# MOLECULAR IMPRINTED CRYOGELS FOR DEPLETION OF HEMOGLOBIN FROM HUMAN BLOOD

# İNSAN KANINDAN HEMOGLOBİN UZAKLAŞTIRILMASI İÇİN MOLEKÜLER BASKILANMIŞ KRİYOJELLER

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## ÖZ

Kanın farklı organlar ve dokular ile temasta bulunması ortama yeni proteinlerin ilavesine, bazı proteinlerin uzaklaşmasına ve var olan proteinlerin modifikasyonuna neden olmaktadır. Bu proteinler bazı fizyolojik ya da patolojik durumlar değişime uğrayabilir. Bu nedenle plazma proteinlerinin analizi (proteomu), teşhise dayalı biyoişaretleyicilerin ve tedavi hedeflerinin belirlenmesi için ideal bir kaynaktır. Ancak plazma proteomu, yapısında yer alan proteinlerin sayısı ve geniş derişim aralığı nedeniyle oldukça karmaşıktır. Tüm plazma proteinlerinin % 90'ını 10 protein, % 9'unu ise 12 protein oluşturur. Hastalıklara özgü proteinler geriye kalan % 1'lik kısımda yer alır. Çok miktarda bulunan proteinler, az miktardaki hastalık yapan proteinleri analiz sırasında maskelediği için bu proteinlerin kandan uzaklaştırılmaları gerekmektedir. Moleküler baskılanmış polimerler hazırlanması kolay, dayanıklı, ucuz ve moleküler tanıma yeteneği olan polimerlerdir. Moleküler baskılanmış polimerler afinite ayırma araçları olarak düşünülebilir. Makrogözenekli monolitik hidrojeller, diğer bir deyişle kriyojeller, geniş gözenek yapısıyla beraber, birbirleriyle bağlantılı gözeneklere sahip ve bundan dolayı düşük basınç düşmesi gösteren yeni nesil kromatografik destek malzemelerdir.

Bu avantajları nedeniyle sunulan tezde hemoglobin baskılanmış kriyojellerin hazırlanması ve bu malzeme ile kandan hemoglobinin uzaklaştırılması amaçlanmıştır. Bu durumda, kanda yer alan düşük derişimli proteinlerin proteom analizlerinin yapılabilmesi mümkün olacaktır.

**ANAHTAR KELİMELER:** Moleküler baskılama, Moleküler tanıma, Hemoglobin uzaklaştırılması, Protein Adsorpsiyonu, Afinite bağlanma.

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**ABSTRACT**

Interaction of blood with different organs and tissues culminates in input of new proteins, separation of some others and modification of existent ones. These proteins may experience some changes in a specific physiological or pathological situation. Thus, analysis (proteome) of plasma proteins is a significant source for diagnostic markers and determination of treatment targets. However, plasmodic proteome is too complex due to the wide dilution aperture of proteins existed in its structure. All 90% of plasmodic proteins comprise 10 proteins, 10% of them comprises 12 and the rest 1% comprises the other proteins peculiar to diseases. Because the abundant proteins mask the proteins causing diseases, these proteins must be removed from the blood during the analysis process. Repressed molecular polymers are the kind of polymers which are easy to prepare, durable and the ones capable of molecular identification. These can be considered as means of affinity separation. Macro-cellular monolithic hydrogels, in other words cryogels, are modern chromatographic stand-by materials which have wide pour diameters and joint pours, thus showing little pressure decrease.

Due to these advantages, it is aimed to prepare hemoglobin-imprinted cryogels for depletion of hemoglobin from the blood, and enabling the proteome analysis of proteins having low dilution rate.

**KEY WORDS:** Molecular imprinting, Molecular recognition, Depletion of hemoglobin, Protein Adsorption, Affinity binding.

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*To the memory of my dear father*

*(Sevgili atamın anısına)*

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## 1. INTRODUCTION

Hemoglobin (Hb) is the iron-containing oxygen transport metalloprotein in the red cells of blood in mammals and other animals. Hemoglobin transports oxygen from the lungs or gills to the rest of the body, such as to the muscles, where it releases the oxygen for cell use. Hb is a globular protein, and structural changes in any of the four molecular subunits that it is comprised of, can result in the manifestation of hereditary diseases such as sickle cell anaemia, thalassaemia and hemoglobinopathies (Ersöz et al., 2005). The laboratory diagnosis of such conditions requires a combination of complicated and time-consuming procedure (Kleinschmidt et al., 1987) whereas MI may offer a rapid, sensitive and selective approach to the screening, diagnosis and monitoring of hemoglobin disorders. In mammals, the protein makes up about 97% of the red blood cell's dry content, and around 35% of the total content (including water).

By the way, peripheral detection of pathological states through serum protein profiles/biomarkers has a wide variety of potential clinical applications. With advances in proteomic methods, a number of research efforts have been initiated to find biomarkers of different diseases (Wulfkuhle et al., 2003; Davidsson et al., 2005) One of the biggest challenges to these proteomic studies is that a few proteins comprise the vast majority of the protein mass of serum. It is estimated that 85% of the protein mass in human serum is comprised of only a few proteins (Anderson et. al., 2002). These high-abundance proteins make detection of lower abundance proteins difficult, no matter which proteomic method is used. To improve sensitivity of serum studies, it is becoming commonplace to remove or deplete high-abundance proteins from serum before proteomic studies (Gernsten et al., 2002; Pieper et al., 2003; Bjorhall et al., 2003; Echan et al., 2005; Zolotarjova et al., 2005). Currently, several methods for depletion of abundant protein exist with several new methods based on affinity removal (Gernsten et al., 2002; Pieper et al., 2003; Bjorhall et al., 2003; Echan et al., 2005; Zolotarjova et al., 2005; Huang et al., 2005) These methods have been shown to have varying degrees of specificity and reproducibility with human serum samples.

However, a few studies were done on molecular recognition based systems (Uysal et al., 2008; Willard M. Freeman et al., 2006). Molecular recognition based separations

have great attention over the past decades. In molecular imprinting technology, both the shape image of the target and alignment of the functional moieties to interact with those in the target, are memorized in the macromolecular matrix for the recognition or separation of the target during formation of the polymeric materials themselves (Anderson et al., 2002). Molecular imprinting has been used succesfully for imprinting of small molecules, metal-ions and proteins (Gernsten et al., 2002; Pieper et al., 2003; Bjorhall et al., 2003; Echan et al., 2005; Zolotarjova et al., 2005; Grant et al., 2003; Clarkson, 1998; Voytko et al., 2004). Due to the small number of publications, quantitative experimental data on bilirubin-imprinted polymers is very limited. Molecularly imprinted polymers (MIP) are easy to prepare, stable and capable of molecular recognition (Hawtin et al., 2005). These materials are also less expensive to synthesize and can be manufactured in large quantities with good reproducibility. Therefore, MIPs can be considered as artificial affinity media. Molecular recognition-based separation techniques have received much attention in various fields because of their high selectivity for target molecules.

The aim of this study is to prepare a histidine containing hydrogels, so called cryogels, for selective and efficient depletion of hemoglobin from blood. The present work was organized in three main parts. In the first part of the study; histidine containing comonomer 2-methacryloylhistidine (MAH) was synthesized by reacting methacryloyl chloride with L-histidine. Then, poly(2-hydroxyethyl methacrylate) [poly(HEMA)] having methacryloyl histidine (MAH) ligand as a comonomer were obtained by free radical polymerization of HEMA and MAH conducted in an aqueous dispersion medium.

The main criteria of selection of poly(HEMA) as the basic component is its inertness, mechanical strength, chemical and biological stability and well tolerability. Cryogel was characterized using pore size analysis, Fourier Transform Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance (NMR), elemental analysis, Scanning Electron Microscope (SEM) and swelling test. Hemoglobin adsorption properties of these adsorbents were investigated at different pH's and different salt types in a continuous system. The last part of the study, desorption of hemoglobin and reusability of these adsorbents were tested.

## 2. GENERAL INFORMATION

### 2.1. Bioaffinity Chromatography

Bioaffinity chromatography has the highest selectivity among affinity techniques. This is due to the complementarity of surface geometry and special arrangement of the ligand and the binding site of the biomolecules. All biological processes depend on specific interactions between molecules. These interactions might occur between a protein and low molecular weight substance (e.g., between substrates or regularity compounds and enzymes; and so on) but biospecific interactions occur even more often between two or several biopolymers, particularly proteins. The interaction of an affinity ligand with a biomolecule increases with the degree of complementarity of the interacting groups.

Therefore substrate analogues, competitive inhibitors, allosteric inducers and coenzymes are the most suitable bioaffinity ligands for enzymes. Examples of biological interactions used in affinity chromatography are listed in Table 2.1.

**Table 2.1.** Examples of biological interactions used in affinity chromatography.

| **Ligand** | **Counter ligand** |
|---|---|
| Antibody | Antigen, virus, cell |
| Inhibitor | Enzyme (ligands are often substrate analogs or cofactor analogs) |
| Lectin | Polysaccharide, glycoprotein, cell surface receptor, membrane protein, cell |
| Nucleic acid | Nucleic acid binding protein (enzyme or histone) |
| Hormone, vitamin | Receptor, carrier protein |
| Sugar | Lectin, enzyme, or other sugar-binding protein |

The term affinity chromatography has been given quite different connotations by different authors. Sometimes it is very broad; including all kinds of adsorption chromatography based on nontraditional ligands, in the extreme all chromatographies except ion exchange. Often it is meant to include immobilized metal ion affinity chromatography (IMAC), covalent chromatography, hydrophobic interaction chromatography, and so on. In other cases it refers only to ligands based on biologically functional pairs, such as enzyme-inhibitor complexes. The term not only to include functional pairs but also the so-called biomimetic ligands, particularly dyes whose binding apparently often occur to active sites of functional enzymes, although the dye molecules themselves of course do not exist in the functional context of the cell. Thus chromatography based on the formation of specific complexes such as enzyme-substrate, enzyme-inhibitor, etc. (i.e on biological recognition), is termed bioaffinity or biospecific chromatography (Porath et al., 1973; O'Carra et al., 1973) and the respective interaction-biospecific adsorption or bioaffinity (O'Carra, 1974; Barry and O'Carra, 1973). The original term "affinity chromatography" acquired a broader meaning also including hydrophobic chromatography (Shaltiel, 1974), covalent chromatography (Blumberg and Strominger, 1974), metal-chelate chromatography, chromatography on synthetic ligands, etc., i.e chromatography procedures based on different, less specific types of interaction. The broad scope of the various applications of affinity has generated the development of subspecialty techniques, many of which are now recognized by their own nomenclature. Table 2.2 summarizes some of these techniques. As can be seen from Table 2.2, some of these subcategories have become accepted useful techniques (Wilcheck and Miron, 1999).

**Table 2.2.** Subcategories of affinity chromatography

| | |
|---|---|
| Affinity Chromatography | Hydrophobic Chromatography |
| | Immunoaffinity Chromatography |
| | Covalent AC |
| | Metal-Chelate AC |
| | Molecular Imprinting Affinity |
| | Membrane-Based AC |
| | Affinity Tails Chromatography |
| | Lectin Affinity |
| | Dye-Ligand AC |
| | Reseptor Affinity |
| | Weak AC |
| | Perfusion AC |
| | Thiophilic Chromatography |
| | High Performance AC |
| | Affinity Density Pertubation |
| | Library-Derived Affinity |
| | Affinity Partitioning |
| | Affinity Electrophoresis |
| | Affinity Capillary Electrophoresis |
| | Centrifuged AC |
| | Affinity Repulsion Chromatography |

Schematic representation of the main steps in affinity chromatography are shown in Figure 2.1. Affinity chromatography demonstrated in this figure is based on the simple principle that every biomolecule usually recognize 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.

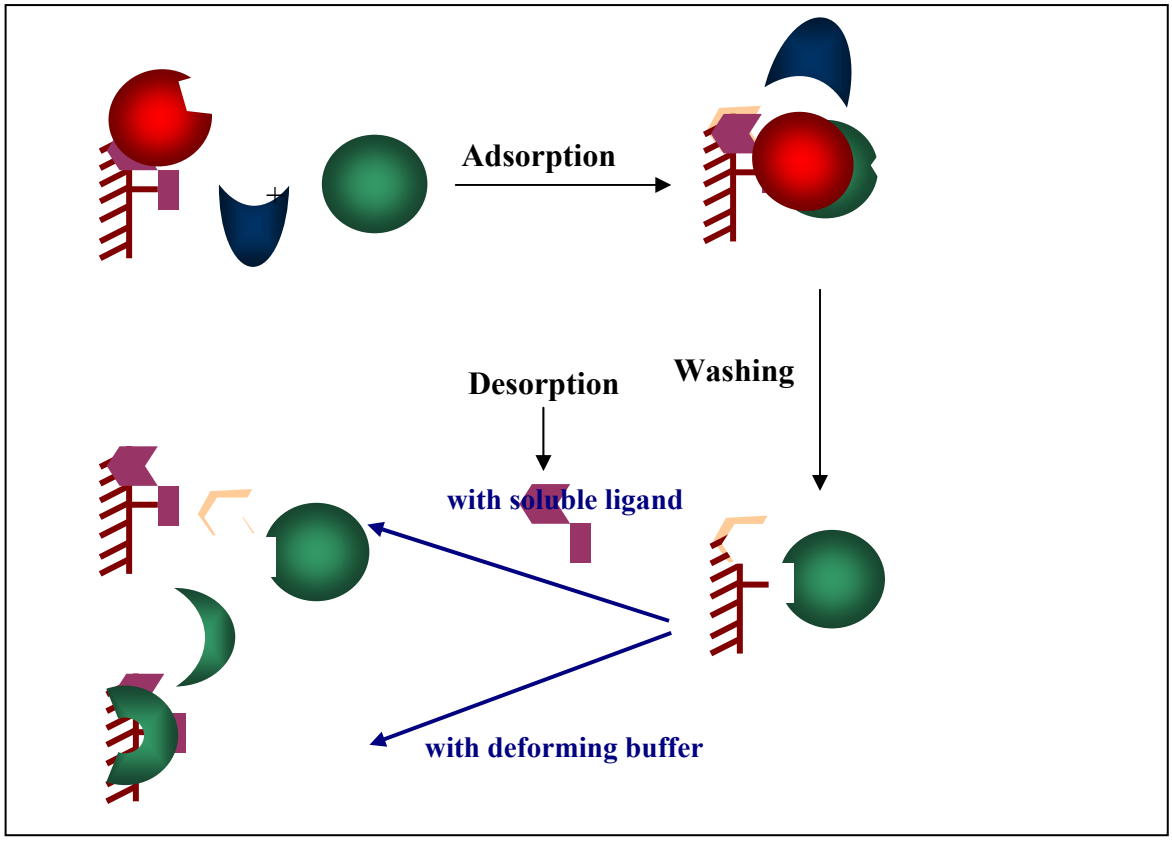


**Figure 2.1.** Schematic representation of the main steps in affinity chromatography.

In such a system, only the protein molecules which selectively bind to the immobilized ligand will be retained on the column. Washing the column with a suitable buffer will flush out all unbound molecules. There are several techniques permit to desorb the product to be purified from the immobilized ligand.

Because affinity chromatography proper relies on the functional properties, active and inactive forms can often be separated. This is however, not unique to affinity methods. Covalent chromatography can do the same thing when the activity depends on a functional thiol group in the protein. By affinity elution, ion-exchange chromatography is also able to separate according to functional properties. These are, however, exceptions to what is a rule for the affinity methods.

Affinity chromatography has proved to be of great value also in the fractionation of nucleic acids, where complementary base sequences can be used as ligands, and in

the separation of cells, where cell surface receptors are the basis of the affinity. Its main use has, however, been in the context of protein purification.

A field that has been so successful that it is often treated separately is affinity based on antigen-antibody interactions, called immunosorption. Sometimes this is the only available route to the purification of a protein and is especially attractive when there is a suitable monoclonal antibody at hand.

Very often the use of affinity chromatography requires that the investigator synthesizes the adsorbent. The methods for doing this, which are described later, are well worked out and are also easily adopted for those not skilled in synthetic organic chemistry. To further simplify the task, activated gel matrices ready for the reaction with a ligand are commercially available. The immobilization of a ligand can, in the best cases, be a very simple affair. In addition, immobilizations are just as easy for proteins as for small molecules.

A property that needs special consideration is the association strength between ligand and counterligand. If it is too weak there will be no adsorption, whereas if it is too strong it will be difficult to elute the protein adsorbed. It is always important to find conditions, such as pH, salt concentration, or inclusion of, for example, detergent or other substances, that promote the dissociation of the complex without destroying the active protein at the same time. It is often here that the major difficulties with affinity methods are encountered. Ligands can be extremely selective, but they may also be only group specific. The latter type includes glycoprotein-lectin interactions, several dye-enzyme interactions, and interactions with immobilized cofactors. However, these interactions have also proved to be extremely helpful in solving many separation problems. Good examples are ligands that are group selective against immunoglobulins (e.g., staphylococcal protein A or streptococcal protein G) (Janson and Ryden, 1998).

### 2.2. Design of an Affinity Chromatography Process

Each protein separation process must be individually optimized and no general rules can predict the factors and parameters for a specific separation. However, some

general factors that influence the design of an affinity chromatography process and its incorporation in a final purification protocol are well known (Labrou and Clonis, 1994; Lowe, 2001). These factors are mainly determined by the nature and quality of desired product and its intended use (Lowe et al., 2001; Sadana and Beelaram, 1994; Narayanan, 1994).

For example proteins for industrial use need not always be absolutely pure whereas therapeutic proteins need to be extremely pure (>99,9%) to minimise the risk of side effects or immunogenic response (Lowe, 2001; Turkova, 1999).

Also, the desired amount of the final product must be defined as it may influence the process. For example, a final dose of pharmaceutical is not known until the end of clinical trials (Sadana et al., 1994).

Certain factors determine the success of an affinity chromatographic step. Some of them are;

1) the ligand selectivity,
2) recovery,
3) through put,
4) reproducibility,
5) stability, maintenance and economy

The selectivity of the ligand perhaps is the most important parameter. Recovery is determined by the amount of biological active protein that is eluted after the end of the process, and throughput is among the several factors that influence dramatically the cost effectiveness of the process. Reproducibility must be determined in order for the process get validation. Differences in purity should be taken into consideration before a purification method is incorporated in large scale protocol. Ligand stability is also an important factor which determines the durability of the column itself and the cost of the process (Lowe, 2001).

