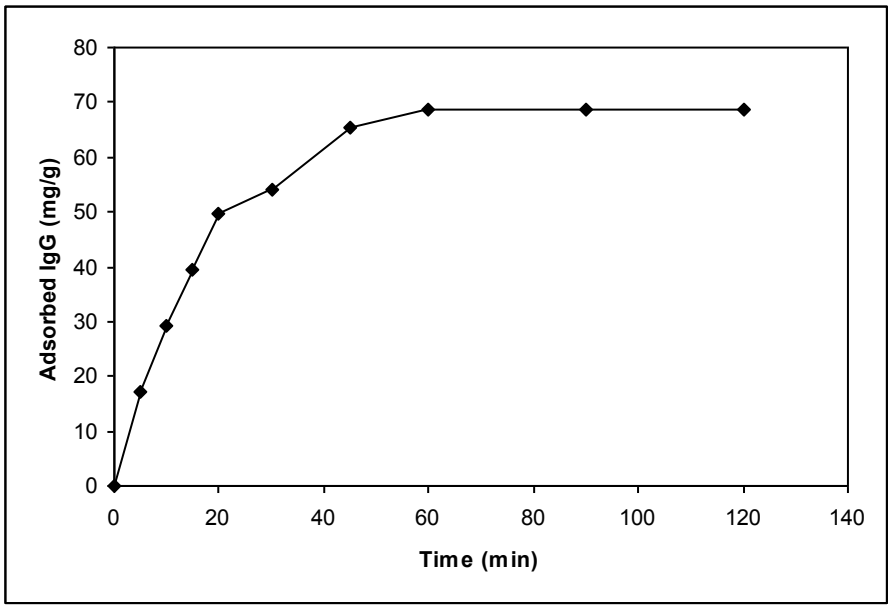


Figure. 4.11. Effect of time on IgG adsorption: IgG concentration: 0.5 mg/mL in 20mM pH 8.0 HEPES buffer containing 0.250 M Na_2SO_4; T: 25°C.

4.2.7. Adsorption Kinetics Modelling

In order to examine the controlling mechanism of adsorption process such as mass transfer and chemical reaction, kinetic models were used to test experimental data. The kinetic models (Pseudo-first- and pseudo second-order equations) can be used in this case assuming that the measured concentrations are equal to adsorbent surface concentrations. The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution. It may be represented as follows:

$$dq_t/dt=k_1(q_{eq}-q_t) \quad (4.4)$$

where k_1 is the rate constant of pseudo-first order adsorption (1/min) and q_{eq} and q_t denote the amounts of adsorbed protein at equilibrium and at time t (mg/g), respectively. After integration by applying boundary conditions, $q_t=0$ at t=0 and $q_t=q_t$ at t=t, gives

$$\log [q_{eq}/(q_{eq}-q_t)]= (k_1t)/2.303 \quad (4.5)$$

Equation 4.5 can be rearranged to obtain a linear form;

$$\log (q_{eq}-q_t)= \log (q_{eq}) - (k_1 t)/2.303 \quad (4.6)$$

A plot of log $(q_{eq}-q_t)$ versus t should give a straight line to confirm the applicability of the kinetic model. In a true first-order process $\log q_{eq}$ should be equal to the interception point of a plot of log $(q_{eq}-q_t)$ versus t.

In addition, a pseudo-second order equation based on equilibrium adsorption capacity may be expressed in the form

$$dq_t/dt = k_2 (q_{eq}-q_t)^2 \quad (4.7)$$

where k_2 (g/mg·min) is the rate constant of pseudo-second order adsorption process. Integrating Equation 4.7 and applying the boundary conditions, $q_t=0$ at t=0 and $q_t=q_t$ at t=t, leads to

