PREPARATION OF CRYOGEL COLUMNS FOR DEPLETION OF HEMOGLOBIN FROM HUMAN BLOOD

İNSAN KANINDAN HEMOGLOBİN UZAKLAŞTIRILMASI İÇİN KRİYOJEL KOLONLARININ HAZIRLANMASI

ALI DERAZSHAMSHIR

Prof. Dr. ADİL DENİZLİ
Supervisor

Submitted to Institute of Graduate Studies in Science
Of Hacettepe University as a partial fulfillmen
to the requirements for the award of the degree of
DOCTOR OF PHILOSOPHY
in
CHEMISTRY

2013

This study named **“Preparation of Cryogel Columns for Depletion of Hemoglobin from Human Blood”** by **ALİ DERAZSHAMSHİR** has been accepted as a thesis for the degree of **DOCTOR OF PHILOSOPHY IN CHEMISTRY** by the below mentioned examining committee members.

Head (Supervisor)

Prof. Dr. Adil DENİZLİ

Member

Prof. Dr. Serap ŞENEL

Member

Assoc. Prof. Handan YAVUZ ALAGÖZ

Member

Assoc. Prof. Lokman UZUN

Member

Assoc. Prof. Ali KARA

This thesis has been approved as a thesis for the degree of **DOCTOR OF PHILOSOPHY IN CHEMISTRY** by the Board of Directors of the Institute of Graduate in Science of Hacettepe University.

Prof. Dr. Fatma SEVİN DÜZ

Director of the Institute of

Graduate Studies in Science

ETHICS

In this thesis study, prepared in accordance with the spelling rules of Institute of Graduate Studies in Science of Hacettepe University,

I declare that

- all the information and documents have been obtained in the base of the academic rules
- all audio-visual and written information and results have been presented according to the rules of scientific ethics
- in case of using others works, related studies have been cited in accordance with the scientific standards
- all cited studies have been fully referenced
- I did not any distortion in the data set
- and any part of this thesis has not been presented as another thesis study at this or any other university.

26/09/2013

Ali DERAZSHAMSHIR

To my family

ABSTRACT

PREPARATION OF CRYOGEL COLUMNS FOR DEPLETION OF HEMOGLOBIN FROM HUMAN BLOOD

ALI DERAZSHAMSHIR

Philosophy of Doctorate, Chemistry Department

Supervisor: Prof. Dr. Adil Denizli

September 2013, 95 pages

Proteins are vital molecules for the living organisms. They carry out many important physiological metabolic pathways. Therefore they have always been the study of interest. The term proteome defines the large-scale study of proteins including the proteins produced by any organism as well as their modifications made after the synthesis. An evolving field of the proteomics is to identify disease linked novel proteins within the erythrocyte proteins. As in the all proteomic studies of the blood plasma, high abundant proteins in erythrocytes, i.e. hemoglobin and carbonic anhydrase, interfere with the analysis of low abundant and disease related proteins. Thus it is essential to remove these proteins before detailed analysis of cytosolic proteins of erythrocytes. In the previous studies nickel affinity chromatography has been investigated for its efficiency for hemoglobin depletion and shown to be effective for increasing the number of detectable proteins.

In this thesis we aimed the preparation of the metal chelate affinity cryogels for the hemoglobin depletion. PHEMA cryogels are selected as base matrix because of

their blood compatibility, osmotic, chemical and mechanical stability. Cryogels are also useful when working with the viscous samples such as blood, thanks to their interconnected macroporous structure. PHEMA cryogels are prepared at -12°C for 24 hours. IDA, the chelating agent, was covalently coupled to PHEMA cryogels after activation with the epichlorohydrin. Then Cu^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} ions were chelated to the IDA-bound cryogels. Characterization of the adsorbent was performed with swelling tests, elemental analysis, FTIR, SEM, surface area measurements and blood compatibility studies. IDA attachment was found as 8.27 mmol/g. Specific surface area of the cryogels was 24.7 m^2/g. Swelling degree and porosity of the cryogel was 8.49 g H_2O/g and 74.1% for PHEMA and 63.5% for PHEMA-IDA cryogels, respectively. The effect of pH, Hb concentration, and ionic strength was investigated. The depletion of the Hb from hemolysate was shown by SDS-PAGE. The FPLC studies confirmed that the prepared adsorbent has a good resolution values with respect to other blood proteins.

Keywords: Hemoglobin depletion, metal chelate affinity sorbents, PHEMA cryogel, IDA.

ÖZET

İNSAN KANINDAN HEMOGLOBİN UZAKLAŞTIRILMASI İÇİN KRİYOJEL KOLONLARININ HAZIRLANMASI

ALI DERAZSHAMSHIR

Doktora Tezi, Kimya Bölümü

Danışman: Prof. Dr. Adil Denizli

Eylül 2013, 95 sayfa

Proteinler canlı organizmalar için yaşamsal öneme sahip moleküllerdir. Çok sayıda önemli fizyolojik metabolik yolakları yürütürler. Dolayısıyla her zaman yoğun bir ilgiyle çalışılmaktadırlar. Proteom terimi herhangi bir organizmanın ürettiği proteinlerin, sentezlendikten sonraki modifikasyonlar da dahil, çalışılmasını ifade etmektedir. Proteomik çalışmalarının gelişen alanlarından bir tanesi de eritrosit proteinleri içerisinde hastalıklarla ilişkili yeni olanların tanımlanmasıdır. Kan plazmasının proteomik analizinde olduğu gibi, eritrositlerdeki fazla miktarda bulunan proteinler, hemoglobin ve karbonik anhidraz gibi, az miktarda bulunan ve hastalıklarla ilişkili proteinlerin görülmesini engellemektedir. Daha önceki çalışmalarda nikel afinite kromatografisi hemoglobin uzaklaştırma etkinliği bakımından incelenmiş ve tayin edilebilir protein sayısının artması için etkili olduğu gösterilmiştir.

Bu tez çalışmasında, hemoglobin uzaklaştırılması için metal şelat afinite kriyojellerin hazırlanması amaçlanmıştır. PHEMA kriyojeller, kan uyumluluğu, ozmotik, kimyasal

ve mekanik kararlılığı ve birbiri ile bağlı geniş gözenek yapısı nedeniyle kan gibi viskoz sıvılarla çalışmaya uygunluğu nedeniyle seçilmiştir. PHEMA kriyojeller -12°C'de 24 saat süre ile sentezlenmiştir. Şelatlama ajanı İDA, epiklorohidrin aktivasyonunun ardından kriyojellere kovalent olarak bağlanmıştır. Daha sonra Cu^{2+}, Ni^{2+}, Zn^{2+} ve Co^{2+} iyonları İDA bağlı kriyojellere bağlanmıştır. Adsorbentin karakterizasyonu şişme testleri, elementel analiz, FTIR, SEM, yüzey alanı ölçümleri ve kan uyumluluğu testleri ile karaterize edilmiştir. PHEMA kriyojellere IDA bağlanması 8.27 mmol/g'dır. Kriyojellerin yüzey alanı 24.7 m^2/g olarak belirlenmiştir. Şişme derecesi 8.49 g H_2O/g ve makrogözeneklilik PHEMA için % 74.1, PHEMA-IDA kriyojeller için % 63.5 olarak bulunmuştur. Hemoglobin derişiminin, pH'nın ve iyonik derişimin hemoglobin bağlanmasına etkileri incelenmiştir. Hemolizattan Hb ayrılması SDS-PAGE ile gösterilmiştir. FPLC çalışmaları da hazırlanan adsorbentin diğer kan proteinlerine göre Hb için iyi bir çözünürlük değerine sahip olduğunu göstermektedir.

Anahtar kelimeler: Hemoglobin uzaklaştırılması, metal şelat afinite sorbentleri, PHEMA, kriyojel, IDA

ACKNOWLEDGEMENT

It is with particular pleasure that express my affectionate and deeply-felt gratitude to my supervisor Prof. Dr. Adil Denizli for his continual encouragement, unflagging cheer and sensibility, has given the meticolous attention and his help with difficult problems of analysis and description, were invaluable, and have resulted in a large number of important corrections and improvement.

In addition, I would like to express my gratitude to Assoc. Prof. Handan Yavuz Alagöz and Assoc. Prof. Dr. Lokman Uzun for their comments and suggestions about this research.

I am so deeply indebted to Nilay Bereli, Müge Andaç and Fatma Yılmaz for their an enormous contribution the clarity, accuracy and completeness of the finished my thesis.

Special thanks to my dear friends Bahar Ergün, İlker Koç and Gözde Baydemir Peşint.

I should also like to thank all of the Biochemistry Research Group, Dr. Deniz Türkmen, Dr. Darius Majidi, Dr. Erkut Yılmaz, Recep Üzek, Canan Armutcu, Mehmet Emin Çorman, Gülsu Şener, Erdoğan Özgür, Engin Bayram, Işık Perçin Demirçelik, Ahmet Hamdi Demirçelik, Sevgi Aslıyüce, Emel Tamahkar, Yeşeren Saylan, Duygu Çimen, Ilgım Göktürk, Gizem Ertürk, Mitra Jalilzade, Kemal Çetin, Türkan Mammadzade, Tuğba Doğan, Fatma Kartal, Esma Sarı and Semra Akgönüllü for their help, trustful and providing me a friendly atmosphere to work in.

I am especially grateful to Esra Erdem for her kindly help during my study.

Finally to my family, I am grateful for their patience and understanding...

Ali DERAZSHAMSHİR

Eylül, 201

CONTENTS

Pages

ABSTRACT i
ÖZET iii
ACKNOWLEDGEMENT v
CONTENTS vi
FIGURE LEGEND viii
TABLE LEGENDS ix
1. INTRODUCTION 1
2. GENERAL INFORMATION 3
2.1. Bioaffinity Chromatography 3
2.2. Design of an Affinity Chromatography Process 7
2.2.1. Affinity Interactions 8
2.2.2. Choice of Matrix 9
2.2.3. Choice of Ligand 11
2.2.3.1. Classification of Ligands 11
2.2.3.1.1. Monospecific Low Molecular Weight Ligands 12
2.2.3.1.2. Group-Specific Low Molecular Weight Ligands 12
2.2.4. Choice of Spacer Arm 13
2.2.5. Immobilization Techniques 14
2.3. Immobilized Metal Affinty Chromatography (IMAC) 16
2.3.1. General Definition of IMAC 16
2.3.2. Versatility of IMAC 18
2.3.3. Mechanism, Ligands, Ions, and Techniques in IMAC 18
2.3.4. Some Advantages of IMAC 24
2.3.4.1. Use Under Denaturing Conditions and In Situ Refolding 24
2.3.4.2. Immobilization on the Column 25
2.3.5. Some Disadvantages and Problems of IMAC 25
2.3.5.1. Oxidation and Reduction Conditions Inside the Column and Metal-Induced Cleavage 25
2.3.6. Future of IMAC 27
2.4. Hemoglobin 29
2.4.1. Functional Class 29
2.4.2. Occurrence 29
2.4.3. Biological Function 30
2.4.4. Amino Acid Sequence Information 32
2.4.5. Purification and Molecular Characterization 33
2.4.6. Hemoglobin Variants 33

2.5. Hydrogels, so called Cryogels 34
3. EXPERIMENTAL 37
3.1. Materials 37
3.2. Preparation of Metal-Chelated PHEMA Monolithic Cryogels 37
3.2.1. Preparation of PHEMA Cryogels 37
3.2.2 IDA-Attached PHEMA (PHEMA-IDA) cryogel 37
3.2.3. Chelation of Metal Ions (Cu2+, Zn2+, Ni2+, Co2+) 38
3.3. Characterization of PHEMA Cryogels 39
3.3.1. Swelling Test 39
3.3.2. Surface Morphology 41
3.3.3. Surface Area Measurements 41
3.3.4. FTIR Studies 41
3.4. Blood Compatibility Studies 41
3.4.1. Coagulation Time (CT) 41
3.4.2. Activated Partial Thromboplastin Time (APTT) 41
3.4.3. Prothrombin Time (PT) 42
3.4.4. Cell Adhesion Studies 42
3.5. Adsorption-Desorption studies 42
3.5.1. Adsorption of Hemoglobin from Aqueous Solutions 42
3.6. Desorption and Repeated Use 44
3.6.1 Depletion of Hemoglobin from Human Blood 45
3.7. FPLC studies 45
4. RESULTS AND DISCUSSION 47
4.1. Preparation of PHEMA-IDA-Me Cryogels 47
4.2. Characterization of PHEMA Cryogel 50
4.2.1. Swelling Tests 50
4.2.2. Surface Morphology 51
4.2.3. Surface Area Measurements 53
4.2.4. FTIR Studies 54
4.3. Blood Compatibility Studies 55
4.3.1. Coagulation Times 56
4.3.2. Cell Adhesion Studies 57
4.4. Adsorption of Hb from Aqueous Solutions 58
4.4.1. Effect of pH 58
4.4.2. Effect of Flow Rate 58
4.4.3. Effect of Equilibrium Concentration 59
4.4.4. Effect of Ionic Strength 60
4.4.5. Adsorption Isotherms 61
4.5. SDS-PAGE analysis of human hemolysate 68
4.6. Desorption and Reusability 69
4.7. FPLC studies 70
5. CONCLUSIONS 71
REFERENCES 74

FIGURE LEGEND

Pages

Figure 2.1. Schematic representation of the main steps in affinity chromatography 6
Figure 2.2. Immobilization of ligands to different activated carriers: 15
Figure 2.3. Putative structures of some representative chelators in complex with usually used metal ions 24
Figure 2.4. Schematic representation of the quaternary structure of deoxy T state Hb 30
Figure 2.5. Comparison between oxy R states (PDB: 1HHO) and the deoxy T (PDB code: HGA) highlighting the shifts associated with the quaternary transition. 32
Figure 2.6.a. Cryogel formation. 35
Figure 2.6.b. Different forms of cryogels 36
Figure 3.1. Schematic presentation of PHEMA-IDA-Ni2+ cryogel synthesis 39
Figure 3.3. Continuous system used for adsorption experiments. 44
Figure 3.4. Picture of FPLC system. 46
Figure 4.1. Effect of initial metal concentration on metal loading 49
Figure 4.2. Loading of metals on cryogels. 50
Figure 4.3. SEM photographs of PHEMA cryogels 52
Figure 4.4. The FTIR spectra of PHEMA, PHEMA-IDA and PHEMA-Ni cryogels. 54
Figure 4.5. The role of adsorbed proteins in body fluid biomaterial interaction..... 56
Figure 4.6. Effect of pH on Hb adsorption onto metal-chelated PHEMA cryogels. 58
Figure 4.7. Effect of flow-rate on Hb adsorption onto cryogels. 59
Figure 4.8. Effect of hemoglobin concentration on adsorption capacity. 60
Figure 4.9. Effect of ionic strength on hemoglobin adsorption 61
Figure 4.10. Langmuir adsorption isotherms of the PHEMA cryogel. 62
Figure 4.11. Freundlich adsorption isotherms of PHEMA cryogel. 63
Figure 4.12. Pseudo-first-order kinetic of the experimental data for the adsorbent 66
Figure 4.13. Pseudo-second-order kinetic of the experimental data for the adsorbent. 66
Figure 4.14. SDS-PAGE visualization for hemoglobin depletion from human hemolysate. 68
Figure 4.15. Adsorption-desorption cycle showing the reusability potential of a columns 69

TABLE LEGENDS

Pages

Table 2.1. Examples of biological interactions used in affinity chromatography. 3
Table 2.2. Subcategories of affinity chromatography 5
Table 2.3. Comparision of IMAC with other adsorption principle. 17
Table 2.4. Some chelating compound in use for immobilization in IMAC. 22
Table 4.1. Physicochemical properties of the PHEMA cryogel. 53
Table 4.2. Coagulation times of human plasma (reported in sec)*. 57
Table 4.3. Platelet and leukocyte adhesion with cryogels*. 57
Table 4.3. Langmuir and Freundlich adsorption constants and correlation coefficients for PHEMA cryogel. 64
Table 4.4. The first and second order kinetic constants for MIP cryogel. 67
Table 4.5. Chromatographic separation data from human red blood cell hemolysate. 70

1. INTRODUCTION

After the accomplishment of Human Genome Project a new age has started in the life sciences era; researches on proteins encoded by genes to further understand their biological functions increased exponentially. Proteomic technologies are applied to study function, organization, diversity and dynamic state of a cell or whole tissue.

Erythrocytes (red blood cells) are the most abundant cells in vertebrates and they involve in a numerous processes by interacting with other red blood cells, endothelial cells etc. Because of its important role in vertebrates, many researchers heavily study them. There are many diseases related with the red blood cells such as sickle cell anemia, malaria, type-2 diabetes, thalassemia, aplastic anemia etc. However, there are very few studies published on the proteomic studies of red blood cells.

As in the all proteomic studies of the blood plasma, high abundant proteins in erythrocytes, i.e. hemoglobin and carbonic anhydrase, interfere with the analysis of low abundant and disease related proteins. Hemoglobin constitutes 95% of the total cytosolic proteins of erythrocytes and masks observation and the detection of the low abundant proteins in 2D gel electrophoresis. Thus it is essential to remove these proteins before detailed analysis of cytosolic proteins of erythrocytes. In the previous studies metal chelate affinity chromatography has been investigated for its efficiency for hemoglobin depletion and shown to be effective for increasing the number of detectable proteins.

Immobilized metal ion affinity chromatography (IMAC) is a widely used analytical and preparative separation method for many biomolecules. It depends on the formation of stable complexes with the metal ions and molecules bearing O, N, and S by ion-dipole interactions. Mostly used metal ions for this purpose are the transition metal ions such as (Zn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{3+} and Co^{2+}) incorporated with any metal chelating compound to the polymer matrix.

Cryogels are supermacroporous gels. They can be prepared at subzero temperatures by the cryotropic gelation technique. The solvent (water in this case)

is frozen at this temperature and polymerization continues in nonfrozen liquid phase. After completion of cryopolymerization, melting ice crystals at room temperature forms interconnected pores which allows flow of mobile phase with minimal flow resistance. Macropore size within cryogels differs from tens or hundreds of micrometers to only a few micrometers. Cryogels have also micropores that are formed in polymeric phase. The large interconnected pores offer advantages when working with viscous media like blood, plasma and plant or animal tissue extract. They provide low pressure drop, short diffusion path, and very short residence time for adsorption and elution.

The main criteria of selection of poly(HEMA) as the basic component is its inertness, mechanical strength, chemical and biological stability and well tolerability.

In this thesis, PHEMA cryogels were prepared and metal chelating agent IDA was covalently coupled to the cryogels. Then, four different metal ions (i.e. Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+}) were investigated for their performance to remove hemoglobin from red blood cell hemolysate. The results are presented here.

2. GENERAL INFORMATION

2.1. Bioaffinity Chromatography

The complementarity of surface geometry and special arrangement of the ligand and the binding site of the biomolecules makes bioaffinity chromatography the most selective one among affinity techniques. Specific interactions between molecules designate the biological processes. The mentioned interactions can be between a protein and low molecular weight substance (e.g., between substrates or regularity compounds and enzymes; and so on) while biospecific interactions occur even more often between two or several biopolymers, particularly proteins. As the degree of complementarity of the interacting groups increase, the interaction between an affinity ligand and a biomolecule increases.

Regarding this, the most suitable bioaffinity ligands for enzymes are substrate analogues, competitive inhibitors, allosteric inducers and coenzymes. 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

Different investigators define the term “affinity chromatography” in quite different ways. These definitions can sometimes be very broad; including all kinds of adsorption chromatography based on non-conventional ligands. Generally, these definitions are meant to include immobilized metal ion affinity chromatography (IMAC), covalent chromatography, hydrophobic interaction chromatography, and so on. And sometimes they refer only to ligands based on biologically functional pairs, such as enzyme-inhibitor complexes. The term includes the so-called biomimetic ligands, particularly dyes whose binding obviously often occur to active sites of functional enzymes, in addition to functional pairs. It must be kept in mind that; dye molecules themselves do not exist in the functional context of the cell. Thus, bioaffinity or biospecific chromatography is the chromatography based on the formation of specific complexes such as enzyme-substrate, enzyme-inhibitor, etc. (i.e on biological recognition), [1]. while the original term “affinity chromatography” gain a broader definition also including hydrophobic chromatography [2], covalent chromatography [3], metal-chelate chromatography, chromatography on synthetic ligands, etc., i.e chromatography procedures based on different, less specific types of interaction. Subspecialty techniques, many of which are now known by their own nomenclature has been developed by various applications of affinity. 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 [4].

Table 2.2. Subcategories of affinity chromatography

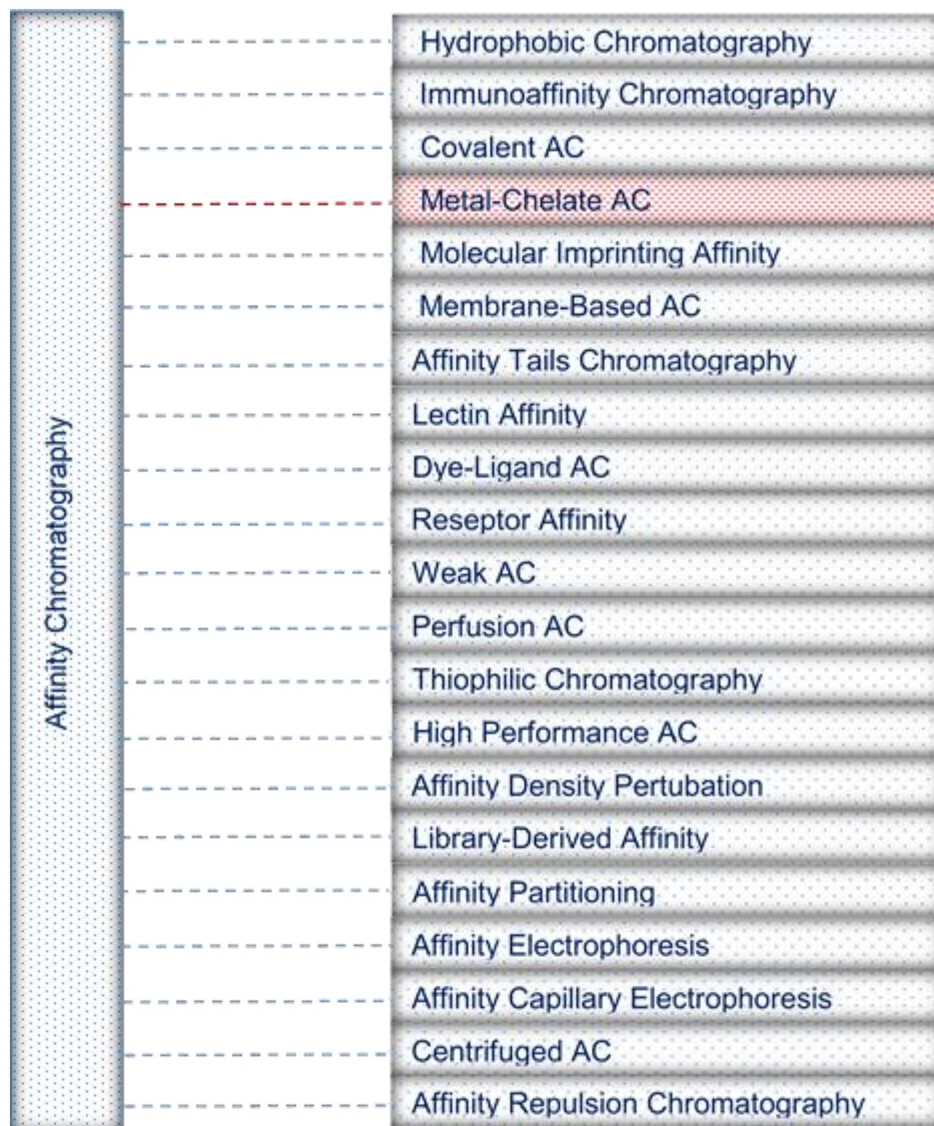


Figure 2.1. shows main steps in affinity chromatography. The simple principle of affinity chromatography demonstrated in this figure is that every biomolecule recognize another natural or artificial molecule. A broad type of ligands may be packed into a chromatographic column via the attachment of these ligands to an inert matrix.

