PREPARATION OF FAB FRAGMENT IMMOBILIZED IMMUNOAFFINITY CRYOGELS AND ALBUMIN PURIFICATION

FAB FRAGMENT İMMOBİLİZE İMMUNOAFİNİTE KRİYOJELLERİN HAZIRLANMASI VE ALBÜMİN SAFLAŞTIRILMASI

Sabina HÜSEYNLİ

PROF. DR. ADİL DENİZLİ

Thesis Supervisor

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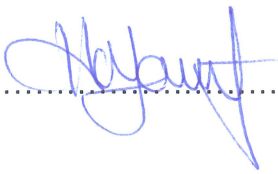
This work named **"Preparation of Fab Fragment Immobilized Immunoaffinity Cryogels and Albumin Purification"** by **SABİNA HÜSEYNLİ** has been approved as a thesis for the Degree of **DOCTOR OF PHILOSOPHY IN CHEMISTRY** by the below mentioned Examining Committee Members.

Prof. Dr. Handan YAVUZ ALAGÖZ
Head ..... 

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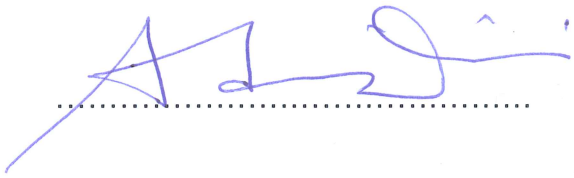
Supervisor ..... 

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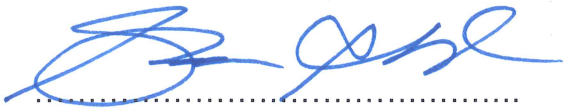
Member ..... 

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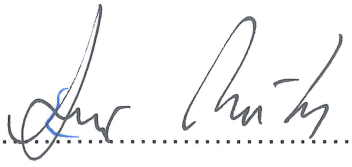
Member ..... 

Assist. Prof. Dr. Fatma YILMAZ
Member ..... 

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- I did not do any distortion in the data set
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ÖZET

FAB FRAGMENT İMMOBİLİZE İMMUNOAFİNİTE KRİYOJELLERİN HAZIRLANMASI VE ALBÜMİN SAFLAŞTIRILMASI

Sabina HÜSEYNLİ

Doktora, Kimya Anabilim Dalı

Tez Danışmanı: Prof. Dr. Adil DENİZLİ

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585 aminoasitli tek bir zincirden oluşan albümin karaciğerde sentez edilir. İnsan ve diğer memeli hayvanların kan plazmasında bulunan en yaygın proteindir. Kanda bulunan proteinlerin %60'ını oluşturur. Ayrıca, doku sıvılarında, özellikle kas ve deride, az miktarda gözyaşı, ter, mide suları ve safrada da bulunur. Vücuttaki toplam albüminin %30-40'ı kandadır. Yağ asitleri ve çeşitli başka maddeleri kanda taşımasının yanı sıra en önemli işlevi, kan ile doku sıvıları arasında suyun dengelenmesini sağlamaktır. Albumin kandaki onkotik basıncı düzenleyen ve dokular arası madde alışverişini sağlayan taşıyıcı proteindir.

Afinite kromatografisinde söz konusu etkileşim antikorların antijenlerle, enzimlerin substrat anologlarıyla ve de hormonların reseptörleri ile olan etkileşimleri türündendir. Afinite kromatografisinin bir çeşidi olan immunoafinite kromatografisi son yıllarda sıklıkla kullanılan kromatografik bir tekniktir. İmmunoafinite kromatografisinde ligand olarak kullanılan antikorlar antijenlerin saflaştırılmasında kullanılır.

Bu çalışma kapsamında insan serum albümin (HSA) saflaştırılması için Fab fragmenti immobilize edilmiş kriyojel kolonlar hazırlanmıştır. Bu amaçla öncelikle anti insan serum albümini (Anti-HSA) moleküllerinin papain enzimi ile enzimatik hidrolizi gerçekleştirilmiş ve bunun sonucunda elde edilen Fab fragmenti kriyojel kolon içerisine immobilize edilmiştir. Kontrol amaçlı Fab fragmenti immobilize edilmemiş PHEMAC kriyojel kolonu da hazırlanmıştır. Hazırlanan kolonların karakterizasyon çalışması (SEM, BET, FTIR-ATR, şişme) yapılmıştır. Daha sonra albümin saflaştırılması işleminde çeşitli parametrelerin (pH taraması, derişim, akış hızı vs.) etkisi incelenmiştir.

HSA seçiciliği Fab bölgesindeki değişimlere bağlı olduğu için, molekülün bütününü immobilize etmektense antijen bağlanmasından asıl sorumlu olan işlevsel Fab bölgesi immobilize edilmiştir. Bu sayede hem immobilizasyon işlemi kolaylaştırılmış, hem de kolonun etkinliği arttırılmıştır. PHEMA temelli kriyojeller proteinlerle biyouyumluluk göstermektedir. Çalışma kapsamında sulu çözeltiden seçici bir şekilde HSA saflaştırılması gerçekleştirilmiştir.

Anahtar Kelimeler: Kriyojel, İmmunoafinite Kromatografisi, İnsan Serum Albümini, Anti İnsan Serum Albümini.

ABSTRACT

PREPARATION OF FAB FRAGMENT IMMOBILIZED IMMUNOAFFINITY CRYOGELS AND ALBUMIN PURIFICATION

Sabina HÜSEYNLİ

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Albumin, composed of a single chain of 585 amino acids, is synthesized in the liver. It is the most common protein found in blood plasma of humans and other mammals. It constitutes 60% of the proteins found in blood. It is also found in tissue fluids, especially in muscle and in the skin, small amounts in tears, sweat, stomach juices and in the gall. 30-40% of the total albumin in the body is in the blood. The most important function is to balance the water between the blood and the tissue fluids, as well as to keep the fat acids and various other substances moving. Albumin is carrier protein that organizes oncotonic pressures in the body and provides inter-tissue substance transport.

In affinity chromatography, the interaction of the corresponding antibodies with antigens, enzymes with substrate anologues and receptors of hormones is the type of interaction. Immunoaffinity chromatography, a type of affinity chromatography, is a chromatographic technique frequently used in recent years. Antibodies used as ligands in immunoaffinity chromatography are used for the purification of antigens.

In this work, cryogel columns immobilized Fab fragments were prepared for human serum albumin (HSA) purification. For this purpose, firstly enzymatic hydrolysis of anti human serum albumin (Anti-HSA) molecules with papain enzyme was performed and the resulting Fab fragments were immobilized in the cryogel column. For control purposes, Fab fragment immobilized PHEMAC cryogel column was prepared. Characterization studies of prepared columns (SEM, BET, FTIR-ATR swelling) were performed. The effect of various parameters (pH scanning, concentration, flow rate, etc.) on the albumin purification process was examined.

Albumin selectivity depends on changes in the Fab region. Thus, instead of immobilizing the whole of the molecule, the functional Fab region responsible for antigen binding is immobilized. Thus, both the immobilization process is facilitated and the efficiency of the column is increased. PHEMA-based cryogels are biocompatible with proteins. In this study, HSA purification was carried out selectively from aqueous solution.

Key words: Cryogels, Immunoaffinity Chromatography, Human Serum Albumin, Anti Human Serum Albumin.

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SYMBOLS AND ABBREVIATIONS

Symbols

g	Gram
mL	Milliliter
μm	Micrometer
min	Minute
m^2	Square meter
mg	Milligram
M	Molarity

Abbreviations

HSA	Human Serum Albumin
Anti-HSA	Anti Human serum Albumin
FTIR-ATR	Fourier-Transform Infrared Spectroscopy-Attenuated Total Reflectance
SEM	Scanning Electron Microscope
BET	Brunauer-Emmett-Teller
MAC	N-methacryloly-(L) cysteinemethylester
MBAAm	N,N'-Methylenebisacrylamide
HEMA	(Hydroxyethyl)methacrylate
PHEMA	Poly(Hydroxyethyl)methacrylate
PHEMAC	Poly(Hydroxyethyl)methacrylate-N-methacryloly-(L) cysteinemethylester
PHEMAC-Fab	Poly(Hydroxyethyl)methacrylate-N-methacryloly-(L) cysteinemethylester-Fab

1. INTRODUCTION

The proteomic term is a very important concept when we look at the day. When we examine the past, it is Marc Wilkins and his colleagues who used the proteomic term in the 1990s. After the first use, the proteomic term began to be used in many areas. Proteomics of genomic protein identifiers. and genomic areas of application [1].

The use of proteomics in the personalization of proteins has become a more important concept of analysis with its being an important source. The development of new techniques to distinguish the proteins effectively has been achieved as a result of long researches. These techniques have made it easier to diagnose and treat diseases [2,3].