$$[1/ (q_{eq}-q_t)] = (1/q_{eq}) + k_2 t \quad (4.8)$$

Or equivalently for linear form

$$(t/q_t)= (1/k_2 q_{eq}^2) + (1/q_{eq})\, t \quad (4.9)$$

A plot of t/q_t versus t should give a linear relationship for the applicability of the second-order kinetics. The rate constant (k_2) and adsorption capacity at equilibrium (q_{eq}) can be obtained from the intercept and slope, respectively. A comparison of the experimental adsorption capacity and the theoretical values which obtained from Figures 4.12 and Figures 4.13 are presented in Table 4.4. The theoretical q_{eq} value estimated from pseudo-first and second order kinetic models was close to the experimental values and the correlation coefficients were

high. Thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes obeyed the pseudo second order kinetic model.

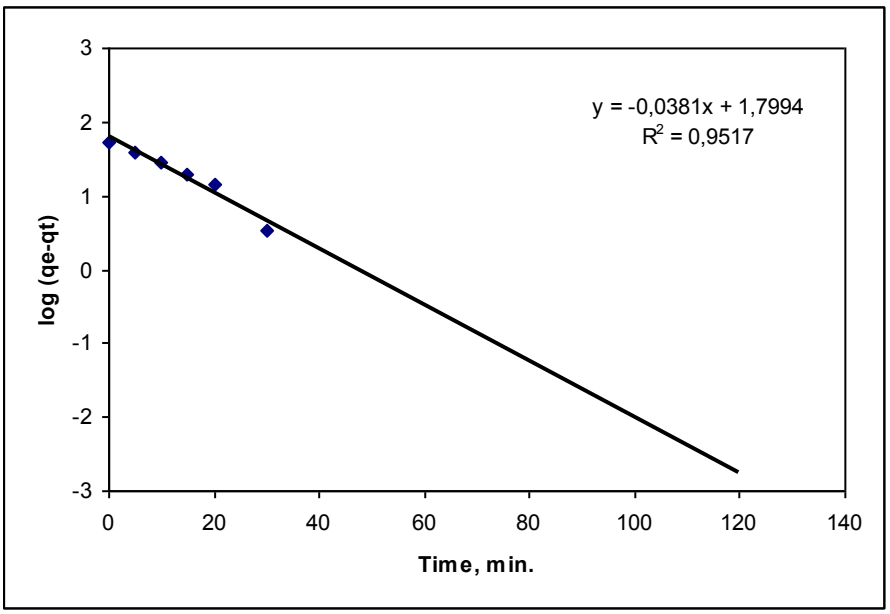


Figure. 4.12. Pseudo-first order kinetic of the experimental data for thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes.

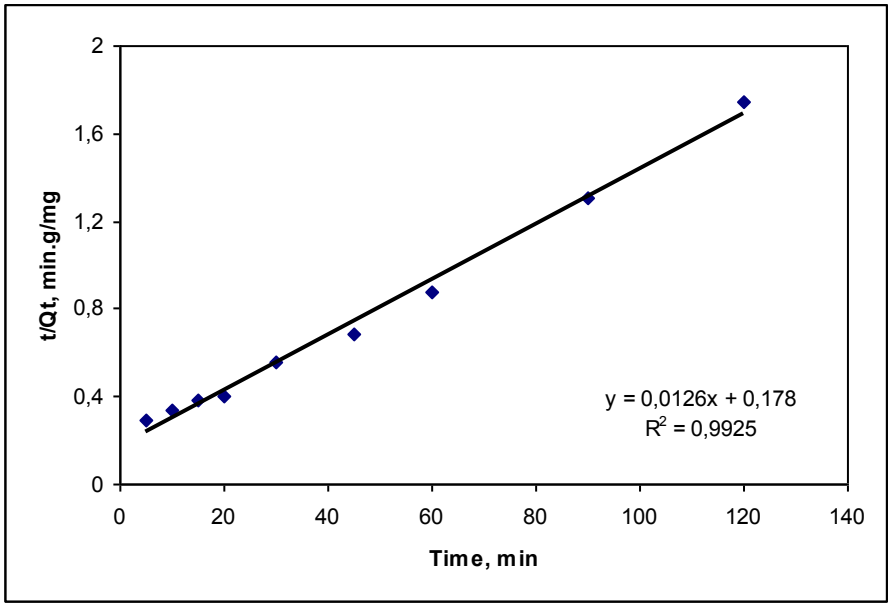


Figure. 4.13. Pseudo-second order kinetic of the experimental data for thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes.