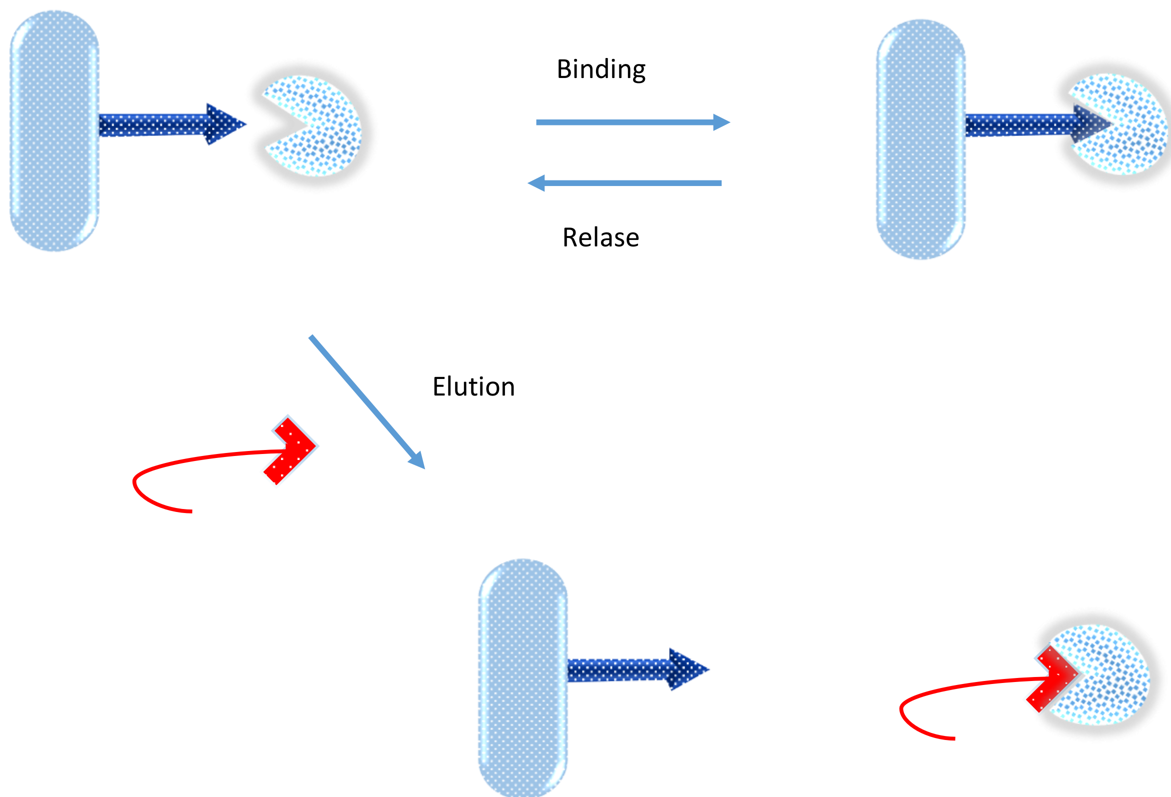


Figure 2.1. Schematic representation of the main steps in affinity chromatography.

In such a system, the column will adsorb only the protein molecules which selectively bind to the immobilized ligand. All unbound molecules will be flushed out by the suitable washing buffer. Various techniques allow the desorption of molecules to be purified from the ligand.

Active and inactive forms can often be separated since affinity chromatography counts on functional properties, however, this is not a private property for affinity methods. For example, covalent chromatography can do the same thing when the activity depends on a functional thiol group in the protein. When affinity desorption is used, ion-exchange chromatography can also separate according to functional properties. But it must be kept in mind that these are exceptions to what is a rule for the affinity methods.

When complementary base sequences are used as ligands, affinity chromatography is a great tool in the nucleic acids fractionation, similarly; in cell separation where cell surface receptors are ligands, affinity chromatography has again a great value.

Immunoadsorption is another kind of affinity which is based on antigen-antibody interactions and is usually treated separately. Immunosorption can sometimes be the only choice for the purification of a protein when the monoclonal antibody of the target protein is suitable at hand.

Usually the researchers need to synthesize the adsorbent in affinity chromatography. The related methods, which will be discussed in later sections, are well known, easily adoptable methods for those not skilled in synthetic organic chemistry. There are also commercially available activated gel matrices for the reaction with a ligand.

Association strength between ligand and counterligand requires special consideration. It must be kept in mind that a very weak association will lead to an adsorption fail while a very strong one will lead to an elution problem. Finding the most suitable conditions for adsorption-desorption in order to promote the dissociation of the complex without destroying the active protein is always one of the most important parts of affinity chromatography.

Ligands can both be extremely selective or only group specific. The other type of affinity interactions include glycoprotein-lectin interactions, dye-enzyme interactions and interactions with immobilized cofactors. The above interactions are known to be extremely useful in overcoming many separation problems. Ligands that are group selective against immunoglobulins can be given as example (e.g., staphylococcal protein A or streptococcal protein G) [5].

2.2. Design of an Affinity Chromatography Process

It is hard to set up a general rule in order to predict the factors and parameters for a specific separation, so each protein separation process must be individually optimized. Nevertheless, there are a few general factors that influence the design of an affinity chromatography process [6]. The nature and quality of desired product and its intended use are predicted by these factors [7, 8, 9]. For example, there is

no need for an extreme purification for the proteins used in industry whereas at least a >99,9% purification can be needed in therapeutic proteins [10, 11].

The amount of the target product must be well defined since this may influence the process pathway. For example, at the end of a clinical trial, the targeted dose of a pharmaceutical can be determined and the process can be defined [12].

Below are the factors that determine the success of an affinity chromatographic step;

1) the ligand selectivity,

2) recovery,

3) through put,

4) reproducibility,

5) stability, maintenance and economy

Ligand selection is the most important part in affinity chromatography. Recovery rate and correspondingly the cost effectiveness of the process is determined by the amount of desorbed protein. Number of repeated use must be determined for the process validation. Prior to the transfer of the process into a large scale, purity differences must carefully be considered. One of the most important parameters which determine the durability and the cost of the process is ligand stability [13].

Since the purity requirements are low, downstream processing costs are generally low in the case of industrial enzymes while the cost of pharmaceutical protein purifications which should have a purity of >99.9% are much more higher. When high purity is needed, purification and recovery costs can account for as much as 80% of the total process cost. As the number of steps in a process increases, the cost also increases due to the capital and operating costs and also due to the decrease in overall yield.

2.2.1. Affinity Interactions

Reactive groups in the receptor and ligand interact with each other mostly via non-covalent interactions. Different types of interactions depending on the spatial configuration, charge and hydrophobicity of biological molecules regulate the affinity

reactions between these molecules. These 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.

Solid support characteristics and the activity and functional availability of the solid phase-bound protein also affects binding affinity [11], such that affinity K_d varies between 10^{-3} and 10^{-7} M. Non-specific interactions between the solid phase and ligand are unreconciled and impossible to foretell and therefore are complex elution profiles [15].

Effective interaction between free ligand and immobilized protein can sterically be hindered by the physical structure of the solid phase polymer, including high k_d values. Using spacer arms may reduce the mentioned problems [16, 17]. In developing an appropriate elution system, for a particular biological interaction k_d can be used as a guide [18]. The elution buffer can be strict leading to partial denaturation [19] and activity loss for the released target protein. On the other hand, weak interactions can lead to a decrease in binding specificity and low purification factors, although this may also be an advantage.

2.2.2. Choice of Matrix

Maximizing the capacity of the affinity adsorbent requires a large surface area per unit column volume in all adsorption chromatography. Hydrophilic gels with a high surface area are very stable as matrices. In affinity chromatography, the following characteristics should be met by the ideal gel material;

- Should be macroporous to accommodate the free interaction of large molecular weight proteins with ligands
- Hydrophilic and neutral in order to prevent the nonspecific interaction between proteins and gel matrix
- Contain functional groups to allow derivatization by a wide range of chemical reactions.

- Chemically stable to resist harsh conditions during derivatization, regeneration, and maintenance.
- Physically stable in order to resist hydrodynamic stress in packed beds and, when applicable, autoclave sterilization.
- Have a low cost and be readily available for industrial applications.

These characteristics directs polymeric gels to highly substituted with alcohol hydroxyls and thus to polysaccharides. Indeed, gel-forming galactan agarose meets most of the characteristics of an ideal matrix for affinity chromatography. Chemical and physical instability of agarose gel is its major weakness, however, this weakness is compensated for by chemical cross-linking of the physically cross-linked so-called junction zones in the agarose gel structure. 4% agarose has been the most popular matrix for affinity chromatography since its introduction by Cuatrecasas, Wilchek, and Anfinsen in 1968 [20, 15]. Simple, convenient and even commercially available coupling methods developed to agarose has a wide contribution to the popularity of agarose gels.

Cellulose, cross-linked dextran, polyacrylamide, and silica are relatively less used gel matrices. Commercially available matrices made of mixtures of polyacrylamide and agarose (Ultragel) and polymerized tris (hydroxymethyl) acrylamide (Trisacrl, Biosepra, France) can also be included in this group. Having too small pore diameters, even after derivatization with affinity ligands, is the most important disadvantage of gels based on cross-linked dextran and polyacrylamide. There are also other methods available for immobilization on polyacrylamide.

Beads having a diameter of approximately 100 µm are standard in traditional low-pressure affinity chromatography systems, however, in high-performance liquid affinity chromatography (HPLAC), in which higher pressure drops are often required, which also means a demand for higher gel rigidity beads, beads having diameters 5 to 30 µm are used. This accounts for the reason for why the first HPLAC applications were based on modified and derivatized porous silica. One of the most important reasons for using smaller particles is to decrease the diffusion path lengths and increase the interphase area between the stationary and the mobile

liquids and in this way increasing the chromatographic efficiency. Small-diameter agarose beads are alternate to silica for HPLC applications. Synthetic organic polymers having adequate porosity and rigidity and which are highly substituted with alcohol by hydroxyls adequate porosity and rigidity are also interesting matrices. Some of these are commercially available [22].

2.2.3. Choice of Ligand

The rapid and efficient generation of ligands for affinity chromatography have been feasible with the growth of bioinformatics and molecular docking techniques in addition to the introduction of combinatorial methods of systematic generation and screening of large numbers of novel compounds [6, 24, 25]. A good affinity ligand should meet the following criteria;

- The ligand and the target molecule should form a reversible complexes
- The specificity must be appropriate up to the application
- The complex constant should be high enough to form a stable complex or to give sufficient retardation
- The dissociation between ligand and target molecule should be easy by only a simple change in medium and should not affect the target molecule
- Ligand should easily be immobilized to matrix.

There is always various ligands for a target protein to be purified by affinity chromatography. In addition to monoclonal antibodies, other naturally occurring biospecific pairs such as enzyme-substrate (analogs), enzyme-cofactor and enzyme-inhibitor complexes can be used. Immobilized carbohydrates can be used for the purification of glycoproteins. Several groups of enzymes, plasma proteins, and other proteins such as interferons can also be purified by using immobilized biomimetic dyes.

2.2.3.1. Classification of Ligands

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

2.2.3.1.1. Monospecific Low Molecular Weight Ligands

Steroid hormones, vitamins and certain enzyme inhibitors can be classified under this group. Since these ligands bind to a single or a very small number of proteins, they are named as monospecific ligans. Nevertheless, nonspecific adsorption may still occur which can be due to interaction with the ligand or with residues from the immobilization reaction or the spacer arm. This can be overcome by using a second adsorbent without the ligand itself and allowing the desorbed material from the ligand-containing adsorbent to pass this under identical conditions [26].

2.2.3.1.2. Group-Specific Low Molecular Weight Ligands

This is the largest group of ligands which contain enzymes, cofactors and their analogs. Biomimetic dyes, boronic acid derivatives and a number of amino acids and vitamins also belong to this group. Despite this group's relatively broad specificity, by using specific elution processes with either soluble cofactors or by ternary complex formation using a combination of cofactor and substrate, very high purification factors may be obtained. Adequate separation can also be obtained by the ligand-enzyme association with a soluble cofactor.

High molecular weight ligands such as proteins, which have large number of suitable functional groups can be immobilized without adversely influencing structure or function while in low molecular weight ligands the coupling results in a relatively large change in the molecule. On condition that the affinity interaction decreases, to provide a ligand with an appropriate functional group for immobilization, a chemical modification may be needed.

In order to prevent the leakage of the ligand, functional group used should form a stable covalent bond. This is particularly important for small ligands where the attachment is always a "single-point attachment" where “multipoint attachment" between ligand and matrix is more common for proteins. In these affinity adsorbents, the stability of each individual bond is less critical.

The ligand must remain unaffected during the immobilization and stable enough to allow the process to be carried out. This can be a problem at high pH coupling of proteins. The ligand must be at highest possible purity and must not contain

competitive substances. Proteins should be subjected to gel filtration to remove low molecular weight substances such as ammonium sulfate [27].

2.2.4. Choice of Spacer Arm

Due to steric hindrance, sometimes an affinity adsorbent might function poorly. This is usually a case with low molecular weight ligands while this rarely happens with high molecular weight ligands. This problem can be solved by the use of “spacer arms” which are generally aliphatic, linear hydrocarbon chains with two functional groups located at each end of the chain. One of the groups which is generally a primary amine, is attached to the matrix, whereas the group at the opposing side, which is also called the terminal group and is usually a carboxyl (-COOH) or amino group, is selected on the basis of the ligand to be bound. 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) [6, 29].

Chemical nature of the spacer arm is another important parameter in affinity chromatography. Hydrophobic arms are readily available and relatively simple compounds but have some disadvantages such as creating non-specific interactions. The most encountered hydrophilic spacer arms are generally synthetic polypeptides but they may induce non-specific ionic interactions. Polypeptides, particularly glycine oligomers, are examples of hydrophilic spacers. These, however, might bind proteins by nonspecific interactions [29].

Although there is a possibility that longer spacer arms can give rise to unwanted nonspecific interactions, it is possible to lengthen spacer arms by firstly immobilizing a spacer arm with a terminal primary amine and then increasing the length of the arm by a reaction with succinic anhydride [30].

The use of a spacer arm is directly related to the sterical approach between the ligand and the target molecule. Thus, the length of the spacer arm is one of the most important parameters which is case specific. In case the spacer arm is short, steric effects may not be eliminated, on the other hand, non-specific binding may occur when long arms are used.

2.2.5. Immobilization Techniques

Ligands are generally immobilized onto matrixes via several intermediate steps [29, 32]. The immobilization consists of following steps;

- Matrix activation to make it reactive towards the functional group of the ligand.
- Ligand coupling
- Residual active groups are deactivated or blocked by a large excess of a suitable low molecular weight substance such as ethanolamine.

In order to minimize the interference on the specific interaction between the ligand and the target molecule, ligand should be immobilized through the least critical region (not from the active site) [33]. The activation 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.

An electrophilic group containing ligand can be immobilized to a matrix containing a nucleophilic group although it is not a common approach. The coupling can be made both immediately after activation or following a storage period of activated matrix. Isourea linkage can be obtained by subsequent coupling of ligands, however, due to charge, isourea linkage of the ligand may cause nonspecific binding and due to instability of the isourea bond, ligand leakage can be seen.

Active esters have also been used for immobilizing ligands. Their preparation requires matrixes containing carboxylic groups which can be easily obtained from agarose by activation of the hydroxyl groups with different reagents such as cyanogen bromide, activated carbonates, etc. (Figure 2.2). Reaction of the hydroxyl groups with α-amino acids of different sizes depending on the length of spacer arm is another method of obtaining carboxylic group containing matrixes. The NHS ester is then prepared by mixing the carboxylic matrix with dicyclohexylcarbodiimide and NHS. Due to a stability problem [34], a new method was introduced based on N’,N’,N’,N’-tetramethyl (succinimido) uranium tetrafluoroborate.

Polysaccharides can also be activated by using N'N-disuccinimidyl carbonate (DSC), which forms highly reactive carbonate derivatives with hydroxyl containing polymers. These carbonate derivatives react nucleophiles and the procedure results in a stable carbamate linkage of the ligand coupled to the carrier.

The molecular weight ratio of the ligand and target molecule is of primary importance for gel adsortion capacity since affinity involves the adsorption of one target molecule on one ligand molecule. Thus, the ligand size, the ligand specificity and the gel porosity are also important parameters. If a macromolecule is separated by using a low molecular weight ligand, the degree of substitution may be very low. In such a case, if the method of immobilizing the ligand and the porosity of the gel are well chosen, the molecular weight ratios is reflected by a high capacity.

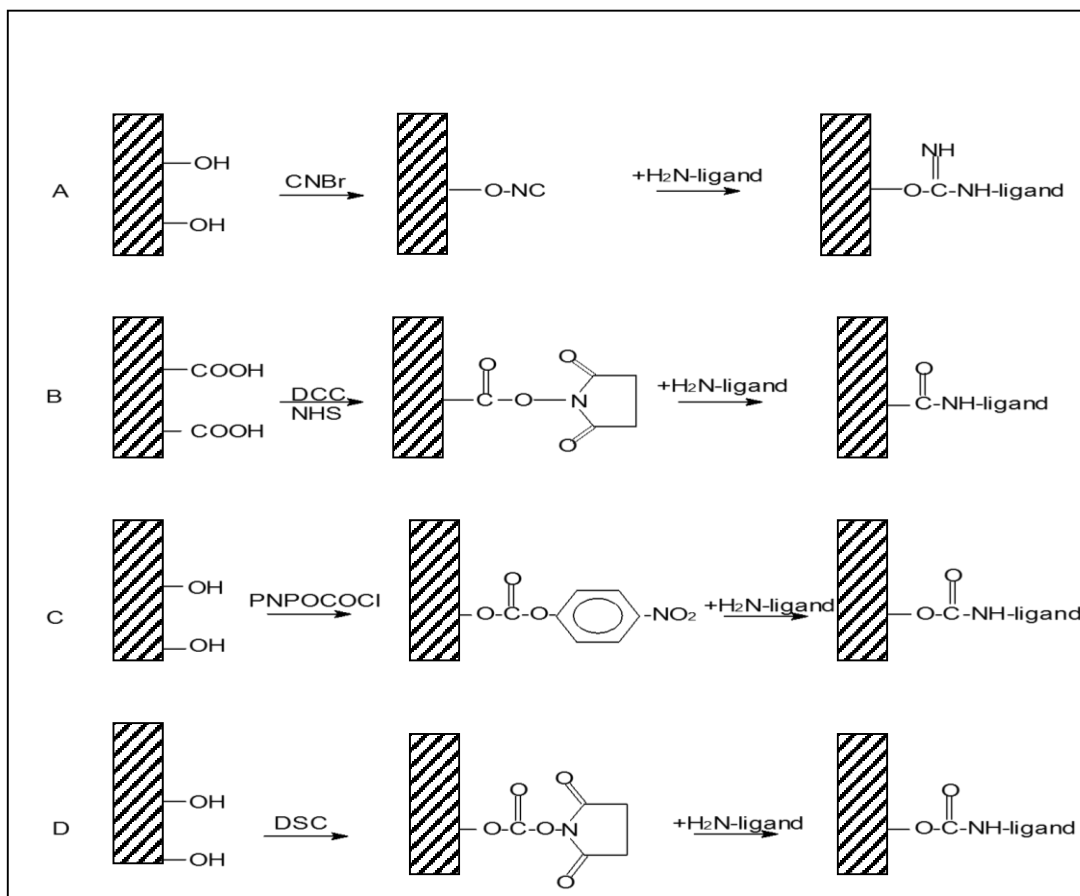


Figure 2.2. Immobilization of ligands to different activated carriers:

(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 [35].

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. Immobilized Metal Affinty Chromatography (IMAC)

2.3.1. General Definition of IMAC

Immobilized Metal Ion Affinity Chromatography, IMAC, is a separation technique that uses covalently bound chelating compounds on solid chromatographic supports to entrap metal ions, which serve as affinity ligands for various proteins, making use of coordinative binding of some amino acid residues exposed on the surface [5]. Separation principle depends on utilizing the differential affinity of proteins for immobilized metal ions to effect their separation. This differential affinity derives from the coordination bonds formed between metal ions and certain amino acid side chains exposed on the surface of the protein molecules. Since the interaction between the immobilized metal ions and the side chains of amino acids has a readily reversible character, it can be utilized for adsorption and then be disrupted using mild i.e., non-denaturing conditions [37]. IMAC represents a relatively new separation technique that is primarily appropriate for the purification of proteins with natural surface-exposed histidine residues and for recombinant proteins with engineered histidine tags or histidine clusters. Recently, because the method has gained broad popularity in recent years, the main recent developments have been occured in the field of new sorbents, techniques and possible applications.

As with other forms of affinity chromatography, IMAC is used in cases where rapid purification and substantial purity of the product are necessary, although compared to other affinity separation technologies it cannot be classified as highly specific, but only moderately so. On the other hand, IMAC holds a number of advantages over biospecific affinity chromatographic techniques, which have a similar order of affinity constants and exploit affinities between enzymes and their cofactors or inhibitors, receptors and their ligands or between antigens and antibodies. The benefits of IMAC—ligand stability, high protein loading, mild elution conditions, simple regeneration and low cost are decisive when developing large-scale purification procedures for industrial applications [38]. Table 2.3. presents a comparison between IMAC and a number of other adsorption principles. This shows that IMAC occupies a space between "true affinity" chromatography and other adsorption principles, and so complements them rather well.

Table 2.3. Comparision of IMAC with other adsorption principle.

Property	**IMAC**	**Affinity**	**IEC**	**HIC**
Capacity	High (Medium)	Low	High	High (Medium)
Recovery	High	Medium	High	Medium
Loading	Mild	Mild	Mild	Sometime Harsh
Elution	Mild	Harsh	Mild	Mild
Regeneration	Complete	Incomplete	Complete	Incomplete
Selectivity	Medium-High	High	Low-Medium	Low-Medium
Cost	Low	High	Low	Low

2.3.2. Versatility of IMAC

Since IMAC is applicable under a wide range of conditions, one would expect it to be incorporated in a significant number of purification procedures, either as a capture step and/or as a final polishing step in the purification of wild-type and recombinant proteins. The wide range of conditions will be discussed in more detail below, but one could point out here that IMAC, with intermediate and hard metal ions, covers almost completely the biologically relevant pH range (i.e., pH 4–10). Furthermore, IMAC can be utilized in the presence or absence of chaotropic salts as well as a number of structure-forming reagents. It is compatible with strong denaturing reagents, such as urea and guanidinium–HCl, as well as a large number of non-ionic detergents, making it extremely useful in the initial steps of purification immediately after the extraction/ isolation procedure. IMAC adsorbents can also be utilized as ion exchangers. While this feature was undesired during the initial development of the technique, it can deliver additional selectivity or capacity for solving particular separation problems. For example, modulation of binding/elution of proteins to intermediate [39] and hard metal ions can be obtained by simultaneous change of salt and pH (unpublished data). IMAC adsorbents can also be used for negative adsorption as exemplified by the purification of horseradish peroxidase on a tandem column of Cu^{2+}-IDA and thiophilic adsorbent. The broad range of conditions under which IMAC can be carried out have also proved useful in the isolation of proteins from organisms that live in extreme natural environments. For example, immobilized Fe^{3+} ions have been used at high salt concentrations (1.5–3 M NaCl) for the purification of proteins from halophilic microorganisms [40].