The most common fluid used in proteomic analysis is the blood plasma, in addition, with 10% protein and minerals in it is the most working biological fluid in our bodies [4]. One of the benefits of serum proteins is that they facilitate disease diagnosis, but because of their large protein spectrum, the diagnosis of serum proteins is not easy at all [5].

Plasma albumin in human blood is a protein used by many people for therapeutic purposes, as well as a higher level of albumin in human blood than other proteins. Albumin has been studied for many years, especially on the physiological and pharmacological structure. Increasing or decreasing the albumin level in the blood affects human health. Different diseases such as protein deficiency, respiratory insufficiency, hemodialysis, jaundice, hepatic failure, gallstones and pancreatitis were occur due to albumin change in the blood.

By increasing the efficiency in the purification of albumin, it is a point we want to achieve in this work to synthesize highly sensitive and reusable affinity membranes and determine the optimum level of albumin adsorption conditions.

The studies planned to be carried out within the scope of the thesis can be grouped under six groups:

1. The formation of anti-HSA antibody fragments by papain enzyme,
2. Anti-HSA antibody fragment used as a matrix for immobilized cryogel HSA purification,
3. Preparation of cryogel polymers,
4. Characterization studies of synthesized cryogels (SEM, BET, FTIR-ATR, swelling),
5. Examination of various parameters in HSA purification process (pH, concentration, ionic strength etc.),
6. HSA adsorption from artificial plasma.

2. GENERAL INFORMATION

2.1. Genomics and Proteomics

The proteomic word that was first introduced in 1995 was first spoken at a two-dimensional electrophoresis meeting held in Siena, Italy by Marc Wilkins and others. It was accepted in the scientific world. A new approach, proteomics, has spread across a wide range of applications [1]. Due to the fact that the function of the genes is not easy to understand, researching the human genome structure map has led to long work and studies. After long studies, the human genome structure map has been completed in the last 60 years. For this reason, solving protein structures has become a priority. The human genome, expressed as the genetic structure of an organism, consists of 24 chromosomes. The number of genes distributed in these chromosomes is about 35 thousand. The concept of proteomics produced by the "Human Genome Project" started in 1990 started to develop rapidly [6].

It contains all the peptide/protein based gene derivatives synthesized by the proteome genome and is also known as genomic protein complement [7]. The main tasks of proteomics are to examine multiple protein systems and to ensure that the proteins, structural properties and functions of the produced proteins are determined in different situations [8]. The development of protein characterization methods has led to the emergence and development of proteomic analysis [9]. Environmental conditions can completely alter the structure of the proteome, including the entire protein of the organism [10, 11].

2.1.1. Applications of Proteomics

Proteins are more effective in diagnosing diseases [12]. The use of biomarker proteins is very common, but biomarker proteins must be distinguished as healthy and diseased before use [13]. Potential biological markers provide many benefits. identification of cancer types, diagnosis of hemophilia, and cardiovascular diseases. Biomarker proteins, in addition to these benefits, are an important element in the development of drugs [14].

Protein analysis is used to diagnose disease-causing genes. Protein analysis has a more complex structure than DNA analysis and is more important when we compare DNA analysis with protein analysis. While DNA consists of only four building blocks, natural proteins are composed of 20 different amino acids. An organism is a genome, but there are many proteomes. Proteins that are used effectively in the diagnosis of diseases are widely used in almost all fields of medicine.

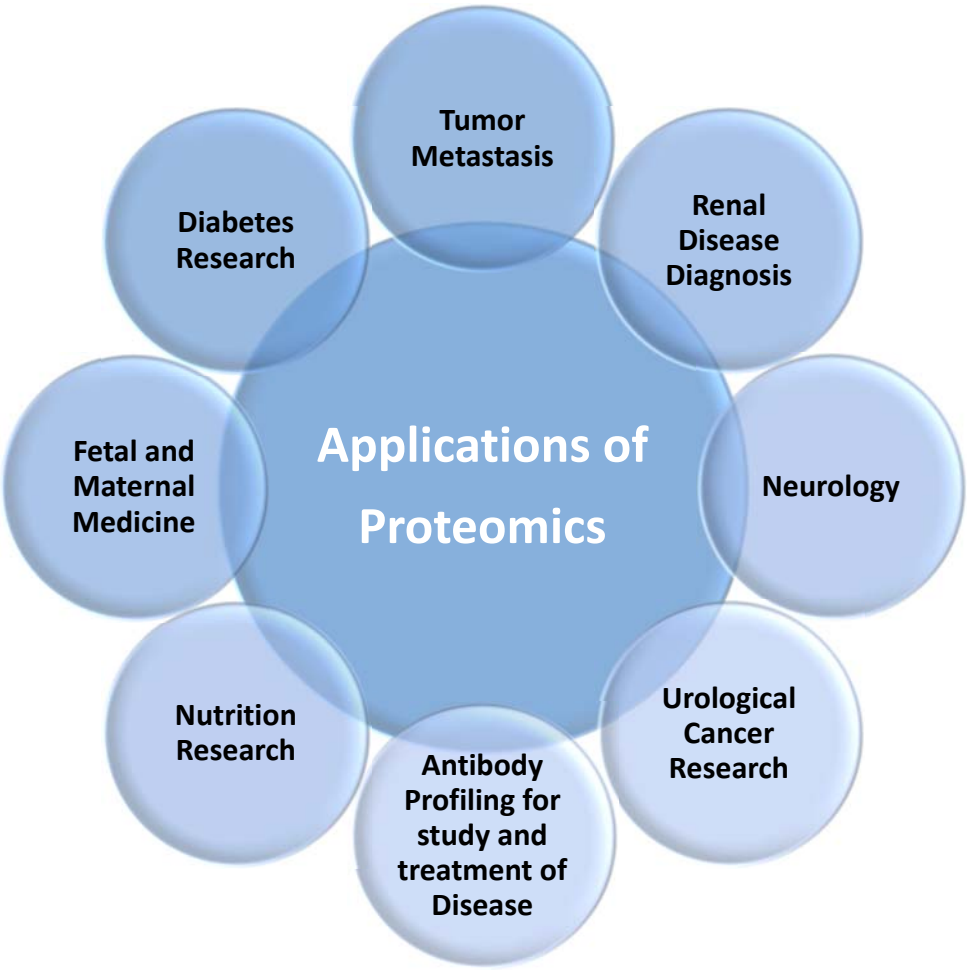


Figure 2.1. Applications of proteomics.

Proteomic research is becoming increasingly important not only in the medical field (oncology, pharmacology, immunology, biochemistry, microbiology, neurology) but also in the field of agricultural biology and environmental science. It facilitates the work of scientists, especially in forensic sciences, since it allows the separation and quantification of samples using very small quantities.

2.1.1.1. Proteins

When we examine the structure of proteins, we can see that the proteins are amino acid polymers. The amino acid of a specific number is arranged in covalent bonds in the polypeptide chain. It is estimated that there are about one million protein species in all living things on Earth. At the same time, many functioned proteins are found in all cells. All the vital functions of living things depend on the proteins. For example, enzymes and hormones are used to regulate metabolism. Muscle proteins enable movement. The collagen found in the bone structure allows the storage of calcium phosphate crystals, which are of great importance to the human body. While doing albumin and hemoglobin transport duty; immunoglobulins are involved in the inhibition of bacteria and viruses.

2.1.2. Plasma Proteins

In the blood plasma, there are important proteins, which are the most studied biological fluids [15]. Plasma, which is a liquid component, forms the half of the blood structure. This result has been accomplished by a lot of research in the field of human plasma proteins. Approximately 90% of the plasma, which accounts for about half of the total blood, is water, while the remaining 10% are protein, minerals, waste, clotting factors. Most of the dissolved substances in blood plasma are proteins. Plasma protein consists of about 3.5 to about 5.0 g/dl serum albumin, about 2.5-3.2 g/dl globulins. Without blood plasma, blood cells could not move comfortably in the body. The process of separating the blood plasma from the blood is quite simple. Sedimentation is carried out by centrifugation or the anticoagulant substance is added to the human blood. Thus, heavy blood cells collapse and plasma comes up. The part that occurs after blood clotting is serum.