Table. 4.4. The first and second order kinetic constants for thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes.

Initial Conc. (mg/mL)	Experimental	First-order kinetics			Second-order kinetics		
		slope	intercept		Intercept	slope	
	Q_{eq} (mg/g)	k_1 (1/min)	q_{eq} (mg/g)	R^2	k_2 (g/mg.min)	q_{eq} (mg/g)	R^2
1.0	68.76	0.0877	63.01	0.952	0.00089	79.37	0.993

4.3. Desorption and repeated use

In the last step of the affinity separation, the main concern was to desorb the adsorbed protein in the shortest time and at the highest amount possible. It was thus necessary to evaluate the regeneration efficiency of the affinity adsorbents after each cycle. In this study, more than 95 % of the adsorbed IgG molecules was removed easily when 1 M NaCl was used as desorption agent for 2 h .

In order to show the reusability of thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes, the adsorption-desorption cycle was repeated ten times using the same polymeric cryogels. It is observed that the adsorption behavior of IgG onto the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes little changed over ten cycles. At the end of ten adsorption-desorption cycles, there was no remarkable change in the adsorption capacity. The IgG adsorption capacity decreased, from 51.0 mg/g to 44.4 mg/g**.**

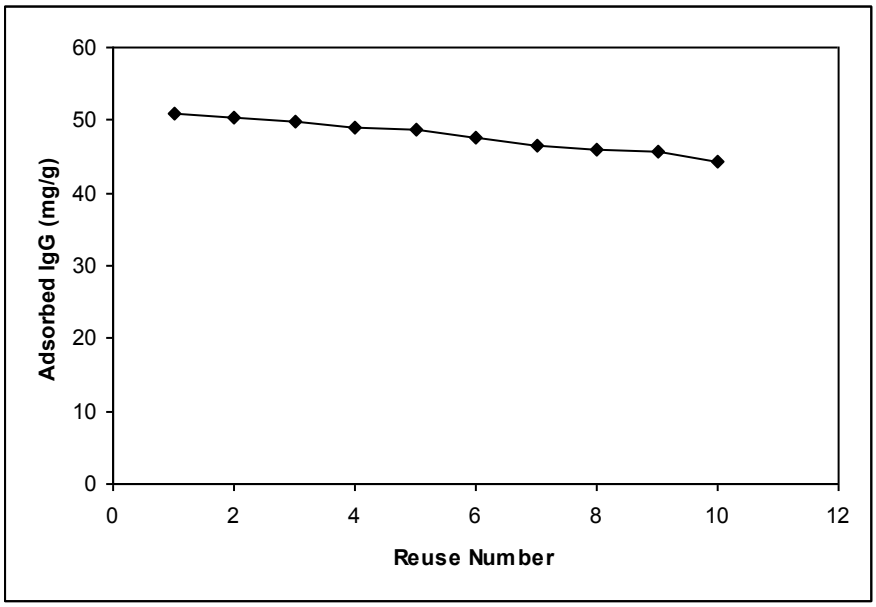


Figure. 4.14. Reusability of the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes: IgG concentration: 0.5 mg/mL in 20mM pH 8.0 HEPES buffer containing 0.250 M Na_2SO_4; T: 25°C.

4.4. Adsorption of Human IgG from human plasma

Table 4.5 gives the adsorption data. As seen here, there was a pronounced adsorption of IgG (up to 137.4 mg/g) onto the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes and 74.8 mg/g onto thiophilic P(HEMA) cryogel membranes for plasma diluted with Phosphate Buffered Saline(PBS) (1:2 diluted plasma (PBS pH: 7.4)).