Downstream processing costs can be minimal in the case of industrial enzymes, because the purity requirements are low. In contrast, the purity of pharmaceutical proteins, especially those to be administered should be very high (99,9%).

In such cases, purification and recovery costs can account for as much as 80% of the total manufactured cost. Product cost increases with the number of steps involved in the process, not only because of the capital and operating costs but also because the overall yield decreases with the number of steps.

### 2.2.1. Affinity Interactions

The chemistry of biological interactions involves a variety of mostly non-covalent contacts between reactive groups in the receptor and ligand. Affinity reactions between biological molecules are regulated by different types of interactions which depend on their spatial configuration, their charge, their hydrophobicity, etc. These various forces may be complementary and additive, their sum determining the dissociation constant ($k_d$), or affinity of the binding such that, $k_d$= [A][B]/[AB] where A is for instance, an antibody, B is an antigen and AB is the complex formed between them.

Binding affinity is also influenced by solid support characteristics and the activity and functional availability of the solid phase-bound protein (Cuatrecasas, 1972) such that the Kd for affinity chromatography varies between $10^{-3}$ and $10^{-7}$ M. Both the chemical and mechanical properties of the solid phase are important in this regard. Non-specific interactions between the solid phase and ligand are inconsistent and unpredictable and therefore complicate elution profiles (Fleminger et al., 1990).

On the other hand the physical structure of the solid phase polymer may sterically hinder effective interaction between free ligand and immobilized protein even at high $k_d$ values. Both of these problems can be significantly reduced by prudent use of inert spacer arms (Soltys and Etzel, 2000; Scouten, 1981).

Ultimately, the $k_d$ for a particular biological interaction can only be used as a guide to developing an appropriate elution system. For very high affinities ($10^{-8}$ M) such as hormone-receptor, high affinity monoclonal antibody-hapten and immobilized dye ligand interactions (Cochet et al., 1994).

The elution buffer may need to be very stringent. This can lead to partial denaturation (Narhi et al., 1997) and loss of activity of the released target protein. Alternatively,

weak interactions may result in lack of binding specificity and low purification factors, although this can also be used to advantage.

### 2.2.2. Choice of Matrix

As in all adsorption chromatography, an adsorbent with a large surface area per unit column volume is desirable to maximize the capacity of the affinity adsorbent. Hydrophilic gels with a high surface-to-volume ratio are very stable as matrices. For affinity chromatography applications, the ideal gel material should meet the following characteristics.

1. Macroporous to accommodate the free interaction of large molecular weight proteins with ligands that could themselves be proteins or other macromolecules.
2. Hydrophilic and neutral to prevent the proteins from interacting nonspecifically with the gel matrix itself.
3. Contain functional groups to allow derivatization by a wide variety of chemical reactions.
4. Chemically stable to withstand harsh conditions during derivatization, regeneration, and maintenance.
5. Physically stable to withstand hydrodynamic stress in packed beds and, when applicable, sterilization by autoclaving.
6. Readily available at low cost to facilitate industrial applications.

These characteristics point to gels based on polymers highly substituted with alcohol hydroxyls and thus to polysaccharides. Among the latter, the spontaneously gel-forming galactan agarose indeed possesses most of the characteristics of an ideal matrix for affinity chromatography. The major weakness of native agarose is its chemical and physical instability, which, however, has been largely compensated for by chemical cross-linking of the physically cross-linked so-called junction zones in the agarose gel structure. Ever since its introduction in 1968 by Cuatrecasas, Wilchek, and Anfinsen, 4% agarose has been the most popular matrix for affinity chromatography (Cuatrecasas, 1972; Wilchek and Anfinsen, 1968). A contributing reason this popularity, in addition to the advantageous matrix properties as such, is

that there were early simple and convenient coupling methods developed for agarose and even commercially available.

Less frequently used gel matrices for affinity chromatography are cellulose, cross-linked dextran, polyacrylamide, and silica. To this group also belong the potentially interesting and commercially available matrices made of mixtures of polyacrylamide and agarose (Ultragel) and polymerized tris (hydroxymethyl) acrylamide (Trisacrl, Biosepra, France). Gels based on cross-linked dextran and polyacrylamide both suffer from the serious disadvantage of having too small pore diameters, which become still smaller after derivatization with affinity ligands. Also, fewer methods are available for immobilization on polyacrylamide.

In traditional low-pressure affinity chromatography systems, beads with a diameter of approximately 100 μm are usually standard. However, beads with diameters in the range 5 to 30 μm are used in so-called high-performance liquid affinity chromatography (HPLAC). In this variety of affinity chromatography, higher pressure drops are often required, which also means a demand for higher gel rigidity. This is why the first HPLAC applications were based on modified and derivatized porous silica. The major reason for using smaller particles is to increase the chromatographic efficiency by decreasing the diffusion path lengths and increasing the interphase area between the stationary and the mobile liquids. An alternative to silica for HPLC applications, which has also proven useful in HPLAC, is small-diameter agarose beads. Synthetic organic polymers, highly substituted with alcohol by hydroxyls and with adequate porosity and rigidity, should also present interesting matrices. Some of these are now commercially available (Janson and Ryden, 1998).

### 2.2.3. Choice of Ligand

The rapid growth of bioinformatics and molecular docking techniques and the introduction of combinatorial methods of systematic generation and screening of large numbers of novel compounds have made feasible the rapid and efficient generation of ligands for affinity chromatography (Spalding, 1991; Labrou, 2002; Clonis, 1990). A good affinity ligand should possess the following characteristics:

1. The ligand must be able to form reversible complexes with the protein to be isolated or separated.
2. The specificity must be appropriate for the planned application.
3. The complex constant should be high enough for the formation of stable complexes or to give sufficient retardation in the chromatographic procedure.
4. It should be easy to dissociate the complex by a simple change in the medium, without irreversibly affecting the protein to be isolated or the ligand.
5. It should have chemical properties that allow easy immobilization to a matrix.

For any particular protein intended to be purified by affinity chromatography, there is often a choice of several different ligands. In addition to the obvious choice of using a monoclonal antibody of appropriate affinity, which is generally applicable to all immunogenic solutes, one may look for components of naturally occurring biospecific pairs such as enzyme-substrate (analogs), enzyme-cofactor and enzyme-inhibitor complexes. For glycoproteins, there is the possibility of using immobilized carbohydrates. Considerable interest has been focused immobilized biomimetic dyes, which show a wide variety of specificities applicable to several groups of enzymes, plasma proteins, and other proteins such as interferons.

#### 2.2.3.1. Classification of Ligands

Generally ligands may be classified as either monospecific or group specific, each of which in turn may be into low molecular weight or macromolecular.

##### 2.2.3.1.1. Monospecific Low Molecular Weight Ligands

This group includes ligands such as steroid hormones, vitamins and certain enzyme inhibitors. The term monospecific refers to the fact that these ligands bind to a single or a very small number of proteins in any particular cell extract or body fluid. Despite the high specificity, nonspecific adsorption may occur. This can be due to interaction with the ligand or with residues from the immobilization reaction or the spacer arm. One way to cope this problem is to make a second adsorbent lacking only the ligand itself and allow to the desorbed material from the ligand-containing adsorbent to pass this under identical conditions (Kula, 1979).

#### 2.2.3.1.2. Group-Specific Low Molecular Weight Ligands

This is the largest group of ligands containing a wide variety of enzyme, cofactor and their analogs. This group also includes biomimetic dyes, boronic acid derivatives, and a number of amino acids and vitamins. Despite its relatively broad specificity, very high purification factors may be obtained using specific elution protocols with either soluble cofactors or by ternary complex formation using a combination of cofactor and substrate. Alternatively, the ligand-enzyme association with a soluble cofactor may result in adequate separation.

Ligands of a high molecular weight type (e.g., proteins) with a large number of suitable functional groups can normally be immobilized without adversely influencing structure or function. In low molecular weight ligands the coupling of course results in a relatively large change in the molecule. If the affinity interaction decreases, a chemical modification of the ligand may be necessary to provide it with an appropriate functional group for immobilization.

The functional group used should permit the formation of a stable covalent bond so that the ligand is not released from the matrix. This is particularly important for small ligands where "single-point attachment" is often the case. For proteins, “multipoint attachment" between ligand and matrix is rather common. In such affinity adsorbents, the stability of each individual bond is less critical.

It is, of course, also essential that the ligand remains intact during the immobilization procedure and that it is sufficiently stable to allow the planned affinity chromatography to be carried out.

This might be a problem when proteins are coupled at high pH. It is essential that the ligand reagent is as pure as possible and, in particular, does not contain substances with functional groups that can react competitively in the immobilization. Proteins should be subjected to gel filtration to remove low molecular weight substances such as ammonium sulfate (Janson and Ryden, 1998).

### 2.2.4. Choice of Spacer Arm

Occasionally, an affinity adsorbent might show poor function due to low steric availability of the ligand. This rarely happens with high molecular weight ligands but may occur with low molecular weight ligands.

The use of a "spacer arm" in many cases solves this problem. Commonly used spacer arms are aliphatic, linear hydrocarbon chains with two functional groups located at each end of the chain. One of the groups (often a primary amine, $-NH_2$) is attached to the matrix, whereas the group at the other end is selected on the basis of the ligand to be bound. The latter group, which is also called the terminal group, is usually a carboxyl (-COOH) or amino group, ($-NH_2$). The most common spacer arms are 6-aminohexanoic acid [$H_2N-(CH_2)-COOH$], hexaethylene diamine [$H_2N-(CH_2)-NH_2$], and 1,7-diamino-4-azoheptane (3,3-diamino dipropylamine) (Cuatrecasas, 1970, Lowe et al., 1974)

It is important to consider the chemical nature of the spacer arm and particularly its hydrophilic or hydrophobic character. Hydrophobic arms have the advantage of being available and relatively simple compounds, but may create non-specific interactions. The most encountered hydrophilic spacer arms are generally synthetic polypeptides but they may induce non-specific ionic interactions.

Longer spacer arms can be introduced by first immobilizing a spacer arm with a terminal primary amine and then increasing the length of the arm by reaction with succinic anhydride (Cuatrecasas, 1970) but a drawback with the hydrophobic arms, especially the longer ones, is that they can give rise to unwanted nonspecific interactions. Polypeptides, particularly glycine oligomers, are examples of hydrophilic spacers. These, however, might bind proteins by nonspecific interactions (Lowe et al, 1974).

The essential purpose of the use of spacer arm is to increase sterical approach between the immobilized ligand and the product to be separated. The length of the spacer arm is thus an important parameter which must be decided according to each case.

If short spacer arms do not systematically eliminate steric effects, long spacers may favor non-specific adsorptions, particularly when the hydrocarbon chain has a hydrophobic character.

### 2.2.5. Immobilization Techniques

There are many methods for immobilization of ligand molecules onto the support matrix, in which usually several intermediate steps are followed (Turkova, 1993; Matejtschuk, 1997). The immobilization procedure consists of three steps;

1- Activation of the matrix to make it reactive toward the functional group of the ligand,
2- Coupling of the ligand,
3- Deactivation or blocking of residual active groups by a large excess of a suitable low molecular weight substance such as ethanolamine.

The main points for a successful ligand immobilization are given. Note that the correct choice of coupling method and conditions depends on both the matrix and the ligand.

First of all immobilization should be attempted through the least critical region (not from the active site) of the ligand molecule, to ensure minimal interference on the specific interaction between the immobilized ligand and the target molecules (Denizli et al., 2001). The activation normally consists of the introduction of an electrophilic group into the matrix. This group later reacts with nucleophilic groups, such as $NH_2$ (amino), SH (thiol), and OH (hydroxyl) in the ligand.

Alternatively, a matrix with nucleophilic groups can be used to immobilize a ligand containing an electrophilic group, although such an approach is less common. The activated structure is sometimes stable enough for the activated matrix to be isolated and stored until the coupling of ligand is performed. In other cases the coupling procedure has to be performed immediately after activation.

In the following, we will describe a few of them and the most representative. The most frequently used method is the cyanogen bromide-activation of agarose (activated resin is commercially available), which leads to a highly reactive cyanate ester. Subsequent coupling of ligands to the activated matrix results in an isourea linkage. Despite the popularity of this method, the isourea linkage of the ligand causes several problems during the purification procedure, including nonspecific binding due to charge and leakage of ligand due to instability of the isourea bond.

Since the early days of affinity chromatography, active esters, in particular N-hydroxy-succinimide (NHS) esters, have also been used for immobilizing ligands. The preparation of active esters requires a matrix that contains carboxylic groups. Such matrices can be easily obtained from agarose by activation of the hydroxyl groups with different reagents, including cyanogen bromide, activated carbonates, etc. (Figure 2.2), and successive reaction with $\alpha$-amino acids of different sizes depending on the length of spacer arm required. The NHS ester is then prepared by mixing the carboxylic matrix with dicyclohexylcarbodiimide and NHS. Due to a stability problem (Wilchek and Miron, 1987), a new method was introduced based on N',N',N'-tetramethyl (succinimido) uranium tetrafluoroborate.

The covalent attachment of ligands to such activated carriers proceeds through the production of stable amide bonds.

Another method for activating polysaccharides is use of N'N-disuccinimidyl carbonate (DSC), which forms highly reactive carbonate derivatives with polymers containing hydroxyl groups. These derivatives react nucleophiles under mild, physiological conditions (pH 7.4), and the procedure results in a stable carbamate linkage of the ligand coupled to the carrier.

The immobilization of different ligands (e.g., enzymes, enzyme inhibitors, antigens and antibodies) to activated carbonate carriers has been achieved, together with excellent maintenance of biological activity of the proteins (Wilchek and Miron, 1999).

It must be remembered that in certain cases, high degree of substitution may favor non-specific adsorptions. The multipoint binding of an antigen molecule results in changes in

its conformation that leads to weakening of the interactions between the antigen and the active site of the antibody.

Since affinity involves the adsorption of one molecule of product to be separated by one molecule of immobilized ligand, the ratio of their molecular weigths is of primary importance for gel adsortion capacity. The degree of immobilization must take into account; the ligand size, the ligand specificity and the gel porosity. If the ligand is allow molecular weight compound and the product to be separated is a macromolecule, the degree of substitution may be very low. In this case, the ratio of molecular weights is reflected by a high capacity, providing that the method of immobilizing the ligand and the porosity of the gel were well chosen.

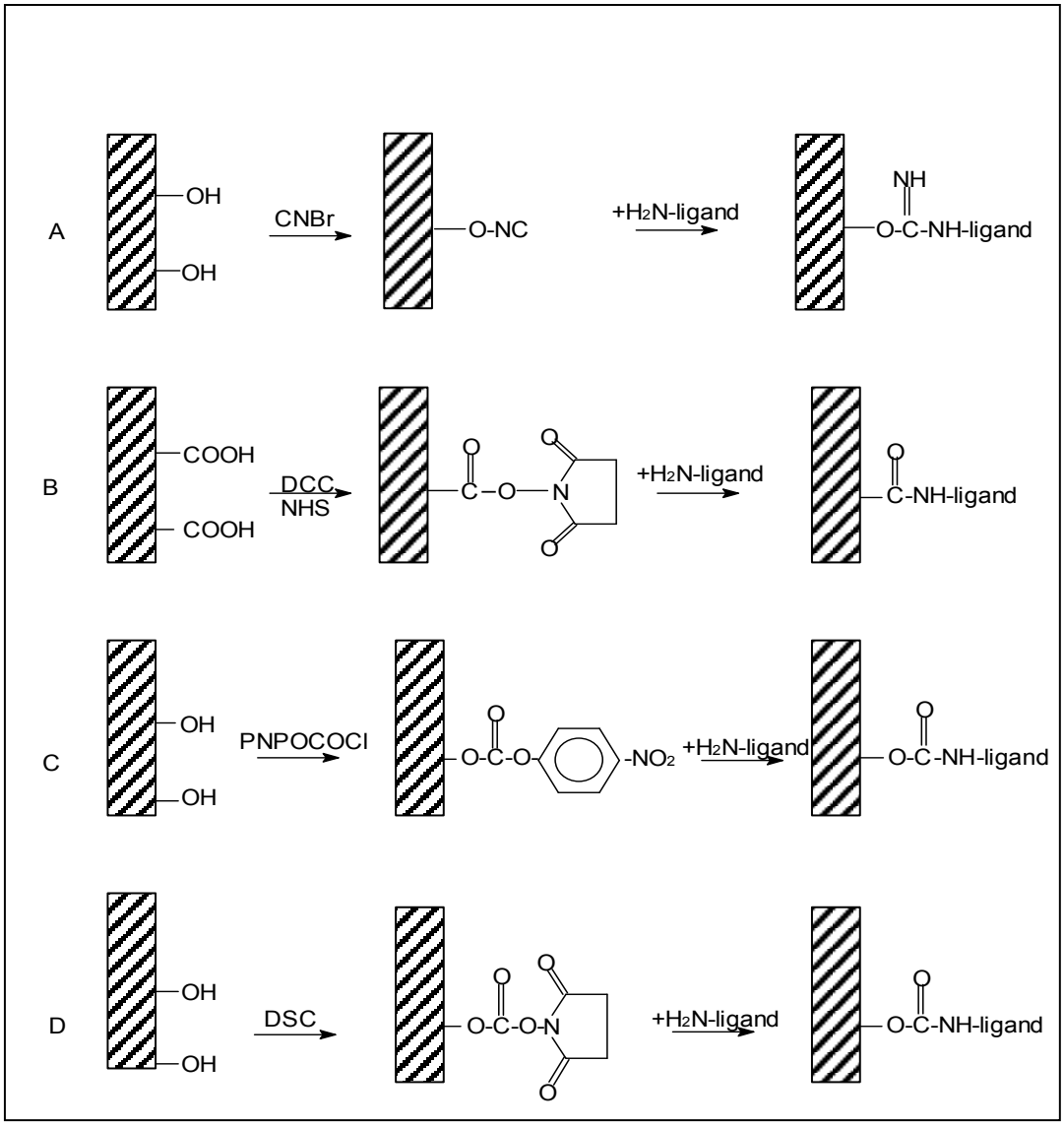


**Figure 2.2.** Immobilization of ligands to different activated carriers:

(A) cyanate ester, obtained by activation with cyanogen bromide, leading to an isourea derivative; (B) N-hydroxysuccinimide ester yielding an amide derivative; (C) and (D) p-nitro-phenyl and N-hydroxysuccinimide carbonate obtained by activation with p-nitro phenylchloroformate or DSC, giving carbamate derivatives been achieved, together with excellent maintenance of biological activity of the proteins (Wilchek and Miron, 1999).