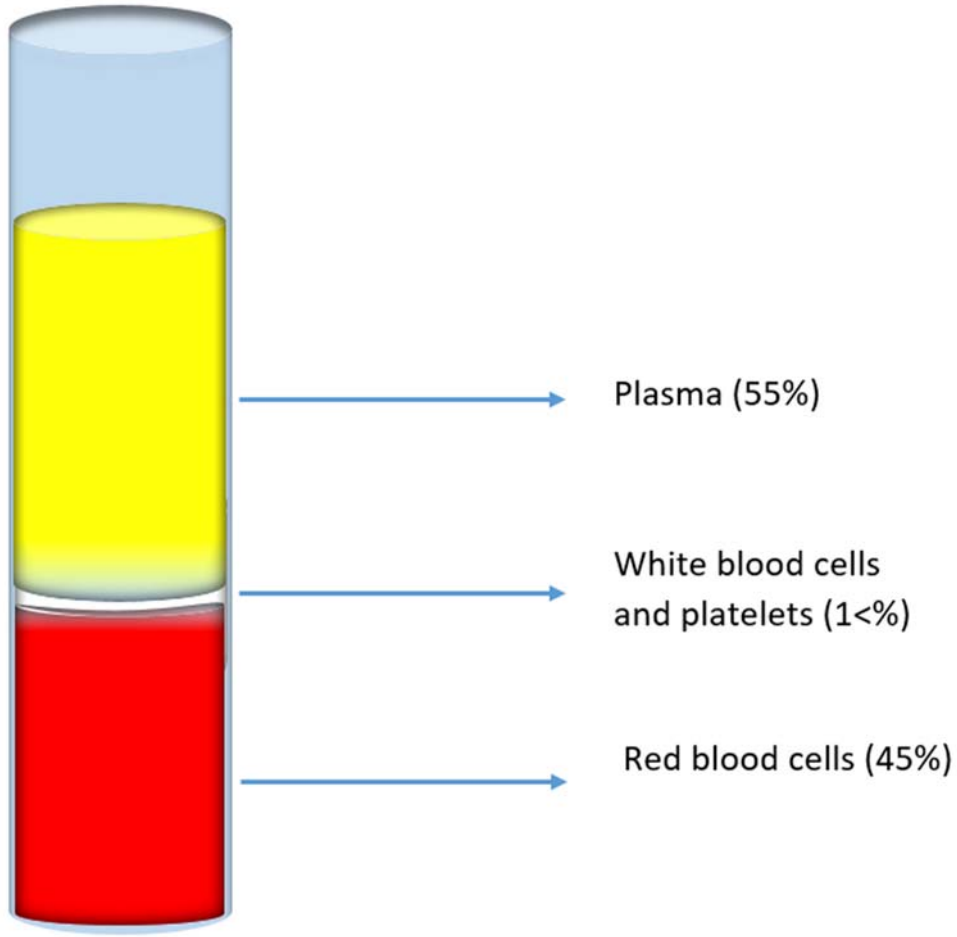


Figure 2.2. Plasma proteins.

There is no fibrinogen in the structure of the serum. This is the difference between serum and plasma. In normal conditions, plasma is yellowish but according to human health this color may be different. It leaves the substances useful for the cells of the body and receives waste products for processing. The components of the plasma are always renewed [16]. When we examine plasma proteins, we see that there are 3 main groups separated. These are albumin, globulin and fibrinogen. Cells are used to produce proteins of their own amino acids, so plasma proteins do not separate from plasma. However, the identification of these biomarkers requires very strong analytical techniques. For example, albumin alone, with a concentration of 30-50 mg/ml, constitutes more than half of plasma proteins. Therefore, quantification of all proteins by a single technique is extremely difficult.

There are used for albumin removing dyes and specific antibodies [17]. After development, preparation of super macro porous monolithic columns for the purification/removal of biological molecules is in the literature. Low pressures are essential in the affinity column of the reconstituted liquid blood plasma. It is expected that the plasma passing through the column at high flow rate will have at least 90% porogen structure. Due to the porogen structure of the plasma, the ratio between the increase in surface area and affinity is in direct proportion [18].

2.2. Albumin

When the amount of protein is taken into consideration, the blood plasma is rich in the body and at the same time the most complex proteome. Albumin is the plentiful protein in human plasma, also an essential constituent of blood plasma. In the light of various studies, we can say that blood plasma is protein rich in its many aspects. The most important and richest protein is human serum albumin (HSA) and albumin is simple globular protein according to its structure. Albumin is soluble in water and aqueous salt solutions; denatured by heat. The ovalbumin that found in the egg white, the eleven serum albumin in the blood, and the milk albumin lactalbumin are animal origin albumins; legumele in legumes family, and leucosin in the cereals are vegetal origin albumins. The albumin has important physiological functions and interferes to protein analysis in low amounts [19, 20].

Putnam analyzed the amount of protein in the plasma when examining the structure of the proteins [21]. Albumin is one of the longest and probably the most studied of all known proteins [22]. When we look at the mammalian blood serum angle, albumin is the richest protein [23].

There are many applications of albumin in clinical and basic research areas. The availability of various functions of albumin has made it the focus of interest for scientists and physicians. The most abundant protein in all body systems with vertebrate structure is plasma proteins, and albumin is as large as 60% section [24]. Albumin checks up the osmotic pressure and water diffusion [25]. Almost all of the plasma proteins are glycoproteins: these proteins contain N- and O-linked oligosaccharides or both. Albumin is not glycoprotein. Each plasma protein has a specific half-life: (albumin-20 days). The liver produces 12 g of albumin per day. The albumin/globulin ratio in the plasma is reduced in liver diseases.

2.2.1. History of the Albumin

The discovery of proteins in plasma is based on the knowledge of the existence of genes. When we examine the meaning of albumin, we mean albumin in white in latin, and in german it means egg white. During the first stages of protein identification, the class of proteins that are soluble in water and coagulate in hot climates have been given this name. Since the most common protein in the serum carries these properties, it is called "serum albumin". Proteins such as the ovalbumin in the egg and the lactic acid in the milk, which are also in this respect, are similarly named, but they do not have any other partnerships with serum albumin.

The first known feature of the albumin was clotting when warmed. In an observation published in 1765, Domenico Cotugno observed that the urine remained clean when warmed under normal conditions, but when an edematous patient's urine was warmed, he observed that the urine was clotted. In the following years, other researchers have examined this phenomenon and finally Richard Bright declared that urine coagulation, edema and renal failure (glomerulonephritis) were related to each other in 1827. Bright simplifies the urine test by saying: ‘‘An easy way to figure out whether or not the albumin is present is to put a little urine on a spoon and warm the urine with the help of a candle. If there is albumin in the urine, the urine will lose its transparency before reaching the boiling point. A very similar appearance occurs at the tip of the spoon and then it collapses’’ [26].

The French doctor Antoine Fourcroy, in 1800, made three major animal tissues, albumin, fibrin and gelatin, as a result of research and chemical tests. In addition, the Swiss physicist G. Kander again found that the protein taken from blood serum was close to globulin he claimed to be albumin. In the early 19th century, various researchers studied on body fluids and albumin. They are the British physician John Bostock, the French Louis-Jacques Thenard, the British Alexander Marcet and the Swedish J.J. Berzelius. Berzelius measured the amount of albumin present in the serum in a precise manner in the light of detailed studies he performed in 1812.

The first use of protein wording was realized by Gerit Jan Mülder in 1839 when he published the elemental combination of serum albumin. The amino acid sequence of albumin was solved in 1976, and in 1992 it was found that the three-dimensional structure is heart-shaped [26, 27].

2.2.2. The Structure of Albumin

HSA is a small (66.500 Da) globular protein with 585 amino acids [28]. In the presence of a small number of tryptophan and methionine residues, high amounts of residues such as lysine & aspartic acid and prosthesis groups do not contain carbohydrates. When X-ray crystallography shows that albumin has a tertiary structure similar to the heart, it has been observed that the structure of HSA is in ellipsoidal form in solution phase [29, 30]. Approximately 67% of the tertiary structure of HSA consists of alpha helixs [31]. There are nine pairs of rings formed by seventeen disulfide bridges [32]. The homologous domains are subdivided into subgroups, respectively, called IAB, IC, IIAB, IIC and IIIAB, IIIC [33]. When we examine the common properties of these subgroups, we observe that basic amino acids have hydrophobic facies and at the same time they can contain proline residues [34]. This grouping can be seen under X-rays [35]. Fatty acids are also linked to the ligands by the copper and nickel variants of the indole derivatives other than the warfarin binding region, various dyes and drugs, and others [36].

Domen II binds ligands such as short chain fatty acids, bilirubin, dyes, indole derivatives and steroids. It is also referred to as indole binding site. Domen III is different from domen II. Domen III binds ligands such as diazepam, long chain fatty acids [34]. Due to the mixed and different connection points, its role in transporting and storing various endogenous and exogenous substances is of great importance [37].