The purity of IgG was assayed by SDS-PAGE. The purity of IgG obtained was found to be 89%. It is worth to point that the adsorption of IgG onto the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes was higher than those obtained for the studies in which aqueous solutions were used. This is due to the high initial concentration of IgG in the plasma. IgG, has a molecular mass of 150 000 and consists of four peptide chains; two identical light chains. These chains are linked by strong disulphide bonds into a Y- or T-shaped structure with hinge-like flexible arms. Thus an IgG molecule would expand and contract significantly with the variation of the ionizable groups in the molecule.

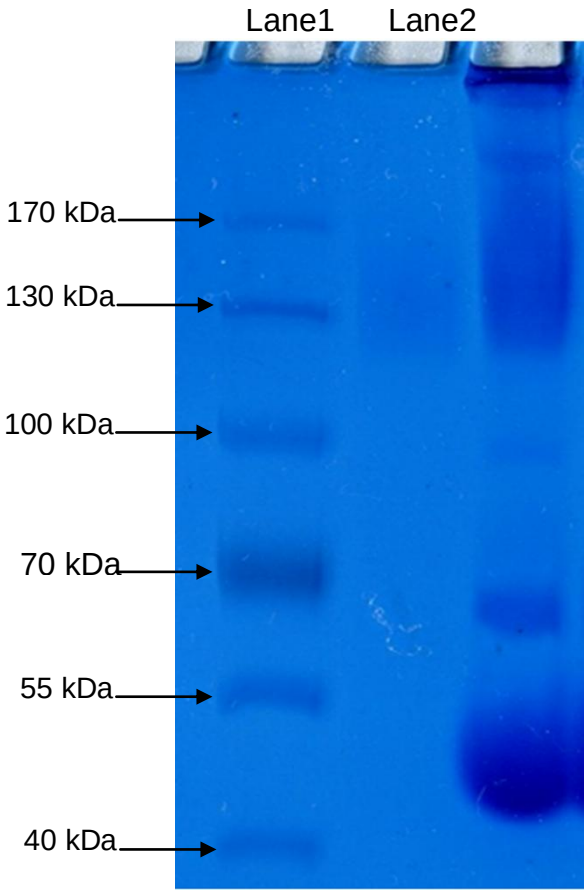


Figure. 4.15. SDS-PAGE of plasma fractions. The fractions were assayed by SDS-PAGE using 10% separating gel (18x16 cm). Separating gels were stained with 0.25% (w/v) Coomassie Brillant Blue R 250 in acetic acid-methanol-water (1:5:5, v/v/v) and destained in ethanol-acetic acid-water (1:4:6, v/v/v). Lane 1, biomarker (Fermentas); Lane 2, eluted sample; Lane 3, 1/8 diluted plasma. Equal amounts of samples were applied to each line.

Table. 4.5. IgG adsorption from human plasma: IgG concentration before dilution: 14.1 mg/mL; T: 25°C.

	Dilution Ratio	Adsorption Capacity (mg/g)
PE	1:2 diluted plasma (PBS pH: 7.4)	137.4± 1.72
	1:8 diluted plasma (PBS pH: 7.4)	35.3± 0.84
P	1:2 diluted plasma (PBS pH: 7.4)	74.8± 1.02
	1:8 diluted plasma (PBS pH: 7.4)	21.6± 0.55

Adsorption of albumin (5.22 mg/g) and fibrinogen (1.56 mg/g) was also determined. There was a pronounced adsorption of IgG onto the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes for undiluted plasma (137.4 mg/g; 1:2 diluted plasma (PBS pH: 7.4)).

5. CONCLUSION

- Water swelling ratio of the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) based cryogel, thiophilic P(HEMA) cryogel membranes and P(HEMA) were found to be 9.01 gH_2O/g polymer, 8.41 gH_2O/g polymer,7.79 gH_2O/g polymer, respectively.