Such a broad environmental compatibility enables the incorporation of IMAC steps before or after any other adsorption step or Size Exclusion Chromatography (SEC), or, for that matter, even as a part of such a step. [41] were among the first to suggest the use of IMAC columns in such cascade-mode multi-affinity chromatography experiments (CASMAC)

2.3.3. Mechanism, Ligands, Ions, and Techniques in IMAC

In IMAC, the adsorption of proteins is based on the coordination between an immobilized metal ion and electron donor groups from the protein surface. Most

commonly used are the transition-metal ions Cu(II), Ni(II), Zn(II), Co(II), Fe(III) , which are electron-pair acceptors and can be considered as Lewis acids. Electron-donor atoms N, S, O present in the chelating compounds that are attached to the chromatographic support are capable of coordinating metal ions and forming metal chelates, which can be bidentate, tridentate, etc., depending on the number of occupied coordination bonds.

The remaining metal coordination sites are normally occupied by water molecules and can be exchanged with suitable electron-donor groups from the protein. In addition to the amino terminus, some amino acids are especially suitable for binding due to electron donor atoms in their side chains. Although many residues, such as Glu, Asp, Tyr, Cys, His, Arg, Lys and Met, can participate in binding, the actual protein retention in IMAC is based primarily on the availability of histidyl residues. Free cysteines that could also contribute to binding to chelated metal ions are rarely available in the appropriate, reduced state. However, aromatic side chains of Trp, Phe and Tyr appear to contribute to retention, if they are in the vicinity of accessible histidine residues [42, 43].

Adsorption of a protein to the IMAC support is performed at a pH at which imidazole nitrogens in histidyl residues are in the nonprotonated form, normally in neutral or slightly basic medium. Usually relatively high-ionic-strength buffers (containing 0.1 to 1.0 M NaCl) are used to reduce nonspecific electrostatic interactions, while the buffer itself should not coordinatively bind to the chelated metal ion. Elution of the target protein is achieved by protonation, ligand exchange or extraction of the metal ion by a stronger chelator, like EDTA. Elution buffers with lower pH or lowering pH gradients are widely used for elution of the target protein. However, for proteins sensitive to low pH, ligand exchange, e.g., with imidazole, at nearly neutral pH is more favorable. In this case, the IMAC columns must be saturated and equilibrated with imidazole prior to chromatographic separation to avoid the pH drop caused by the imidazole proton pump effect [89]. Application of a strong chelating agent, such as EDTA, also results in elution of the bound proteins, although the binding properties are also destroyed and the column must be recharged with metal ions prior to the next separation.

Selectivity in protein separation can be effected through various approaches: by choice of the metal ligand, through variation of the structure of the chelating compound, by variation of the spacer arms, ligand density, concentration of salts and competing agents, etc. For example, in the case of human growth hormone, reduction of ligand IDA–Cu(II) density on chelating sorbent resulted in higher protein purity and increased yield [90]. The apparent affinity of a protein for a metal chelate depends strongly on the metal ion involved in coordination. In the case of the iminodiacetic acid IDA chelator, the affinities of many retained proteins and their respective retention times are in the following order: Cu(II) > Ni(II) > Zn(II) > Co(II). In contrast to these currently most commonly used metal ions, which have a preference for extra-nitrogen-containing amino acids, hard Lewis metal ions, such as Al(III), Ca(II), Fe(III), Yb(III), prefer oxygen-rich groups of aspartic and glutamic acid or phosphate groups and this provides an opportunity to engineer new affinity handles, based on glutamic- or aspartic-acid-rich affinity tails [44].

IDA is by far the most widely used chelating compound. It is commercially available from many producers, although in the past several years, other chelators have also been tried for immobilization to support particles. Some chelating compounds and coordination principles are shown in Table 2.4. In general, tetradentate ligands, such as NTA and TALON (trade name for carboxymethylated aspartic acid: CM-Asp), have higher affinities for metal ions than the tridentate chelator IDA, but they exhibit lower protein binding due to the loss of one coordination site. This is even more pronounced in a pentadentate TED chelating ligand, where in an octahedral arrangement around a divalent metal ion only one coordination site is left for protein binding. Putative structures of metal ion complexes and most popular chelators are shown in Figure 2.2.

Classical stationary phases are based on soft-gel matrices, such as agarose or cross-linked dextran. While polysaccharides are biologically compatible and easily activated, they exhibit low mechanical strength and a large pressure drop, which limits their use in large-scale industry. On the other hand, inorganic adsorbents, like silica, have excellent mechanical properties but exhibit irreversible nonspecific adsorption of proteins. Combination of polysaccharide on inorganic beads has

resulted in several new supports that are also applicable to the expanded-bed adsorption (EBA) technique, which enables the recovery of proteins directly from unclarified cell suspensions and homogenates. In Streamline Chelating (Amersham Pharmacia Biotech) the defined particle distribution is achieved by combining macroporous, cross-linked agarose with a crystalline quartz core and dextran coating, while UpFront EBA adsorbents (UpFront Chromatography, Copenhagen) are based on highly cross-linked agarose beads with a central core of high-density glass. Silica-based or synthetic polymer-based particles, such as Ni–NTA silica (Qiagen), Prosep-Chelating (Bioprocessing) or TSKgel Chelate (TosoHaas) can withstand high pressures in HPLC systems and can also be used for expanded-bed technology, which is especially appropriate for large-scale separations due to the reduced number of steps and consequently decreased process time. To increase the efficiency of protein binding, tentacle gels were developed, e.g., Fractogel EMD Chelate (Merck). Coupling of a chelator molecule, such as IDA, to the linear polymer chains (tentacles), results in higher functional-group density and better steric accessibility dueto increased flexibility of polymer chains as compared to the conventional spacer technology. As a result, higher protein capacity and stronger binding of the protein is achieved.

Membranes consisting of a hydrophylic copolymer and carrying metal chelating groups, the so-called Immobilized-Metal Affinity Membrane Adsorbers (IMA-MA), represent an interesting alternative to conventional chromatography, especially in terms of speed and simple scale-up [45]. Some years ago these membranes were commercially available from Sartorius (Goettingen) but they are not marketed any more. IDA was also bound to the surface of the Bioran-M glass hollow fiber microfiltration membranes (Schott Glass, Mainz), which exhibited higher metal loading capacity than conventional IMAC matrices but lower protein binding [46]. Recovery of serum proteins using porous IDA–Cu(II) cellulose affinity membranes was also demonstrated [47]. The role of a spacer element between the polymeric backbone and chelating group IDA was studied on microporous sheets obtained from Arbor Tech (Ann Arbor, MI). Membranes with no spacer and with 14-atom spacing element (1,4-butanediol diglycidyl) ether, bearing IDA, were prepared and

charged with Cu(II). Equilibrium adsorption of lysozyme was similar for both membrane types. However, dynamic adsorption was much higher when the spacer was included [48].

Table 2.4. Some chelating compound in use for immobilization in IMAC.

Chelating Compound	**Coordination**	**Metal Ions**
Aminohydroxamic acid	Bidentate	Fe(III)
Salicylaldehyde	Bidentate	Cu(II)
8-hydroxy-quinoline (8-HQ)	Bidentate	Al(III), Fe(III),Yb(III)
Iminodiacetic acid (IDA)	Tridentate	Cu(II), Zn(II), Ni(II), Co(II)
Dipicolylamine (DPA)	Tridentate	Zn(II), Ni(II)
Ortho-phosphoserine (OPS)	Tridentate	Al(III), Fe(III), Ca(II), Yb(III)
N-(2-pyrimidylmethyl) aminoacetate	Tridentate	Cu(II)
2,6-Diaminomethylpyridine	Tridentate	Cu(II)
Nitrilotriacetic acid	Tetradentate	Ni(II)
Carboxymethylated aspartic acid (CM-Asp)	Tetradentate	Ca(II), Co(II)
N,N,N′-tris(carboxymethyl) ethylene diamine (TED)	Pentadentate	Cu(II)

Recently, an interesting application of IDA–Cu(II) polysulfone hollow-fibre membrane for fractionation of the commercial pectic enzyme has been reported [49]. Pectinlyase, useful for large-scale fruit-juice clarification, passed through the membrane, while pectinesterase, responsible for undesirable methanol production, was retained. An article on the combined dye and metal chelate affinity membranes has also been published [50]. Cibacron Blue F3GA was covalently bound to microporous poly 2-hydroxyethyl methacrylate membrane and charged with Fe (III) ions to study adsorption/desorption behavior of various pure proteins. The same type of membranes was used for immobilization of glucose oxidase and the potential for the construction of glucose biosensors was demonstrated [51]. Cellulose membranes with imidazole as a ligand for Cu (II) immobilization have also been described [52]. In general, affinity membranes operate in convective mode, which can significantly reduce diffusion limitations commonly encountered in column chromatography. As a result, higher throughput and faster processing times are possible in membrane systems. Membranes are also capable of handling unclarified solutions and thus can be applied in the earlier stages of downstream processing. Therefore, wide applicability of IMA membranes for high-speed purification and analyses as well as for capture of trace amounts of histidine-bearing impurities, would be expected. However, to our knowledge, there are no commercially IMA membranes available. Another example of novel IMAC stationary phases are various metalloprotopor phyrins, covalently bound to silica supports, which have demonstrated a fundamental advantage of stable metal binding over the normally used stationary phases with weakly anchored metal ions [53].

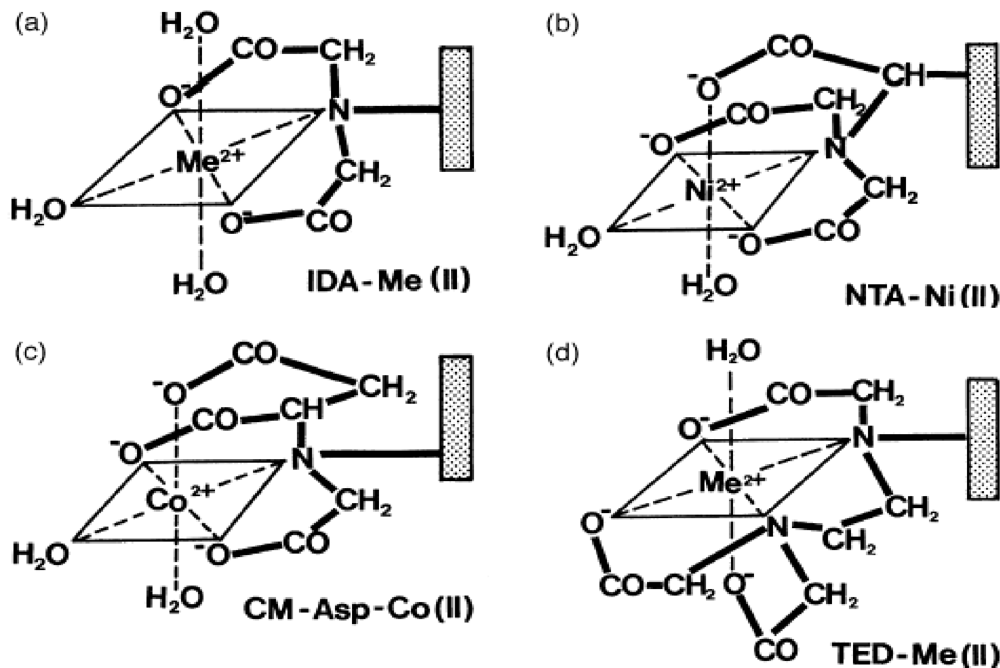


Figure 2.3. Putative structures of some representative chelators in complex with usually used metal ions: IDA–Me(II), NTA–Ni(II), CM–Asp–Co(II), TED–Me(II) . Me(II) stands for Cu(II), Ni(II), Zn(II) or Co(II). Spacers to the solid support are not specified, but may vary in length and chemical structure, which also affects chromatographic behavior.

Water molecules can be replaced by other ligands, usually histidines exposed on the protein surface. This represents the major binding interaction of the protein towards the IMAC matrix, provided that unspecific, residual interactions, e.g., ionic or hydrophobic, are minimized by selection of appropriate matrix material and buffer composition.

2.3.4. Some Advantages of IMAC

2.3.4.1. Use Under Denaturing Conditions and In Situ Refolding

Today, many recombinant proteins are produced by intracellular expression of heterologous genes in genetically engineered *E. coli* strains. However, recombinant proteins, accumulated intracellularly, are frequently deposited in the form of insoluble aggregates of misfolded proteins in inclusion bodies. The production of pure, biologically active proteins involves denaturation and refolding/renaturation, which is classically accomplished by the low-efficiency techniques of dialysis or dilution.

IMAC chromatography has the advantage of enabling histidine-tagged proteins to be separated efficiently in the presence of denaturing concentrations of urea or guanidine–HCl. Additionally, affinity tagging by consecutive histidines offers the possibility of efficient purification and refolding in a single IMAC step. GATA-1 protein, antigens of Mycobacterium tuberculosus, and two different membrane proteins are some examples of His-tagged fusion proteins, purified under denaturing conditions, renatured while still bound to the solid phase by lowering the concentration of chaotropic agent and then eluted from IMAC columns in the renatured, biologically active form [54, 55, 56].

2.3.4.2. Immobilization on the Column

Due to the relatively high affinity of histidine tags for special IMAC matrices these can serve for the reversible immobilization of proteins. An interesting example is the isolation of the multimeric chaperonin GroEL, containing His6 tail on the C-terminus, from a crude extract of recombinant *E. coli* and its subsequent immobilization on Ni-chelate Cellulofine. The protein retained its ability to mediate protein refolding when attached to the affinity-chelating matrix.

Similarly, IMAC matrices were used for the reversible immobilization of 2-haloacid dehalogenase (57) and β-galactosidase (58), which retained their enzymatic activities. As the affinity of the His6-tagged proteins for Ni–NTA matrix is higher than the affinity between most antibodies and antigens, the recombinant protein can be immobilized on such columns and serve for the affinity purification of specific antibodies [59].

2.3.5. Some Disadvantages and Problems of IMAC

2.3.5.1. Oxidation and Reduction Conditions Inside the Column and Metal-Induced Cleavage

Several amino acids, especially histidine, lysine, cysteine, proline, arginine, and methionine, are susceptible to metal-catalyzed oxidation reactions that produce highly reactive radical intermediates, which can damage a variety of proteins. Taking into account that metal chelates as well as Cu(II) ions themselves can be used for the site-specific cleavage of proteins, it is not surprising that destruction of amino acid side chains and cleavage of the protein backbone can also be provoked during

IMAC chromatography. In such cases, the replacement of high redox-active Cu(II) with a less active metal ion, such as Zn II , may prevent, or at least minimize, protein damage [60].

The majority of routine IMAC separations are carried out under aerobic, mildly oxidative conditions, due to oxygen dissolved in the sample and buffers. Potential damage to proteins, caused by reactive oxygen species or metal-catalyzed reactions inside the IMAC column, has not been studied enough. In experiments under forced conditions, e.g., when hydrogen peroxide or ascorbate - and especially a combination of both - were added to elution buffers, a significant loss of protein activity was demonstrated on Cu(II)-IDA columns. This was ascribed to reactions of hydroxyl radicals, which are produced by the Fenton reaction;

$$M^{n+} + H_2O_2 \longrightarrow OH^{\cdot} + OH^{-} + M^{(n+1)+}$$

Experiments with short model peptides confirmed that histidines react readily in this type of reaction. Thus, in real IMAC columns, the potential for oxidative radical damage exists, especially in cases where extensive elution with widely used detergents, such as Tween or Triton X-100, contaminated with hydroperoxy compounds, is performed. Low in-column contact times and use of superflow matrices would be preferable in such cases [61].

When IMAC is used under denaturing conditions, reducing agents, such as β-mercaptoethanol (β-ME) or dithiothreitol (DTT), are usually added to the buffers used to dissolve inclusion bodies and also during chromatography to prevent formation of disulfide-bonded aggregates. With some IMAC matrices, low concentrations of reducing agents, usually β-ME in the millimolar range, are permitted, although the column capacity is rapidly diminished and chromatographic performance becomes irreproducible after several applications of the sample. In this respect, TALON with immobilized Co(II) should be the matrix of choice, since, according to the manufacturer, it tolerates up to 30 mM β-ME under native

conditions. Although IMAC was also used for the resolution of sulfitolyzed proteins, no data exist for the highest concentrations of sulfite allowed [62, 63].

2.3.6. Future of IMAC

With the completion of the Human Genome Project there will be a strong need for tools to carry out massive parallel protein expression experiments. One could envision the use of poly-histidine tags for high-throughput HT purification in such experiments to be very broad indeed. The use of a small tag for purification purposes is very advantageous. The poly-histidine tag can be fused to the target protein either on the N-or on the C-terminus. It is expected to be more accessible for cleavage than larger tags (sometimes comprising full-length proteins) if its removal is required. As compared to the GST fusion tag, the poly-histidine tag has been shown to be less susceptible to aggregation. Moreover, protein expression of histidine tagged recombinants results in little or no fragmentation [64]. The poly-histidine tag has also been shown to be better suited for purification of proteins from transfected mammalian cells than the strep-tag [65]. The development of HT purification procedures puts the following requirements on the type of the adsorbent and the layout:

- Good physical stability of the adsorbent.
- The adsorbent has to be ready for use, preferably pre-charged with metal ion.
- The adsorbent would have to withstand high flow rates.
- The adsorbent has to have high selectivity.
- The adsorbent should be compatible with crude samples.
- A high throughput format for the layout of the adsorbent columns has to be developed.
- A generic protocol that suits the requirements of the majority of the tagged proteins, including extraction, equilibration, loading and elution of the target proteins has to be developed. Such a protocol will preferably contain a simple capture step that would yield high recovery and purity of the target proteins (Fig. 5a and b; unpublished data).

Already, a number of groups and companies are developing or have available HT systems. Qiagen for example has an HT purification system based on Ni^{2+}-NTA magnetic beads that is compatible with robotic manipulation. This system has a capacity per separation well of 5–30 mg of tagged protein. Our own group is developing a vacuum-based HT system, which would permit parallel purification of analytical amounts (up to 1 mg/column) of poly-histidine tagged proteins.

A novel application of IMAC might be in the use of solid phases with immobilized metal ions as anchors of histidine-tagged recombinant proteins or DNA for studies of protein–protein and protein–DNA interactions [66].

It is inevitable that IMAC will find much broader use for pre-fractionation of complex protein mixtures prior to analyses by MS in the Proteomics area. One envisions the use of two IMAC columns in tandem, with affinities for two different tags in conjunction with MS that can be utilized for rapid analyses of drug effects on protein expression. Such a system could also be used to analyze the phosphorylation state of a particular target protein.

The incorporation of an IMAC step in large-scale purification procedures will continue with increased speed because of the versatility of the principle and the necessity for quick development of purification procedures. It is certain that sooner rather than later the use of purification tags will be accepted broadly for protein products. The pressure of the market for shorter product release times on companies producing proteins is already causing a number of separation groups to pay closer attention to the possible incorporation of purification tags in their downstream processes.

The future of IMAC is bright and the number of its applications will continue to grow. It is of great importance that the fundamental development of the principle continues hand in hand with its applied use. New chelating ligands and metal ions will deliver novel specificity and selectivity. This will ensure the application of IMAC in yet new and exciting areas of biology, biotechnology and pharmaceutical science.

2.4. Hemoglobin

Haemoglobin (Hb) is the iron-containing oxygen transport metalloprotein, which can be found in the red cells of blood of mammals and other animals. Hb is a globular protein, and structural changes in any of the four molecular subunits that it is made up of, may result in the manifestation of hereditary diseases such as thalassaemia, sickle cell, anaemia, and haemoglobinopathies [67]. The laboratory diagnosis of such conditions necessiate a combination of complicated and time-consuming procedure [68], whereas MI may offer a quick, sensitive and selective approach to the visualization, diagnosis and monitoring of haemoglobin disorders.

2.4.1. Functional Class

Oxygen transport protein; heme containing protein connected to myoglobin is know as hemoglobin (Hb). Hemoglobins (Hbs) are heme-containing proteins. They both share a well known 'globin fold' and reversibly bind molecular oxygen. They may be found in various species from bacteria [69] to man. Their existance can be like monomers, dimmers, tetramers or even higher molecular weight assemblies. Polypeptide chains, which consist of duplicated globin domains, have also been found [70]. The oligomerization of Hb was a vital step in evolution, by which Hb acquired its allosteric properties, which are essential for efficient oxygen transport.

2.4.2. Occurrence

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

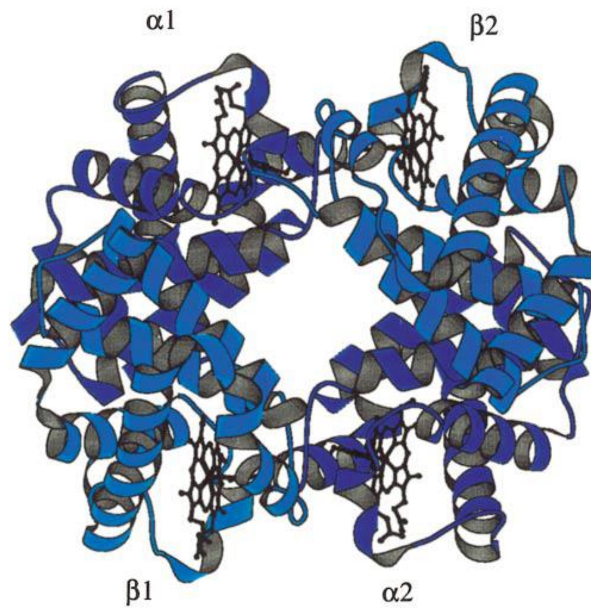


Figure 2.4. Schematic representation of the quaternary structure of deoxy T state Hb (PDB code: 1HGA). The α-subunites are blue-colored, while the β-subunits are cyan-colored; the heme groups are shown in a black ball and stick model. The cavity is seen at the center of the schema. In its T state conformation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

2.4.3. Biological Function

The physiological functions of vertebrate Hbs are the transportation of oxygen from the lungs (or gills in fish) to the body tissues, increasing of carbon dioxide transport in the reverse direction and regulation of blood pH (acid-base balance). The partial oxygen pressure in the lungs (alveolar oxygen pressure) is approximately 100 mmHg in humans and Hb becomes 98% saturated with oxygen at that pressure. The partial oxygen pressure in mixed venous blood (blood returning to the lungs) is approximately 40 mmHg and under these conditions Hb is only 70-75% saturated with oxygen [71]. Therefore, approximately one quarter of the blood oxygen is delivered to the tissues.