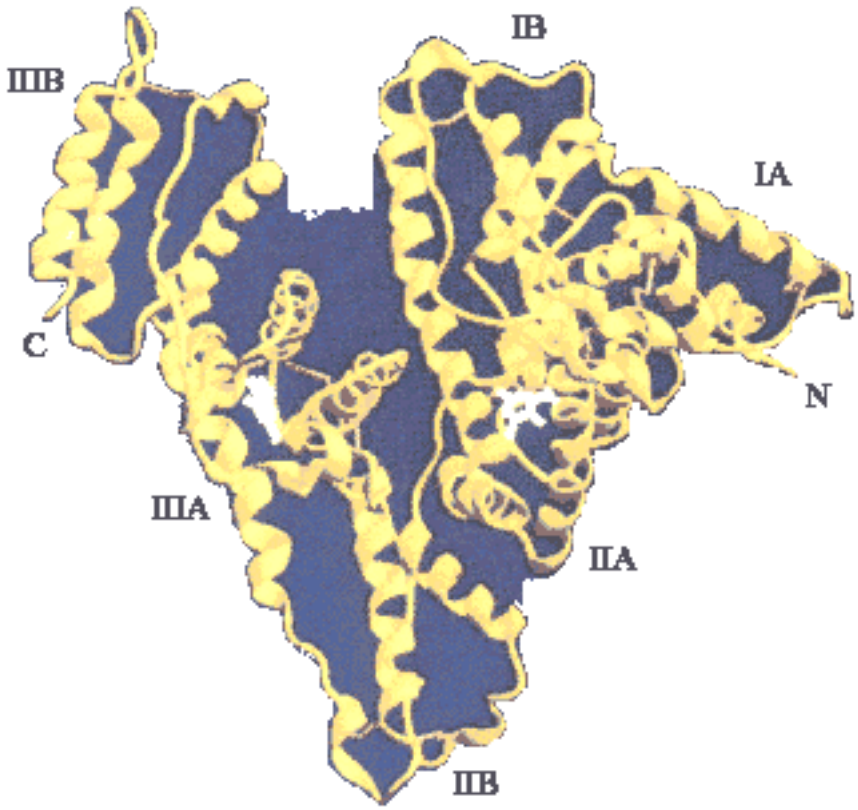


Figure 2.3. Albumin structure.

HSA contains 35 cysteine residues. These residues constitute disulfide bridges and total tertiary structure [38]. Physiologically, HSA is found predominantly in a low form (i.e. free thiol is present in the form of HSA-SH) and is known as mercaptoalbumin [39, 40].

2.2.3. Function of Albumin

As a result of the researches and applications related to the albumin of the last 20 years, the albumin has taken its place in the clinical research literature. The level of the albumin in human body is an important factor to specify the person's health status [41]. If the amount of albumin falls below the normal level, the patient should be evaluated and the patient treated. The increase in the amount of albumin is a rare occurrence [42].

The albumin provides 70% of the inoculative pressure. Liquid balance in the veins of the albumin and outside the veins shows how important the albumin is. The Starling law specifies the movement of fluid within the body. The law describes the vascular transformation of the osmotic pressure body fluid [43]. But there is a low amount of fluid that has not been passed through, leaked, and returned back to the lymphatic system. Large proteins, which do not pass through the capillaries, regulate the bending tendencies of the fluid. In this case the originating albumin causes the colloid osmotic pressure or the oncotic pressure regulating main material to leak water or water-soluble substances into the tissues from the capillaries [44].

The albumin carries a wide variety of substances. It is responsible for binding and control of oxygen free radicals except albuminurine, the main carrier of fatty acids with low solubility in water. It converts toxic metabolism such as bilirubin, which is activated by the separation of oxygen free radicals into the seed molecule, by binding to some water insoluble substances [45]. Albumin is one of the different properties of albumin that can bind to a variety of metal ions that can be toxic in high concentrations. In the majority of physiological processes, nitric oxide (NO) is also the main carrier of albumin. Albumin binds these substances together to reduce their concentration in harmless levels, it carries the duty of carrying these items [46].

When we look at the binding area, two of the albumins consist of tight, four non-tight areas. They can be optionally added with long fatty acid molecules (oleic, linoleic, linolenic, interdidic, palmitic and myristic acid). Albumin acts as a carrier in transporting these fatty acids to the cells and these acids are carried in the cells [47]. Multicyclic

compounds containing small aromatic carboxylic acids and negative charges are sites where small organic ions can be bound, except where fatty acids are bound. Thyroid hormones, other steroid hormones and bilirubin can be transported in these areas. The drugs we use for the treatment of the albumin are also related, and even the transport of pyridoxal (B6 vitamins) is made by albumin [48].

Apart from the compounds to which we have indicated the albumin is bound to be able to control the concentrations bound to many heavy metal ions [49]. When binding direction is examined, the albumin protein has two metal ion binding sites. The binding sites can be linked to various ions such as zinc, copper, cadmium, mercury, gold, silver and nickel [50]. As well as these ions, albumin is also associated with calcium and magnesium, which can also affect the relative concentrations of these ions [51]. Another feature of the albumin is that it can partially buffer the blood pH.

2.2.4. Reasons of Albumin Lack

The number of hypoalbuminemi is the number of albumin which is less than 3.5 g/dL. The reason for the low number of albumin is related to the low number of albumin production or the rapid loss of albumin. If there is a decrease in body protein reserves, this may be due to low albumin level [52]. Advanced liver disorders (cirrhosis), amino acid absorption imbalances and nutritional deficiencies cause the body to produce less albumin. Albumin is produced in the liver. Because of this, serum albumin testing is a test that controls liver function [53]. Kidney diseases lead to rapid albumin loss. Apart from this, the cause of low albumin in the body is caused by other skin burns, which is why we store albumin in our skin. The amount of albumin in our body may be low due to the very uncommon musculature. Low albumin level causes edema in the body (such as sweating) can increase the level of the albumin [54].

Albumin production occurs in the liver. The liver produces half of the amount of albumin that it can produce. Hormones (thyroid, corticosteroids, insulin and growth), the osmotic pressure and the amino acid levels on the side affect the regulation of the production of albumin.

Most of the albumin is destroyed in the kidney. This process takes the albumin of kidney cells and separates them into small peptides in lysosomes, and the peptides are removed from the body with the help of urine [55]. When it comes to albumin problems in the body (deficiency of albumin), serum protein electrophoresis method is used. Various tests are applied to see albumin in the urine. The tests also include albumin-specific monoclonal antibodies and high pressure liquid chromatography.

2.2.5. Cutting of Albumin with Papain Enzyme

Papain enzyme can be separated albumin molecule into two proteolytic fragments from the hinge region. These fragments are Fab and Fc region. Fab includes the antigen binding activity of the antibody. The Fc region is the constant region. Papain is a cysteine protease. Its structure consists of three disulfide bridges and one sulfhydryl group.

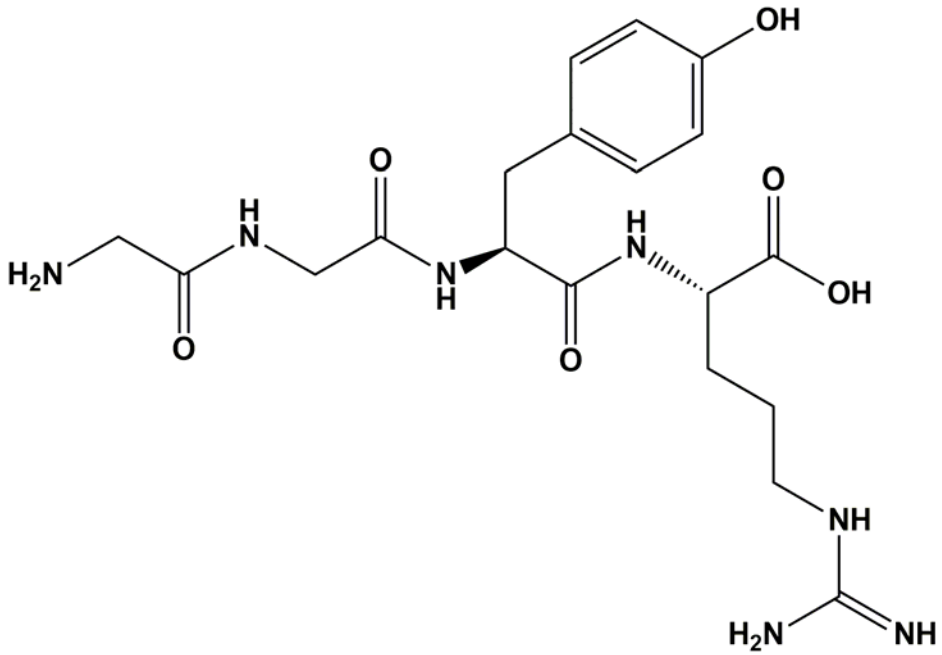


Figure 2.4. Molecular structure of papain enzyme.

2.3. Chromatographic Methods and Proteins

Since the physico-chemical properties of polysaccharides and nucleic acids differ from proteins, the existing isolation, separation/separation methods have begun to decrease due to these differences. Chromatography as a physical method is to separate and purify the substances in the mixture at fixed and mobile phase without comparison [56].