The degree of immobilization depends on activation and coupling procedures:
1- quantity of activating agent,
2- reaction time,
3- pH of the medium,
4- reaction temperature,
5- ligand concentration.

### 2.3. Molecular Imprinting of Biological Macromolecules and Assemblies

Design and synthesis of novel materials which are capable of recognizing proteins and other biological assemblies, such as whole cells and viruses, have potential fundamental and practical applications (Jacobs, 1994). Specific applications of such materials would encompass down-stream bioprocess for the purification of biopharmaceuticals, drug delivery, diagnostics, sensors, etc. Currently, biologically derived recognition agents, like antibodies, enzymes, receptors, etc., are being used for some of these applications (Chaiken, 1983). In the area of bioseparation, much of the cost of producing an enzyme or hormone occurs in product recovery. Extremely high selectivity for a specific biological macromolecule can be obtained through interactions such as antigen-antibody reactions, enzyme-substrate/inhibitor binding and hormone-receptor binding. Unfortunately, these methods tend to be very expensive and involve labile systems. Hence there is an incentive to develop inexpensive, robust and reusable replacements for these expensive and labile recognition agents.

Molecular imprinting technology, which is based on matching spatial distribution of complementary functional groups on the surface of receptor polymers to those of target molecules, has been largely confined to small organic molecules as the

substrates. On the other hand, literature on the development of imprinted polymers for the recognition of proteins and other biological species is relatively scarce. This is due to the fact that the problem of designing imprinted polymers for biopolymers is a difficult mission compared to small molecules. Some of the limitations include the relative complexities of protein and cell surfaces, which carry large numbers of competing binding sites. Furthermore, their sensitivity to temperature, pH, the nature of the solvent, biocompatibility and large molecular sizes are some of the major factors which need consideration while designing molecular imprinting polymerization processes for these substrates. This implies that there is an incentive to develop very innovative chemistries to prepare imprinted polymers as receptors to selectively recognize and bind biological macromolecules and assemblies.

In spite of the above limitations, efforts have been made in recent years to prepare imprinted polymers for the recognition of proteins. The first example in this direction was reported by Mosbach and co-workers (Glad et al., 1985). They were able to prepare a thin layer of polymer coating on the surface of porous silica beads, which was selective for the glycoprotein transferrin. Transferrin contains two identical carbohydrate moieties, each with two branches ending in a sialic acid group. Since boronic acid is known to interact covalently with cis-diol groups (e.g. those in carbohydrate moieties of glycoproteins) (Benes, 1993), the boronic acid-diol interaction was used as the binding site for preparing said polymer. They prepared a boronate silane functional monomer, N-[2-hydroxy-3-(tripropoxy silyl)propoxy]propyl-3-amino benzene boronic acid (I), which was allowed to equilibrate with the protein. Subsequently a mixture of several other organic silanes was added and the mixture was polymerized. Polysiloxanes were prepared with both transferrin and BSA as the template. A control polymer was prepared in the absence of a protein. The selectivity of the polymers for transferrin was tested in chromatographic experiments and was reported as the relative retention (relative retention = elution volume for the transferrin/ elution volume of BSA). The relative retention of transferrin imprinted polymer was found to be 2.16. In contrast, the relative retention for both control and BSA imprinted polymers was found to be 1.22. This suggests that the transferrin imprinted polymer showed a higher affinity for transferrin when compared to BSA (Glad et al., 1985).

Arnold and co-workers proposed a novel and generic approach to prepare molecularly imprinted matrices for the selective recognition and binding of proteins (Mallik et al., 1994) They used metal coordination interaction to induce complementary binding between the matrix bound metal chelates, with the coordinating ligands present on the surface of proteins. The rationale to this approach was derived from the fact that, due to the complexities of protein surfaces, an effective and general recognition would depend on the strategic placement of as few specific functional groups as possible. Thus, incorporation of strong and specific binding interactions is crucial for distinguishing proteins from one another when in contact with the receptor. Metal coordination interaction has been considered to meet most of these needs. Surface exposed metal coordinating residues such as histidines exhibit high affinity for different metal ions (viz. $Cu^{2+}$, $Zn^{2+}$, $Hg^{2+}$, etc.). This affinity between metal chelates and proteins has been the basis for protein purification using immobilized metal affinity chromatography (Arnold, 1991).

In order to address this problem in a systematic manner, they set out to prove this concept using structural analogues of proteins at the beginning (Dhal et al., 1992). Towards this end, they synthesized a number of small molecule bis-imidazole derivatives as protein analogues. Metal coordinating functional monomers were prepared and cross-linking polymerization reactions were carried out in the presence of these bis-imidazole derivatives as the templates. After removal of the templates, substrate selectivities of these polymers were tested under batch and chromatographic conditions (Dhal et al., 1995).. Another approach devised by this group to design synthetic receptors for proteins was the synthesis of two-dimensional matrices such as liposomes and bilayers (Mallik et al., 1994). By using metal coordination mediated vesicle formation (Figure 2.3) they prepared two-dimensional receptors. A good deal of effort was made to understand the properties of these interfacial functional matrices with metal coordination sites, with an aim towards the rational design of polymeric receptors for selective recognition of proteins.

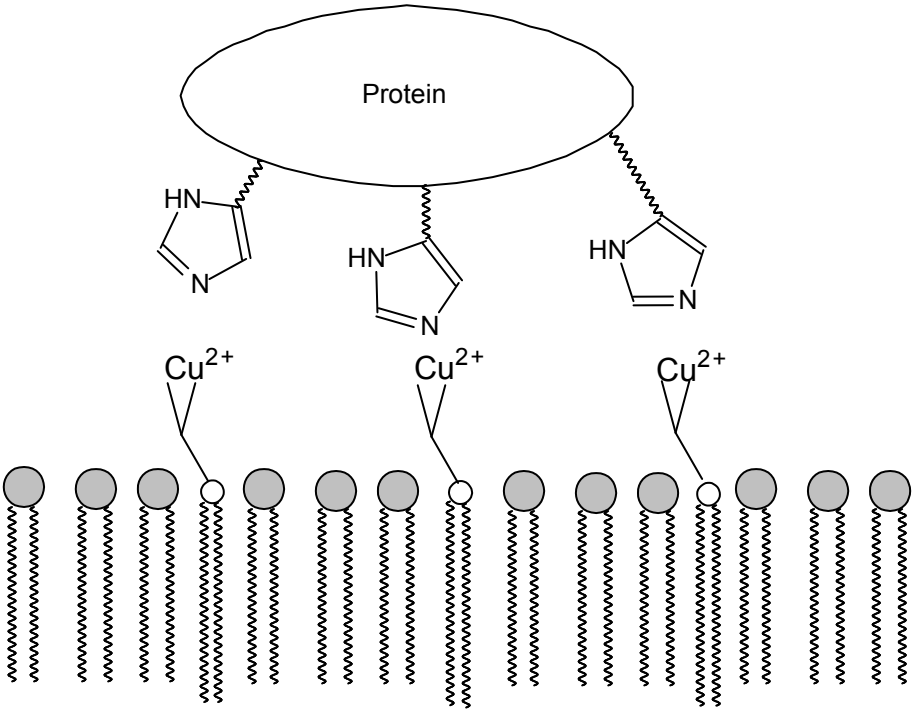


**Figure 2.3.** Schematic illustration of metal coordination mediated two-dimensional assemblies for protein recognition.

Mosbach and co-workers also attempted to use metal coordination interactions to prepare polymeric adsorbents for selective recognition of proteins (Kempe et al., 1995). They polymerized the protein-monomer complex on methacrylate derivatized silica particles in the presence of ribonuclease A as the template protein. After removing the template, the imprinted functional silica particles were used as packing adsorbents in an HPLC column and the separation of lysozyme and ribonuclease A was carried out using this column. An enhanced affinity for the ribonuclease A was observed.

Venton and co-workers carried out the synthesis of functional sol-gel matrices in the presence of protein-based templates to prepare imprinted polysiloxanes for protein recognition (Venton and Gudipati, 1995). They proposed that organic functional side chains on silanol monomers tended to associate with complementary functional groups on the protein surface during polymerization. This phenomenon would lead to complementary binding pockets for the proteins inside the polymer matrices. 3-aminopropyl triethoxysilane was used as the functional monomer and tetraethyl orthosilicate as the cross-linker. Sol-gel synthesis was carried out using urease and

BSA as the template proteins. After formation of the matrices, removal of the templates was accomplished by pronase digestion. The template free silica prepared in the presence of urease as the template showed preferential binding affinity towards its template over the non-templated protein BSA. However, using the closely analogous proteins hemoglobin and myoglobin as templates, the resulting polymers were unable to distinguish the template from non-templates. This further reinforces the need to devise matrices based on highly specific binding interactions.

Hjerten and co-workers prepared substrate-specific polyacrylamide gels by carrying out cross-linking polymerization in the presence of proteins (Liao et al., 1996). Polymerization of acrylamide and N, N'-methylenebisacrylamide was carried out in the presence of cytochrome C and hemoglobin as the templates. After removal of the templates using surfactants and acetic acid, substrate selectivity of the polymer gels were tested by chromatographic methods. The imprinted polymers reportedly showed preferential affinities for their templates.

Burow and Minoura performed a similar kind of investigation to prepare protein imprinted polymers (Burow and Minoura, 1996). They used methacrylate modified silica particles as the carrier matrix on which imprinted sites were created. Using acrylic acid as the functional monomer and *N,N'*-1,2-diethylene bisacrylamide as the cross-linker, template polymerization was carried out in the presence of glucose oxidase. This approach led to formation of a thin layer of cross-linked polymer film on the silica surface. After removing the template protein, substrate selectivity of the polymer was tested. Preferential affinity of the polymer for its template suggests the formation of substrate-selective binding sites in the polymer matrix.

In the approaches mentioned above, the capacity of receptors to bind enzymes was low, probably because only the weak non-covalent interactions between the enzyme and monomers were exploited to create the receptor sites. Another reason for this low uptake could be that, while the tertiary structure of enzyme is three dimensional, the imprinting was restricted to the two-dimensional surface of the silica-based support, which can recognize only the superficial surface topography of an enzyme. Thus, there is a need to develop a methodology which will provide a stronger interaction between the receptor and the enzyme.

Inhibitors contain functional groups which possess strong affinity for the active site of enzymes. These interactions have been exploited in affinity precipitation and affinity ultrafiltration techniques for selective recovery of enzymes (Benes, 1993). Thus, utilising this principle of biospecific inhibitor-enzyme interaction, Vaidya et al. have developed an elegant imprinting methodology for the recognition of enzymes (Vaidya et al., 1999). By using this “affinity-imprinting" (which is a combination of molecular imprinting and affinity chromatography), novel protein receptors were designed. For this purpose, N-acryloyl-para-aminobenzamidine was used as the affinity monomer and the monomer was allowed to form a complex with the active site of trypsin. The optimal ratio of the affinity monomer to trypsin was arrived at from active site titration. This complex was polymerized with the comonomer acrylamide and the cross-linker N, N’-methylenebisacrylamide to form gels. After polymerization trypsin was removed. This created a polymer matrix possessing cavities with shapes specific for trypsin. The trypsin imprinted gel behaved as a true receptor, as evidenced by the fact that it exhibited a linear Scatchard plot for the uptake of trypsin. The corresponding plot for the non-imprinted gel was non-linear, manifesting simple affinity chromatographic behavior. In a batch experiment, the trypsin imprinted gel exhibited exclusive uptake of trypsin, while a closely related enzyme, chymotrypsin, was not taken up. In competitive binding experiments using a mixture containing trypsin and chymotrypsin, the trypsin imprinted gel took up trypsin exclusively. In terms of capacity, this gel had an uptake capacity of 0.62 mg trypsin/g of polymer, which is much higher than the capacity of the imprinted silica gel for glucose oxidase (5.6 μg/g) reported by Burow and Minoura (Burow and Minoura, 1996).

## 2.4. Hemoglobin

Haemoglobin (Hb) is the iron-containing oxygen transport metalloprotein in the red cells of blood in mammals and other animals. Hb is a globular protein, and structural changes in any of the four molecular subunits that it is comprised of, can result in the manifestation of hereditary diseases such as sickle cell anaemia, thalassaemia and haemoglobinopathies (Nagari et al., 1987). The laboratory diagnosis of such conditions requires a combination of complicated and time-consuming procedure (Kleinschmidt et al., 1987), whereas MI may offer a rapid, sensitive and selective approach to the screening, diagnosis and monitoring of haemoglobin disorders.

### 2.4.1. Functional Class

Oxygen transport protein; heme containing protein related to myoglobin; know as hemoglobin (Hb). Hemoglobins (Hbs) are heme-containing proteins that share a well-known ‘globin fold’ and reversibly bind molecular oxygen. They are found in diverse species from bacteria (Wakabayashi et al., 1986) to man. They exist as monomers, dimmers, tetramers or even higher molecular weight assemblies. Polypeptide chains consisting of duplicated globin domains have also been found (Baere et al., 1992). The oligomerization of Hb was a crucial step in evolution, by which Hb acquired its allosteric properties essential for efficient oxygen transport.

### 2.4.2. Occurrence

They are found in erythrocytes (red blood cells). The concentration of Hb in human erythrocyte is approximately 30% (w/v) or 20 mM in heme. Erythrocytes take up approximately 40-45% of the blood volume (hematocrit) and hence 100 ml of human blood contains approximately 15 g of Hb.

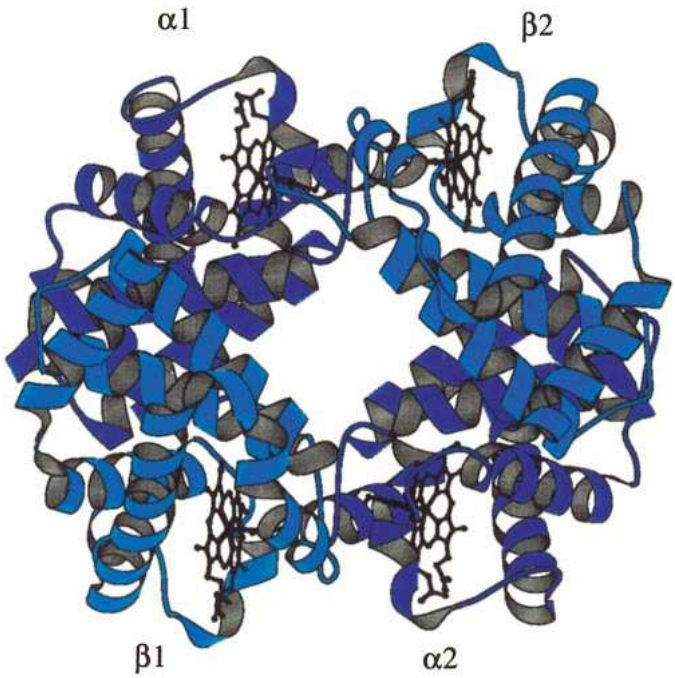


**Figure 2.4.** Schematic representation of the quaternary structure of deoxy T state Hb (PDB code: 1HGA). The α-subunites are in blue, while the β-subunits are in cyan; the heme groups are shown in a black ball and stick model. The cavity present at the center of the tetramer in its T state conformation is apparent.

### 2.4.3. Biological Function

The physiological function of vertebrate Hbs are the transport of oxygen from the lungs (or gills in fish) to the body tissues, enhancement of carbon dioxide transport in the opposite direction and regulation of blood pH (acid-base balance). In humans, the partial oxygen pressure in the lungs (alveolar oxygen pressure) is approximately 100 mmHg and Hb becomes 98% saturated with oxygen at this pressure. The partial pressure of oxygen in mixed venus blood (blood returning to the lungs) is approximately 40 mmHg and under these conditions Hb is only 70-75% saturated with oxygen (Kilmartin and Bernardi, 1973). Approximately one quarter of the blood oxygen is therefore delivered to the tissues.

Hb contains two α- and two β- subunits, each containing a heme or Fe(II)-protoporphyrin IX. Since each heme can bind one oxygen molecule, four molecules of oxygen bind to a Hb tetramer (3D structure). The affinity for the first oxygen is low but

the affinity rises with the number of bound oxygen molecules. This is known as the heme-heme interaction or homotropic allosteric effect.

Early observation that oxygenated and deoxygenated Hbs crystallize in different crystal forms suggested that Hb undergoes a structural change upon binding to oxygen (Haurowitz et al., 1938). The elucidation of the crystal structure of horse met (ferric) and deoxy and human deoxy Hb by Perutz and his coworkers revealed the structural differences between deoxy and oxyHb in detail (Perutz, 1970). Perutz proposed a stereochemical mechanism of cooperative oxygen binding based on these structures. The structures of the deoxy and various liganded forms of Hbs are now known at high resolution.

As a first approximation, the oxygen binding properties of Hb can be described by the two-state allosteric model proposed by Monod, Wyman and Changeux (Monod et. al., 1965). They proposed that the Hb molecule is in equilibrium between two alternative quaternary structures (arrangement of the four subunits) (Figure 2.4). The T(tense), or deoxy structure has a low oxygen affinity and the R(relaxed), or oxy structure has high oxygen affinity. Since deoxy Hb is predominantly in the T state, the first oxygen binds with low affinity. The binding of oxygen stabilizes the R state, and thus the oxygen affinity increases with the number of the bound oxygens. The equilibrium between the T and R states is also affected by the concentration of other metabolites within erythrocytes. Purified Hb(stripped Hb) shows much higher oxygen affinity than whole blood and is unable to unload sufficient amount of oxygen to the tissues. An intermediate of the glycolytic pathway, 2,3-diphosphoglycerate(2,3-DPG), is present in equimolar amount to the Hb tetramer in human erythrocytes. It reduces the oxygen affinity of Hb by preferentially binding to the deoxy form. Below pH 6.0, the oxygen affinity is raised by protons. This is called the acid Bohr Effect and reversed Bohr Effect. Above pH 6.0, the oxygen affinity is reduced by protons and this is called the alkaline Bohr Effect. This effect is explained by release of protons associated with oxygen binding. Active tissues produce $CO_2$, which dissolves into water forming a bicarbonate ion and a proton, thereby lowering the pH. The effect of $CO_2$ is two-fold: the lowering of pH reduces the oxygen affinity and free $CO_2$ binds to the T state more tightly than to the R state, also reducing the oxygen affinity. Kilmartin and Rossi-Bernard (Kilmartin and Bernardi, 1973). showed the direct binding of $CO_2$ to the α-

amino groups of both α and β chains by forming carbamino groups. The allosteric properties of the Hb therefore allow preferential unloading of oxygen to the tissues, but Hb plays an important role in the transport of $CO_2$ because not only does it directly convey a fraction of $CO_2$ to the lungs mainly as bicarbonate ions, but also enhances bicarbonate formation through proton uptake upon deoxygenation.