Hb contains two α- and two β- subunits, each of them contains a heme or Fe(II)-protoporphyrin IX. Each heme can bind one oxygen molecule, for this reason four molecules of oxygen bind a Hb tetramer (3D structure). The affinity for the first oxygen is low however the affinity begins to rise with the number of bound oxygen molecules. This is known either as homotropic allosteric effect or the heme-heme interaction.

Early observation that oxygenated and deoxygenated Hbs crystallize in different crystal forms proposed that Hb exposes a structural change upon binding to oxygen [72]. 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 [73]. Perutz proposed a stereochemical mechanism of cooperative oxygen binding which is based on these structures. Various liganded forms of Hbs and the structures of the deoxy are now known at high resolution.

As a first approximation, the oxygen binding properties of Hb can be portrayed by the two-state allosteric model proposed by Monod, Wyman and Changeux [74]. They proposed that the Hb molecule is in balance between two alternative quaternary structures (arrangement of the four subunits) (Figure 2.3).The T(tense), or deoxy structure has a low oxygen affinity however the R(relaxed), or oxy structure has high oxygen affinity. Since deoxyHb is predominantly in the T state, the first oxygen naturally binds with low affinity. The binding of oxygen couses stabilization on the R state, and therefore the oxygen affinity increases with the number of the bound oxygens. The equilibrium between the T and R states is affected by the concentration of other metabolites within erythrocytes as well. Purified Hb (stripped Hb) is unable to unload sufficient amount of oxygen to the tissues and shows much higher oxygen affinity than whole blood. An intermediate of the glycolytic pathway, 2,3-diphosphoglycerate (2,3-DPG), is present in equimolar amount to the Hb tetramer in human erythrocytes. By preferentially binding to the deoxy form, it reduces the oxygen affinity of Hb. Protons below pH 6.0 raise the oxygen affinity. This is both called as the acid Bohr effect and reversed Bohr effect. Above pH 6.0, protons reduce the oxygen affinity and this is called the alkaline Bohr effect. This effect is explained by release of protons that are associated with oxygen binding. Active tissues produce CO_2, which dissolves into water forming a proton and a bicarbonate ion, 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, [75] by forming carbamino groups, displayed the direct binding of CO_2 to the α-amino

groups of both α and β chains. The allosteric properties of the Hb therefore allow preferential unloading of oxygen to the tissues, however Hb plays an crucial 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.

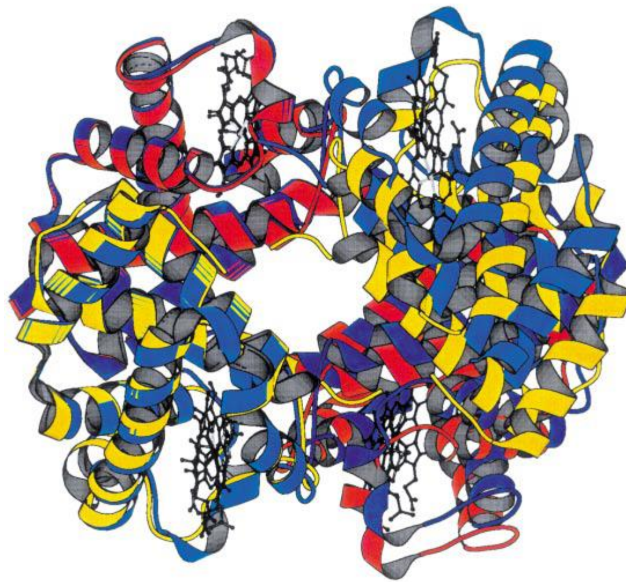


Figure 2.5. Comparison between oxy R states (PDB: 1HHO) and the deoxy T (PDB code: HGA) highlighting the shifts associated with the quaternary transition. Deoxy T state α subunits are shown in blue, while deoxy T state β-subunites are shown in cyan; oxy R state α-subunits are shown in red and oxy R state β-subunites are shown in orange. The T state hemes are colored in black while the R state hemes in gray. Cylinders represent helices. 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 conversion and translation of the $\alpha_2\beta_2$ dimer with respect to the $\alpha_1\beta_1$ dimer are obvious.

2.4.4. Amino Acid Sequence Information

Hbs, which are from a considerable number of vertebrate species, have been sequenced and this has provided significant insights into molecular evolution of proteins. The majority of these sequences have been ascertained by Braunitzer, who devoted his life to sequence determination of Hbs from rare species [76] The

first cDNA to be cloned and sequenced consist of the cDNA of human α and β globin chains [77]. The sequence identity between the most distantly related vertebrate Hbs contains small insertions and deletions. Hbs are post-translationally modified in some species. Most common modification is the acetylation of the N-terminus. Human blood involves minor glycosylated components. Diabetic patients have rising levels of glycosylated Hbs, which are used for diagnostic aims [78].

2.4.5. Purification and Molecular Characterization

Hb can be produced readily from vertebrate blood. Freshly drawn blood is immediately mixed with either citrate or heparin in order 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 taken out by centrifugation after addition of NaCl to 3% (w/v). Hb represents 98% of protein in the hemolysate and no further decontamination 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) that consists of two α- and two γ-subunits. DeoxyHb F does not bind DPG greatly and therefore 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 them are genetic mutations. Nearly 8% of African Americans carry the HbS trait and 2.3% African Americans carry HbC as well. In sub-Saharan Africa, predominance of these two is up to one-third of all patients. In Southeast Asia, HbE can be as high as 30%. Many hemoglobinopathies, including HbSC disease, sickle cell disease, homozygous HbC disease and β-thalassemia, frequently show increased amounts of 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. In diabetic patients, chemically modified Hbs may be chronically present. Most commonly encountered of these is the CarbamylatedHb [79]. Facilities that are performing A_{1C} testing should be aware of interferences, which are 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. 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) [80]. 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 [81].

Conventional packed-bed columns possess some inherent limitations such as the slow diffusional mass transfer and the large void volume between the beads [89]. Although some new stationary phases such as the non-porous polymeric beads [90] and perfusion chromatography packings are designed to resolve these problems, these limitations cannot be overcome in essence [91]. 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 [82].

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 2.5.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 2.5.b different forms of cryogels can be seen.

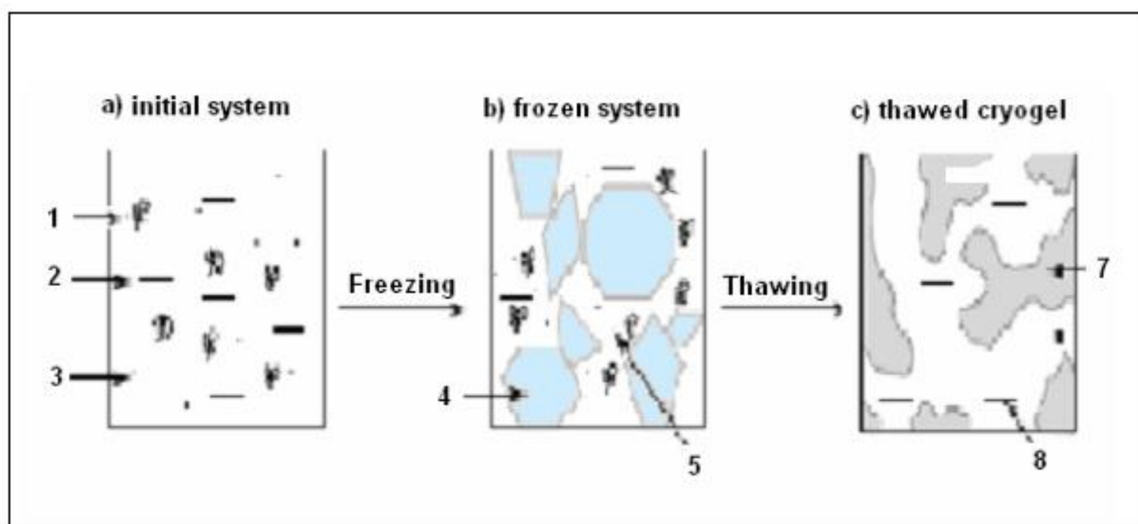


Figure 2.6.a. Cryogel formation. 1-Macromolecules, 2-Solution, 3-Solvent, 4-Polycrystals of freezed solvent, 5-Unfreezed solvent, 6-Cryogel 7-Macropores, 8-Solvent.

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

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 (MBA), and N,N,N',N'-tetramethylene diamine (TEMED) were also obtained from Sigma. Hemoglobin (Hb) from bovine was 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).

3.2. Preparation of Metal-Chelated PHEMA Monolithic Cryogels

3.2.1. Preparation of PHEMA Cryogels

Supermacroporous monolithic PHEMA cryogel was prepared by bulk polymerization. 283 mg of MBAAm was dissolved in 15 ml deionized water and 1.3 ml HEMA was added. The cryogel was produced by free radical polymerization initiated by TEMED (15 µl) and APS (150 µl) (10% (w/v)). After adding APS, the solution was cooled in an ice bath for 2-3 min. after adding TEMED, the reaction mixture was stirred for 1 min and then, this mixture was poured into a plastic syringe (5 mL, id. 1.0 cm) with closed outled at the bottom. The polymerization solution in the syringe was frozen at -12°C for 24 h and then thawed at room temperature. After washing, the cryogel was stored in buffer containing 0.02% sodium azide at 4°C until use.

3.2.2 IDA-Attached PHEMA (PHEMA-IDA) cryogel

PHEMA cryogel was first activated with epichlorohydrin. For this, a solution of 18.5 ml epichlorohydrin,172.5 ml 2 M NaOH, and 637 mg sodium borohydride passed through the cryogel column overnight at room temperature and then washed successively with water and 0.1 M acetic acid, respectively. In order to prepare the

IMAC support material, IDA was used as a chelating agent. IDA was covalently attached to the cryogel in a continuous system. In order to investigate the effect of initial IDA concentration on IDA immobilization onto the PHEMA cryogel, the PHEMA cryogel was treated with different amounts of IDA solution, which were 15, 30, 50 and 100 mg in 30 ml of 2.0 M Na_2CO_3 (pH=11), respectively, by using a peristaltic pump at 70 °C for 12 h. After immobilization reaction, IDA functionalized cryogels were washed with acetic acid (5%) and distilled water to remove the unreacted IDA.

3.2.3. Chelation of Metal Ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+})

Chelates of metal ions with IDA-attached PHEMA cryogels were prepared as follows: four metal ions **(Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+})** were prepared in different concentrations (10 ppm, 25 ppm, 50 ppm and 100 ppm in 10 mM HEPES buffer solution), separately, and passed through the IDA-attached PHEMA cryogels in a continuous system for 2 hours. Nitrate salts were used for the preparation of standard metal-ion solutions. The concentration of the metal ions in the resulting solutions was determined with a graphite furnace atomic absorption spectrometer (AA800, Perkin Elmer, Bodenseewerk, Germany). The amounts of adsorbed metal ions were calculated by using the concentrations of the metal ions in the initial solution and in the equilibrium.

Metal leakage from the PHEMA cryogels were investigated with media pH (5.0-8.0), and also in a medium containing 1.0 M NaCl. It should be also noted that immobilized metal containing cryogels were stored at 4°C in the 0.1 M phosphate buffer (pH 7.4) with 0.02% sodium azide to prevent microbial contamination.

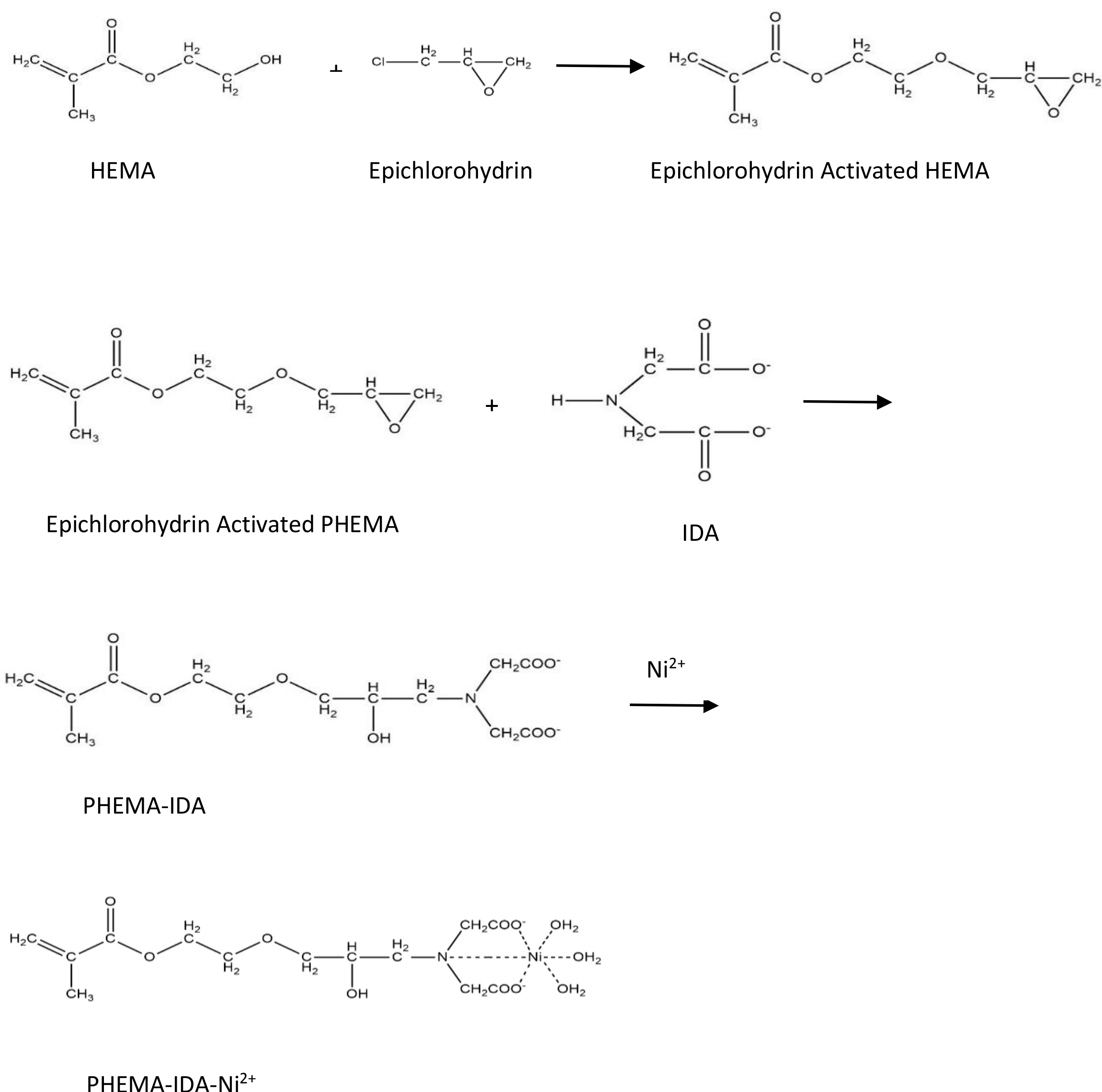


Figure 3.1. Schematic presentation of PHEMA-IDA-Ni^{2+} cryogel synthesis

3.3. Characterization of PHEMA 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 hours 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: $(m_{swollen\ gel} - m_{squeezed\ gel})/m_{swollen\ gel} \times 100\%$. The percent of swollen gel weight is also calculated as $(m_{swollen\ gel} - m_{dried})/m_{swollen\ gel} \times 100\%$. 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.

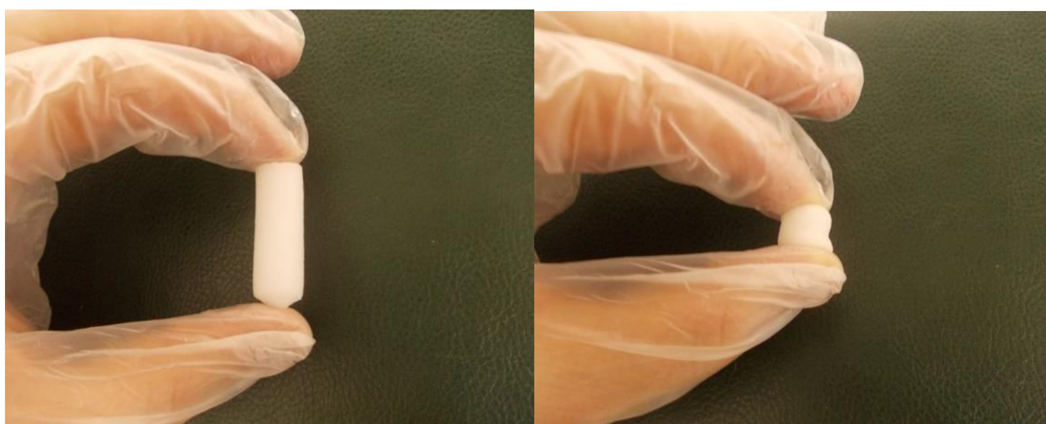


Figure 3.2. Swelling properties of cryogels

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 cryogel 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. FTIR Studies

FTIR spectra of PHEMA, PHEMA-IDA and PHEMA-IDA-Ni 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. Blood Compatibility Studies

3.4.1. Coagulation Time (CT)

PHEMA cryogels were incubated with 0.1 M phosphate buffer solution (pH: 7.4) for 24 h at room temperature and washed with 0.5 M NaCl solution and distilled water. Fresh frozen pooled human plasma (0.1 mL) was preheated to 37°C for 2 min and then 250 mg of dried and powdered cryogel was added into this medium and mixed immediately. The clotting time was measured by using fibrometer method [83].

3.4.2. Activated Partial Thromboplastin Time (APTT)

PHEMA cryogel was incubated with 0.1 M phosphate buffer solution (pH: 7.4) for 24 h at room temperature and washed with 0.5 M NaCl solution and distilled water. Fresh frozen pooled human plasma (0.1 mL) was preheated to 37°C for 2 min. The partial thromboplastin (0.3 mL, bioMerieux, Marcy-l'Etoile, France) was also preheated to 37°C for 2 min and was added to preheated human plasma. Then, 250 mg of dried and powdered cryogel was added into this medium. Thirty seconds later,

$CaCl_2$ (0.1 mL, 0.025 M) was added, then, the active partial thromboplastin time (APTT) was determined by using the fibrometer method [84].

3.4.3. Prothrombin Time (PT)

In order to determine prothrombin time (PT), one-stage prothrombin method was used [85]. PHEMA cryogel was incubated in 0.1 M phosphate buffer solution (pH: 7.4) for 24 h at room temperature. Fresh frozen pooled human plasma (0.1 M) was preheated to 37°C for 2 min. The thromboplastin (0.2 mL, bioMerieux, Marcy-l'Etoile, France) was also preheated to 37°C for 2 min and was added to preheated human plasma. Then, 250 mg of dried and powdered cryogel was added into this medium. Thirty seconds later, $CaCl_2$ (0.1 mL, 0.025 M) was transferred into the medium. After these operations, the prothrombin time was measured by using fibrometer method [85].

3.4.4. Cell Adhesion Studies

Human blood (heparinized, 500 IU/kg) was contacted with PHEMA cryogel at in-vitro system. It should be noted that prior to the blood contact, cryogels were washed with 0.1 M KCl in buffer until no further impurities (monitored by the absorbance at 280 nm) was detected in the washing solution. Cryogels were incubated with blood for 1 h. Blood samples were withdrawn at the beginning and at the end of the procedure, and the platelet and leukocyte count of samples were determined by microscopy.

3.5. Adsorption-Desorption studies

3.5.1. Adsorption of Hemoglobin from Aqueous Solutions

The hemoglobin adsorption studies were carried out in a recirculation system. Figure 3.2 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 7.4) 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 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 0.01-0,08 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 0.01 mg/mL of hemoglobin solution in 0.1 M phosphate buffer (pH 7.4, 30 mL). The effect of pH on the adsorption capacity was determined by changing pH of the solution between 5.0 and 8.0. 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.

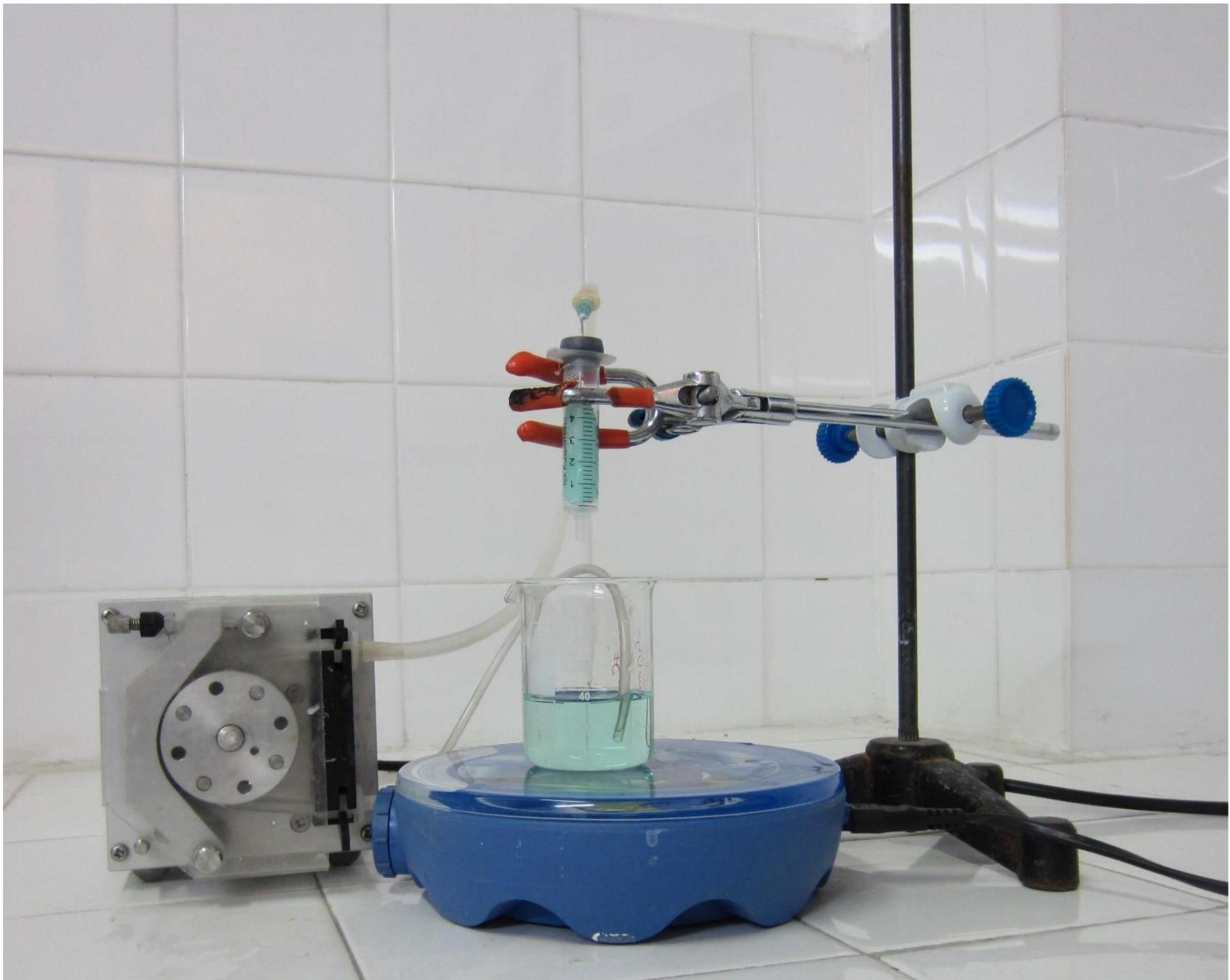


Figure 3.3. Continuous system used for adsorption experiments.