Chromatography was described in 1906 with the contributions of the Russian botanist Tswett. Over the years, much attention was paid to it in the following years, particularly in the 1960s [57, 58]. Unstable proteins can be affected by high temperatures, pH conditions, natural solvent conditions, and degradation. Today's chromatographic methods can be divided into phase types by hydrophobic chromatography, such as affinity chromatography, ion exchange chromatography, polar and non-polar proteins, for example, when the application fields are taken into consideration [59].

2.3.1. Affinity Chromatography

The ability to bind molecules bilaterally is an important and characteristic feature of biological macromolecules. This is the case where the complexes with the substrates or inhibitors and the antibodies are linked to the antigens [60]. Affinity chromatography has become more and more important in the past as compared to the past. Importance has also led to various discussions. It is not easy to find the difference between the complexity specific to the biological field and the non-specific complexity associated with hydrophobic bonds. A substance bound to a carrier may form specific complexes with macromolecules, forming only non-specific hydrophobic interactions with the other [61]. Campbell and colleagues introduced affinity chromatography using solid matrix covalently bound ligands. This is why the 1951 covalently bound antigen was used to isolate antibodies in the cellulose column. Lerman was the first person to use affinity chromatography in 1953 and used it for the isolation of enzymes. It has not been used for a long time because affinity techniques appeared to be undesirable. Later, Porath et al. brought the method of chromatography again using cyanogen bromide as the ligand to activate the agarose. In the 1960s, Yagi and colleagues found new clues about the affinity antigen-antibody relationship [62].

Biospecific affinity chromatography (also referred to as affinity chromatography) is associated with the ability to bind biologically active substances to other substances, specifically called affinity ligands [63]. Affinity chromatography based on molecular recognition [64]. Affinity chromatography, which is a separation method, is used to analyze and characterize very small protein molecules to their finest detail. With a biodegradation method, a substrate molecule is chemically reacted to a carrier such as agarose, such as polysaccharide, which is then placed in the column. When a protein mixture is passed through the collet, the substrate catches and retains the self-specific protein in the mixture; other proteins pass through the orbit. Affinity

chromatography is divided into various sub-branches such as immunoaffinity, lectin affinity, biomimetic affinity, and metal ion affinity. These methods can be used for protein, enzyme, antigen and antibody purification [65]. Affinity chromatography differs from other methods of separation because of the advantages it offers, such as the purification of the targeted protein in one step, its high selectivity, its rapidity and its ease of use [66].

2.3.1.1. Ligands

In addition to being biologically predominant, some synthetic ligands are also present. We see ligands in two groups as ligands with high specificity (monospecific) and other general purpose ligands (specific group). When we examine monospecific ligands, are closely related to each other in relation to each other. These may include antibodies, enzyme inhibitors or cofactors, nucleic acids. The group specific ligands bind to molecules of a class associated with each other, thereby separating the compounds. Group specific ligands may be exemplified by lectins, synthetic dyes, metal chelates [67, 68]. Since the monospecific ligand allows purification of a single compound, group specific ligands are generally preferred [69]. The specific and recyclable complex property of the substance to be purified is a requirement for the affinity ligands to be present in carrying and functional groups.

2.3.1.2. Matrix

The column matrix must have a hydrophilic, porous, hard, inert and stable structure in order to be accepted as a good column. When we examine high performance affinity chromatography, we can see that auxiliary materials consisting of small and hard particles, which are synthetic polymers with high flow rate, are used [67, 68]. Good ligands are achieved by affinity chromatography with solid ligand binding of the appropriate ligands. It is important that the selected immobilization technique is suitable for determining ligand activity [64]. In general, the immobilization method consists of two levels. The first is the activation phase. At this stage, the support material is ligated chemically. The appropriate functional groups are selected for this step. The functional groups to be selected are comprised of amines, sulfhydryls, aldehydes, carboxylic acids and hydroxyls. The second step is the step of coupling the selected ligand to the auxiliary material [70]. In affinity chromatography, biospecific and bilateral interactions are used. There are a large number of affinity chromatography;

Immunoaffinity Chromatography, Membrane, Metal Chelate, Covalent, Protein A Chromatography, Dye Affinity Chromatography [64].

2.3.2. Immunoaffinity Chromatography

Immunoaffinity chromatography is the meticulousness and selectivity in the purification of biological structures such as antibodies and antigens which make the chromatography different from the other methods. In the field of immunoaffinity chromatography has many advantages. These are the conservation and economically viability of the protein biological activity beyond capacity and high activity.

Purification of a protein by binding as a precursor to a target protein is defined as immunoaffinity. The use of the method is as follows: the antibody is immobilized on a column material, in which case the column is bound to the protein as a precursor if there is any leakage from the column. Protein is removed from the collet. The adsorbent, which is called as the adsorbent, is applied to the immunoglobulins of the antigens called antigens. The adsorbents which are based on the monoclonal and polyclonal antibodies are selected. The monoclonal antibodies are antibodies which only react to one epitope and are obtained from only 1 β-lymphocyte based cell colonies. Polyclonal antibodies are antibodies that are produced by several different β-lymphocyte colonies, all of which bind to different determinants of the same antigen. The anti-HSA antibody we used throughout the thesis is the polyclonal antibody.

Immobilization in immunoaffinity chromatography is more precisely, immobilization of the antibody is very important. The pores that help to properly bind the surface-embedded target molecules to the active binding sites cause an increase in surface area, as well as an increase in cryogel binding capacity [71, 72].

Normal antibody-antigen binding procedures are immobilized at many different locations where the antibodies may interfere with the formation of the antibody-antigen complex [73, 74].

Antibody binding sites present in immobilization regions should be immobilized as competently as possible so that they properly match the antigen binding site. The good aspects of the proper immobilization process are the proper attachment of the antibody-antigen and stable structure [75].

2.4. Polymeric Gels

Following their development, the use of polymeric gels is now seen in various areas of biotechnology. However, new requirements have come to the fore due to the development of polymer gels. These can be said to be commercialized by the development of polymeric gels. Polymeric gels, which are amongst efficient carrier substances during the immobilization of biomolecules and cells, are polymeric stabilizer systems composed of 3D networks [76]. The solvent has the property of increasing diffusion and gel stability in the polymeric gel system [77].

2.4.1. Classification of Polymeric Gels

Polymeric gel is classified in several main categories:

Kemotrophic gels: gels resulting in the formation of 3D covalent networks. This gel is generally used in the field of biotechnology.

Solvotropic gels: Examples include cellulose nitrate or cellulose acetate. Gels are involved in the non-covalent polymer-polymer interaction. There is no biotechnology application yet.

Ionotrophic gels: Ion-exchange reactions occur with the formation of time-stable ionic (salt) bonds. This gel is stable in certain context, but can easily be resolved when the content of the environment changes.

Thermotropic gels: Gelling occurs by heating the starting polymer.

Chelatotrophic gels: formed by stable coordination bonds.

Physicotropic gels: For example, gelatin, starch, agarose, agar agar, carrageenan gel can be given. Physicotropic gels are well known.

Cryotrophic gels (Cryogels): Freezes the starting system.

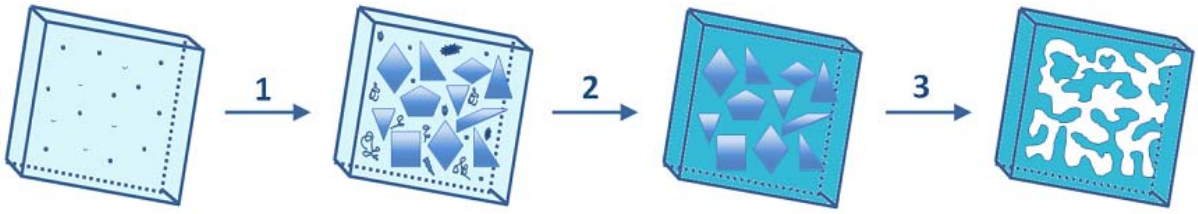
2.4.1.1. Cryotrophic Gels or Cryogels

Cryogel is a type of solution prepared at low temperatures from the melting point of the solution. It is also the gel system of the precursor species. When we examine the structure of cryogels, we observe that they are composed of large pores or very large pores physically connected to each other [78, 79]. Cryogels having a spongy and porous morphological structure are composed of covalently cross-linked polymer networks. Cryogel derived from greek word thats mean cryos-ice or frozen.

2.4.1.1.1. Advantages of Cryogels

When we look at it in the first place, cryogels are treated as a concept only, but this idea is based on 50 years ago. Cryogels have become important in the field of biotechnology in recent years as they have developed. As we look at the use of cryogels in biotechnology, osmotic, chemical and mechanical balance are related. [80, 81]. Cryogels are a very good alternative to chromatographic support materials in biotechnology since they have many advantages [82]. One of the most important advantages of cryogels is that large pores in the structure are also beneficial to mass transfer of nano- or micro-sized particles at the same time, as well as cryogels cause efficient diffusion [82, 83].