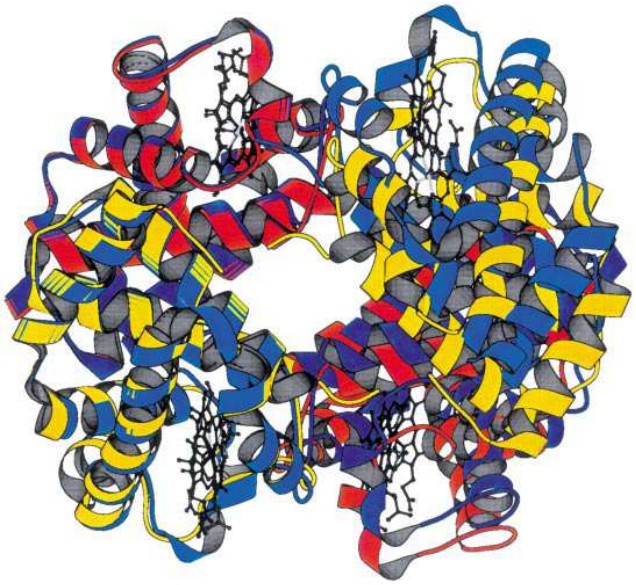

**Figure 2.5.** Comparison between the deoxy T (PDB code: HGA) and oxy R states (PDB: 1HHO) highlighting the shifts associated with the quaternary transition. Deoxy T state α subunits are shown in blue, while deoxy T state β-subunites are in cyan; oxy R state α-subunits are in red and oxy R state β-subunites in orange. The T state hemes are colored in black while the R state hemes in gray. Helices are represented by cylinders. The structures were superimposed by least squares fitting the main chain atoms of the B, G and H helices of the $\alpha_1\beta_1$ dimer. Some of the movements of rotation and translation of the $\alpha_2\beta_2$ dimer with respect to the $\alpha_1\beta_1$ dimer are apparent.

### 2.4.4. Amino Acid Sequence Information

Hbs from a considerable number of vertebrate species have been sequenced and this has provided important insights into molecular evolution of proteins. The majority of these sequences have been determined by Braunitzer, who devoted his life to sequence determination of Hbs from rare species (Kleinschmidt and Sgouros, 1987). The cDNA of human α and β globin chains were the first cDNA to be cloned and sequenced (Niehaus and Maniatis, 1987). Numerous Hb sequences can be found in

protein databases (Swiss Prot URL: www.expasy.ch/srot). The sequence identity between the most distantly related vertebrate Hbs contain small deletions and insertions. Hbs are post-translationally modified in some species. The acetylation of the N-terminus is the most common modification. Human blood contains minor glycosylated components. Diabetic patients have elevated levels of glycosylated Hbs, which are used for diagnostic purposes (McDonald et al., 1979).

### 2.4.5. Purification and Molecular Characterization

Hb can be prepared readily from vertebrate blood. Freshly drawn blood is immediately mixed with either heparin or citrate to prevent coagulation and blood cells are separated from plasma by centrifugation. After washing the cells with isotonic saline (0.9% NaCl(w/v)), an equal volume of water is added to lyse the erythrocytes and Hb is released from the cells. Cell debris can be removed by centrifugation after addition of NaCl to 3% (w/v). Hb represens 98% of protein in the hemolysate and no further purification is necessary.

Approximately 2% of Hb in adult bood is Hb $A_2$ consisting of two α- and two δ-subunits. Human fetal blood contains Hb F (fetal Hb) consisting of two α- and two γ-subunits. Deoxy Hb F does not bind DPG strongly and hence Hb F has higher oxygen affinity than Hb A in its presence.

### 2.4.6. Hemoglobin Variants

More than 700 characterized Hb variants have been reported, the majority of which are genetic mutations. Nearly 8% of African Americans carry the HbS trait and 2.3% carry HbC. In sub-Saharan Africa, prevalence of these two is up to one-third of all patients. HbE can be as high as 30% in Southeast Asia. Many hemoglobinopathies, including sickle cell disease, homozygous HbC disease, HbSC disease, and β-thalassemia, frequently show increased amounts minor species, i.e. $HbA_2$ and HbF. HbF can reach 30% in individuals with hereditary persistence and 20% in β-thalassemia and sickle cell patients. Chemically modified Hbs may be chronically present in diabetic patients. Carbamylated Hb is the most commonly encountered of

these (Bry et al., 2001). Facilities performing $A_{1C}$ testing should be aware of interferences produced in such assays from genetic variants such as HbS, HbC, and HbE and chemically modified derivatives such as carbamyl-Hb of hemoglobin.

## 2.5. Polymerization Techniques

The main polymerization techniques are bulk, solution, suspension and emulsion polymerization. Plasma polymerization and gas phase polymerization are other possibilities for polymerization. To produce spherical beads, emulsion or suspension or dispersion polymerization is performed. The size of resulting beads is less than 1 μm and ranges from 100 μm to 5 mm, if emulsion or suspension technique is followed, respectively. Suspension polymerization proceeds by dispersing the monomer in water with a suspending agent and then converting the dispersed droplets into polymer by reaction with an initiator that is normally soluble in the monomer.

The monomer droplets are usually more finally dispersed in emulsion polymerization, with diameters ranging from a few microns to >10 μm. Unlike suspension polymerization, the emulsion process does not simply involve polymerization of the monomer droplets. Nucleation and growth of smaller particles is a significant mechanism in emulsion polymerization (Billmeyer, 1971).

### 2.5.1. Free Radical Polymerization Technique

Free radical polymerization is the most widespread method of polymerization of vinylic monomers. Free radical polymerizations are chain reactions in which every polymer chain grows by addition of a monomer to the terminal free radical reactive site called “active center”. The addition of the monomer to this site induces the transfer of the active center to the newly created chain end. Free radical polymerization is characterized by many attractive features, such as applicability for a wide range of polymerizable groups including styrenic, vinylic, acrylic and methacrylic derivatives, as well as tolerance to many solvents, small amounts of impurities and many functional groups present in the monomers. However, classical free radical polymerization has some limitations, inherent to its mechanism. In particular, it is difficult to control molar

masses and polydispersities as well as to introduce defined end-groups, or to prepare special macromolecular architectures such as block copolymers. In order to overcome these limitations, new strategies in free radical polymerization have emerged recently, often referred to as “controlled” free radical polymerization (Fischer, 2001).

Characteristically, these methods use reagents that transform reversibly the propagating radicals into a dormant species. So in average, the polymer chains grow simultaneously, and no more one after the other. Thus, chain growth and monomer consumption takes place at a comparable rate. Processes that have received most attention are: i) nitroxide-mediated radical polymerization (NMRP) (Georges, 1993; Hawker et al., 2001), ii) Atom Transfer Radical Polymerization (ATRP) (Matyjaszweski, 2001; Kamigaito et al., 2001) catalysed by transition metal complexes, and more recently iii) polymerization via Reversible Addition-Fragmentation chain Transfer (RAFT) (Le et al.,1998; Rizzardo et al., 1999; Moad et al., 2000) or Macromolecular Design via Interchange of Xanthates (MADIX) (Charmot et al D. 1999, Destarac et al., 2002). In the case of xanthates are used as chain transfer agents. Within these methods, the first two ones are based on reversibly blocking the growing polymer radical, whereas the last one is based on degenerative chain transfer (Jagur and Grodzinski, 2001, Matyjaszweski, 2003). The RAFT process seems to be well adapted to numerous classes of monomers, solvents (a priori, this process is also compatible with water), functional moieties and to a large temperature window. It enables in many systems the control of the molar mass and of the polydispersities, and allows preparing complex architectures e.g. stars (Mayadunne et al., 2002; Barner et al., 2003) and comb-like graft block copolymers (Quinn et al., 2002). In order to understand the advantages of the RAFT process on a classical free radical polymerization we will describe the mechanism of free radical polymerization for comparison.

### 2.5.2. Stages of Free Radical Polymerization

The mechanism of the free radical polymerization (Chiefari et al., 1998; Hawthorne et al., 1999) can be divided into three distinct stages referenced as initiation, propagation and termination. Initiation: The initiation step is considered to involve two reactions creating the free radical active center. The first event is the production of free radicals.

This can be done by several mechanisms such as thermal, redox or photochemical reactions. The most common case is the homolytic dissociation of an initiator I to yield two radicals $R_1$• often referred as primary radical. The subsequent event is the addition of this radical to a monomer molecule M producing the initial propagating species $P_1^{\bullet}$.

Equation (2.1; 2.2):

$$I \xrightarrow{k_d} 2R_1^{\bullet} \qquad V_d=2k_d[I] \qquad (2.1)$$

$$2R_1^{\bullet} + M \xrightarrow{k_i} P_1^{\bullet} \qquad V_i=k_i[M].\,[R_1^{\bullet}] \qquad (2.2)$$

Since the formation of R1• proceeds more slowly that the reaction of R1• with monomer, the first step is rate-determining, so that Vi can be neglected. Propagation: Propagation consists in the successive addition of a large number of monomer molecules M to the primary radical Pn•. Each rapid sequential addition of a monomer molecule creates a new radical Pn+1• that is chemically identical to the previous one, except that it is larger by one monomer unit. The successive additions are represented in a general equation by: Equation (2.3):

$$P_n^{\bullet} + M \xrightarrow{k_p} P_{n+1}^{\bullet} \qquad V_p = k_p\,[M]\,[P^{\bullet}] \qquad (2.3)$$

where $k_p$ is the constant rate for propagation and $[P^{\bullet}] = \Sigma_i\,[P_i^{\bullet}]$. Usually, the propagation takes place rapidly. The value of $k_p$ for most monomers is in the range 102-104 $l \cdot mol^{-1} \cdot s^{-1}$. Typically, the time required for the addition of a monomer to a growing chain is about a millisecond. In fact, thousands of addition can occur within few seconds. Termination: In this step, the growth of the polymer chain is terminated irreversibly. The two most common mechanisms for termination involve bimolecular reactions. The first one, combination, is the reaction between two growing polymer chains leading to the annihilation of the radical active centers and the formation of a single polymer chain. Termination by combination is represented by the equation: Equation (2.4):

$$P_n^{\bullet} + P_m^{\bullet} \xrightarrow{k_{tc}} P_{n+m} \qquad V_t = k_{tc}(P_n^{\bullet})^2 \qquad (2.4)$$

where $k_{tc}$ is the rate constant for combination. The second one is the termination via disproportionation. A hydrogen atom is abstracted from a growing chain and transferred to another one. This reaction leads to two terminated polymer chains, one with an unsaturated end-group and the other one with a saturated end-group. Equation (2.5):

$$P_n^{\bullet} + P_m^{\bullet} \xrightarrow{k_{td}} P_n + P_m^{\bullet} \qquad V_t = k_{td}(P^{\bullet})^2 \qquad (2.5)$$

This equation represents the termination by disproportionation where $k_{td}$ is the rate constant. Because in a conventional free radical polymerization, combination and disproportionation prevail, the overall rate constant for termination for which the mode of termination is not specified can be approximated as $k_t = k_{tc} + k_{td}$. Typical rate constant values for termination are in the range of 106-108 $l \cdot mol^{-1} \cdot s^{-1}$, i.e. they are very high. The termination step leads unavoidably to *dead polymer*: the growth of the polymer chain is terminated and the active centers are irreversibly annihilated. This implies that it is impossible to form block copolymers by adding a new monomer and re-activating the polymerization system.

### 2.6. Hydrogels, so called Cryogels

At present, polymeric gels have application in many different areas of biotechnology including use as chromatographic materials, carriers for the immobilization of molecules and cells, matrices for electrophoresis and immunodiffusion, and as a gel basis for solid cultural media. A variety of problems associated with using polymer gels, as well as the broad range of biological objects encountered, lead to new, often contradictory, requirements for the gels. These requirements stimulate the development and commercialization of new gel materials for biological applications. One of the new types of polymer gels with considerable potential in biotechnology is ‘cryogels’ (from the greek kryos meaning frost or ice) (Lozinsky, 2002). Cryogels were first reported nearly 40 years ago and their properties, which are rather unusual for polymer gels, soon attracted attention. The biomedical and biotechnological potential

of these materials has now been recognized (Lozinsky, 2002; Nambu, 1990; Kaetsu, 1993; Suziki, and Hirasa, 1993; Lozinsky, 1998; Lozinsky and Plieva 1998; Hassan and Peppas 1998; Lozinsky et al., 2001).

Conventional packed-bed columns possess some inherent limitations such as the slow diffusional mass transfer and the large void volume between the beads (McCoy et al., 1996). Although some new stationary phases such as the non-porous polymeric beads (Denizli et al., 2000) and perfusion chromatography packings are designed to resolve these problems, these limitations cannot be overcome in essence (Özkara et al., 2003). Recently, cryogel materials are considered as a novel generation of stationary phases in the separation science because of their easy preparations, excellent flow properties and high performances compared to conventional beads for the separation of biomolecules (Lozinsky et al., 2003; Arvidsson et al., 2003; Lozinsky et al., 2002; Arvidsson, et al., 2002; Babaç et al., 2006; Dainiak et al., 2006; Hanora et al., 2006).

Porous cryogels are a very good alternative to protein separation with many advantages. Several potential advantages of cryogels are large pores, short diffusion path, low pressure drop and very short residence time for both adsorption and elution.

Cryogels are gel matrices that are formed in moderately frozen solutions of monomeric or polymeric precursors. Cryogels typically have interconnected macropores (or supermacropores), allowing unhindered diffusion of solutes of practically any size, as well as mass transport of nano and even microparticles. The unique structure of cryogels, incombination with their osmotic, chemical and mechanical stability, makes them attractive matrices for chromatography of biological nanoparticles (plasmids, viruses, cell organelles) and even whole cells. Polymeric cryogels are efficient carriers for the immobilization of biomolecules and cells. Cryogels are formed as a result of cryogenic treatment (freezing, storage in the frozen state for a definite time and defrosting) of low or high molecular weight precursors, as well as colloid systems all capable of gelling.

Production is carried out in partially frozen reaction system where ice crystals perform as pore-forming material (porogen) and the gelation proceeds in non-frozen

microphase of the apparently frozen reaction system. Figure 2.6.a shows cryogel formation.

When gelation in microphase is completed, melting of the reaction system results in a system of large pores (the space previously occupied by ice crystals) surrounded by walls of dense hydrogel formed in the unfrozen microphase. In Figure 2.6.b different forms of cryogels can be seen.

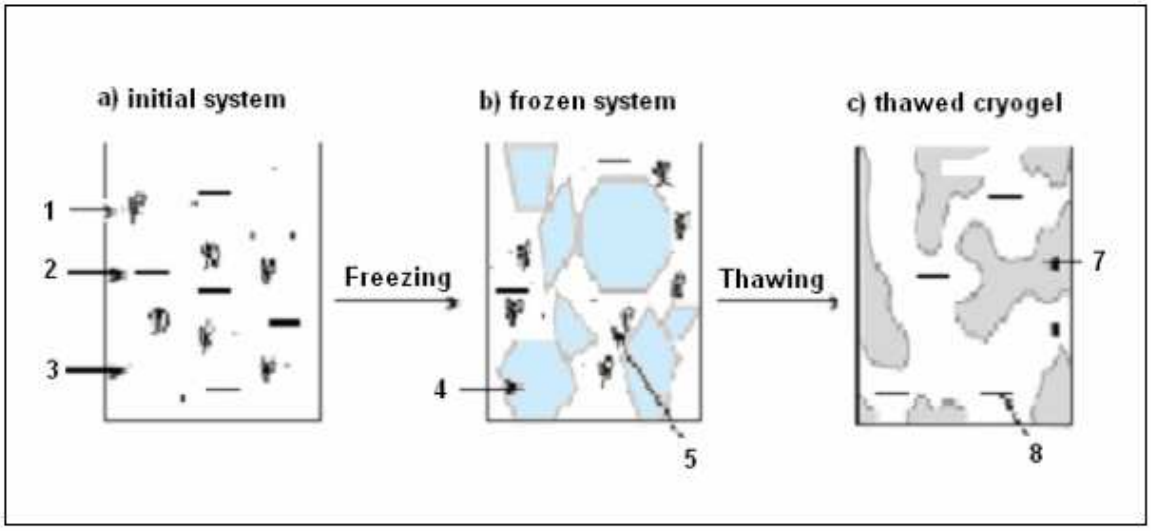


**Figure 2.6.a.** Cryogel formation. 1-Macromolecules, 2-Solution, 3-Solvent, 4-Polycrystals of freezed solvent, 5-Unfreezed solvent, 6-Cryogel 7-Macropores, 8-Solvent.

Cryogels can be prepared in different forms; Beads, monolithic columns or membrane disks**.**

**Figure 2.6.b.** Different forms of cryogels.

## 3. EXPERIMENTAL

### 3.1. Materials

L-histidine and methacryloyl chloride were supplied by Sigma (St. Louis, MO). Hydroxyethyl methacrylate were obtained from Sigma, distilled under reduced pressure in the presence of a hydroquinone inhibitor, and stored at 4 °C until use. Ammonium persulfate (APS), methylene bisacrylamide (mBAAm), and N,N,N',N'-tetramethylene diamine (TEMED) were also obtained from Sigma. Hemoglobin (Hb) from bovine, myoglobin (Myb) from equine skeletal muscle and albumin (Alb) from bovine serum were purchased from Sigma with purity of 95-100%. All other chemicals were reagent grade and were purchased from Merck AG (Darmstadt, Germany). All water used in the experiments was purified using a Barnstead (Dubuque, IA) ROpure LP reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731), followed by exposure to a Barnstead D3804 NANOpure organic/colloid removal and ion exchange packed bed system. Buffer and sample solutions were prefiltered through a 0.2-μm membrane (Sartorius, Göttingen, Germany). All glassware was extensively washed with dilute nitric acid before use**.**

### 3.2. Preparation of Cryogels

#### 3.2.1. Synthesis of N-methacryloyl-(L)-histidine (MAH)

The following experimental procedure was applied for the synthesis of N-methacryloyl-(L)-histidine (MAH). 5.0 g of L-histidine hydrochloride and 0.2 g of hydroquinone were dissolved in 100 ml of dichloromethane solution. This solution was cooled down to 0°C. 12.7 g triethylamine was added to the solution. 5.0 ml of methacryloyl chloride was poured slowly into this solution and then this solution was stirred magnetically at room temperature for 2 h. At the end of the chemical reaction period, hydroquinone and unreacted methacryloyl chloride were extracted with 10% NaOH solution. Aqueous phase was evaporated in a rotary evaporator. The residue (i.e., MAH) was crystallized in an ether-cyclohexane mixture and then dissolved in ethyl alcohol.