3.6. Desorption and Repeated Use

Desorption of the adsorbed hemoglobin from PHEMA cryogel was studied in continuous experimental setup. Hb adsorbed PHEMA cryogel was desorbed with 0.1 M phosphate buffer (pH=8, include 0.5 M urea, 1 M sodium chloride) for 2.0 hour at room temperature. The final hemoglobin concentration in desorption medium was determined by spectrophotometer at 406 nm. The desorption ratio for hemoglobin was calculated with the following expression:

$$\text{Desorption ratio (\%)} = \frac{\text{Amount of hemoglobin released}}{\text{Amount of hemoglobin adsorbed}} \times 100 \qquad (3.4)$$

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

3.6.1 Depletion of Hemoglobin from Human Blood

Depletion of hemoglobin from human blood was adapted from the following procedure [86]. The collected fresh blood was centrifuged for 10 min at 3 000 rpm. The plasma was removed and remaining packed red blood cells (RBCs) were washed thrice with normal saline to remove the buffy coat. Hemolysis was performed by pipetting out 1 mL of washed red blood suspension in ice-cold distilled water. Erythrocyte ghosts were sedimented in a high-speed refrigerated centrifuge at 12 000 rpm for 40 min. The cell content was separated out carefully and used for Hb depletion experiments. For this purpose, 10 mL of hemolysate solution was carefully pumped through the PHEMA-IDA-Ni cryogel under recirculation for 2.0 h with 1.0 mL/min of flow rate at 20ºC. The adsorption from hemolysate was detected by photometric method at Mesa Hospital (Ankara, Turkey). The amount of Hb depletion in hemolysate per unit mass of dry polymer in cryogel was calculated using the mass balance.

The depletion of hemoglobin was imaged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For this purpose, 50 mg of sample solution was run on a 5–12% stacking PAGE gel. A wide-range molecular weight marker (205–36.5 kDa) was used (Wide Range Sigma Marker, Sigma, USA). The gel was stained with colloidal Coomassie Blue method and monitored by ImageQuant 300 (Amersham, USA).

3.7. FPLC studies

FPLC separation was performed using an AKTA-FPLC (Amersham Bioscience, Uppsala, Sweden) system equipped with a UV detection system. The system includes M-925 mixer, P-920 pump, UPC-900 monitor, INV-907 injection valve and Frac920 fraction collector. Separation was carried out at GE Healthcare column (10/10, 19-5001-01) that packed with PHEMA-IDA-Ni^{2+} cryogel. FPLC mobile phases A and B were prepared using 0.1 M phosphate buffer (pH 7.4) and 0.1 M phosphate buffer (pH 8, include 0.5 M urea, 1 M sodium chloride), respectively. The chromatographic separation was performed using a linear gradient at 1.0 mL/min

flow rate. After a 5.0 min starting period with 100% mobile phase A, a linear gradient started from 0% B to 100% B in 3.0 min, continued with 7.0 min 100% eluent B and finished last 10 min 100% buffer A. All buffers and protein solutions were filtered before use. 1 mL protein mixture was applied to the column. Absorbance was monitored at 280 nm. The separation was performed at room temperature. KBr was used as the void marker. Capacity factor (k') and separation factor (α) were calculated as $k' = (t_R - t_o)/t_o$, $\alpha = k_2'/k_1'$, where tR is the retention time of the protein and to is the retention time of the void marker (KBr), k_2' is the capacity factor for Hb and k1' is the capacity factor for other proteins. The resolution (Rs) and total theoretical plate numbers (N) were calculated using the following equations:

$$N = 5.54 \, (t_R / w0.5)^2 \qquad (3.5)$$

$$Rs = 2 \, (t_{R,2} - t_{R,1}) / (w_2 + w_1) \qquad (3.6)$$

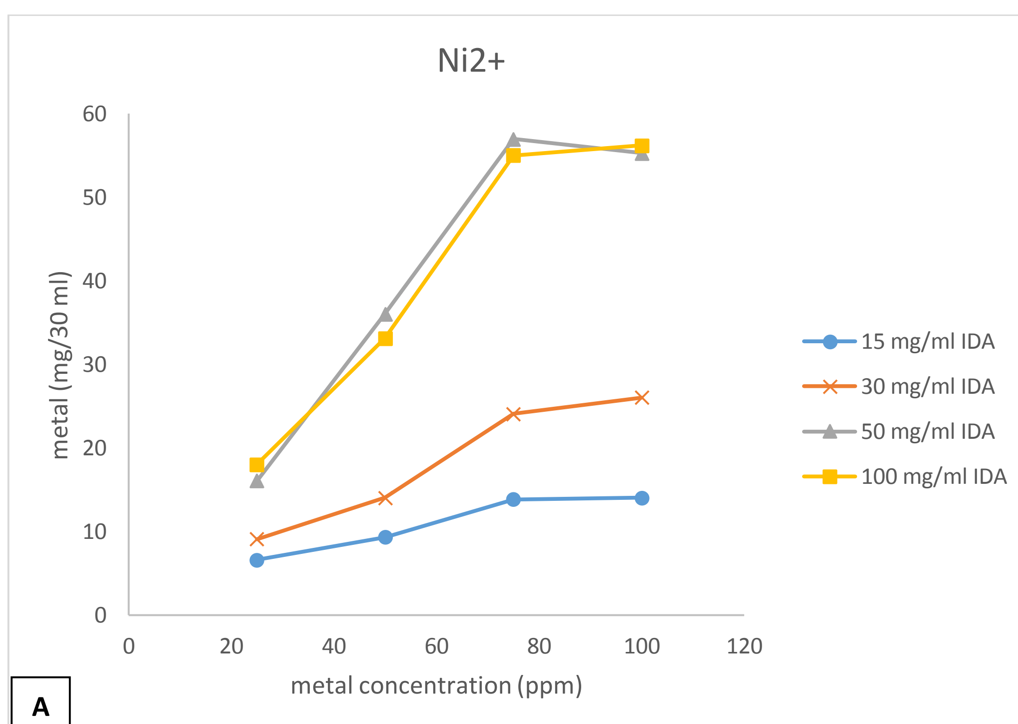
where w0.5 is the peak width at the corresponding peak height fraction, $t_{R,1}$ and $t_{R,2}$ are the retention times of two adjacent peaks, w_1 and w_2 are the widths of the two adjacent peaks at the baseline.

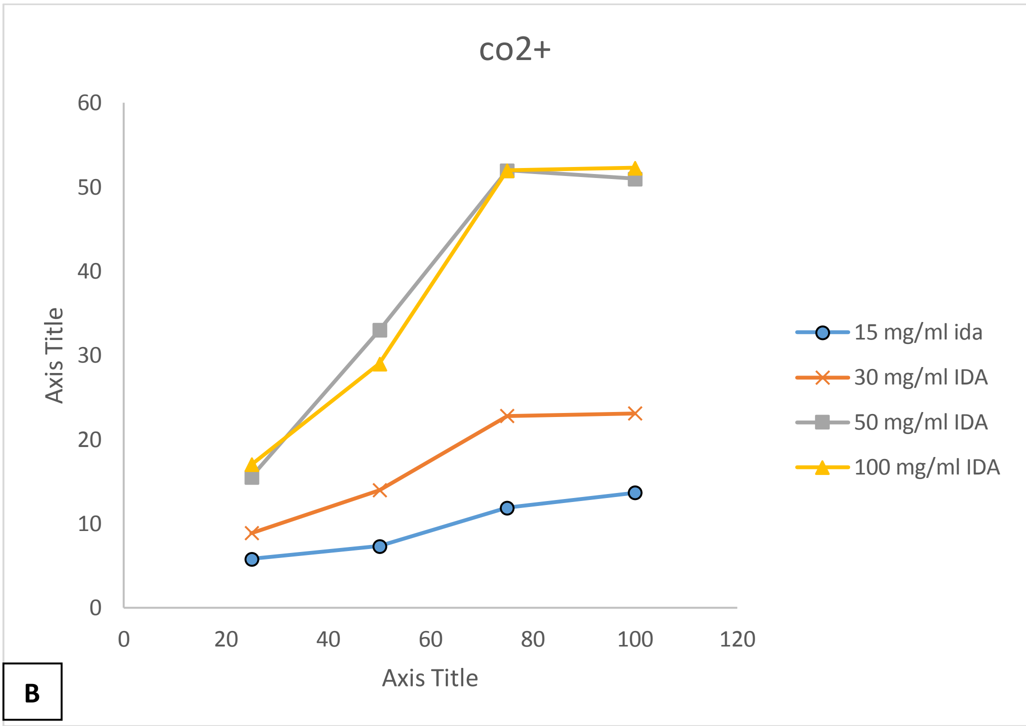
Figure 3.4. Picture of FPLC system.

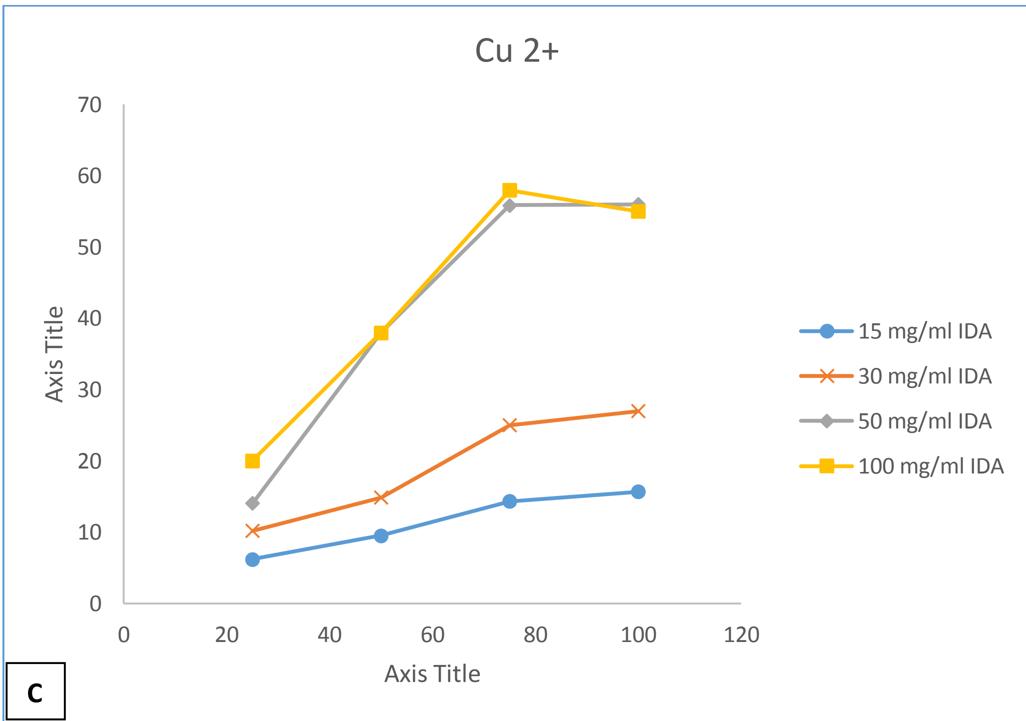
4. RESULTS AND DISCUSSION

4.1. Preparation of PHEMA-IDA-Me Cryogels

For preparation of PHEMA-IDA-Me cryogels, four different metal ions (Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}) were used with different amounts of IDA attachment. As seen in Figures 4.1., (A-B-C-D), the maximum amount for four different metal ions immobilized onto PHEMA-IDA cryogel was obtained with 50 mg/mL of IDA initial concentration. The highest metal loading onto PHEMA-IDA cryogel by this IDA initial concentration was obtained as 57 mg/g cryogel for Ni ions. Therefore, the following study was implemented for PHEMA-IDA-Ni cryogel. In figures 4.1., the optic photographs of the covalently attachment of four different metal ions (Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}) can been seen apparently.




co2+
Axis Title
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Axis Title
15 mg/ml ida
30 mg/ml IDA
50 mg/ml IDA
100 mg/ml IDA
B


Cu 2+
Axis Title
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
Axis Title
15 mg/ml IDA
30 mg/ml IDA
50 mg/ml IDA
100 mg/ml IDA
C

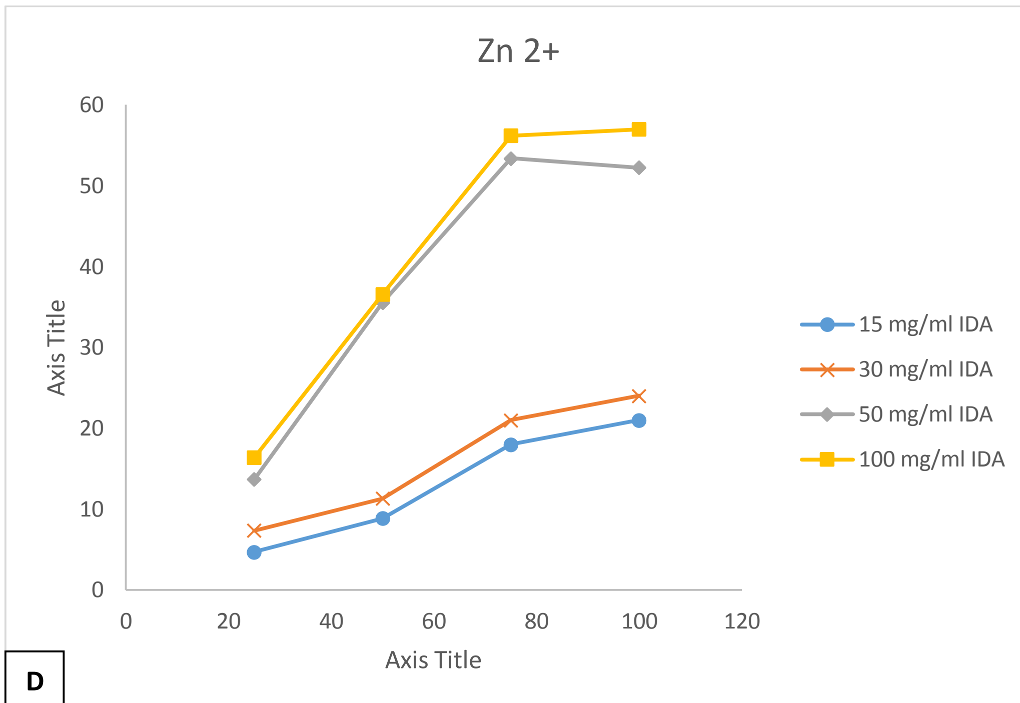


Figure 4.1. Effect of initial metal concentration on metal loading with different IDA attachment. Experimental conditions: Flow rate =1mL/min, Buffer solution: pH 7.0 HEPES, Loading time: 2 hours, Temperature: 20 °C. A: Ni^{2+}, B: Co^{2+}, C: Cu^{2+}, D: Zn^{2+}

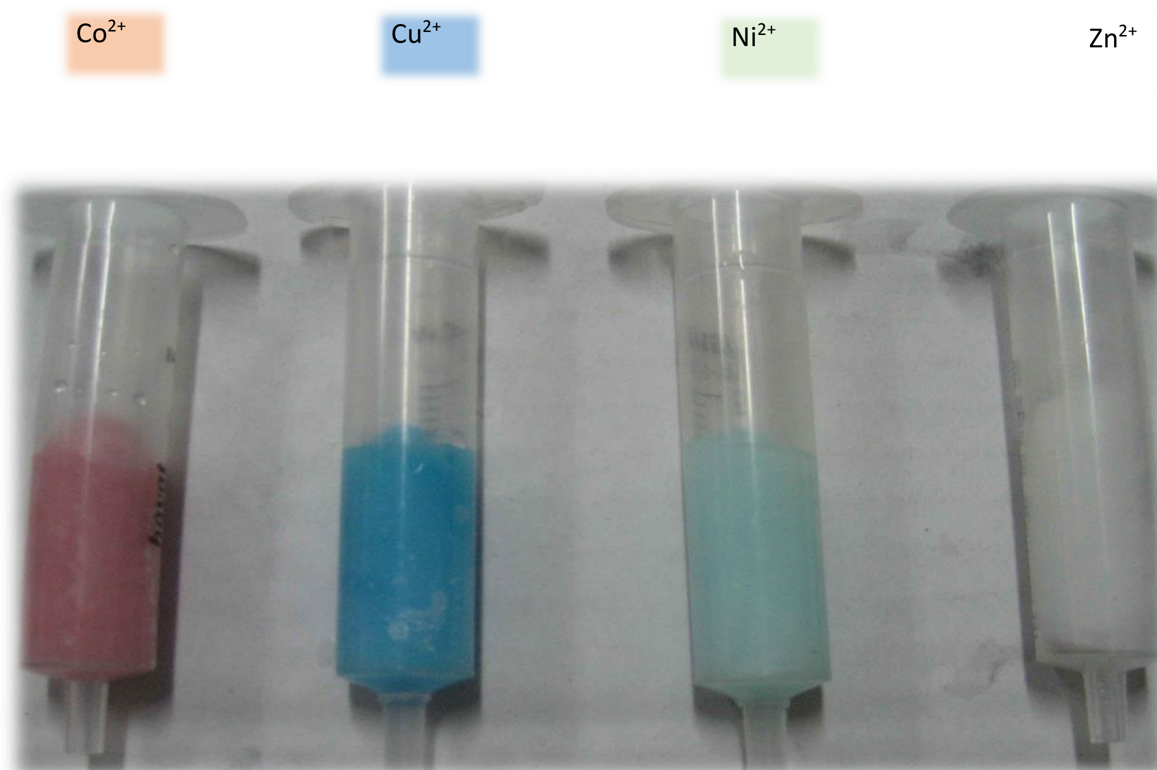


Figure 4.2. Loading of metals on cryogels.

4.2. Characterization of PHEMA Cryogel

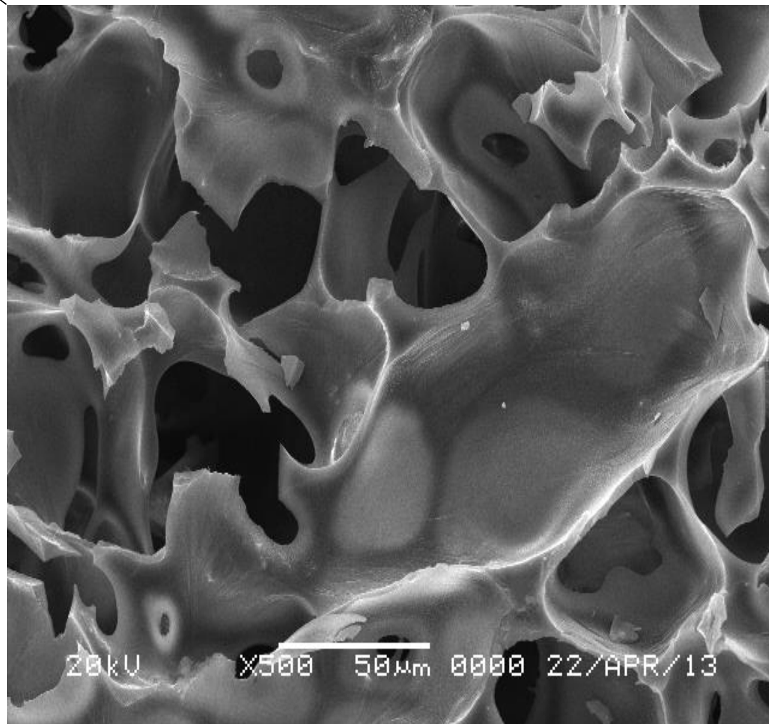
4.2.1. Swelling Tests

PHEMA cryogels prepared in this study are hydrophilic structures, i.e., hydrogels. Hydrogels are water swollen, cross-linked polymeric structures produced by the simple reaction of one or more monomers or by association bonds such as hydrogen bonds and strong van der Waals interactions between chains. An integral part of the physical behavior in water, since upon preparation they must be brought in contact with water to yield the final, solved network structure. A dry, hydrophilic cross-linked network is placed in water. Then, the macromolecular chains interact with the solvent molecules owing to the relatively good thermodynamic compatibility. Thus, the network expands to the solvated state. Hydrogels have received significant attention, especially in the past 30 years, because of their exceptional promise in biomedical applications [87].

4.2.2. Surface Morphology

The surface morphology of PHEMA cryogel was exemplified by the scanning electron micrographs. As clearly seen from Figure 4.1, the polymeric cryogel has macropores which formed during the polymerization procedure. After 24 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 PHEMA cryogels 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 cryogels produced in such a way has large continuous interconnected pores (10–200 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.

A



B

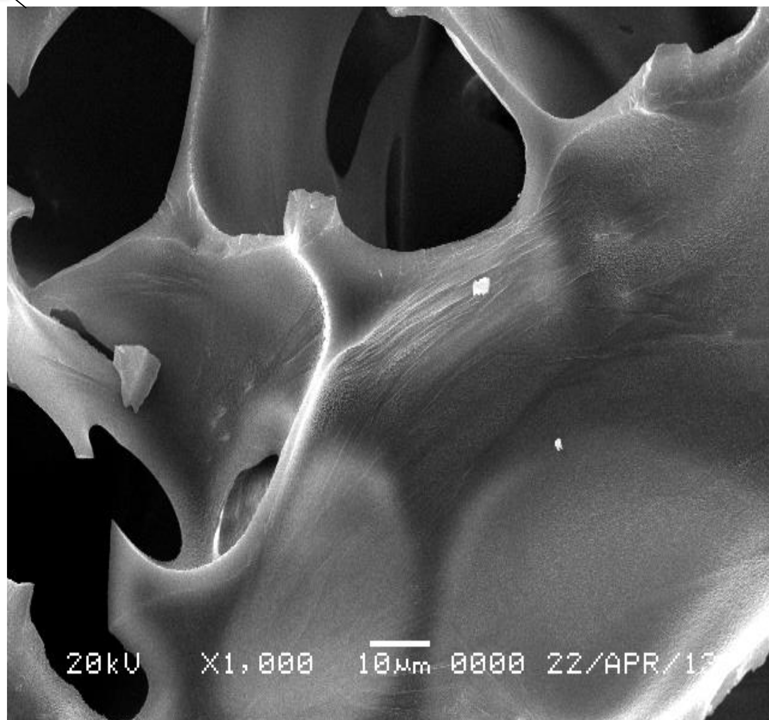


Figure 4.3. SEM photographs of PHEMA cryogels with different magnifications. A: 500, B: 1000

4.2.3. Surface Area Measurements

The surface area of the cryogels was found to be 24.7 m^2/g by BET method. An advantage of cryogels is that they have large and highly interconnected pores. Although this provides cryogels with low backpressures 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 [88].

Table 4.1. Physicochemical properties of the PHEMA cryogel.