- The average size of the polymeric microbeads, P(HEMA-EGDMA) obtained mainly falls between 50-63μm.
- The specific surface area of the P(HEMA-EGDMA) microbeads was 47.86 m^2/g.

- Scanning electron micrographs showed that, the polymeric microbeads have a spherical form and rough surface due to the pores which formed during the polymerization procedure. The roughness of the microbead surface should be considered as a factor providing an increase in the surface area.

- The specific surface area of the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes and thiophilic P(HEMA) cryogel membranes were found to be 32.7 m^2/g and 18.48 m^2/g, respectively.

- FTIR spectrum of P(HEMA) cryogel and thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes were observed as CH stretching (alkyl, 2978 cm^{-1}), broad band for OH group of carboxylic acid (3400 cm^{-1}), stretching of C=O of acid (1729 cm^{-1}), C=C stretching (1657 cm^{-1}), and C=O bending of acid (1387cm^{-1}) in both spectra. Different bands, -SO_2 stretching asymmetric (1274 cm^{-1}), -SO_2 stretching symmetric (1155 cm^{-1}), -SO stretching (S-O double bond, 1074 cm^{-1}), -CS stretching (broad, 700-685 cm^{-1}) showed the occurrence of sulfone and 2-mercaptoethanol in structure of thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes.

- IgG adsorption for thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes seemed to depend on the buffer system used. To clarify this point adsorption studies were carried out using HEPES and acetate-phosphate buffers within their respective buffering ranges. In HEPES buffer, adsorption capacity of IgG from aqueous solutions is high. Maximum adsorption capacity was observed at pH: 8.0 for HEPES (51.0mg/g).

- When thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes were used, IgG adsorption capacity significantly increased up to 68,7 mg/g and IgG adsorption capacity for thiophilic P(HEMA) cryogel membranes was 27,5 mg/mL from IgG aqueous solution.

- The corresponding transformations of the equilibrium data for IgG gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems. The maximum adsorption capacity (Q_{max}) for the adsorption of IgG was obtained from the experimental data. The Langmuir adsorption model can be applied in this affinity adsorbent system. The maximum adsorption capacity (Q_{max}) was found to be 73.52mg/g IgG with thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes (HEPES buffer at pH 8.0).

- IgG adsorption capacity decreased with the increasing salt (NaCl) concentration, in HEPES buffer. The adsorption capacity of IgG decreases by about 2.75 mg/g for HEPES, as the NaCl concentration changed from 0.001 M to 0.1 M.

- IgG adsorption capacity increased with the increasing salt (Na_2SO_4) concentration, in HEPES buffer. The adsorption capasity of IgG increased by about 51.5mg/g for HEPES, as the Na_2SO_4 concentration changed from 0.001 M to 0.400 M.

- The effect of temperature on IgG adsorption was studied in the range of 4-37°C. The equilibrium adsorption of IgG onto the thiophilic P(HEMA-EGDMA) microbeads embedded P(HEMA) cryogel membranes decreased with increasing temperature.

- Desorption of IgG from P(HEMA-EGDMA) microbeads embedded P(HEMA) based cryogel was carried out in batch experiments. The highest desorption ratio was obtained by using 1 M NaCl at pH 5.0. After one adsorption-desorption cycle the IgG adsorption capacity decreased, from 51.0 mg/g to 50.4 mg/g. At the end of ten adsorption-desorption cycles, there was no remarkable decrease in the adsorption capacity. The IgG adsorption capacity decreased, from 51.0 mg/g to 44.4 mg/g proving the reusability of the matrix (6.6 mg/g decrease in IgG adsorption capacity).

- SDS-PAGE analysis proved the purity of IgG. It was found to be 89%. Adsorption of albumin (5.22 mg/g) and fibrinogen (1.56 mg/g) was also determined.

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