### 3.2.2. Preparation of Hb-Imprinted Cryogels (MIPs)

Firstly, a Hb-MAH complex was prepared. For this purpose, 1 mmol of hemoglobin was added to 1 mmol of MAH which was dissolved in deionized water. This mixture was incubated for 3 hours for the formation of a complex between Hb and MAH monomer before the polymerization process. The monomer mixture was prepared by adding 1.3 mL of HEMA into 10 ml of mBAAm (0,283 g) solution. After MAH-Hb complex was added to monomer mixture, the final monomer solution was degassed under vacuum for about 5 min to eliminate soluble oxygen. The free radical polymerization was initiated by adding APS and TEMED (1% (w/v) of the total monomers) in an ice bath at 0°C. Immediately, the reaction mixture was poured into 4 plastic syringe (5 ml, id. 0.8 cm) with closed outled at the bottom and was frozen at -16°C for 24 hours. The hemoglobin-imprinted cryogels (MIPs) were thawed at room temperature to form supermacropores. The non-imprinted cryogels (NIPs, p(HEMA-MAH) cryogel) were prepared in the same manner without using Hb as template. After washing with 200 ml of water, the cryogels were stored in buffer containing 0.02% sodium azide at 4°C until use.

The effect of the template amount in mg Hb/g polymer and the monomer concentration on pore size, flow resistance and swelling degree was investigated by altering the amount of template (Hb) and monomer concentration with eight different cryogels.

Table 3.1 shows the amount of template (Hb) used in mg/g cryogel and total monomer concentration per 100 mL monomer solution.

**Table 3.1.** Composition of the cryogels

| Notation for Cryogels | Amount of template (Hb) (mg)/g cryogel | %monomer in total concentration |
|---|---|---|
| MIP1 | 5 | 10 |
| MIP2 | 10 | 10 |
| MIP3 | 22 | 10 |
| MIP4 | 5 | 12 |
| MIP5 | 10 | 12 |
| MIP6 | 22 | 12 |
| NIP1 | 0 | 10 |
| NIP2 | 0 | 12 |

### 3.2.3. Removal of the Template (Hb)

In order to remove unreacted monomers and other ingredients, MIP cryogel was extensively washed with methanol/water solution (60/40, v/v). After cleaning procedure, the template (Hb) was removed by 0.1 M acetate buffer containing %10 ethylene glycol, (pH 4.0) solution passed through the Hb-imprinted cryogel column, by a peristaltic pump for 48 h at room temperature. This procedure was repeated until no Hb leakage was observed. Then, the column was washed with 50 mM NaOH for the sterilization. The amount of hemoglobin extracted from the structure was determined at 406 nm by spectrophotometer(UV-1601, Shimadzu, Japan). A calibration curve was prepared using hemoglobin solutions as standards. The experiments were performed in replicates of three, and the samples were analyzed in replicates of three as well.

### 3.3. Characterization of Cryogels

#### 3.3.1. Swelling Test

Water uptake ratios of the cryogels were determined in distilled water. The experiment was conducted as follows: initially dry cryogel were carefully weighed before being placed in a 40 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 sample was taken out from the water, wiped using a filter paper, and weighed. In different times the weight of cryogel was recorded. After 24 hour the final weight of cryogel was recorded. The weight ratio of dry and wet samples was recorded. The water content of the cryogel was calculated by using the following expression;

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

$W_o$ and $W_s$ are the weights of cryogel before and after uptake of water, respectively.

The gelation yield was determined as follows: the swollen cryogel sample (1 ml) was put in an oven at 60 °C for drying. After drying till constant weight, the mass of the dried sample was determined ($m_{dried}$). The gel fraction yield was defined as ($m_{dried}/m_t$) x100%, where $m_t$ is the total mass of the monomers in the feed mixture.

$$\text{Gel Fraction Yield} = (m_{dried}/m_t) \times 100\% \quad (3.2)$$

The total volume of macropores in the swollen cryogel was roughly estimated as follows: the weight of the sample ($m_{squeezed\ gel}$) was determined after squeezing the free water from the swollen gel matrix, the porosity was calculated as follows: ($m_{swollen\ gel} - m_{squeezed\ gel}$)/$m_{swollen\ gel}$ × 100%. The percent of swollen gel weight is also calculated as ($m_{swollen\ gel} - m_{dried}$)/$m_{swollen\ gel}$ × 100%. All measurements were done in triplicate and the average values are presented.

The flow-rate of water passing through the columns was measured at the constant hydrostatic pressure equal to 100 cm of water-column corresponding to a pressure of ca. 0.01 MPa. At least three measurements were done for each sample.

### 3.3.2. Surface Morphology

The surface morphology of the monolithic cryogel was examined using scanning electron microscopy (SEM). The sample was fixed in 2.5% glutaraldehyde for overnight. Then the sample was dehydrated at -50 °C in lyophilizate. (Lyophilizer, Christ Alpha 1-2 LD plus, Germany). Finally, it was coated with gold-palladium (40:60) and examined using a JEOL JSM 5600 scanning electron microscope (Tokyo, Japan).

### 3.3.3. Surface Area Measurements

The specific surface area of both MIP and NIP cryogels was measured according to the Brunauer-Emmett-Teller (BET) model using multi point analysis and a Flowsorb II 2300 from Micromeritics Instrument Corporation, Norcross, GA.

### 3.3.4. $^{1}$H-NMR and FTIR Studies

The proton NMR spectrum of MAH monomer was taken in $CDCl_3$ on a JEOL GX-400-300 MHz instrument. The residual non-deuterated solvent (TMS) served as an internal reference. Chemical shifts were reported in ppm (δ) downfield relative to TMS.

FTIR spectra of MAH monomer, Hb-MAH complex, MIP and NIP cryogels were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry particles (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.

## 3.4. Adsorption-Desorption studies

### 3.4.1. Adsorption of Hemoglobin from Aqueous Solutions

The hemoglobin adsorption studies were carried out in a recirculation system equipped with a water jacket for temperature control. Figure 3.1 shows the experimental set-up schematically. The cryogel was washed with 30 mL of water and

then equilibrated with 0.1 M phosphate buffer (pH 6.0) for 30 min. Then, the hemoglobin solution was pumped through the column under recirculation for 2 hour. The adsorption was followed by monitoring the decrease in absorbance at 406 nm. Effects of hemoglobin concentration, flow rate, pH of the medium, temperature and ionic strength on the adsorption amount were studied. The effect of the initial concentration of hemoglobin on adsorption capacity was studied by changing the concentration of hemoglobin between 0.1-2.0 mg/mL. The effect of flow rate on adsorption capacity was investigated at different flow rates changing between 0.5-4 mL/min pumped through the column under recirculation for 2.0 hour with 0.75 mg/mL of hemoglobin solution in 50 mM phosphate buffer (pH 6.0, 30 mL). The effect of pH on the adsorption capacity was determined by changing pH of the solution between 4.0 and 7.0. The observation of the effect of ionic strength was carried out in solutions containing different amounts of NaCl.

The amount of adsorbed hemoglobin was calculated as in Eq (3.3);

$$q= [(C_o-C)\ V]/m \qquad (3.3)$$

Here, q is the amount of hemoglobin adsorbed onto unit mass of cryogel (mg/g); $C_o$ and C are the concentrations of hemoglobin 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 used (g). Each experiment was performed three times for quality control and statistical calculations.

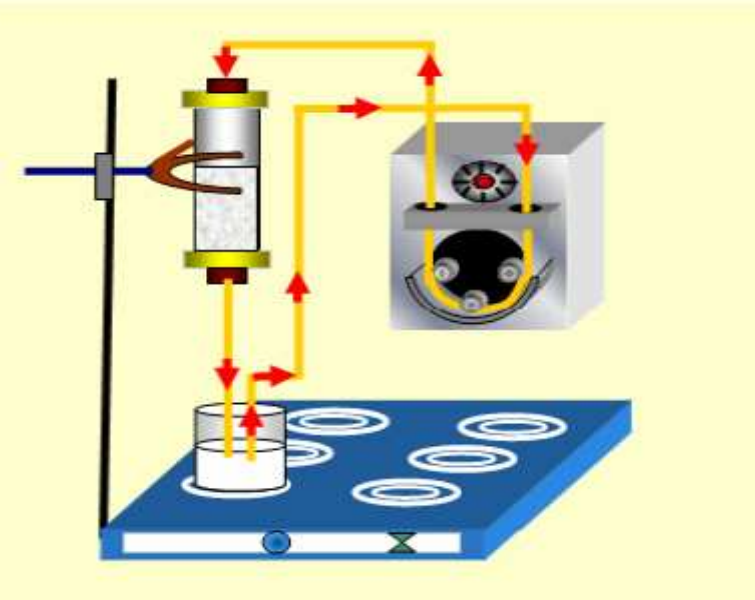

**Figure 3.1.** Continuous system used for adsorption experiments.

### 3.4.2. Selectivity Experiments

The hemoglobin selectivity of MIP cryogel was investigated by competitive adsorptions of Alb and Myb in 0.1 M of pH 6.0 phosphate buffer solution. The selectivity of the MIP cryogel for Hb (Mw: 64.5 kDa, pI: 6.8-7.0) with the competitive proteins Alb (Mw: 67.0 kDa, pI: 4.9) and Myb (Mw: 17.0 kDa, pI: 6.8-7.2) was evaluated in a circulating system with 0.45 g of the MIP cryogel, using a peristaltic pump for 2 h. After competitive adsorption equilibrium was reached, the remaining samples were detected separately for each protein by Analytical LC system (Ultimate-3000, Dionex, USA) equipped with LPG-3000 pump, WPS-3000 Autosampler, TCC-3000 Column Department, PDA-3000 Detector. Separation was carried out by MN-HPLC column (Nucleosil 4000-7 PEI, Length/I.D; 50/4 mm). Mobile phases A and B were prepared using 10 mM TRIS buffer (pH 8.2) and 20 mM TRIS buffer (pH 8.2) with 1.5 M NaCl, respectively. The chromatographic separation was performed using a linear gradient at 1 mL/min flow rate. After a 1 min starting period with 100% mobile phase A, a linear gradient started from 0% B in 2 min, continued with increasing B from 0% to 10% in 10 min and finished with increasing B from 10% to 60% in 20 min. 100 µL of protein solution was injected to the column. Absorbance was monitored at 406 nm for Hb and Myb and 280 for Alb. The separation was performed at the ambient temperature.

The distribution coefficients (Kd) for Alb and Myb with respect to Hb were calculated by the following equation, Eq. (3.4);

$$K_d = [(C_i - C_f)/C_f] \times V/m \quad (3.4)$$

in which $K_d$ represents the distribution coefficient for the Hb(mL/g); $C_i$ and $C_f$ are initial and final concentrations of proteins (mg/mL), respectively. V is the volume of the solution (mL) and m is the weight of the cryogel used in the column (g). The selectivity coefficient (k) for the binding of Hb with competing species (i.e, Alb and Myb) was determined by the following equation, Eq. (3.5);

$$k=K_{d(\text{template})}/K_{d(\text{competing protein})} \quad (3.5)$$

in which, $K_{d(\text{template})}$ is the distribution coefficient of the template protein (Hb) and $K_{d(\text{competing protein})}$ is the distribution coefficient of the competing proteins (Alb and Myb). The relative selectivity coefficient, which was used to estimate the effect of imprinting on protein selectivity, can be defined from the following equation, Eq. (3.6);

$$k=k_{MIP}/ k_{NIP} \quad (3.6)$$

in which $k_{MIP}$ is for MIP cryogel, $k_{NIP}$ is for NIP cryogel.

The selectivity of MIP cryogel was imaged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For this purpose, 50 µg of sample solutions were run on a 5%-12% stacking PAGE gel which was prepared with 12% T, 30% C monomer solution, where T was total acrylamide concentration and C was degree of cross linking. The high range molecular weight marker (205-6.5 kDa) was used (High Range Sigma Marker, Sigma, USA). The gel was stained with Coomassie Blue G250 and monitored by ImageQuant (Amersham, UK).

### 3.5. Desorption and Repeated Use

The desorption of the adsorbed hemoglobin from MIP cryogel was studied in continuous experimental setup. Hb adsorbed MIP cryogel was desorbed with 0.1 M acetate buffer containing %10 ethylene glycol, (pH 4.0) solution for 2.0 hour at room temperature. The final hemoglobin concentration in the desorption medium was determined by spectrophotometer at 406 nm. The desorption ratio for hemoglobin was calculated with the following expression:

$$\text{Desorption ratio (\%)} = \frac{\text{Amount of hemoglobin released}}{\text{Amount of hemoglobin adsorbed}} \text{ X } 100 \qquad (3.7)$$

In order to show the reusability of cryogel, adsorption-desorption cycle was repeated ten times by using the same cryogel.

# 4. RESULTS AND DISCUSSION

## 4.1. Characterization of MIP Cryogel

### 4.1.1. Swelling Tests

Different types of MIP and NIP cryogels were prepared by different polymerization conditions as described in section 3.1. Table 4.1 presents the swelling properties and linear flow resistance (hydrostatic pressure, ca. 0.01 mPa) of the monolithic cryogels. Due to the MIP3 has the maximum flow rate (981cm/h) compared to the other MIPs, it was chosen for working cryogel. As NIP1 has the maximum flow rate (820 cm/h) compared to the NIP2, NIP1 was situated as the control polymer. All MIP1, MIP2, MIP3, MIP4, MIP5, MIP6, and NIP1, NIP2 cryogels have high gelation yield (about %90) with similar swelling properties. The swelling ratio of both MIP and NIP cryogels are about 90% due to its interconnected macroporous structure. It can be concluded that both MIP and NIP cryogels have similar physical characteristics. Moreover, the swelling degree of all cryogels are considerable the same (about 10) compared to cryogels prepared by Bereli et al. (about 9.1) and considerable high compared to embedded cryogels prepared by Baydemir et al. (about 6.1) as a result of embedded particles into the cryogel structure that leads to the filling of gaps instead of the water molecules. The flow rate through the gel matrix is a simple way of estimating superporosity (Arvidson et al., 2003; Bereli et al., 2008, Baydemir et al., 2009).

**Table 4.1.** Swelling properties and flow resistance of cryogels.

| Notations for cryogels | Hb content (mg)/g cryogel | monomer conc. % | Yield % | Swelling ratio % | Swelling degree | Macroporosity | Flow rate (cm/h) |
|---|---|---|---|---|---|---|---|
| MIP1 | 5 | 10 | 86 | 91 | 10,8 | 76,4 | 650 |
| MIP2 | 10 | 10 | 93 | 91 | 10,2 | 78,44 | 240 |
| **MIP3** | **22** | **10** | **94** | **92** | **11,8** | **78,95** | **981** |
| MIP4 | 5 | 12 | 95 | 90 | 9 | 74 | 250 |
| MIP5 | 10 | 12 | 95 | 89 | 8,6 | 78 | 613 |
| MIP6 | 22 | 12 | 96 | 89 | 8,7 | 72 | 118 |
| **NIP1** | **0** | **10** | **98** | **95** | **12** | **81** | **820** |
| NIP2 | 0 | 12 | 96 | 91 | 10 | 75 | 520 |

### 4.1.2. Surface Area Measurements

The surface area of the MIP cryogel was found to be 92.3 $m^2$/g by BET method. An advantage of cryogels is that they have large, highly interconnected pores. Although this provides cryogels with low back pressures but it also gives them much lower surface areas compared to other chromatographic supports. This in turn can result in small amounts of immobilized ligand and low sample capacities when using cryogels in affinity separations (Arvidsson, et al., 2003).

### 4.1.3. Surface Morphology

The surface morphology of both MIP and NIP cryogel was exemplified by the scanning electron micrographs in Figure 4.1.

As clearly seen from Figure 4.1, the polymeric cryogel has macropores which formed during the polymerization procedure. After 16 hours of polymerization process, the formed hydrogels, so called cryogels, were allowed for thawing at room temperature. During this process, the thawed water molecules give place to large holes in the cryogel structure which was shaped in interconnected large pores. The presence of this interconnected supermacroporous structure within the cryogel interior was clearly seen in SEM photographs. It can be concluded that both for MIP and NIP cryogel have

a macroporous interior, in the dry state. These pores reduce diffusional resistance of hemoglobin molecules and facilitate mass transfer because of high pore size. The cryogel produced in such a way has large continuous interconnected pores (10–100 μm in diameter, supermacroporous) that provide channels for the mobile phase to flow through. Moreover, it can be used for samples with high viscosity, such as, body fluids, blood and plasma proteins. Pore size of the matrix is much larger than the size of the protein molecules, allowing them to pass easily. This also provides higher protein adsorption capacity.