Cryogels offers many effective solutions. For example, allow high flow rates and volume elution in a shorter time, also can be applied to cryogel columns without pretreatment [84].



1)initial system 2) frozen system 3) thawed cryogel

Figure 2.5. Cryogel formation.

2.4.1.1.2. Factors Affecting Cryogel Structure

2.4.1.1.2.1. Solvent

Structurally, cryogels are classified as 3-dimensional heterophasic jellies and their task is to find the frozen solvent as a porogen in the polycrystalline gel formation [85]. When the polycrystalline is melting, voids filled with solvent leave macro pores. It is important to prevent the solvent from collapsing during gelling. If the frozen system is homogeneous, it is actually heterogeneous [86].

2.4.1.1.2.2. Freezing temperature

The pore sizes vary in parallel to the freezing temperatures. The pore sizes of cryologists can range from 10 micrometers to a few micrometers. The low freezing temperature causes the formation of small-sized dissolved crystals, and the pores on this surface are smaller in size [87]. At low temperatures, the volume of the non-frozen liquid is reduced, resulting in thinner and dense pore walls. If the freezing temperature is high, the size of the ice crystals becomes bigger, resulting in larger porous cryogels. However, the risk of freezing temperature should also be considered, if the temperature is too high, freezing may not take place [88].

2.4.1.1.2.3. Cross-linker

The use of a large number of crosslinkers in the cryogel formation reduces the rate of swelling in the solvent.

2.4.1.1.2.4. Concentration effect

The cryogenic structure is sensitive to changes to the concentration. The high concentration causes the formation of more fragile matrices and the decrease of the concentration makes the formed matrices flexible and soft [89].

2.4.1.1.3. Cryogel Formation in Polymer Systems

During cryogel formation, water is usually used as a suitable solvent. The process is as follows: first, the dissolved particles are quickly frozen, followed by freezing with the aim of bringing the majority of the dissolved ice crystals to the well. After the reaction is completed, the ice crystals which are brought to room temperature are melted and a porous polymer network called "cryogel" is obtained [90]. Cryogels can be used as beads, spongy discs or monolithic columns [91].

2.4.1.1.4. Application Areas of Cryogels

Firstly, cryogels which have been applied in the chromatographic separation processes of proteins, microorganisms, cells [92,93], in recent years, with the production of many biocompatible polymers with different chemical properties, medical and surgical field has been widely used in cell immobilization [94,95].

- To be prepared in a very simple way,

- Obtaining interconnected large and open pores,

- It can range from 10μm to 200μm, which is crucial in view of the size of the cells, so that they have suitable dimensions for separating the cells without impeding flow,

- The cell suspension moves very smoothly through both the diffusion and transport along the cryogel and the mass transfer is very efficient.

3. MATERIAL AND METHOD

Experimental researches carried out within the scope of the thesis are summarized as following;

- Detection of anti-human serum albumin (anti-HSA) against Fab and Fc region provided by papain enzyme,
- Synthesis of N-methacryloyl-(L)-cysteinemethylester (MAC) functional monomer,
- Preparation of the complex of obtained Fab region by N-methacryloyl-(L)-cysteinemethylester (MAC)
- Preparation and properties of MAC-Fab precomplex-stationary PHEMAC-Fab monolithic columns in different quantities, as well as the preparation and properties of PHEMAC blank columns,
- Analysis of various parameters in purification of human serum albumin with prepared columns and in the purification process (pH, concentration, flow rate).

3.1. Materials

Thesis studies was carried out at Hacettepe University, Faculty of Science, Department of Chemistry, Department of Biochemistry. Anti-HSA was used to obtain Fab fragments. The papain enzyme was used for the enzymatic hydrolysis of the anti-HSA molecule and the anti-HSA molecule was generated from Sigma (St. Louis, USA). In addition, Ethylenediaminetetraacetic acid (EDTA), iodoacetamide, methacryloyl chloride and L-cysteine methyl ester dihydrochloride formed from Sigma (St. Louis, USA). The basic matrix monomer is selected from the group consisting of 2-hydroxyethyl methacrylate (HEMA), N,N,N'-tetraethylenediamine (TEMED) serving as catalyst, N,N-methylene bisacrylamide (MBAAm) and initiator ammonium persulfate (APS) as crosslinkers (Fluka AG, Switzerland). All reagents have been purchased without any purification.

Using ROpure LP® reverse osmosis unit from Barnstead (Dubuque, IA), the water used in the experiments was purified. Then, purified with Barnstead D3804 NANOpure® organic/colloid distillation unit and ion exchange packed column system. Resistance to pure water (deionized water) from the square is 18 MΩ/cm. Before using, all glasses were washed extensively with 4M nitric acid diluted.

Fourier-transform infrared spectroscopy-Attenuated Total Reflectance, FTIR-ATR (Spectrum One™, Perkin Elmer, Massachusetts, USA) spectrophotometer have been used in the experiments; Scanning electron microscope (SEM) (JEOL, JEM 1200EX, Tokyo, Japan); peristaltic pump (Ismatec, Wertheim, Germany) and Brunauer-Emmett-Teller (BET) (Quantachrome NOVA 2000, USA), Lyophilizer (Christ Freeze Dryer-Alpha 1-2 LD, Maryland, USA).

3.2. Experimental Methods

3.2.1. Cleavage of Anti-Human Serum Albumin Molecules with Papain Enzyme

The papain enzyme was used for the purpose of fragmentation of anti-HSA molecule. Thus, consisting of 20 mM EDTA, 20 mM sodium phosphate buffer (pH: 7.0) has been prepared. The enzyme/antibody ratio during separation is 1:20. The mixture which was initiated with papain addition includes 0.5 mg/mL anti-HSA. For 24 hours, the mixture was incubated at 37°C. After the completion of incubation, 30 mM iodoacetamide was added to the mixture in order to stop the reaction.

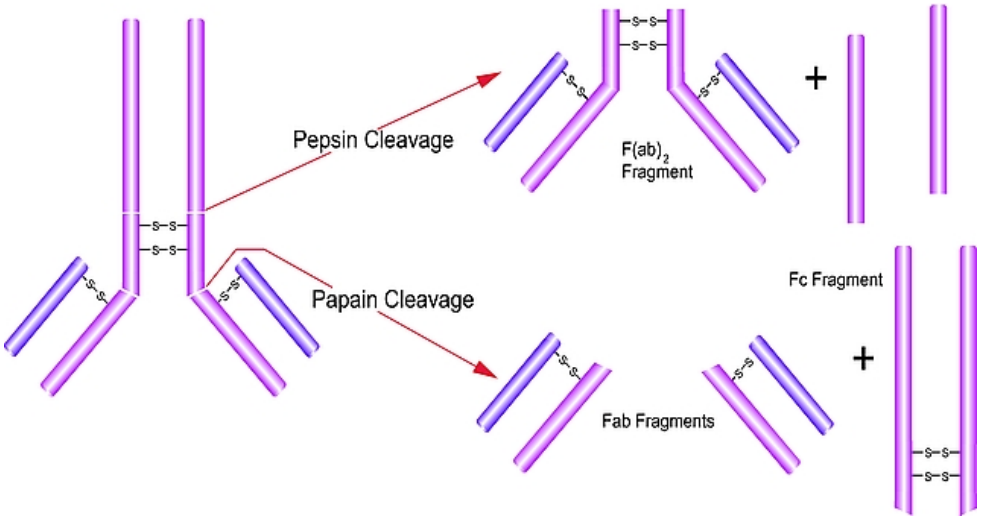


Figure 3.1. Schematic representation of antibody fragments digestion with the aid of pepsin and papain enzymes.

3.2.2. Synthesis of N-methacryloly-(L)-cysteinemethylester (MAC) Monomer

MAC has often been used as a comonomer and ligand. N-methacryloyl-(L)-cysteine methylester (MAC) synthesis and characterization details are elsewhere indicated. Synthesis procedure of N-methacrylol-(L)-cysteine methylester (MAC) co-monomer mentioned in the previous article [96].