IDA attachment	8.27 mmol/g
Specific surface area	24.7 m^2/g
Pore size diameter	10-200 μm
Porosity	74.1% for PHEMA
	63.5% for PHEMA-IDA
Swelling Degree	8.49 g H_2O/g cryogel
Back pressure	0.26 MPa

4.2.4. FTIR Studies

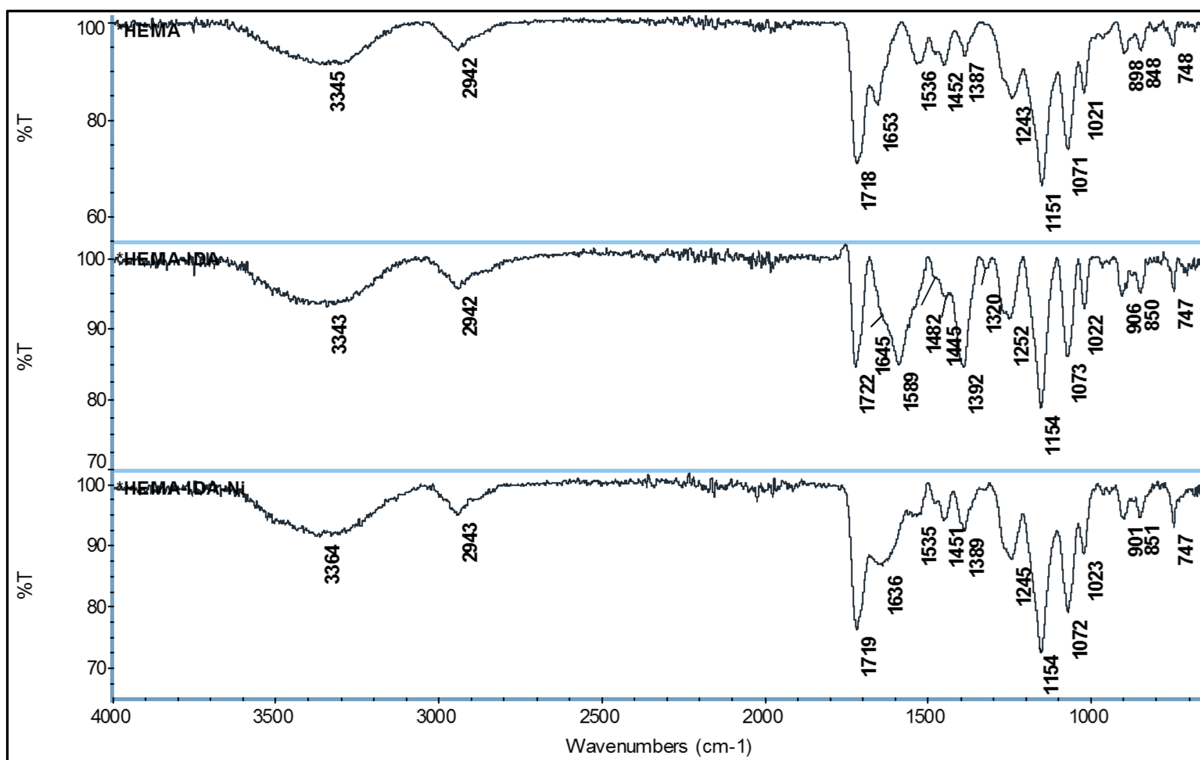


Figure 4.4. The FTIR spectra of PHEMA (top side), PHEMA-IDA (middle side) and PHEMA-Ni (bottom side) cryogels.

As seen in Figure 4.3, the characteristic peaks of PHEMA were observed at 3345 (O-H stretching), 2942 (C-H aliphatic), 1718 (C=O stretching), 1653-1536-1387 (amide I, amide II, amide III bands in MBA) and 1151 (C-O stretching) cm^{-1}. The N-H stretching vibrations of PHEMA after epichlorohydrin activation and IDA attachment evidently increased in terms of intensity in the amide range of PHEMA-IDA spectrum. The amide I, amide II and amide III stretchings at 1645, 1589 and 1392 cm^{-1} found in the FTIR spectrum of PHEMA-IDA showed a right shift with a frequency range of 1636, 1535 and 1389 cm^{-1} in the spectrum of PHEMA-IDA-Ni due to the formation of N-Ni coordination. The right shift in following peak at 1245 cm^{-1} for the C-O stretching vibration in PHEMA-IDA spectrum also supports the O-Ni coordination in PHEMA-IDA-Ni structure. It should be noted that no new peak was observed in the range of 4000-600 cm^{-1} [89, 90].

4.3. Blood Compatibility Studies

A biomaterial is a substance that is used in medical devices or in prostheses designed for contact with the living body for an intended method of application and for an intended period [91]. Synthetic polymers are the most diverse class of biomaterials. Polymeric biomaterials are widely used in both medical and pharmaceutical applications [92]. These applications include a variety of implants or other supporting materials (e.g. vascular grafts, artificial hearts, intraocular lenses, joints, mammary prostheses and sutures), extracorporeal therapeutic and other supporting devices (e.g. hemodialysis, hemoperfusion, blood oxygenation and bags), controlled release systems and clinical diagnostic assays (mainly as carriers) [93].

All biomaterials must meet certain criteria and regulatory requirements before they can be qualified for use in medical applications. Depending on the intended end-use, a biomaterial may be subjected to a set of tests, such as blood-compatibility, tissue-compatibility, carcinogenicity, mutagenicity, biodegradation and mechanical stability [94].

When biomaterials in use come into contact with blood, first small molecules (e.g. water molecules and ions) reach to the surface, which may or may not be adsorbed. This is followed by plasma protein adsorption. The first protein layer adsorbed on the biomaterial surface determines the subsequent events of the coagulation cascade (via the intrinsic pathway), and the complement activation as shown in Figure 4.6 (via the intrinsic-extrinsic pathways) [95].

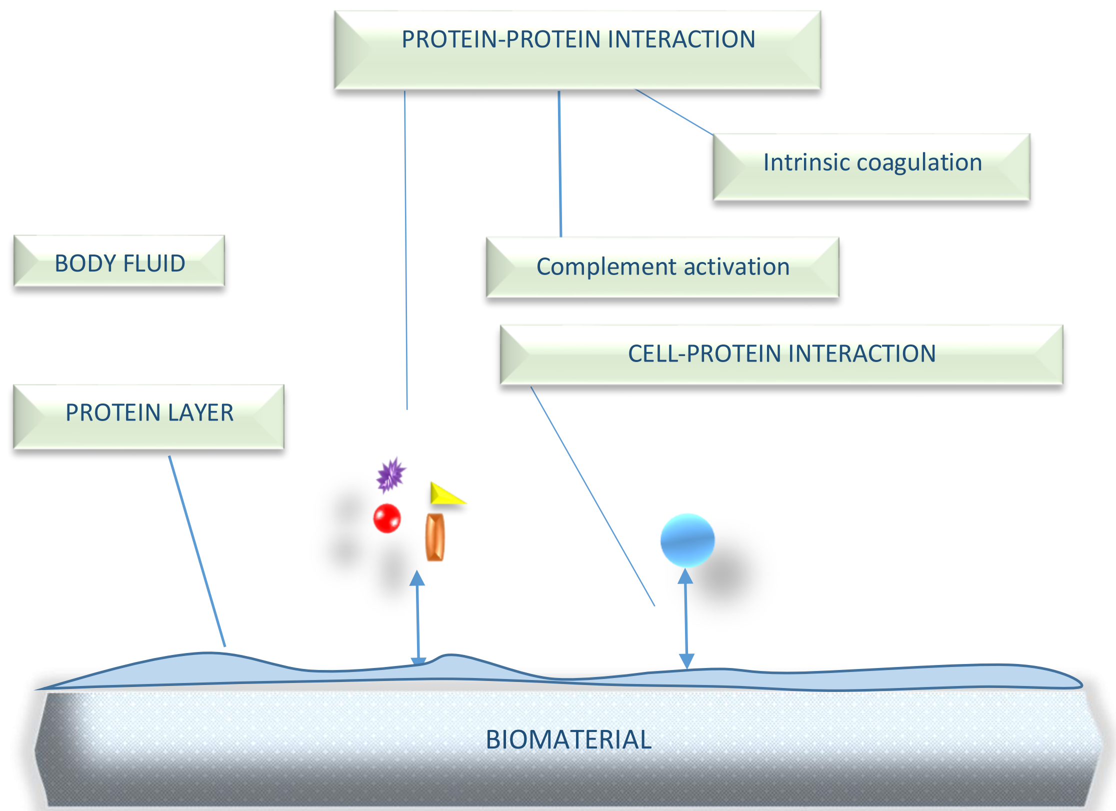


Figure 4.5. The role of adsorbed proteins in body fluid biomaterial interaction.

Much effort has been given upgrading biocompatibility of biomaterials [96]. This includes: (a) device design with good wash out effect, (b) refined fabrication technique, and (c) development of blood compatible polymers. One of the main criteria of selection of PHEMA as the basic component is its good blood tolerability.

4.3.1. Coagulation Times

In order to estimate the blood-compatibility of PHEMA and PHEMA-IDA cryogels, in-vitro coagulation times (CT), activated partial thromboplastin time (APTT) and protrombin time (PT) tests were carried out. It should be mentioned that APTT tests exhibit the bioactivity of intrinsic blood coagulation factors and PT test relates to extrinsic blood coagulation factors on biomaterial surface. CT test shows in-vitro coagulation time. Table 4.2, summarizes the coagulation data obtained in these tests. As can be seen from, all the clotting times for PHEMA CRYOGEL decreased when compared with control plasma. But these decreases are tolerable by the body. Therefore, we concluded that the blood compatibility of PHEMA cryogel was rather

good, and the clotting times were quite reproducible comparing with the values reported in the related literature [97, 98].

4.3.2. Cell Adhesion Studies

Table 4.2, summarizes hematological data obtained in in-vitro blood assay. Lost of platelets with PHEMA and PHEMA-IDA cryogels were 2.3% and 2.3%, respectively. Lost of leukocytes with with PHEMA and PHEMA-IDA cryogels were also 9.1% and 7.2%, respectively. As seen here, there is no significant cell adhesion on the polymeric materials.

These observations showed that surface of PHEMA and PHEMA-IDA cryogels are resistant to adhesion of blood cells (i.e., platelets and leukocytes). In conclusion, because of the good nonthrombogenic properties, macroporous PHEMA and PHEMA-IDA cryogels seem to be very promising immunoaffinity adsorbents for biomedical applications such as extracorporeal immunoadsorption therapy.

Table 4.2. Coagulation times of human plasma (reported in sec)*.

	APTT	PT	CT
Control Plasma	32,00	16,50	19.0
PHEMA	28.3	15.2	17.4
PHEMA-IDA-Ni	27.8	15.0	16.8

* Each result is the average of three parallel studies.

Table 4.3. Platelet and leukocyte adhesion with cryogels*.

	Platelet (x10-3/mm3)		Leukocyte (x10-3/mm3)	
	Initial/Final	Loss(%)	Initial/Final	Loss(%)
PHEMA	264/251	4.9	6.2/6.0	3.2
PHEMA-IDA-Ni	272/253	6.9	6.3/5.9	6.3

* Each result is the average of three parallel studies.

4.4. Adsorption of Hb from Aqueous Solutions

4.4.1. Effect of pH

Protein adsorption onto specific adsorbents is pH dependent. Adsorption of hemoglobin onto the PHEMA-IDA cryogel 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.

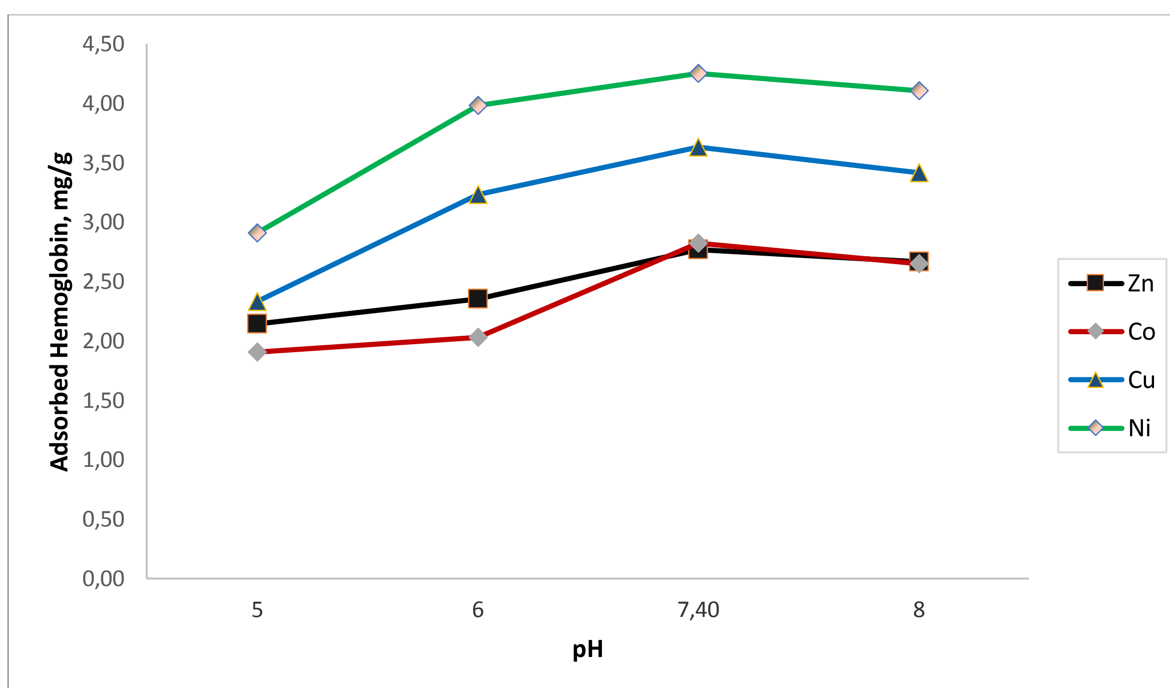


Figure 4.6. Effect of pH on Hb adsorption onto metal-chelated PHEMA cryogels. Running buffer: 0.1 M phosphate buffer(pH=7.4), T: 20°C, flow rate: 0.5 mL/min, Hb Con.: 0.05 mg/ml, $m_{dry\ cryogel}$: 0.41 g.

In these pH ranges, maximum adsorption amount was observed at pH 7.4 as 4.15 mg Hb/g polymer. Maximum adsorption capacity was decreased significantly in more acidic and in more alkaline pH regions.

4.4.2. Effect of Flow Rate

The adsorption amount of metal-chelated PHEMA cryogels for Hb was determined using 0.1 M pH 7.4 phosphate buffer passed through a preequilibrated columns at

different flow rates and plotted in Figures 4.5. The Hb adsorption amount of the cryogels were observed to decrease drastically from 4.15 mg/g to 2 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.

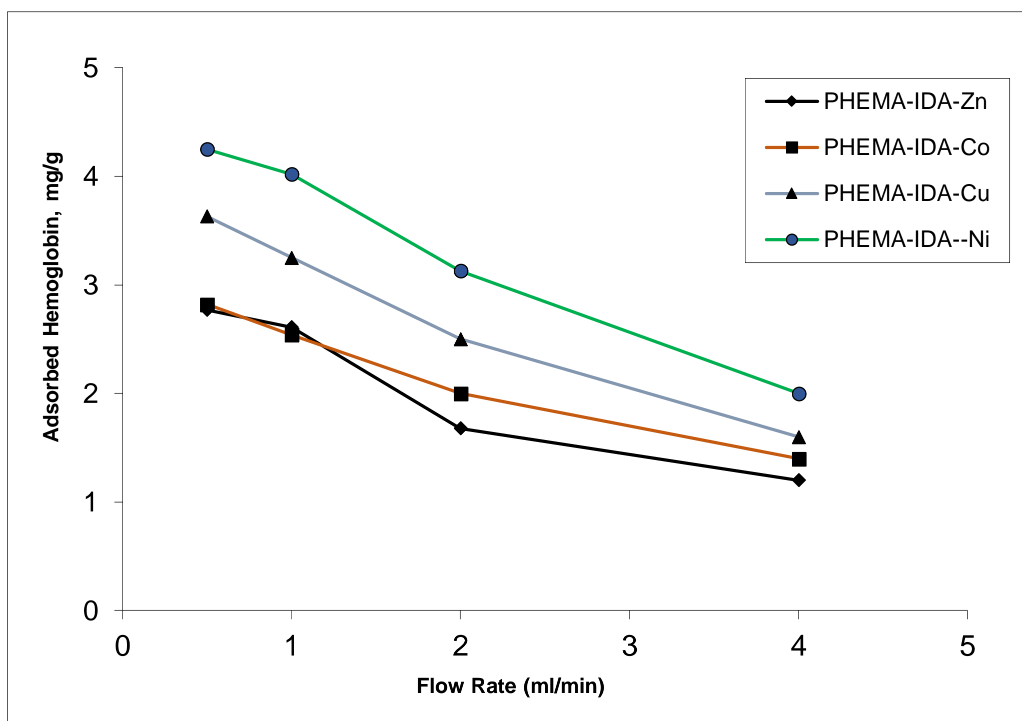


Figure 4.7. Effect of flow-rate on Hb adsorption onto cryogels. running buffer: 0.1 M phosphate buffer(pH=7.4), T: 20ºC, flow rate: 0.5 mL/min, Hb Con.: 0.05 mg/ml, $m_{dry\ cryogel}$: 0.41 g

4.4.3. Effect of Equilibrium Concentration

As seen in Figure 4.6, the Hb adsorption in PHEMA cryogel columns increase as the Hb concentration in buffer solution is increased, and the columns were saturated with excess Hb binding when Hb concentrations above 0.05 mg/mL. The maximum Hb adsorption capacity by the PHEMA-IDA-Ni^{2+} column was found to be 4.25 mg/g polymer on the average. The appropriate choice for the solvent applied during material preparation and recognition events is of high importance.

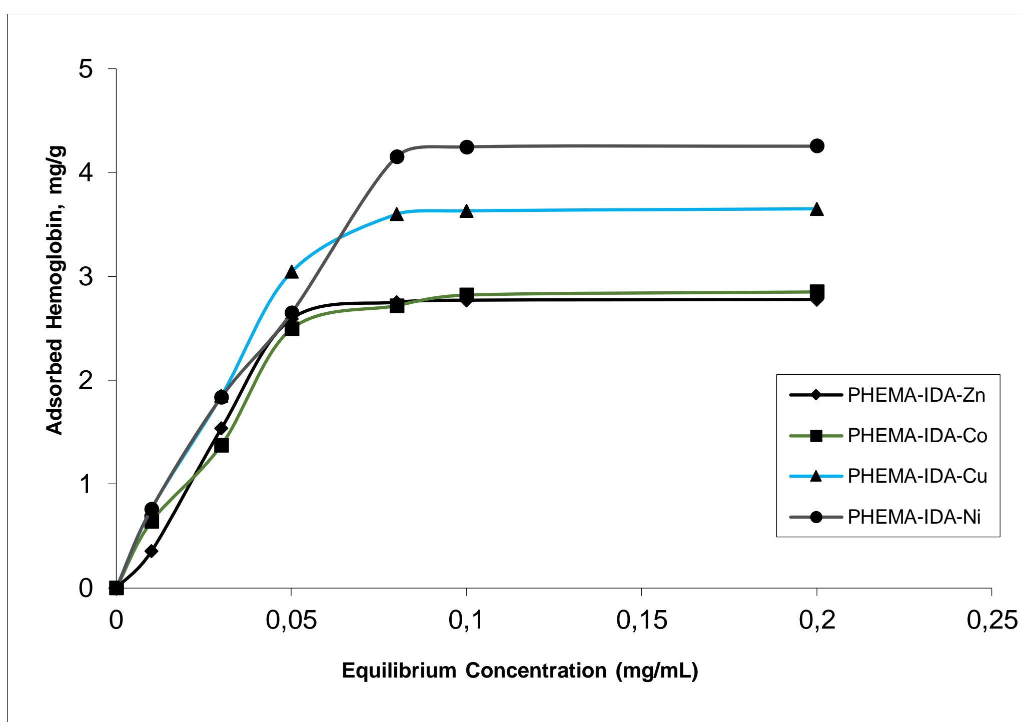


Figure 4.8. Effect of hemoglobin concentration on adsorption capacity. running buffer: 0.1 M phosphate buffer(pH=7.4), T: 20ºC, flow rate: 0.5 mL/min, Hb Con.: 0.05 mg/ml, $m_{dry\ cryogel}$: 0.41 g

4.4.4. 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 4.15 to 1.62 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 cryogel and Hb molecules. 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.

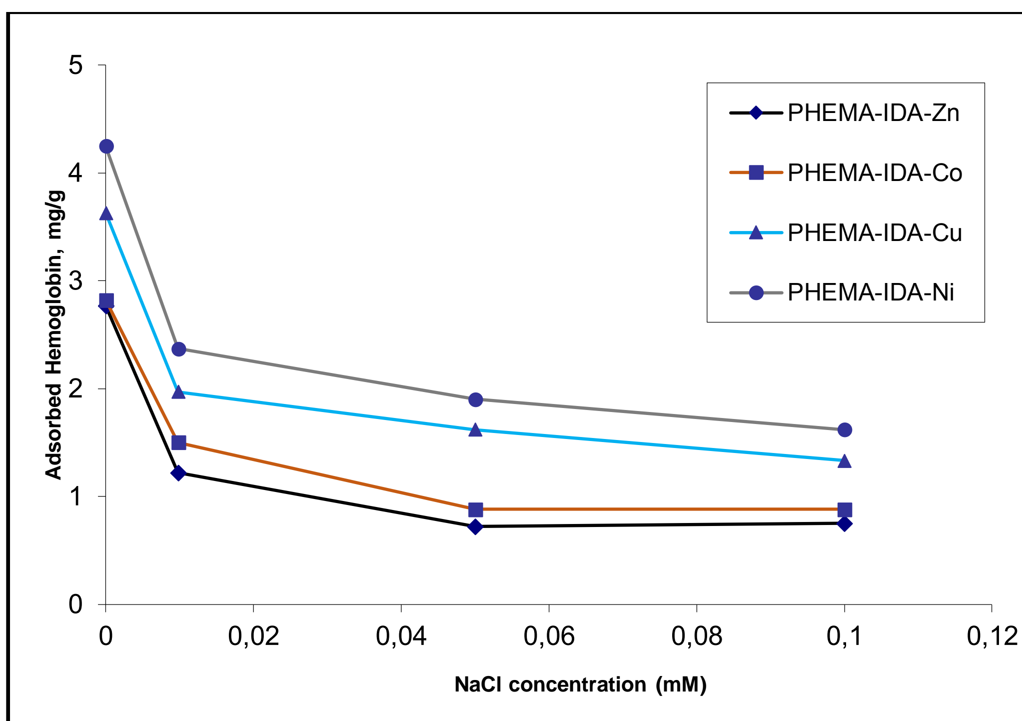


Figure 4.9. Effect of ionic strength on hemoglobin adsorption; running buffer: 0.1 M phosphate buffer (pH=7.4), T: 20ºC, flow rate: 0.5 ml/min, Hb Con.: 0.05 mg/ml, $m_{dry\ cryogel}$: 0.41 g.

4.4.5. 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:

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

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

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

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).