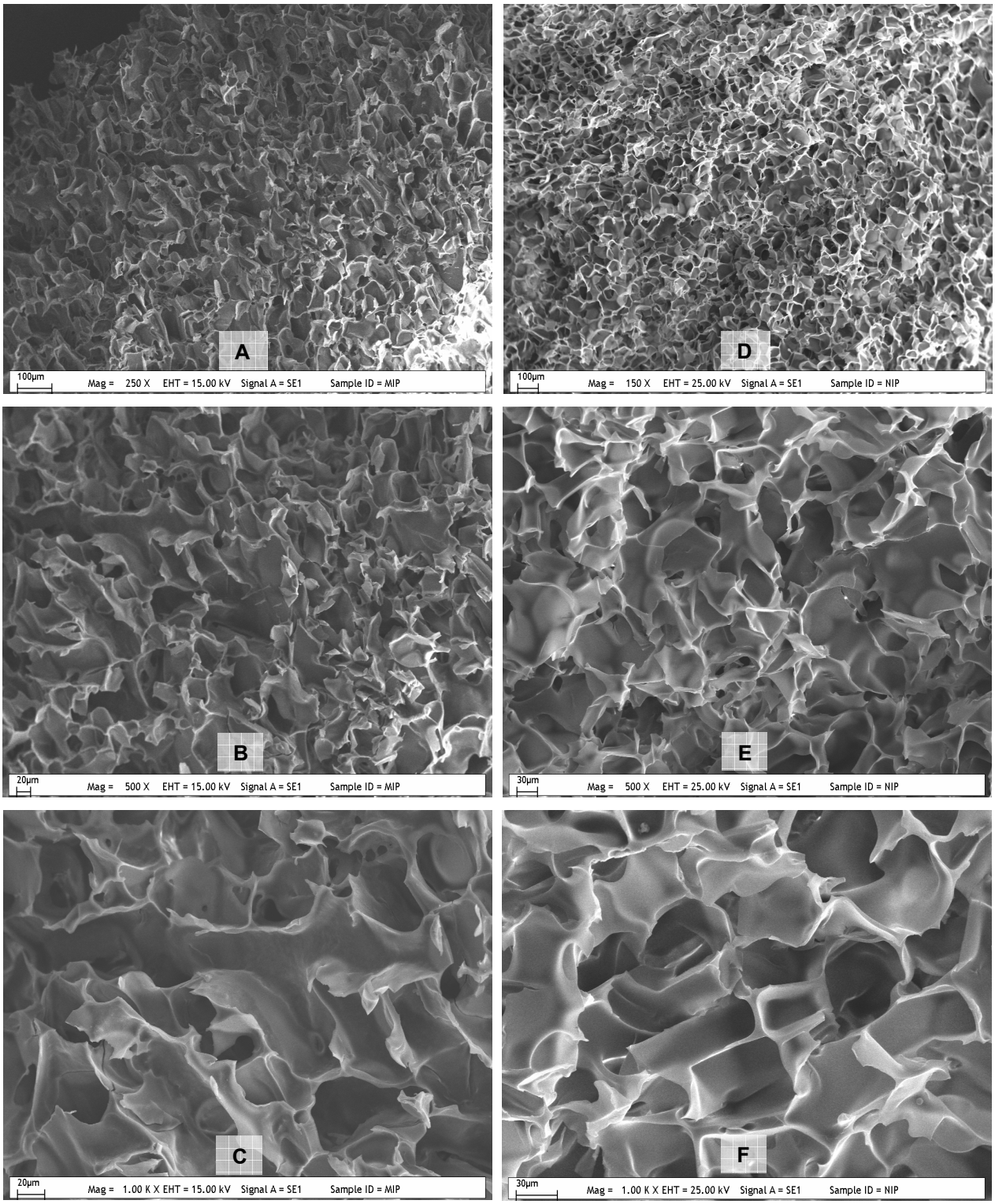


**Figure 4.1.** SEM photographs of MIP and NIP cryogels with different magnifications. A: 250, B: 500 and C: 1000 times magnified for MIP cryogels and D: 250, E: 500, F: 1000 times magnified for NIP cryogels.

### 4.1.4. $^{1}$H-NMR and FTIR Studies

$^{1}$H-NMR was used to determine the MAH structure. Figure 4.2 shows the $^{1}$H-NMR spectrum of MAH. $^{1}$H -NMR spectrum is shown to indicate the characteristic peaks from the groups in MAH monomer. These characteristic peaks are as follows: $^{1}$H -NMR ($CDCl_3$): δ 1.99 (t; 3H, J=7.08 Hz, $CH_3$), 1.42 (m; 2H, $CH_2$), 3.56 (t; 3H, -$OCH_3$) 4.82-4.87 (m; 1H, methine), 5.26 (s; 1H, vinyl H), 5.58 (s; 1H, vinyl); 6.86 (δ; 1H, J=7.4 Hz, NH), 7.82 (δ; 1H, J=8.4 Hz, NH), 6.86-7.52 (m; 5H, aromatic).

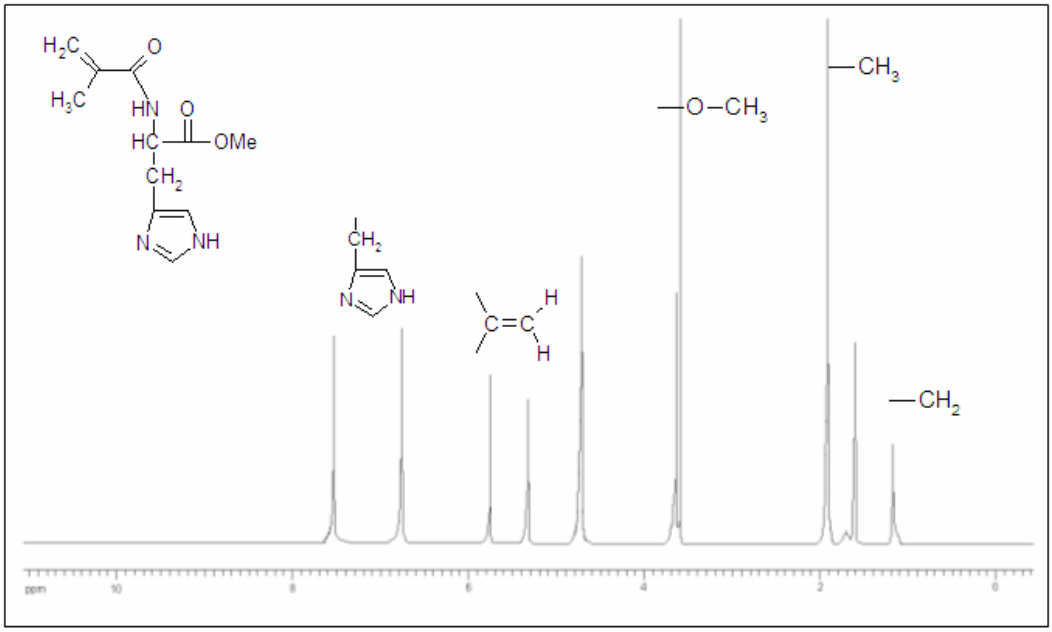


**Figure 4.2.** $^{1}$H-NMR Spectrum of MAH Monomer

Hemoglobin is a globular protein and has structural changes in any of the four molecular subunits. MAH monomer has an imidazole side chain as shown in Figure 4.2. FTIR spectra of both Hb and Hb-MAH complex having the characteristic stretching vibration band amide I and amide II adsorption bands around 1650 $cm^{-1}$ and 1458 $cm^{-1}$. The bands at about 2990 $cm^{-1}$ and 2870 $cm^{-1}$ were assigned to asymmetric and symmetric C–H stretching vibrations. The characteristic broad peak at around 3400 $cm^{-1}$ indicates the O–H stretching vibrations for both Hb and Hb-MAH complex (Figure 4.3.A).

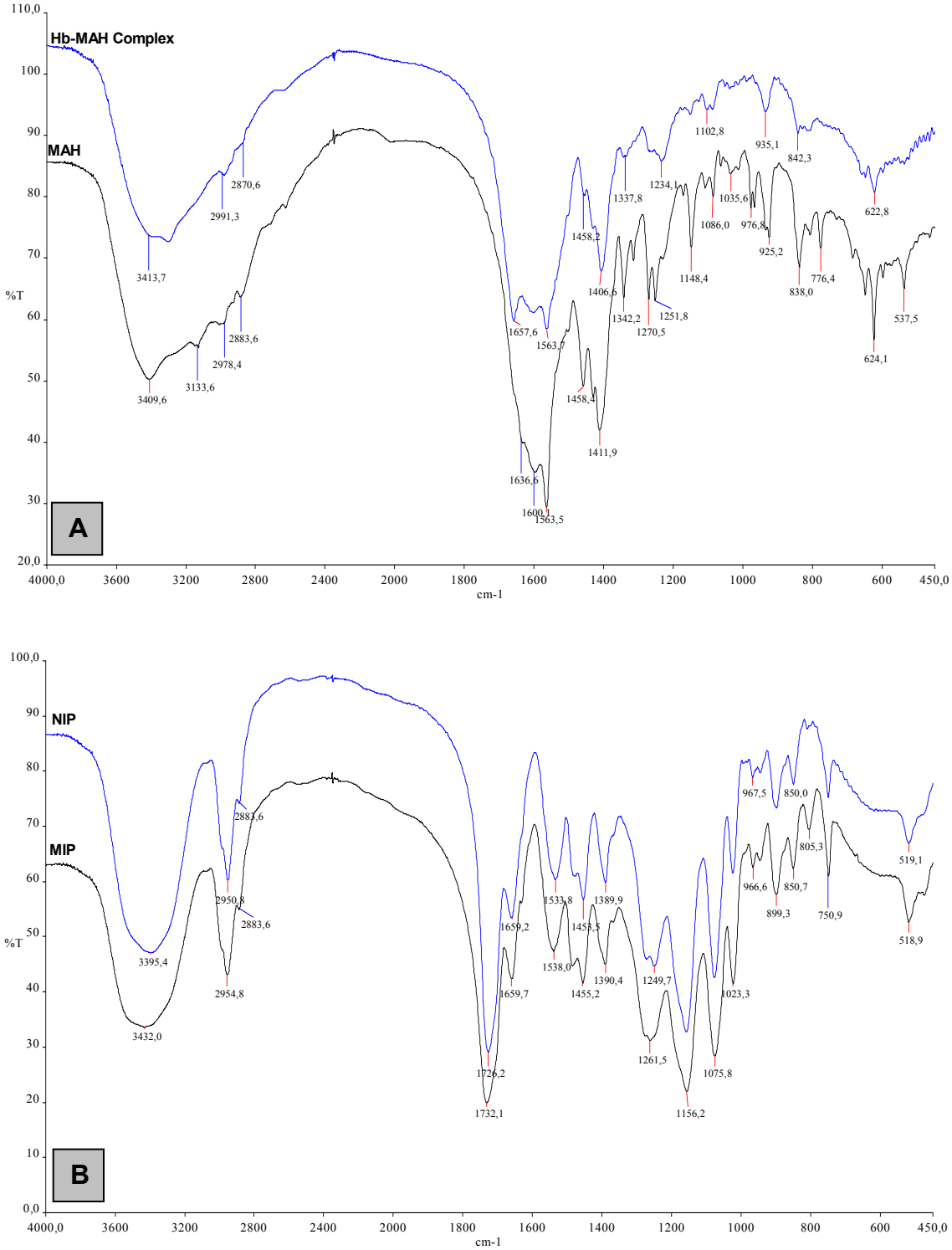


**Figure 4.3.** FTIR spectra of A) MAH, Hb-MAH complex and B) MIP, NIP cryogels.

FTIR spectra of both MIP and NIP cryogels have the characteristic broad peak at around 3400 cm-1, which indicates the O–H stretching vibrations. The bands at about 2950 $cm^{-1}$ and 2883 $cm^{-1}$ were assigned to asymmetric and symmetric C–H stretching vibrations. The presence of the band of medium intensity at 1732 $cm^{-1}$ indicated the presence of C–O stretching vibrations. The amides were characterized by the absorption bands due to N–H and C–O stretching vibrations, and N–H deformation due to a certain mixed vibration (amide bands). The appearance of strong bands at around frequencies of 1659 $cm^{-1}$ (C–O, amide I) and 1538 $cm^{-1}$ (N–H bending, amide II) indicates the incorporation of MAH into the polymer structure.

### 4.2. Adsorption of Hb from Aqueous Solutions

#### 4.2.1. Effect of pH

Protein adsorption onto specific adsorbents is pH dependent. Adsorption of hemoglobin onto the MIP cryogels seemed to be dependent on the buffer system. Adsorption studies were carried out using 0.1 M phosphate buffer in its buffering ranges. Figure 4.4 shows hemoglobin adsorption capacity in phosphate buffer at different pH values.

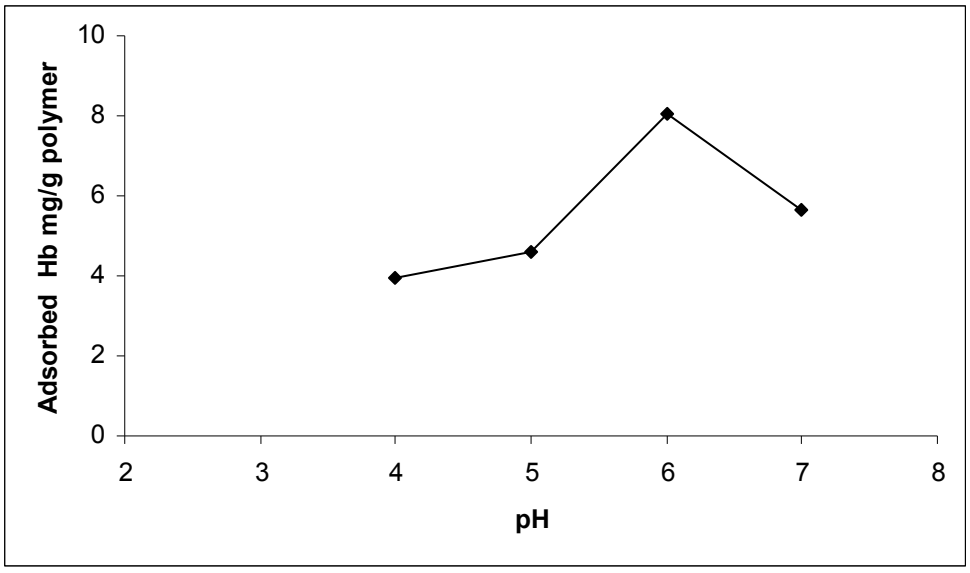


**Figure 4.4.** Effect of pH on Hb adsorption onto MIP cryogel.running buffer: 0.1 M phosphate buffer, T: 20ºC, flow rate: 1.0 mL/min, Hb Conc.: 0.5 mg/ml, $m_{dry\ cryogel}$: 0.45 g

In these pH ranges, maximum adsorption amount was observed at pH 6.0 as 8.05 mg Hb/g polymer. Maximum adsorption capacity was decreased significantly in more acidic and in more alkaline pH regions.

### 4.2.2. Effect of Flow Rate

The adsorption amount of MIP cryogel for Hb was determined using 0.1 M pH 6.0 phosphate buffer passed through a preequilibrated columns at different flow rates and plotted in Figures 4.5. The Hb adsorption amount of the MIP cryogel was observed to decrease drastically from 11.4 mg/g to 8.3 mg/g as the flow rate was increased from 0.5 ml/min to 4.0 ml/min. Ideally, Hb molecules would have more time to diffuse properly into the pores of cryogel and bind to binding sites at lower flow rates, and hence a better adsorption amount is achieved at a flow rate of 0.5 ml/min.

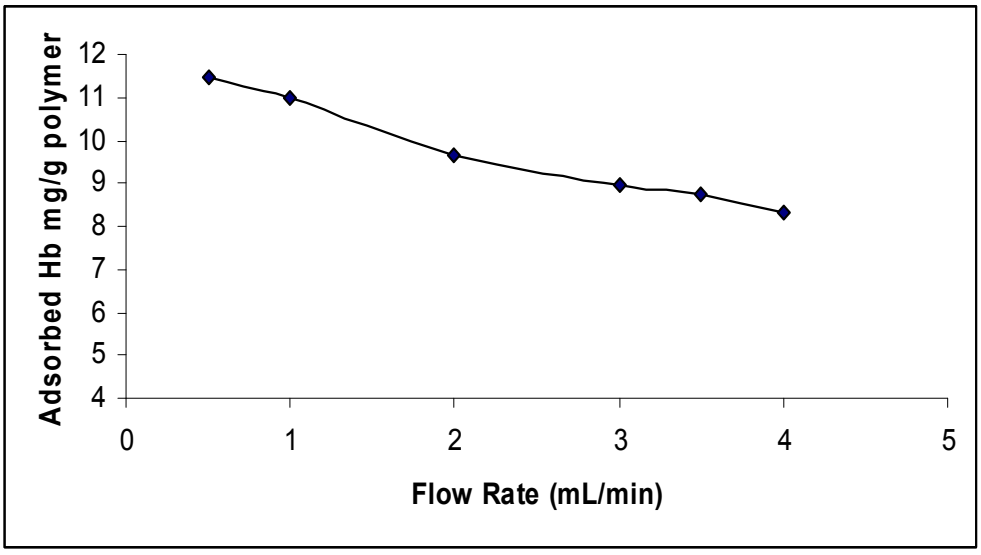


**Figure 4.5.** Effect of flow-rate on Hb adsorption onto MIP cryogel. running buffer: 0.1 M phosphate buffer(pH=6), T: 20ºC, Hb Conc.: 0.75 mg/ml, $m_{dry\ cryogel}$: 0.45 g

### 4.2.3. Effect of Equilibrium Concentration

As seen in Figure 4.6, the Hb adsorption in MIP cryogel column increases as the Hb concentration in buffer solution is increased, and the column was saturated with excess Hb binding when Hb concentrations above 0.75 mg/mL. The maximum Hb adsorption capacity by the MIP cryogel column was found to be 11.01 mg/g polymer on the average. The appropriate choice for the solvent applied during material preparation and recognition events is of high importance (Striegler, 2004). It should be noted that the hemoglobin adsorption onto NIP cryogels were about 1.66 mg/g at the same concentration.