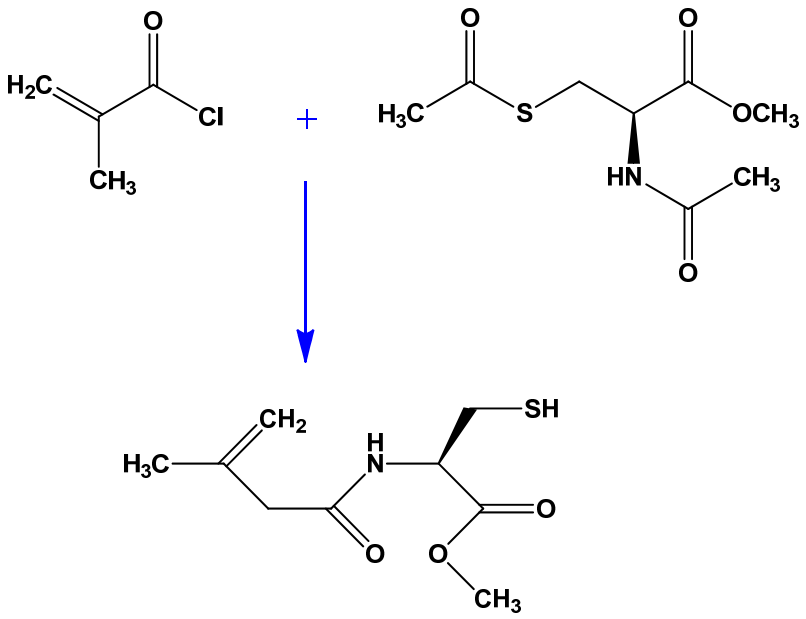


Figure 3.2. Synthesis of N-methacryloly-(L)-cysteinemethylester (MAC) monomer.

3.2.3.Synthesis of N-methacryloyl-(L)-cysteinemethylester (MAC)-Fab Pre-Complex

For a total reaction time of 3 hours, room temperature is needed in the pre-complex reaction. The Fab fragment (template molecule) and functional monomer were prepared in different amounts of MAC, 1:1, 2:1, and 3:1. At room temperature, MAC-Fab complex was stirred for 2 hours.

3.2.4. Determination of Fab fragment by UV-VIS Spectroscopy

Ultraviolet and visible region (UV-Vis) spectra were obtained by scanning the generated complex at a wavelength of λ = 595 nm using SHIMADZU UV-1601 spectrophotometer for optimization of mole ratio of the complex. The device used is shown in Figure 3.3.

Figure 3.3. UV-VIS spectrophotometer (SHIMADZU UV-1601).

3.2.5. Preparation of Fab Immobilized PHEMAC and Fab Non-Immobilized PHEMAC Cryogels

In order to prepare the cryogel, HEMA was used as monomer. N,N-methylene bisacrylamide (MBAAm) was used as the crosslinker. First, 0.283 g MBAA was dissolved in 10 ml of deionized water. In different beakers 1.5 ml of HEMA was dissolved in 5 ml of deionized water. The two mixtures are mixed in the same container. Then MAC-Fab precursor complex added in this mixture. In order to make it homogeneous, the mixture should be mixed in a magnetic stirrer.

Ammonium persulfate (APS), that result in free radical formation for the polymerization, was used as the initiator. N,N,N',N'-tetraethylenediamine (TEMED) was used as a catalyst. 20 mg ammonium persulfate and 20 microliters of N,N,N',N'-tetraethylenediamine was used for polymer formation. The polymers were distributed evenly across the syringes. The tops of the syringes are tightly closed for 24 hours and it's left to polymerize at -14°C. Likewise, the cryogel used for control was synthesized without immobilizing the Fab fragment. After the finishing of polymerization process, the polymers were washed with deionized water.

In this study, the PHEMAC-Fab based cryogels are remain at the forefront with features [97].

A) PHEMAC cryogel B) PHEMAC-Fab cryogel

Figure 3.4. Synthesis of (A) PHEMAC and (B) PHEMAC-Fab cryogel columns.

3.2.6. Characterization of Cryogels

Respectively, FTIR-ATR, BET, SEM tests and swelling experiments have been conducted for the characterization of synthesized PHEMAC-Fab and PHEMAC cryogels.

3.2.6.1. Swelling Test

Cross-linker-including cryogel columns have been prepared in different quantities and their swelling properties were analyzed in detail. Swelling ratios (%) of cryogel columns were calculated as described below;

Drying is needed to weigh cryogel columns with sensitivity of ± 0. 0001. Drying was applied at -60°C in a lyophilizer. The amount of dried weight is calculated as W_d. After, the columns brought to a constant room temperature, were placed in a beaker containing 50 mL of purified water for swelling. Inflation phase lasted 24 hours. Water-swollen monolithic columns were weighed out with water with the aid of filter paper. And this weight was recorded as W_s. Total swelling rate was recorded as (% SR). The equation is as follows:

$$SR\%: [(W_s-W_d) / W_d] \times 100 \quad (3.1)$$

Except the swelling rate, the macro-porosity of the experiments should also be calculated. In order to do this, the weight swell ratio in a beaker including 50 mL of distilled water was recorded as the weight (W_s) of the cryogel columns reaching saturation. By compression and weighed, the water in the macro pores of the columns was removed. The weight of the columns removed from the water in the pores was recorded as W_{sq}. The equation of macroporosity is as follows:

$$Porosity\% = [(W_s-W_{sq}) / W_s] \times 100 \quad (3.2)$$

3.2.6.2. Structural Analysis of Polymers with FTIR-ATR

The bulk structure of PHEMAC-Fab, PHEMAC polymers and MAC monomer have been studied with FTIR-ATR (Spectrum One ™, Perkin Elmer, Massachusetts, USA). Prior to examination, the polymer was dried in a vacuum (50 mbar) in a lyophilizer for

24 hours. In order to bring the FTIR-ATR, the dried polymers have been pulverized (Fourier-Transform Infrared Spectroscopy-Total Weak Reflectivity) spectrum to the plate. The spectra of the samples have been taken at 4000-600 cm^{-1} wave number in the FTIR-ATR device.

3.2.6.3. Surface Area Measurements

In order to detect significant surface areas of PHEMAC-Fab and PHEMAC cryogels, the surface area meter (BET) (Quantachrome NOVA 2000, USA) was used. In the first phase, the weighed columns were degassed in nitrogen at 100°C. Then weighed and placed in the sample chamber. Gas adsorption of cryogel samples was managed at -210ºC and desorption occurred at room temperature.

3.2.6.4. Surface Morphology

In order to detect surfaces of cryogels, scanning electron microscopy (SEM), Fab immobilized PHEMAC and immobilized PHEMAC were used. With this aim, the washed cryogel was connected to a SEM sample plate with a conductive adhesive. The cryogel column was incubated at -60°C in a lyophilizer for cryopreservation. After coating with altpaladium, dry samples were taken with SEM images (JEOL, JEM 1200EX, Tokyo, Japan) (40:60).

3.2.7. Adsorption-Desorption Studies

3.2.7.1. Adsorption of HSA from Aqueous Solutions

The researches have been conducted as regards the interactions of cryogels and HSA via a continuous recirculating system. Cryogels were put in a plastic disk holder column, and the mixture passed through the column via a peristaltic pump (Ismatec, Wertheim, Germany). In the beginning of the each experiment the cryogels were washed with 20 mL deionized water. After, equilibrated with 50 mM phosphate buffer at pH 5.0 for 15 min. The effects of various parameters were analyzed on HSA adsorption on different cryogels. HSA adsorption was evaluated by analyzing their absorbance at 595 nm via a UV spectrophotometer. Then, the amount of adsorption was calculated regarding following mass equation,

$$Q=[(C_i-C_f)xV]/m \quad (3.3)$$

In this equation Q is the adsorption amount of the HSA on to unit mass of cryogels in the unit of mg/g; C_i is the initial HSA concentration of the aqueous solution in the unit of mg/mL; C_f is the final HSA concentration of the aqueous solution which obtained after the treatment of the HSA solution with the cryogels in mg/mL; V is the volume of the aqueous solution in mL; m is the mass of the dried cryogels used during the experiment in (g). Each experiment was performed triplicate for conforming the data.

The role of temperature in determining the effect of concentration, flow rate, pH and adsorption in the various studies was investigated in the same columns. The first one is the effect of pH on the level of HSA adsorption. The pH of the solution is varied between 4.0 and 8.0. The HSA concentration is analyzed between 0.1-2.0 mg/mL and the flow rate is 0.5-2.5 mg/ml.

3.2.7.2. Desorption and Reusability

This process has been investigated in a continuous system. In the present investigation, the desorption process is firstly separated and separated from the adsorbent foreign substances and other bound residues. In order to achieve this treatment, firstly deionized water is added, followed by It should be desorbed with 1 M NaCl (20 mM, pH: 7.0 phosphate buffer) solution. The column is cleaned with deionized water and there is no danger in terms of health in reuse. The equilibration state of the working environment is provided with pH 5.0 phosphate buffer. The desorption ratio for HSA was calculated as follows:

$$\text{Desorption ratio } (\%)=\frac{\text{Amount of HSA released x } 100}{\text{Amount of HSA adsorbed}} \quad (3.4)$$

For determine the reusability of the PHEMAC-Fab, the adsorption-desorption process was repeated at least 10 times using the same column.