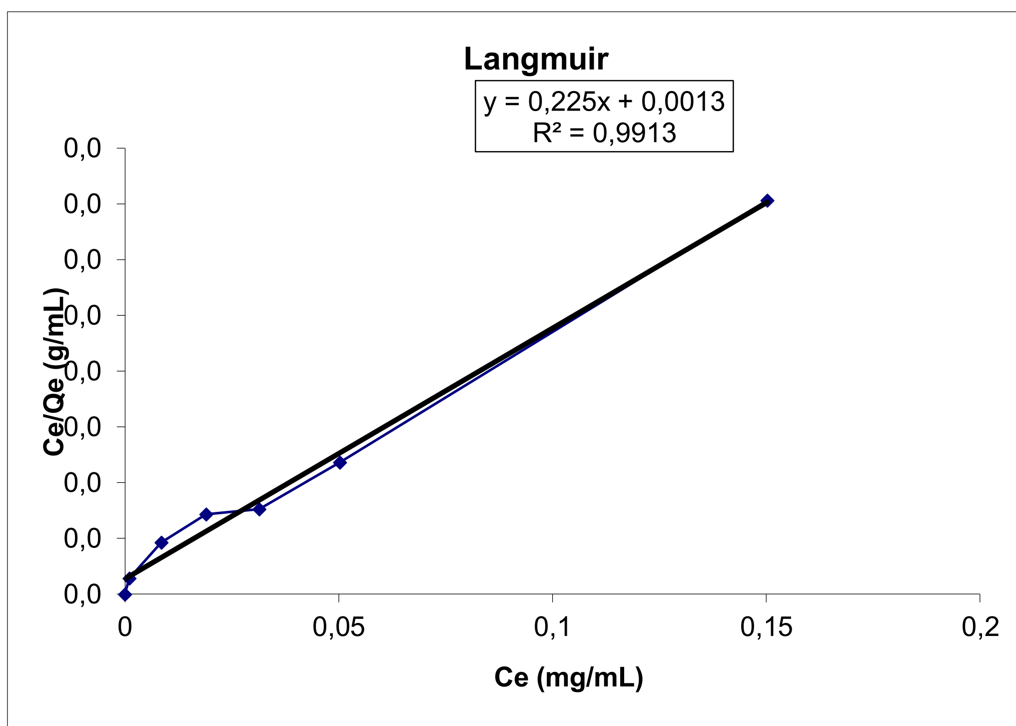


Figure 4.10. Langmuir adsorption isotherms of the PHEMA cryogel.

The maximum adsorption capacity (Q_{max}) data for the adsorption of Hb was obtained from the experimental data. The correlation coefficient (R^2) was 0.9913 at pH 7.4. 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 4.44 mg/g and 17.31 g/mg at pH 7.4, 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).

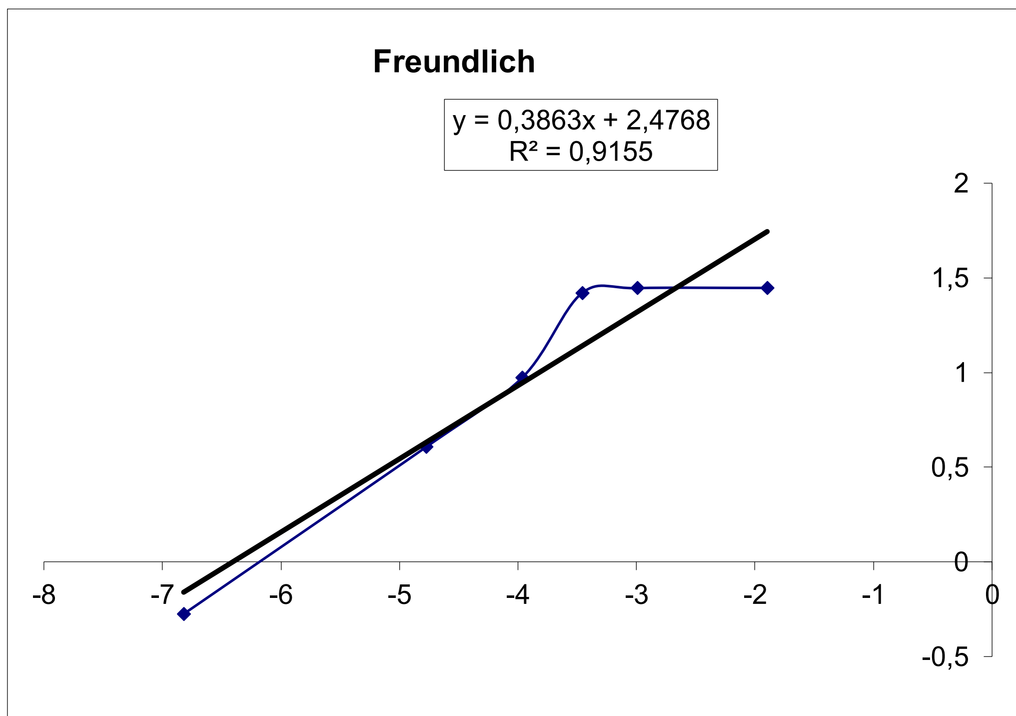


Figure 4.11. Freundlich adsorption isotherms of PHEMA cryogel.

The adsorption isotherm of PHEMA 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 4.3. Langmuir and Freundlich adsorption constants and correlation coefficients for PHEMA cryogel**.**

	Experimental	Langmuir constants			Freundlich constants		
	q_{ex} (mg/g)	Q_{max} (mg /g)	b (g/mg)	R^2	K_f	n	R^2
PHEMA-IDA-Ni	4.25	4.44	17.31	0.99	11.9	2.59	0.91

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. 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

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

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

$$1/(q_{eq}-q_t)] = (1/q_{eq}) + k_2t \qquad (4.9)$$

or equivalently for linear form

$$(t/q_t)= (1/k_2q_{eq}^2) + (1/q_{eq})\ t \qquad (4.10)$$

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, respectivel

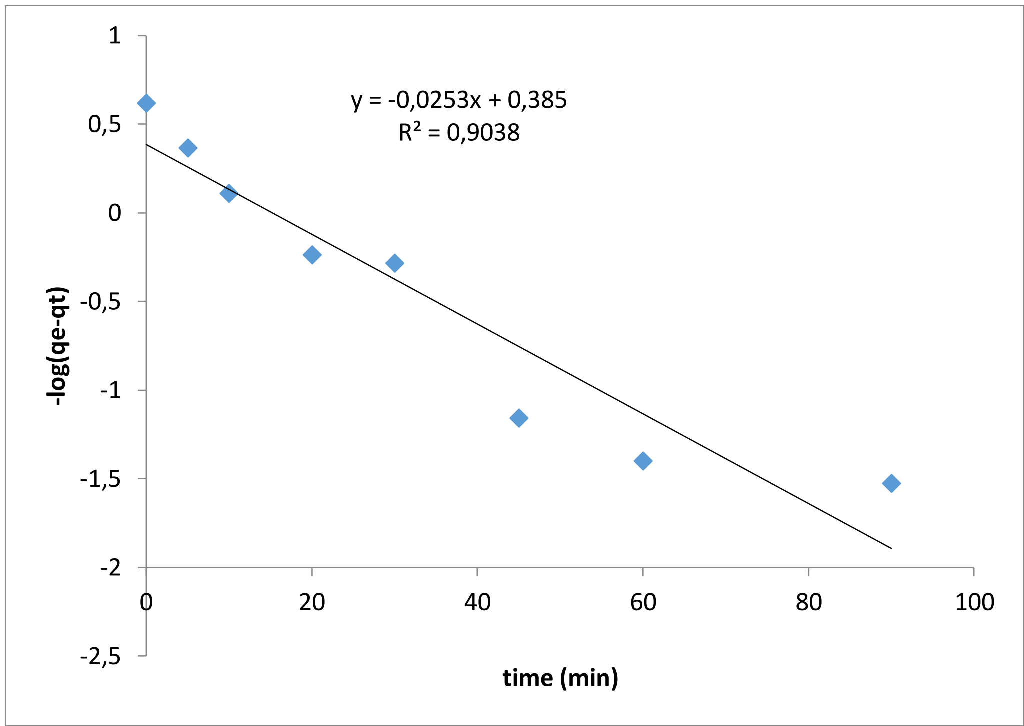


Figure 4.12. Pseudo-first-order kinetic of the experimental data for the adsorbent.

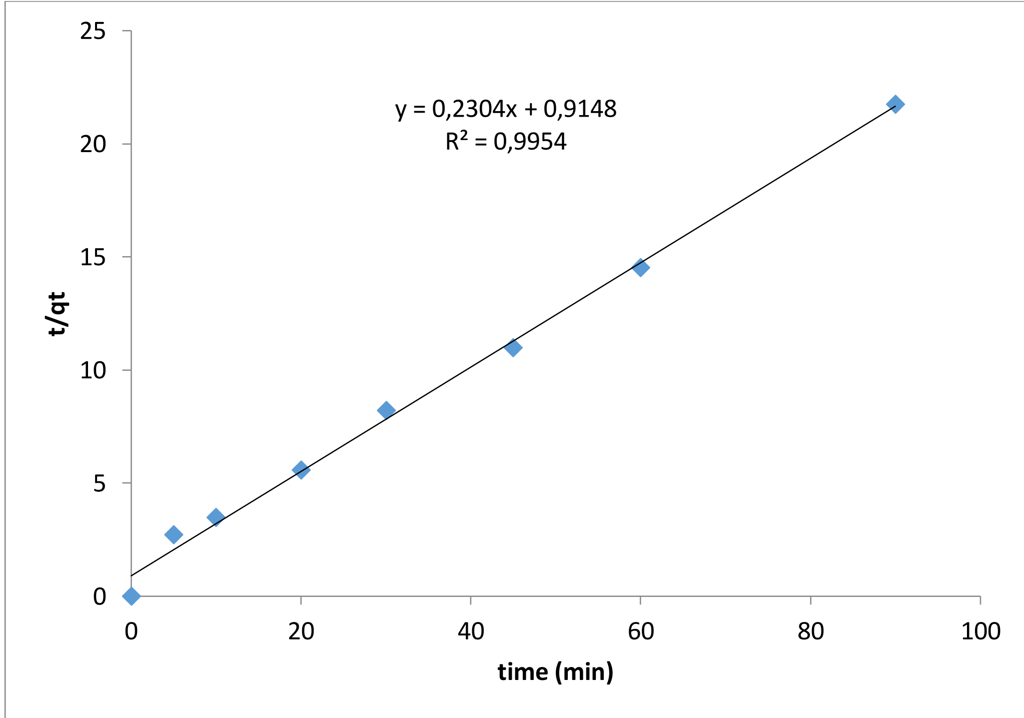


Figure 4.13. Pseudo-second-order kinetic of the experimental data for the adsorbent.

Table 4.4. The first and second order kinetic constants for MIP cryogel.

Equilibrium Conc. (mg/mL)	Experimental Q_{eq} (mg/g)	First-order kinetic k_1 (1/min)	q_{eq} (mg/g)	R^2	Second-order kinetic k_2 (g/mg.min)	q_{eq} (mg/g)	R^2
0.08	4.17	0.058	2.42	0.9038	0.058	4.34	0.99

A comparison of the experimental adsorption capacity and the theoretical values which obtained from Figure 4.12 and 4.13 are presented in Table 4.4. 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 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.

4.5. SDS-PAGE analysis of human hemolysate

Depletion of hemoglobin from human blood with PHEMA-IDA-Ni cryogel was visualized by SDS-PAGE (Figure 4.14).

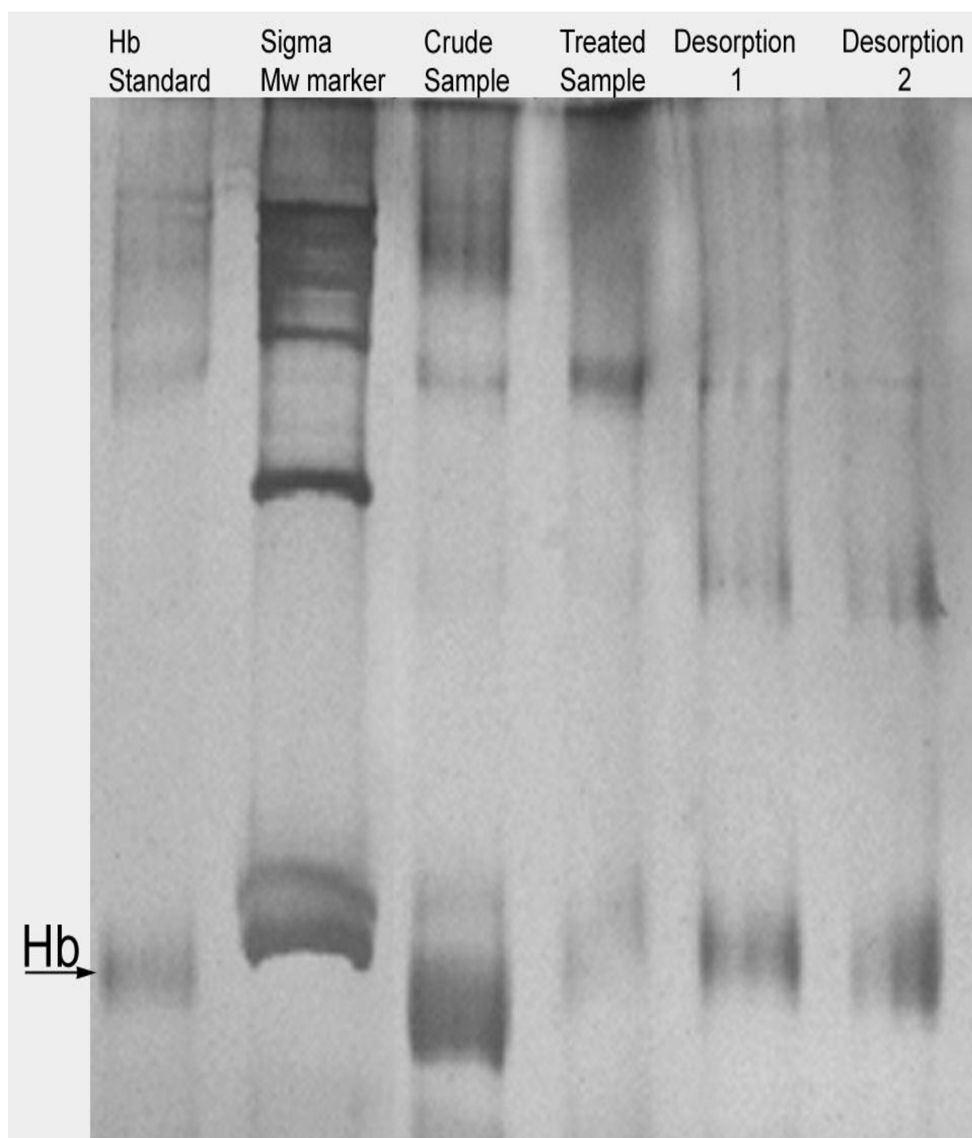


Figure 4.14. SDS-PAGE visualization for hemoglobin depletion from human hemolysate. 5–12% SDS-PAGE, Lane 1: Hb standard, Lane 2: Wide-range Sigma marker, Lane 3: crude sample of hemolysate, Lane 4: treated hemolysate with PHEMA-IDA-Ni cryogel, Lane 5-6: two repeated elutions from the cryogel column.

As clearly seen in Figure 4.13, the hem group of denatured standard Hb solution in Lane 1 was observed around 15 kDa range, which was indicated with black Hb arrow. The bulky Hb band was observed for untreated sample in Lane 3, while this

band disappeared after treatment of hemolysate sample with PHEMA-IDA-Ni cryogel. The elution from PHEMA-IDA-Ni cryogel was repeated two times and results showed that these two elutions contained Hb.

4.6. Desorption and Reusability

Hb adsorbed PHEMA cryogel was desorbed with 0.1 M phosphate buffer (pH=8, include 0.5 M urea, 1 M sodium chloride) for 2.0 hour at room temperature in a continous system. The final hemoglobin concentration in desorption medium was determined by spectrophotometer at 406 nm. In order to test the reusability of the cryogel, Hb adsorption-desorption procedure was repeated ten times by using the same cryogel. At the end of ten adsorption-desorption cycle, there was no remarkable reduce in the adsorption capacity. Recovery of Hemoglobin was calculated as 81% (Figure 4.15).

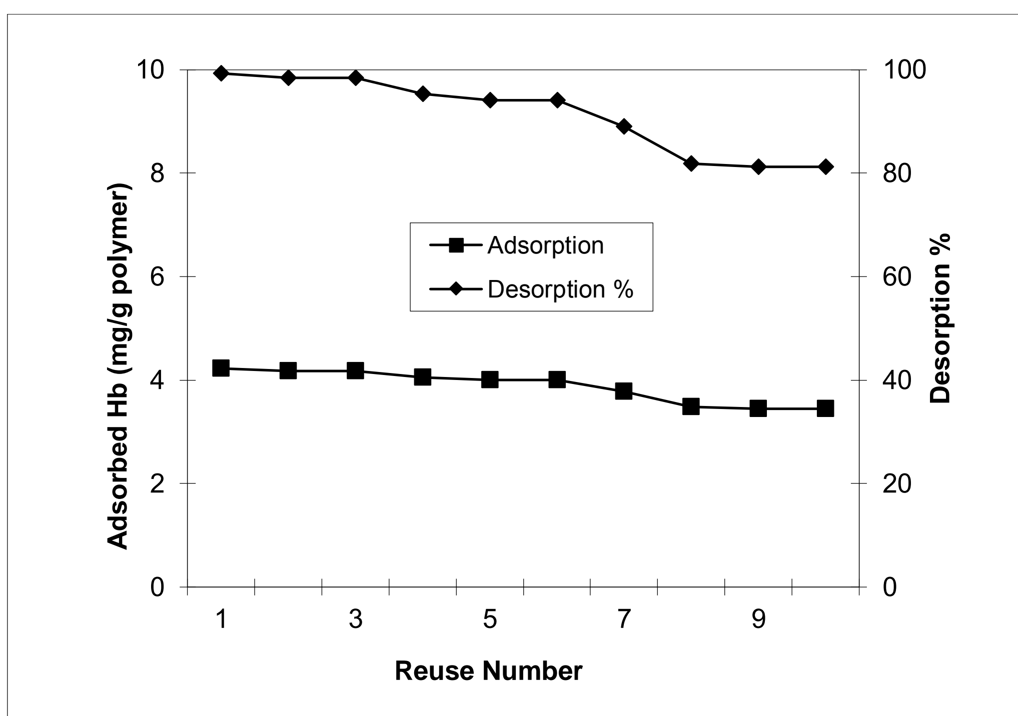


Figure 4.15. Adsorption-desorption cycle showing the reusability potential of a column. running buffer: 0.1 M phosphate buffer(pH=7.4), T: 20ºC, flow rate: 0.5 mL/min, Hb Con.: 0.05 mg/ml, $m_{dry\ cryogel}$: 0.41g

4.7. FPLC studies

As shown in Figure 4.15, separation of Hb and the other proteins in human hemolysate was observed at 11.07 and 4.38 minute, respectively. The t_R, N, k', Rs values are given in Table 1. R_s value was calculated as 1.41 for other proteins in human hemolysate, respectively. Because the Rs value should be higher than 1.0 for a good resolution of two peaks in such a chromatography system the results for the resolution of Hb-other proteins in human hemolysate can be accepted as good resolution values.

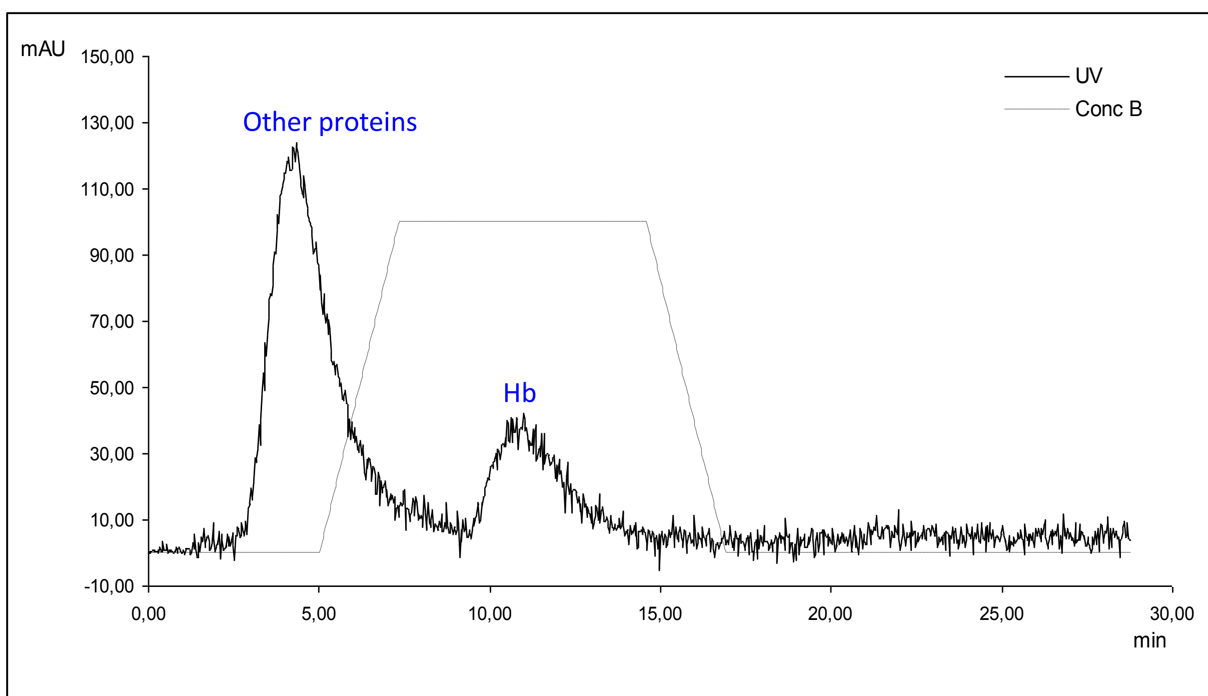


Figure 4.16. FPLC separation of Hb from human hemolysate on a column packed with PHEMA-IDA-Ni^{2+} cryogel: Flow rate: 1.0 ml/min; Detection was performed at 280 nm.

Table 4.5. Chromatographic separation data from human red blood cell hemolysate.

	t_R (min)	N	k'	α	R_s
Hb	11.07	170.58	6.53	-	-
Other proteins	4.38	14.11	1.98	3.29	1.41

5. CONCLUSIONS

- In this thesis, four different metals (Co^{2+}, Cu^{2+} Ni^{2+}, and Zn^{2+}) were immobilized onto PHEMA cryogel for hemoglobin depletion from human blood prior to use in proteome studies by immobilized metal affinity chromatography method.

- Epichlorohydrin activation and iminodiacetic acid attachment to PHEMA cryogel was carried out prior to metal coordination.

- Metals were coordinated to PHEMA-IDA separately via carboxylate and imine groups, and washed extensively with water to remove unbound metals.

- PHEMA-IDA-Metal cryogels were characterizated by a serial process in terms of SEM, swelling studies, surface area, FTIR, blood compatibility, flow resistance and back pressure in different flow rates.

- According to SEM results, PHEMA-IDA-Me cryogels were synthesized with large interconnected macroporous structure in 10-200 µm of pore size range.

- The surface area of the PHEMA-IDA-Me cryogels was found to be 24.7 m^2/g by BET method.

- FTIR results showed that IDA attachment and Ni immobilization onto PHEMA cryogel was occurred successfully.
- Blood compatibility studies showed that PHEMA-IDA-Ni cryogel has a compatibility with blood. This means there is no significant change in good

compatibility of PHEMA with blood after IDA attachment and Ni immobilization onto PHEMA cryogel.