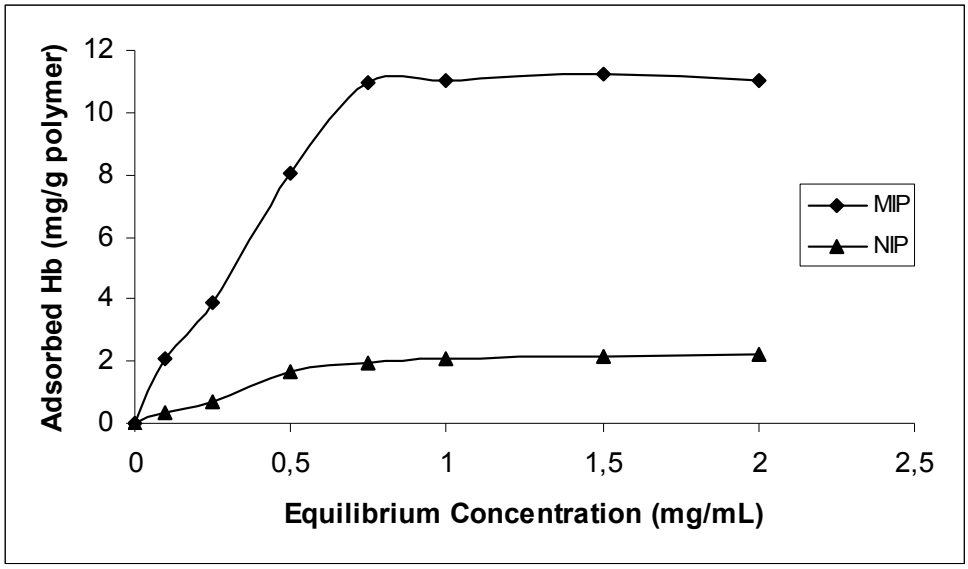


**Figure 4.6.** Effect of hemoglobin concentration on adsorption capacity. running buffer: 0.1 M phosphate buffer(pH=6), T: 20ºC, flow rate: 1.0 mL/min, $m_{dry\ cryogel}$: 0.45 g

### 4.2.4. Effect of Ionic Strength

The effect of NaCl concentration on the Hb adsorption amount was also investigated. High ionic strengths weakened the binding as shown in binding experiments when increasing amounts of NaCl were added to the adsorption solution (Figure 4.7). Hb adsorption amount decreased from 11.01 to 5.67 mg/g with the increasing NaCl concentration. Thus, ionic interactions gave an essential contribution to the recognition and binding process. A possible explanation to this phenomenon could be in two ways: (i) the counter salt ions interact with the Hb molecules via charge–charge inter actions and mask the binding sites and (ii) the decrease in the adsorption amount as

the ionic strength increases can be attributed to the repulsive electrostatic forces between the MIP cryogel and Hb molecules. It should be also noted that a significant decrease of Hb adsorption onto NIP cryogel was not observed. The non-specific interactions between Hb and NIP cryogel may result from the cooperative effect of different mechanisms such as electrostatic interactions. It has been reported that the situation is particularly anxious when electrostatic and hydrophobic interactions occur at the same time, since an increase in the ionic strength of the solution decreases the former type of interaction.

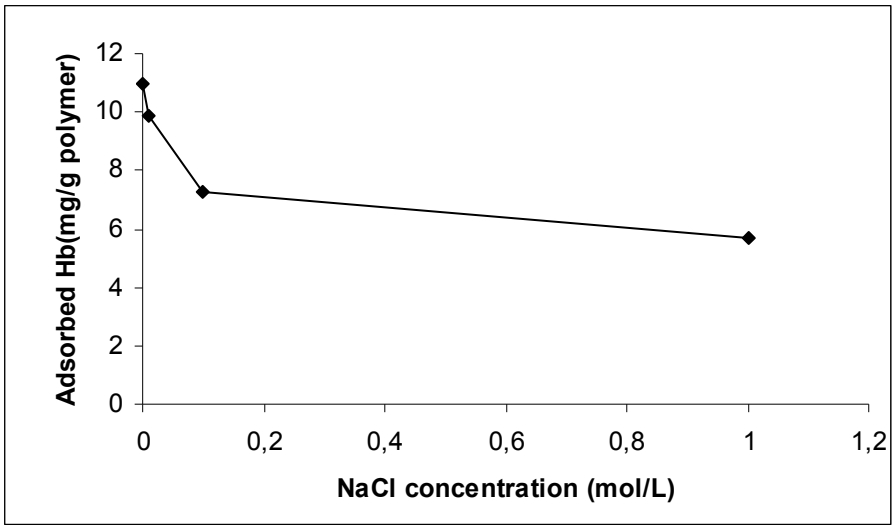


**Figure 4.7.** Effect of ionic strength on hemoglobin adsorption; running buffer: 0.1 M phosphate buffer(pH=6), T: 20ºC, flow rate: 1.0 mL/min, Hb Conc.: 0.75 mg/ml, $m_{dry\ cryogel}$: 0.45 g

### 4.2.5. Adsorption Isotherms

An adsorption isotherm is used to characterize the interactions of each molecule with the adsorbents. This provides a relationship between the concentration of the molecules in the solution and the amount of Hb adsorbed on the solid phase when the two phases are at equilibrium. 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 continuous system experiments, adsorption isotherms were used to evaluate adsorption properties. Equation 4.1 expresses the Langmuir adsorption isotherm. The corresponding transformations of the equilibrium data for Hb gave rise to a linear plot, indicating that the Langmuir model could be applied in these systems and described by the equation:

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

Where, Q is the adsorbed amount of Hb (mg/g), $C_{eq}$ is the equilibrium Hb concentration (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

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

The plot of $C_{eq}$ versus $C_{eq}$ /Q was employed to generate the intercept of $1/Q_{max}.b$ and the slope of $1/Q_{max}$ (Figure 4.8).

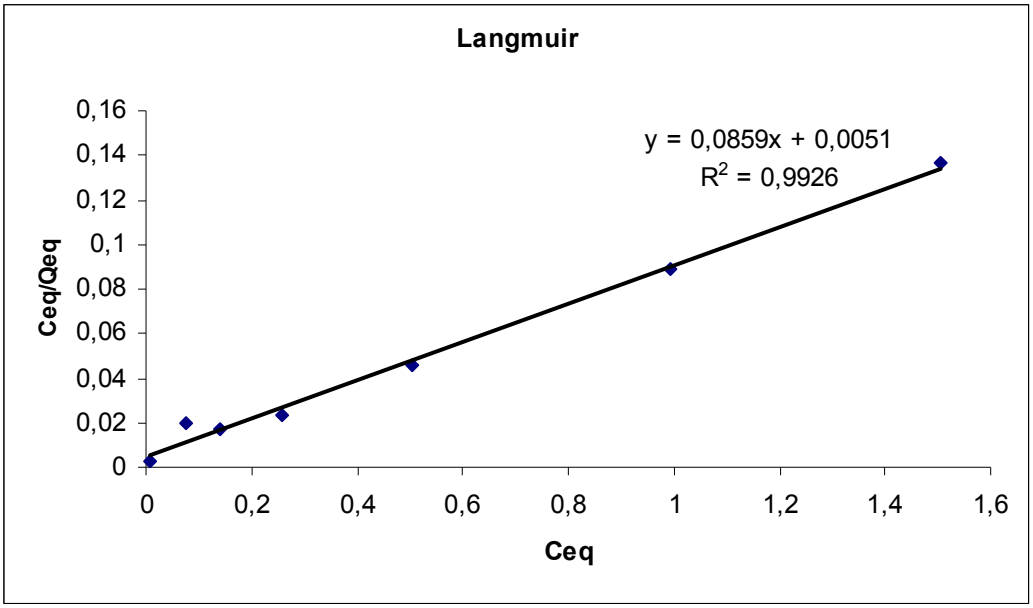


**Figure 4.8.** Langmuir adsorption isotherms of the poly(HEMA-MAH) cryogel.

The maximum adsorption capacity ($Q_{max}$) data for the adsorption of Hb was obtained from the experimental data. The correlation coefficient ($R^2$) was 0.9926 at pH 6.0. The Langmuir adsorption model can be applied in this affinity adsorbent system. It should

be also noted that the maximum adsorption capacities ($Q_{max}$) and the Langmuir constants were found to be 11.64 mg/g and 16.84 g/mg at pH 6.0, respectively.

The other well-known isotherm, which is frequently used to describe adsorption behavior, 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:

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

where, $K_F$ and n are the Freundlich constants. This equation can be linearized so that

$$\ln Q_{eq} = \ln K_F + (n.\ln C_{eq}) \qquad (4.4)$$

The plot of ln $C_{eq}$ versus ln $Q_{eq}$ was employed to generate the intercept of ln $K_F$ and the slope of 'n' (Figure 4.9).

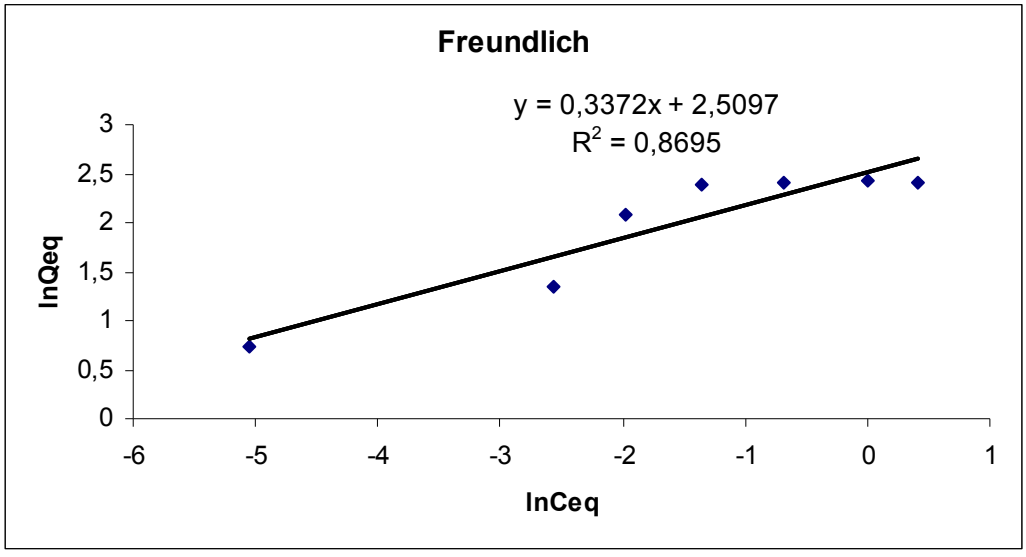


**Figure 4.9.** Freundlich adsorption isotherms of poly(HEMA-MAH) cryogel.

The adsorption isotherm of MIP cryogel was found to be linear over the whole concentration range studies and the correlation coefficients were high. According to the correlation coefficients of isotherms, Langmuir adsorption model is most favorable.

Table 4.2 shows the Langmuir and Freundlich adsorption isotherm constants, n and $K_F$ and the correlation coefficients.

**Table 4.2.** Langmuir and Freundlich adsorption constants and correlation coefficients for MIP cryogel**.**

| | Experimental | Langmuir constants | | | Freundlich constants | | |
|---|---|---|---|---|---|---|---|
| | $q_{ex}$ (mg/g) | $q_{max}$ (mg /g) | $K_d$ (g/mg) | $R^2$ | $K_F$ | n | $R^2$ |
| MIP Cryogel | 11.01 | 11.64 | 16.84 | 0.9926 | 12.30 | 0.33 | 0.8695 |

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 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 (Cheung et al., 2001). It may be represented as follows:

$$\Delta q_t/d_t=k_1(q_{eq}-q_t) \quad (4.5)$$

where $k_1$ is the rate constant of pseudo-first order adsorption ($min^{-1}$) 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

$$\log[q_{eq}/(q_{eq}-q_t)]= (k_1t)/2.303 \quad (4.6)$$

Equation 4.6 can be rearranged to obtain a linear form

$$\log(q_{eq}-q_t)= \log(q_{eq}) - (k_1t)/2.303 \quad (4.7)$$

a plot of log($q_{eq}$) 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($q_{eq}$-$q_t$) via t.

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

$$\Delta q_t/dt = k_2 (q_{eq}-q_t)^2 \qquad (4.8)$$

Where $k_2$ (g $mg^{-1}$ $min^{-1}$) is the rate constant of pseudo-first order adsorption process. Integrating equation 4.8, q and applying the boundary conditions, $q_t$=0 at t=0 and $q_t$=$q_t$ at t=t, leads to

$$1/(q_{eq}-q_t)] = (1/q_{eq}) + k_2 t \qquad (4.9)$$

or equivalently for linear form

$$(t/q_t)= (1/k_2 q_{eq}^2) + (1/q_{eq})\, t \qquad (4.10)$$

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 at equilibrium ($q_{eq}$) can be obtained from the intercept and slope, respectively.

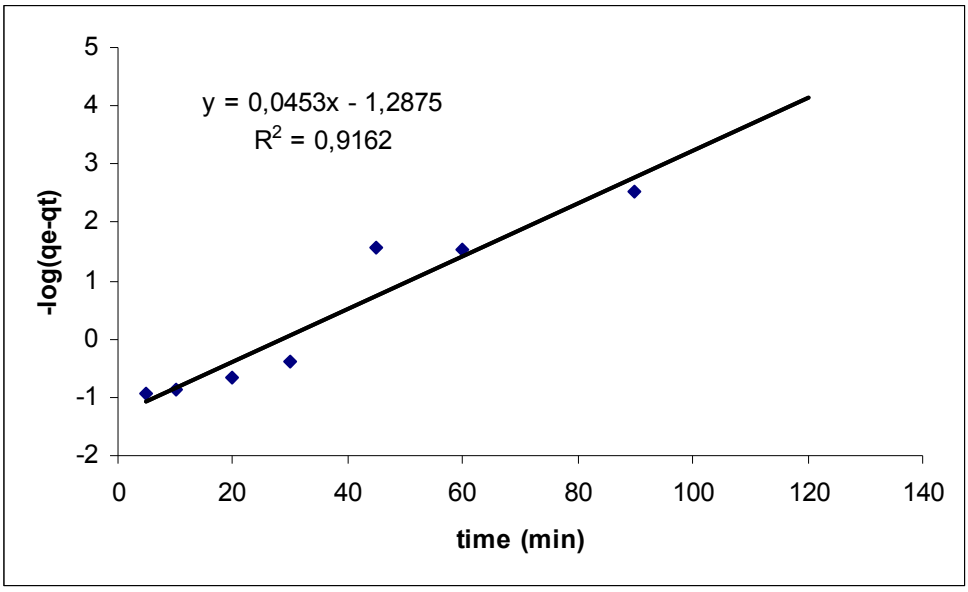


**Figure 4.10.** Pseudo-first-order kinetic of the experimental data for the adsorbent.

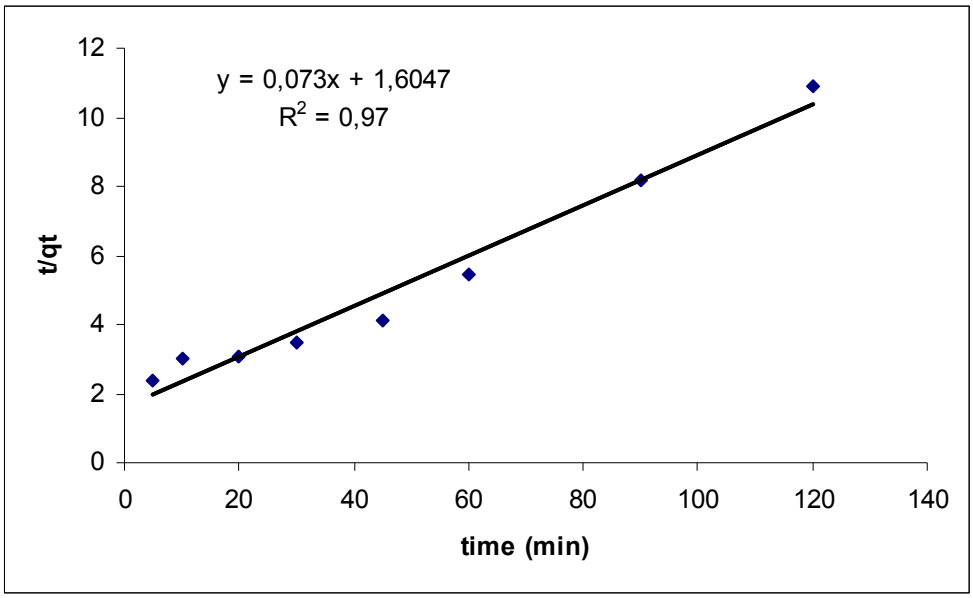


**Figure 4.11.** Pseudo-second-order kinetic of the experimental data for the adsorbent.

**Table 4.3.** The first and second order kinetic constants for MIP cryogel.

| Equilibrium | Experimental | First-order kinetic | | | Second-order kinetic | | |
|---|---|---|---|---|---|---|---|
| **Conc.** (mg/mL) | **$Q_{eq}$** (mg/g) | **$k_1$** (1/min) | **$q_{eq}$** (mg/g) | **$R^2$** | **$k_2$** (g/mg.min) | **$q_{eq}$** (mg/g) | **$R^2$** |
| 0.75 | 11.01 | 0.045 | 1.28 | 0.91 | 1.6 | 0.073 | 0.97 |

A comparison of the experimental adsorption capacity and the theoretical values which obtained from Figure 4.10 and 4.11 are presented in Table 4.3. The correlation coefficient for the linear plot of $-\log(q_{eq}-q_t)$ vs. t for the pseudo-first order equation is lower than the correlation coefficient for the pseudo-second order equation. These values show that this adsorbent system is not so well described by pseudo-first-order kinetic model. By these results, this Hb-imprinted adsorbent system suggested that the pseudo-second order adsorption mechanism is predominant and that the overall rate of the Hb adsorption process appeared to be controlled by chemical process.

## 4.3. Selectivity Studies

The hemoglobin selectivity of MIP cryogel was investigated by competitive adsorptions of Alb and Myb in 0.1 M of pH 6.0 phosphate buffer. The selectivity of the MIP cryogel for Hb (Mw: 64.5 kDa, pI: 6.8-7.0) with the competitive proteins Alb (Mw: 67.0 kDa, pI: 4.9) and Myb (Mw: 17.0 kDa, pI: 6.8-7.2) was evaluated in a circulating system with 0.45 g of the MIP cryogel, using a peristaltic pump for 2 h.

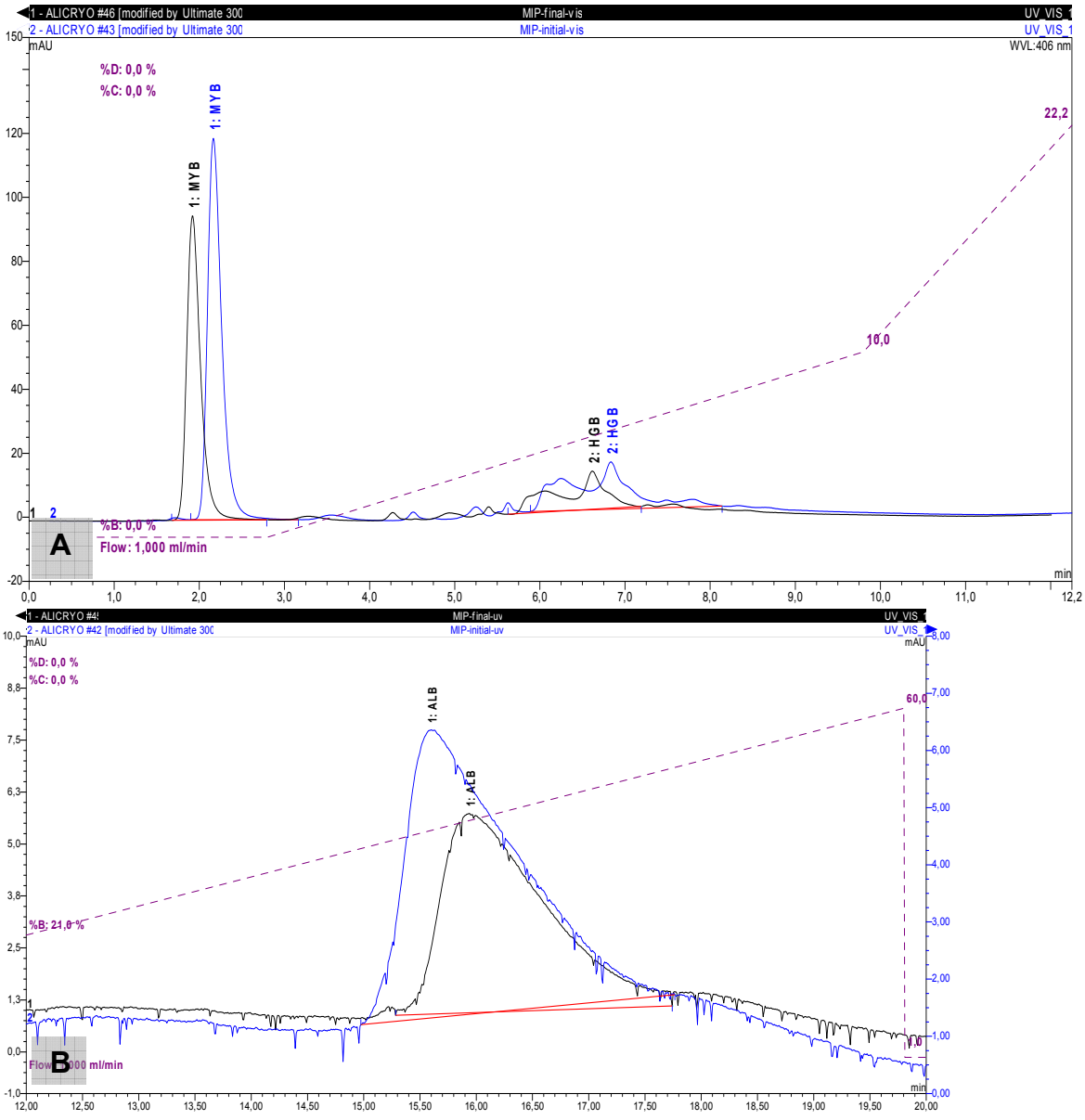


**Figure 4.12.a** HPLC chromatograms of A) Myb and Hb at 406 nm (the time scale was shifted by 2 minutes), B) Alb at 280 nm; which were implemented on MIP cryogels.

The competitive proteins myoglobin which has approximately the same pI and the similar function, albumin as a representative acidic protein (pI: 4.9) were chosen for comparison of selectivity of hemoglobin. The protein mixture was directly applied to MIP cryogel column, and then, the supernatant solution was analyzed by HPLC.

The quantitative selectivity values were obtained by Analytical HPLC System (Dionex) by using a commercial HPLC column (Nucleosil 4000-7 PEI, Length/I.D; 50/4 mm) as described in section 3.3. Figure 4.12 illustrates the separation of Hb, Myb and Alb mixture in HPLC system.

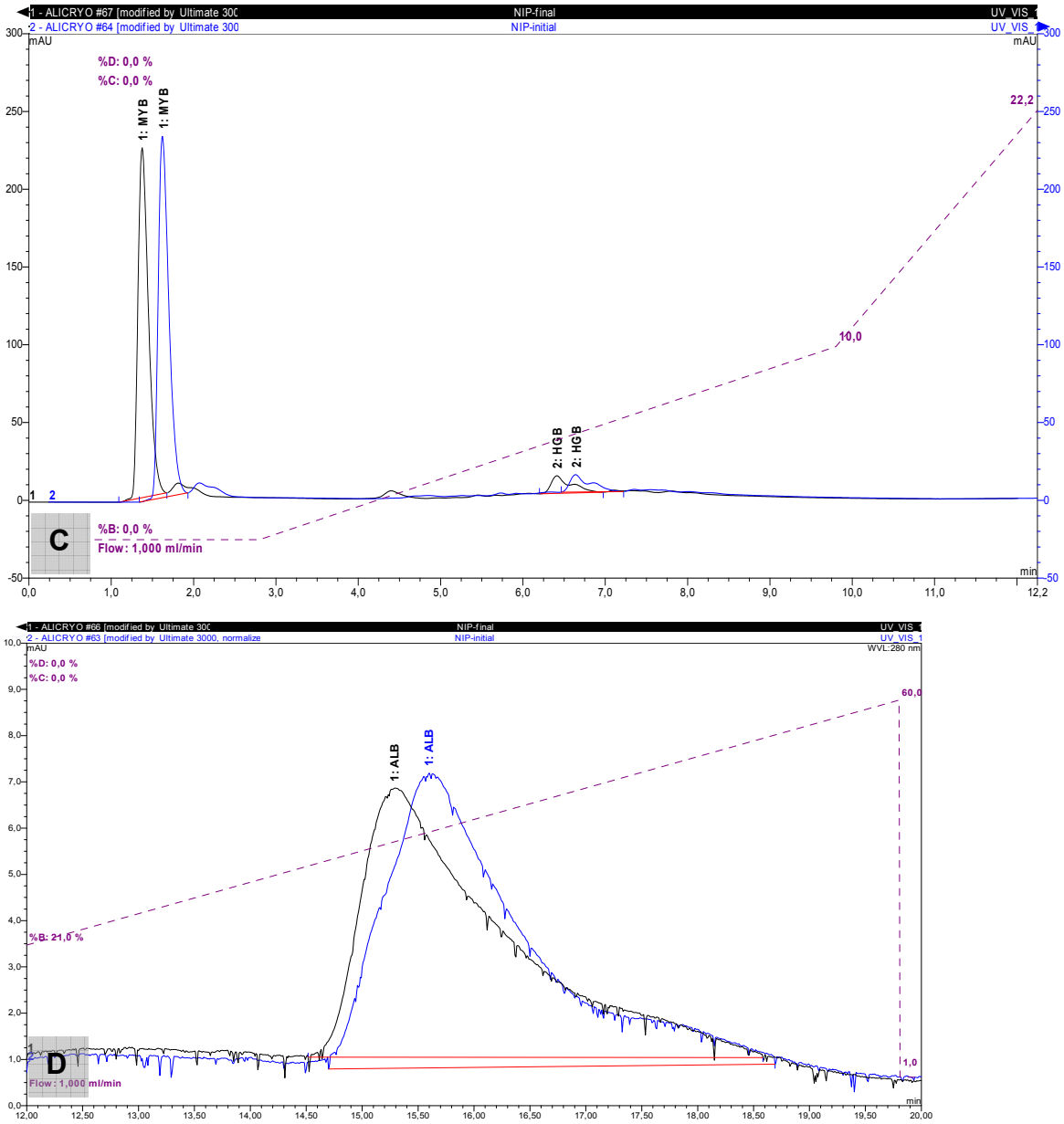


**Figure 4.12.b** HPLC chromatograms of C) Myb and Hb at 406 nm (the time scale was shifted by 2 minutes), D) Alb at 280 nm; which were implemented on NIP cryogels.

The chromatograms shown in Figure 4.12, illustrates the difference between before and after adsorption of protein mixture on MIP and NIP cryogel columns. The decrease in the height of peaks shows the certain amount of protein molecules were adsorbed by the cryogel columns. The correlation of the area of Hb, Myb and Alb peaks gives the amount of these proteins in mg/mL by correlated known standards of each protein solution. All experiments were done in three replicates. Table 4.4 summarizes $K_d$, and k, values of Myb and Alb with respect to Hb. A comparison of the $K_d$ values for MIP cryogels with the NIP cryogels showed an increase in $K_d$ for Hb while $K_d$ decreased for Myb and Alb. The relative selectivity coefficient is an indicator to express protein adsorption affinity of recognition sites of MIP cryogels. These results show that relative selectivity coefficients of MIP cryogels for Hb/Myb and Hb/Alb were 12 and 38 times greater than NIP cryogels, respectively. When myoglobin and albumin was treated with MIP column in the presence of hemoglobin, myoglobin and albumin were hardly adsorbed. The molecular weight of Hb (64.5 kDa) is larger than that of Myb (17.0 kDa) but the isoelectric points are almost the same. Smaller molecules would be inherently expected to more easily gain access to the inside of imprint cavities, but Hb was selectively adsorbed to the MIP cryogel column. It can be concluded that the selectivity is dependent on the shape and size in this manner. On the other hand, binding preference of Hb with respect to albumin, which have similar molecular weight but different pI, was approximately 38 times greater than NIP cryogels. It can be concluded that the selectivity of hemoglobin with respect to albumin was strongly depended on electrostatic/hydrophobic interactions between protein molecule and binding sites of MIP cryogel.

**Table 4.4.** $K_d$, k, and k' values of Myb and Alb with respect to Hb.

| Protein | NIP cryogel | | MIP cryogel | | k' |
|---|---|---|---|---|---|
| | $K_d$ (mL/g) | k | $K_d$ (mL/g) | k | |
| Hb | 1.60 | - | 125.44 | - | - |
| Myb | 7.91 | 0.11 | 51.71 | 4.21 | 38.05 |
| Alb | 14.13 | 0.20 | 29.82 | 2.42 | 12.01 |

The hemoglobin was eluted from MIP cryogel was imaged by SDS-PAGE in the presence of competitive proteins, Myb and Alb. Figure 4.13 shows the electrophoretic separation of Hb (Mw: 64.5 kDa) in the presence of Myb (Mw: 17.0 kDa) and Alb (Mw: 67.0 kDa).

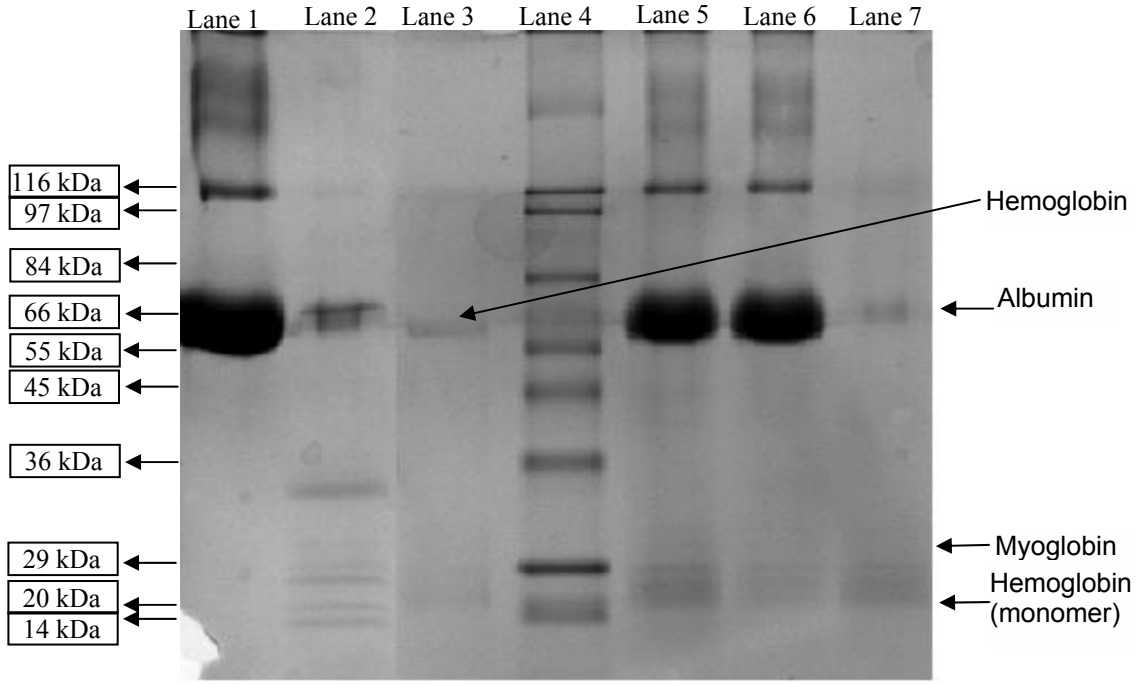


**Figure 4.13.** SDS-PAGE separation for Hb in the presence of Myb and Alb. 12% SDS-PAGE, Lane 1: Alb, Lane 2: Myb, Lane 3: Hb, Lane 4: Sigma Marker, Lane 5: Protein mixture before MIP cryogel column treatment, Lane 6: Protein mixture after MIP cryogel column treatment, Lane 7: Elution from column.

Electrophoretic separation of protein mixture was based on the electrophoretic migration of protein mixture according to their molecular weights, as seen in the protein bands given in Figure 4.13. The fragments of Myb and Hb were also seen in Lane 2 and Lane 3. When the mixture of Hb, Myb and Alb at similar protein concentrations was adsorbed on MIP cryogel column, of the Hb band demonstrated disappeared (see lane 5, 6 in Figure 4.13). The elution from MIP cryogel column in Lane 7 indicates the desorbed Hb after a desired treatment.

### 4.4. Desorption and Reusability

In general, the regeneration of adsorption columns with imprinted polymers is, a crucial step to render these columns more affordable at laboratory and commercial applications. To assess if MIP cryogel columns were reusable, the Hb was desorbed off the by recirculating a desorbing agent through the MIP cryogel. There are several factors affecting the desorption rate of bound protein molecules, including the pH of solution, the polymer microstructure and the binding memory of protein molecule, such as shape, size or isoelectrical point (pI). In this study, the desorption ratio was determined to be as high as 95%. Although the adsorption-desorption cycles for the MIP cryogel column were repeated ten times, the adsorption capacity of the recycled column was about 90% in the 10th cycle as shown in Figure 4.14. It is therefore concluded that the MIP cryogel can be recycled and reused many times with no apparent decrease in their adsorption capacities.

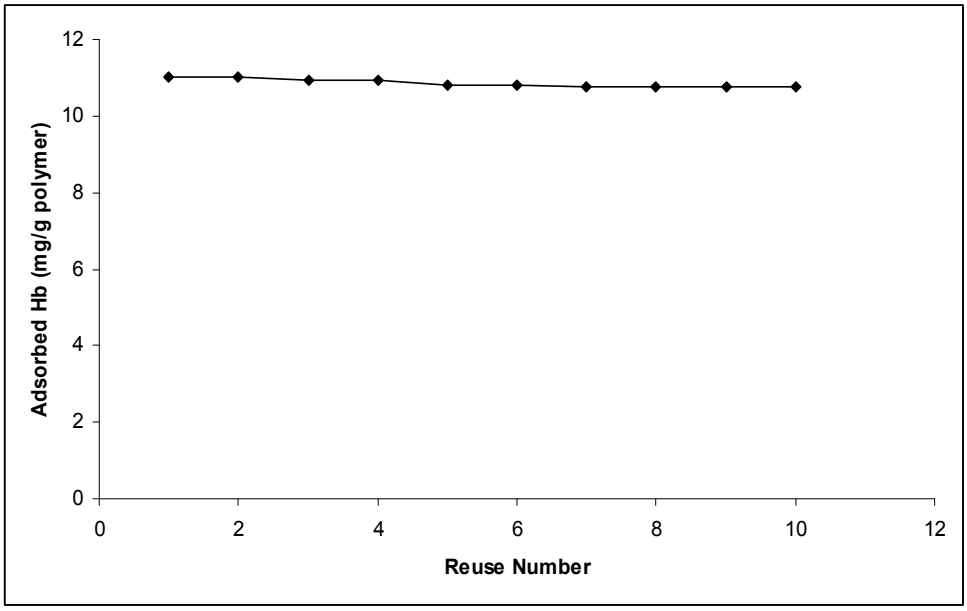


**Figure 4.14.** Adsorption-desorption cycle showing the reusability potential of a column. Running buffer: 0.1 M acetate buffer containing %10 ethylene glycol(pH 4.0); Hb Conc.:0.75 mg/mL, flow rate: 1.0 mL/min, T: 20ºC $m_{dry\ cryogel}$:0.45g

## 5. CONCLUSIONS

- Different types of MIP and NIP cryogels were prepared by different polymerization conditions. It can be concluded that both MIP and NIP cryogels have similar physical characteristics. Moreover, the swelling degree of all cryogels are considerable the same (about 10). Due to the MIP3 has the maximum flow rate (981cm/h) compared to the other MIPs, it was chosen for working cryogel. As NIP1 has the maximum flow rate (820 cm/h) compared to the NIP2, NIP1 was situated as the control polymer.

- Macroporous structure of cryogel reduces diffusional resistance of hemoglobin molecules and facilitates mass transfer because of high pore size. The cryogel produced in such a way has large continuous interconnected pores (10–100 μm in diameter, supermacroporous) that provide channels for the mobile phase to flow through. This also provides higher protein adsorption capacity.

- In desired pH ranges, maximum adsorption capacity was observed at pH 6.0 as 8.05 mg Hb/g polymer. Maximum adsorption capacity was decreased significantly in more acidic and in more alkaline pH regions.

- The Hb adsorption capacity of the MIP cryogel was observed to decrease drastically from 11.4 mg/g to 8.3 mg/g as the flow rate was increased from 0.5 ml/min to 4.0 ml/min.

- The Hb adsorption in MIP cryogel column increases as the Hb concentration in buffer solution is increased, and the column was saturated with excess Hb binding when Hb concentrations above 0.75 mg/mL. The maximum Hb adsorption capacity by the MIP cryogel column was found to be 11.01 mg/g polymer on the average.

- Hb adsorption capacity decreased from 11.01 to 5.67 mg/g with the increasing NaCl concentration.

- The adsorption isotherm of MIP cryogel was found to be linear over the whole concentration range studies and the correlation coefficients were high. According to the correlation coefficients of isotherms, Langmuir adsorption model is most favorable.

- This Hb-imprinted adsorbent system suggested that the pseudo-second order adsorption mechanism is predominant and that the overall rate of the Hb adsorption process appeared to be controlled by chemical process.

- The results show that relative selectivity coefficients of MIP cryogels for Hb/Myb and Hb/Alb were 12 and 38 times greater than NIP cryogels, respectively.

- Finally, the MIP cryogel columns can be used several times without decreasing adsorption capacity significantly.

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