- The affinities of these four different metal ions towards hemoglobin was investigated by means of pH, concentration, flow rate, ionic strength, adsorption time and desorption.

- Among these, PHEMA-IDA-Ni cryogel showed a better adsorption capacity as 4.25 mg/g above 0.05 mg/mL Hb concentration at pH 7.4 with a flow rate of 0.5 mL/min.

- Effect of ionic strength was investigated and results showed that Hb adsorption amount decreased from 4.25 to 1.95 mg/g with the increasing NaCl concentration, which confirmed the interaction between Hb molecules and Ni ions occurred via electrostatic interactions.

- Langmuir and Freundlich isotherms were fitted to equilibrium concentration graph and it was found that Langmuir adsorption model was most favorable with 0.99 correlation coefficient.

- The first and second kinetic constant were also calculated and it was found that the correlation coefficient of second order equation was fitted to kinetic studies.

- SDS-Page and FPLC studies were carried out for visilualize the Hb depletion from human blood. Accoridng to these results, Hb was depleted from human blood successfully.

- In additional, the desorption behavior was investigated for adsorption repeatability of PHEMA-IDA-Ni cryogel and results showed that it can be used several times without decrease in adsorption capacity.

REFERENCES

[1] Porath, J., Sundberg, L., Fornstedt, N., Olson, I., Salting-out in amphiphilic gels as a new approach to Hydrophobic adsorption, *Nature*, 245, 465–466, **1973**.

[2] O'Carra, P., Barry, S., Griffin, T., Spacer arms in affinty chromatography: the need for a more rigorous approach, *Biochemical Society Transactions*, 1, 289-290, **1973**.

[3] Shaltiel, S., Hydrophobic chromatography, *Methods in Enzmology*, 34, 126-140, **1974.**

[4] Blumberg, P.M., Strominger, J.L., Covalent affinity chromatography of penicillin-binding components from bacterial membranes. *Methods in Enzymology*, 34, 401-405, **1974.**

[5] Wilchek, M., Miron, T., Thirty years of affinity chromatography, *Reactive and Functional Polymers*, 41, 263-268, **1999.**

[6] Janson, J.C., Ryden, L., Protein Purification, John Wiley and Sons, Second Edition, New York, USA, 375-442, **1998.**

[7] Labrou, N., Clonis, Y.D., The Affinity technology in downstream processing, Journal of Biotechnology., 37, 95-119, **1994.**

[8] Lowe, C. R., Lowe, A. R., Gupta, G., New developments in affinity chromatography with potential application in the production of biopharmaceuticals. *Journal of Biochemical and Biophysical Methods*, 49, 561-574, **2001.**

[9] Sadana, A., Beelaram, A. M., Efficiency and economics of bioseparation: Some case studies, *Bioseparation*, 4, 221-235, **1994**.

[10] Narayanan, S.R., Preparative affinity chromatography of proteins, *Journal of Chromatography A,* 658, 237-258, **1994.**

[11] Lowe, C.R., Combinatorial approaches to affinity chromatography, *Current Opinion in Chemical Biology*, 5, 248-256, **2001.**

[12] Turkova, J., Oriented immobilization of biologically active proteins as a tool for revealing protein interactions and function, *Journal of chromatography. B, Biomedical Sciences and Applications*, 722 (1-2), 11-31, **1999.**

[13] Sadana, A., Ram, A.B., Fractal Analysis of Antigen-Antibody Binding Kinetics: Biosensor Applications, Biotechnology Progress, 10, 291-298, **1994.**

[14] Lowe, E.J., The natüre of perception, TLS-The Times Literary Supplement, 5151, 24-26, **2001.**

[15] Cuatrecasas, P., Affinity chromatography of macromolecules, *Advances in Enzymology and Related Areas of Molecular Biology*, 36, 29-89, **1972.**

[16] Fleminger, G., Solomon, B., Wolf, T., Hadas, E., Single step oxidative binding of antibodies to hydrazide-modified eupergit C, *Applied Biochemistry and Biotechnology,* 26(3), 231-238, **1990.**

[17] Soltys, P.J., Etzel, M.R., Equilibrium adsorption of LDL and gold immunoconjugates to affinity membranes containing PEG spacers, *Biomaterials*, 21(1), 37-48, **2000.**

[18] Scouten W.H., *Affinity Chromatography: Bioselective Adsorption on Inert Matrices,* New York: John Wiley and Sons, **1981**

[19] Cochet, S., Hasnaoui, M., Debbia, M., Kroviarski, Y., Lambin, P., Cartron, J.P., Bertrand, O., Chromatography of human immunoglobulin G on immobilized drimarene rubine R/K-5BL. Study of mild, efficient elution procedures, *Journal of Chromatography A*, 663(2), 175-186, **1994**

[20] Narhi, L.O., Caughey, D.J, Horan, T. Kita, Y., Arakawa, T., Effect of three elution buffers on the recovery and structure of monoclonal antibodies, *Analytical Biochemistry*, 253(2), 236–245, **1997**

[21] Wilchek, M., Anfinsen, C.B., *Selective Enzyme Purification by Affinity Chromatography*, Proceedings of the National Academy of Sciences of the United States of America, 61(2),636–643, **1968**.

[22] Spalding, B., Pharmaceutical Price control-will governments harmonize regulations J., Bio-Technology, 12, 1312 , **1991.**

[23] Labrou, N. E., Dye-Ligand affinity adsorbents for enzyme purification, Applied Biochemistry and Biotechnology, 20, 77-84. **2002**

[24] Clonis, Y.D., Purification of the glycoprotein allergen Ag7 from mugwort pollen by concanavalin A affinity chromatography Bioprocess technology, 9, 401-445, **1990.**

[25] Kula, M. R., Johnsonn G., Bückmann, A. F., Large-scale isolation of enzymes, *Biochemical Society Transactions*, 7, 1-5, **1979.**

[26] Cuatrecasas, P., Protein purification by affinity chromatography. Derivatizations of agarose and polyacrylamide beads. *The Journal of* Biological Chemistry*,* 245(12), 3059-3065, **1970.**

[27] Lowe, C.R., Harvey, M.J., Dean, P.D.G., Affinity Chromatography on immobilised Adenosine 5′-monophosphate European J. of Biohem., 42, 1-6, **1974.**

[28] Turkova, J., Bioaffnity Chromatography, Elsevier, Amsterdam, **1993**.

[29] Matejtschuk, P., (Ed), Affinity Separations, A Pratical Approach, IRL Press, Oxford, **1997.**

[30] Denizli A., Pişkin, E., Dye-ligand affinity systems, Journal of Biochemical and Biophysical Methods, 49 (1-3), 391-416, **2001.**

[31] Wilchek, M., Miron, T., Limitations of n-hydroxy-succimide esters in affinity chromatography and protein immobilization, *Biochemistry*, 26(8), 2155-2161, **1987.**

[32] Gaberc-Porekar, V., Menart, V., Perspectives of immobilized-metal affinity chromatography, Journal of Biochemical and Biophysical Methods, 49, 335–360, **2001.**

[33] Chaga, G. S., Twenty-five years of immobilized metal ion affinity chromatography: past, present and future, Journal of Biochemical and Biophysical Methods, 49, 313-334, **2001.**

[34] Chaga, G., Andersson, L., Ersson, B., Bera, M., Use of immobilized metal ions as a negative adsorbent for purification of enzymes: Application to phosphoglycerate mutase from chicken muscle extract and horseradish peroxidase.Biomedical Chromatography 6, 172-176, **1992.**

[35] Chaga, G.,Porath J., Illeni, T., Isolation and purification of amyloglucosidase from Halobacterium sodomense. Biomedical Chromatography 7, 251-261, **1993.**

[36] Samaraweera, P., Porath, J. & Law, J. H. Separation of insect hemolymph proteins by cascade-mode multi-affinity chromatography. Arch. Insect Biochem. Physiol. 20, 243–251, **1992.**

[37] Arnold, I., Mesenchymal stem cells. Journal of prthopaedic Research, 9, 5-18, **2005.**

[38] Everson, R.J., Parker, H.E., Zinc binding and synthesis of eight-hydroxy-quinoline-agarose. Bioinorganic Chemistry, 4, 15-20, **1974.**

[39] Sulkowski, E., Immobilized metal-ion affinity chromatography: Imidazole proton pump and chromatographic sequelae. II. Chromatographic sequelae. Journal of Molecular Recognition. 9, 494-498, **1996.**

[40] Liesiene, B., Immobilized metal affinity chromatography of human growth hormone-effect of ligand density. Journal of Chromatography, 764, 27-35, **1997.**

[41] Zachariou, M., Hearn, M.T.W., Protein selectivity in immobilized metal affinity chromatography based on the surface accessibility of aspartic and glutamic acid residues. J. Protein. Chem. 14, 419-430, **1995.**

[42] Reif, O.W.,Nier, V., Bahr, U., Freitag, R., Immobilized metal affinity membrane adsorbers as stationary phases for metal interaction protein separation. Jornal of chromatography A, 664, 13-25, **1994.**

[43] Serafica,G.C., Belfort, G., Pimbley, J., Protein fractionation using fast flow immobilized chelate affinity membranes, Biotechnology and Bioengineering, 43, 21-36, **1994.**

[44] Kubota, N., Nakagawa, Y., Eguchi, Y., Recovery of serum proteins using cellulosic affinity membrane mod,f,ed by immobilization of Cu^{2+}ion, Journal of Applied Polymer science, 62, 1153-1160, **1996.**

[45] Crawford, J.R., Harding, H.I., Cass, J.P., Burns, C.A., adsorption of aqueous heavy metals onto carbonaceous substrates, Colloids and Surfaces A: Physiochemical and Engineering Aspects, 155, 63-68, **1999.**

[46] Camperi, S.A., Grasselli, M., Cascone, O., High-speed pectic enzyme fractionation by immobilised metal ion affinity membranes,Bioseparation, 9, 173-177, **2000.**

[47] Arica, M.Y., Tstereci, H.N., Denizli, A., Dye-ligand and metal chelate poly(2-hydroxyethylmethacrylate) membranes for affinity separation of proteins, Journal of Chromatography A, 13, 83-91, **1998.**

[48] Arica, M.Y., Denizli, A., Baran, T., Hasırcı, V., Dye derived and metal incorporated affinity poly(2-hydroxyethyl methacrylate) membranes for use in enzyme immobilization. Polymer International, 46, 345-352, **1998.**

[49] Hari, P.R., Paul, W., Sharma, C.P., Adsorption of human IgG on Cu(2+)-immobilized cellulose affinity membrane: preliminary study. J Biomed Mater Res. 50(2), 110-113, **2000.**

[50] Xiao, J., Meyerhoff, M.E., Retention behavior of amino acids and peptides on protoporphyrin-silica stationary phases with varying metal ion centers. Analytical Chemistry, 68, 2818-2825, **1996.**

[51] Apezteguia, I., Calligaris, R., Bothardi, S., Santoro, C., Expression, purification, and functional characterization of the two zinc-finger domain of the human GATA-1. Protein Expr Purif. 6, 541-546, **1994.**

[52] Colangeli, R., A. Heijbel, A. M. Williams, C. Manca, J. Chan, K. Lyashchenko, and M. L. Gennaro. Three-step purification of lipopolysaccharide-free, polyhistidine-tagged recombinant antigens of Mycobacterium tuberculosis. J. Chromatogr. B Biomed. Sci. Appl. 714, 223-235, **1998.**

[53] Rogl, H., Kosemund, K., Kühlbrandt, W., Collinson, I., Refolding of Escherichia coli produced membrane protein inclusion bodies immobilised by nickel chelating chromatography. FEBS Lett. 31, 432(1-2), 21-26, **1998.**

[54] Teshima, T., Mashimo, S., Kondo A., Fukuda, H., Affinity purification and immobilization of fusion chaperonin GroEL-(His)6 and its utilization to mediate protein refolding. Journal of Fermentation and Bioengineering, 86, 357-362, **1998.**

[55] Krishnamurthy, R., Madurawe, R.D., Bush, K.D., Lumpkin, J.A., Conditions Promoting Metal-Catalyzed Oxidations during Immobilized Cu—Iminodiacetic Acid Metal Affinity Chromatography. Biotechnology Progress, 11, 643-650, **1995.**

[56] Khossravi, M., Borchardt, R.T., Chemical pathways of peptide degradation: IX. Metal-catalyzed oxidation of histidine in model peptides.Pharm Res., 15, 1096-1102, **1998.**

[57] Oswald, T., Wende, W., Pingoud, A., Rinas, U., Comparison of N-terminal affinity fusion domains: effect on expression level and product heterogeneity

of recombinant restriction endonuclease EcoRV. Applied Microbiology and Biotechnology, 42, 73-77, **1994.**

[58] Wojczyk B.S., Czerwinski, M., Stwora-Wojczyk M.M., Siegel D.L., Abrams W.R., Wunner W.H., Purification of a secreted form of recombinant rabies virus glycoprotein: comparison of two affinity tags. J Chromatogr, B: Biomed Appl 678, 1-14, **1996.**

[59] Lu, T., Van, D.M., Sawadogo, M., Protein-protein interaction studies using immobilized oligohistidine fusion proteins. Anal. Biochem. 213, 318-322, **1993.**

[60] Cooper, R.G., Kleinschmidt, E.J., New products: What separates winners from losers?. Journal of Product Innovation Management. 4, 169-184, **1987.**

[61] Wakabayashi, I., Katsuhiko, H., Kakishita, E., Nagai, K., Diminution of contractile response of the aorta from endotoxin-injected rats. European Journal of Pharmacology, 141, 117-122, **1987.**

[62] Kilmartin, J.V., Breen, J.J., Roberti, G.C.K., Ho, C., Direct Measurement of the pK Values of an Alkaline Bohr Group in Human Hemoglobin. Proc. Nat. Acad. Sci. USA., 70, 1264-1249, **1973.**

[63] Perutz, M.F., Stereochemical Effects of amino acid substitution in abnormal human Heamoglobin. Biochemical Journal, 117, 31-40, **1970.**

[64] Monod, J., Wyman, J., Changeux, J.P., On the natüre of allosteric transitions: A plausible model. Journal of Molecular Biology. 12, 88-118, **1965.**

[65] Kleinschmidt, T., Sgouros, J.G.,. Hemoglobin Sequences. Biological Chemistry Hoppe-Seyler, 368, 579–615. **1987.**

[66] Kratzin, H., Armstrong, V.W, Niehaus, M., Hilschmann, N., Seidel D., Structural Relationship of an apolipoprotein A Phenotype (570-KDA) to plasminogen- Homologous kringle domains are linked by carbohydrate-rich regions. Biological Chemistry HOPPE-SEYLER, 12, 1533-1544, **1987.**

[67] McDonald, J.M., Christie J.D., Lamb, W., A study of stump growth in children with below-knee amputations, J. Bone Joint Surg, 61, 464-465, **1979.**

[68] Bry, L., Chen, P.C., Sacks, D.B., Effects of Hemoglobin Variants and Chemically Modified Derivatives on Assays for Glycohemoglobin. Clin Chem. 47, 153-163, **2001.**

[69] Arvidsson, P., Plieva, F.M., Savina, I.N., Lozinsky, V.I., C hromatography of microbial cells using continuous supermacroporous affinity and ion-exchange columns, Journal of Chromatography A, 977, 27-38, **2002.**

[70] Nambu, M., Rubber-like poly(vinyl alcohol) gel, Kobunshi Robunshu, 47, 695-703, **1990.**

[71] Kaetsu, I., Radiation synthesis of polymeric materials for biomedical and biochemical applications, Advanced In Polymer Science, 105, 81-97, **1993.**

[72] Lozinsky V.I., Cryotropic gelation as an approach to the preparation of supermacroporous hydrogels, Abstracts of Papers of the American Chemical Society, 216, 833-U833, **1998.**

[73] Lozinsky, V.I., Plieva, F.M., Poly(vinyl alcohol) cryogels employed as matrices for cell immobilization. 3. Overview of recent research and developments, Enzyme and Microbial Technology, 23, 227-242, **1998.**

[74] Hassan, C.M., Doyle, F.J., Peppas, N.A., Dynamic behavior of glucose-responsive poly(methacrylic acid-g-ethylene glycol) hydrogels. Macromolecules, 30. 6166-6173, **1997.**

[75] Lozinsky, V.I., Redox-initiated radical polymerisation of acrylamide in moderately frozen water solutions. Macromolecular rapid communications, 22, 1441-1446, **2001.**

[76] Arica, M.Y., Yavuz, H., Patır, S., Denizli, A., Immobilization of glucoamylase onto spacer-arm attached magnetic poly(methylmethacrylate) microspheres: characterization and application to a continuous flow reactor.Journal of Molecular Catalysis B: Enzymatic, 11, 127-138, **2000.**

[77] Özkara, S., Yavuz, H., Denizli A., Purification of immunoglobulin G from human plasma by metal-chelate affinity chromatography, Journal of applied polymer science, 38, 1567-1572, **2003.**

[78] Lozinsky, V.I., Galaev, I.Y., Plieva, F.M., Savina, I.N., Jungvid, H., Mattiasson, B., Polymeric cryogels as promising materials of biotechnological interest, Trends in Biotechnology, 21, 445-451, **2003.**

[79] Arvidsson P., Plieva F. M., Lozinsky I. V., Galaev I. Y., Mattiasson B., Direct chromatographic capture of enzyme from crude homogenate using immobilized metal affinity chromatography on a continuous supermacroporous adsorbent, Journal of Chromatography A, 986, 275-290, **2003.**

[80] Arvidsson P., Plieva F. M., Savina I. N., Lozinsky I. V., Fexby S., Bülow L., Galaev I. Y., Mattiasson B., Chromatography of microbial cells using continuous supermacroporous affinity and ion-exchange columns, Journal of Chromatography A, 977, 27-38, **2002.**

[81] Babac C., Yavuz H., Galaev I. Y., Pişkin E., Denizli A., Binding of antibodies to concanavalin A-modified monolithic cryogel, Reactive and Functional Polymers, 66, 1263-1271, **2006.**

[82] Ahlqvist J., Dainiak M. B., Kumar A., Hörnsten E. G., Galaev I. Y., Mattiasson B., Monitoring the production of inclusion bodies during fermentation and enzyme-linked immunosorbent assay analysis of intact inclusion bodies using cryogel minicolumn plates, Analytical Biochemistry, 354, 229-237, **2006.**

[83] Hanora A., Savina I., Plieva F. M., Izumrudov V. A., Mattiasson B., Galaev I. Y., Direct capture of plasmid DNA from non-clarified bacterial lysate using polycation-grafted monoliths, Journal of Biotechnology, 123, 343-355, **2006.**

[84] Doumas B., Watson W, Biggs H., Albumin standards and measurement of serum albumin with bromcresol green, Clinica Chimica Acta, 31, 87–96, **1971.**

[85] Swedenborg J., Olsson P., Lagergren H., Polyphloretinphosphate, an antithrombogenic substance which inhibits platelet adhesion, Thrombosis Research, 4, 687-698, **1974.**

[86] Song W. C., Brash A. R., Investigation of the allene oxide pathway in the coral Plexaura homomalla: Formation of novel ketols and isomers of prostaglandin A2 from 15-hydroxyeicosatetraenoic acid, Archives of Biochemistry and Biophysics, 290, 427-435, **1991.**

[87] Doumas, B. T., Biggs H. G., Determination of Serum albumin: In standard methods of clinical chemistry (Cooper G.A.), Academic press Inc. New York, 7, 175-179, **1972.**

[88] Odabaşı M., Baydemir G., Karataş M., Derazshamshir A., Preparation and characterization of metal-chelated poly(HEMA-MAH) monolithic cryogels and their use for DNA adsorption , Journal of Applied Polymer Science, 116, 1306-1312, **2010.**

[89] Peppas N. A., Stauffer S. R., Reinforced uncrosslinked poly (vinyl alcohol) gels produced by cyclic freezing-thawing processes: a short review, Journal of Controlled Release, 16, 305-310, **1991.**

[90] Sönmez M., Synthesis and characterization of copper(II), nickel(II), cadmium(II), cobalt(II) and zinc(II) complexes with 2-Benzoyl-3-hydroxy-1-naphthylamino-3-phenyl-2- propen-1-on. Turkish Journal of Chemistry, 25, 181–185, **2001.**

[91] Andac M., Galaev I. Y., Denizli A., Dye attached poly(hydroxyethyl methacrylate) cryogel for albumin depletion from human serum, Journal of Separation Sciences, 35, 1173–1182, **2012.**

[92] Zielke U., Hüttinger K. J., Hoffman W. P., Surface-oxidized carbon fibers: I. Surface structure and chemistry, Carbon, 34, 983-998, **1996.**

[93] Pişkin E., Hoffman A. S., Polymeric biomaterials, Polymers in medicine; Congresses Proceedings, 102-524-944, 258, **1986.**

[94] Song W. C., Brash A. R., Investigation of the allene oxide pathway in the coral Plexaura homomalla: Formation of novel ketols and isomers of prostaglandin A2 from 15-hydroxyeicosatetraenoic acid, Archives of Biochemistry and Biophysics, 290, 427-435, **1991.**

[95] Denizli A., Köktürk G., Yavuz H., Pişkin E., Albumin adsorption from aqueous solutions and human plasma in a packed-bed column with Cibacron Blue F3GA-Zn(II) attached poly(EGDMA-HEMA) microbeads, Reactive and Functional Polymers, 40, 195-203, **1999.**

[96] Inoue K., Yoshizuka K., Baba Y., Wada F., Matsuda T., Solvent extraction of palladium (II) and platinum (IV) from aqueous chloride media with N,N-dioctylglycine, Hydrometallurgy, 25, 271-279, **1990.**

[97] Goosen M. F. A., Sefton M. F., Properties of a heparin-poly(vinyl alcohol) hydrogel coating, Journal of Biomedical Materials Research, 17, 359-373, **1983.**

[98] Kim K. J., Vedula R. R., Glasgow P. D., Jacobs P., Farkas J., Functionalized TPU for advanced media applications, Journal of Magnetism and Magnetic Materials, 155, 73-76, **1996.**

[99] Cheung C. W., Porter J. F., Mckay G., Sorption kinetic analysis for the removal of cadmium ions from effluents using bone char, Water Research, 35, 605-612, **2001.**

CURRICULUM VITAE

Credentials

Name, Surname : Ali DERAZSHAMSHIR

Place of Birth : Bilesavar

Marital Status : Single

E-mail : allinmymind@hotmail.com

Address : Eryaman 4. Etap, Soyak Blokları, 17656 Ada, 3/29 Etimesgut/ANKARA

Education

High School : Dabirestan e Emam Khomeini

BSc. : Daneshgah e Azad e Ardabil

MSc. : Hacettepe University, Institute of Graduate Studies in Science, Chemistry Department

PhD. : Hacettepe University, Institute of Graduate Studies in Science, Chemistry Department

Foreign Languages

English : Upper Intermediate

Persian : Advanced

Azerbaijan : Native

Arabic : Intermediate

Work Experience

Areas of Experiences

Cryogels, chromatographic techniques (HPLC, Nano-LC, CEC, Bio-LC), SDS-PAGE techniques

Projects and Budgets

Publications

Oral and Poster Presentations