4.RESULTS AND DISCUSSIONS

4.1. Characterization Studies

PHEMAC-Fab was originally coupled with MAC-Fab for the purpose of bringing the cryogels into action and then synthesized via column polymerization using MAC-Fab precursor structure. The characterization phase of the rendered columns, surface area measurements (BET), swelling experiments and SEM, FTIR-ATR methods.

4.1.1. FTIR-ATR Spectrum of MAC Monomer

Structurally, the FTIR spectrum of the MAC contains the carbonyl band of the typical strain vibration absorbing bands at 1661 cm^{-1}. On the other hand, since the linear cord is the covalent complex structure, with the aim of determining the typical characteristics of this complex structure, SH-type vibration bands shift to a higher frequency area at 879 cm^{-1} as a result of decreasing the electron density of sulfhydryl group of MAC monomer. The two peaks at 2880, 2973 cm^{-1} may be attributed to symmetric, asymmetric –CH2 stretching respectively. The most intense peak at 3328 cm^{-1} can be assigned to phenol –OH stretching.

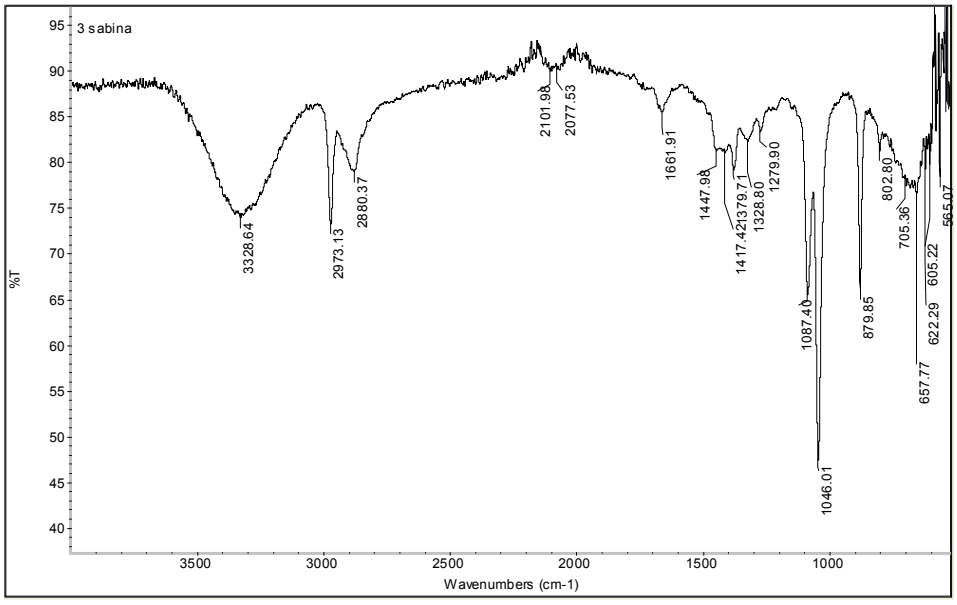


Figure 4.1. FTIR spectrum of MAC monomer.

4.1.2. FTIR-ATR Spectrum of Cryogel Columns

The FTIR-ATR spectra of synthesized PHEMAC and PHEMAC-Fab cryogels are given below;

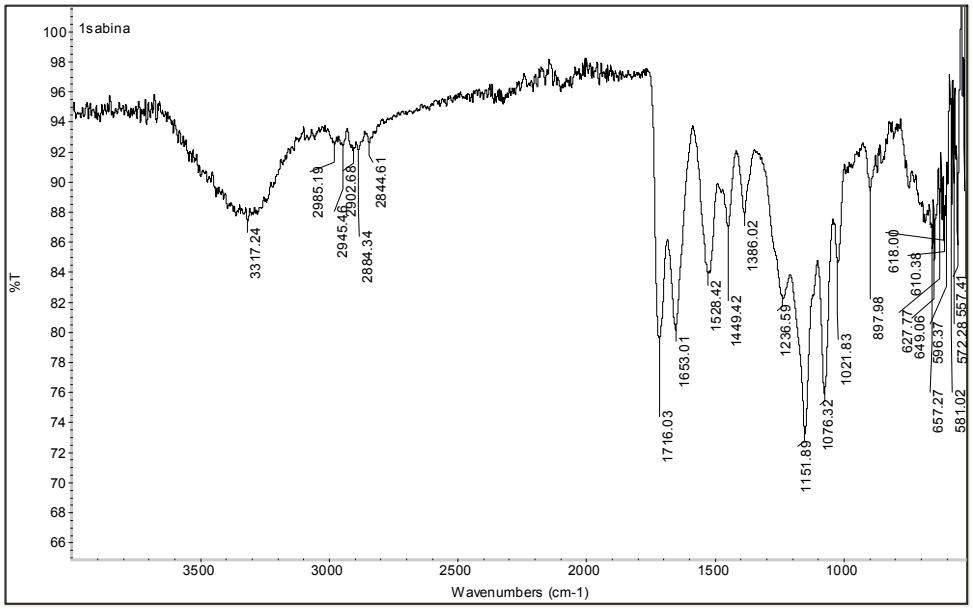


Figure 4.2. FTIR-ATR Spectrum of PHEMAC-Fab cryogel.

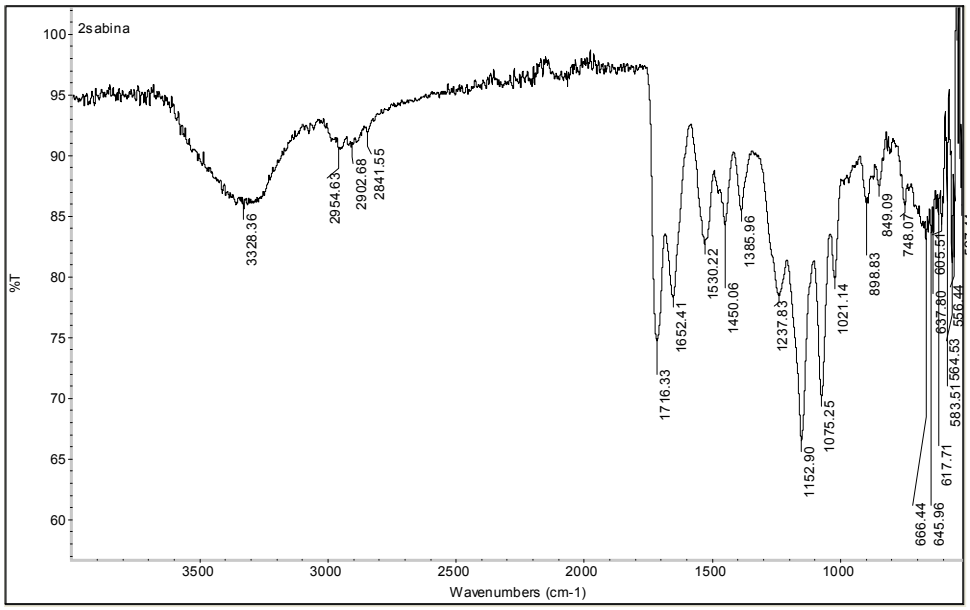


Figure 4.3. FTIR-ATR Spectrum of PHEMAC cryogel.

OH stretching vibrations of PHEMA are important because they have the characteristics of two cryogels. When the structure of PHEMAC was examined, it was found that the PHEMAC-cryogenic structures had OH-induced vibrational peaks around 3328 cm^{-1}. The large OH stress peak (3317 cm^{-1}) of PHEMAC-Fab greatly decreased after the fixation of the Fab fragments. Typical C = O stretching vibrations at 1716 cm^{-1} can be seen in these two cryogogies when we examine two cryogies. 1151 cm^{-1} for PHEMAC-Fab and 1152 cm^{-1} bands S = 0 for PHEMAC. This shows that the MAC monomer enters the structure.

4.1.3. Surface Area Measurement (BET)

Surface area measurement for cryogel is an important parameter. The surface area of the synthesized columns is analyzed by the multi-point BET method. The surface areas of the PHEMAC-Fab are measured via cross-linkers and PHEMAC columns in different levels. When we look at the results after these steps, we see that the surface of the MAC-Fab immobilized columns is 38.6 m^2/g and the surface of the Fab fixed columns is 20.0 m^2/g. The target molecules buried in the surface cause a significant increase in surface area, besides being properly attached to the active binding sites. At the same time, the embedded fragments have increased their pore area with their unique cavities.

4.1.4. Surface Morphology (SEM)

At the figure 4.5 and 4.6, we see the structure of PHEMAC-Fab and PHEMAC. The point to be noted remains the same after surface morphology stabilization. To avoid pore loss of the polymers, they are freeze-dried at -53°C in a lyophilizer. Structural examination of the PHEMAC-Fab shows us that cryo-porous and non-thick polymer walls have wide and always internally interconnected pores (20-80 μm in diameter). At the same time, the level of Fab fixation and HSA adsorption is increased the capacity.

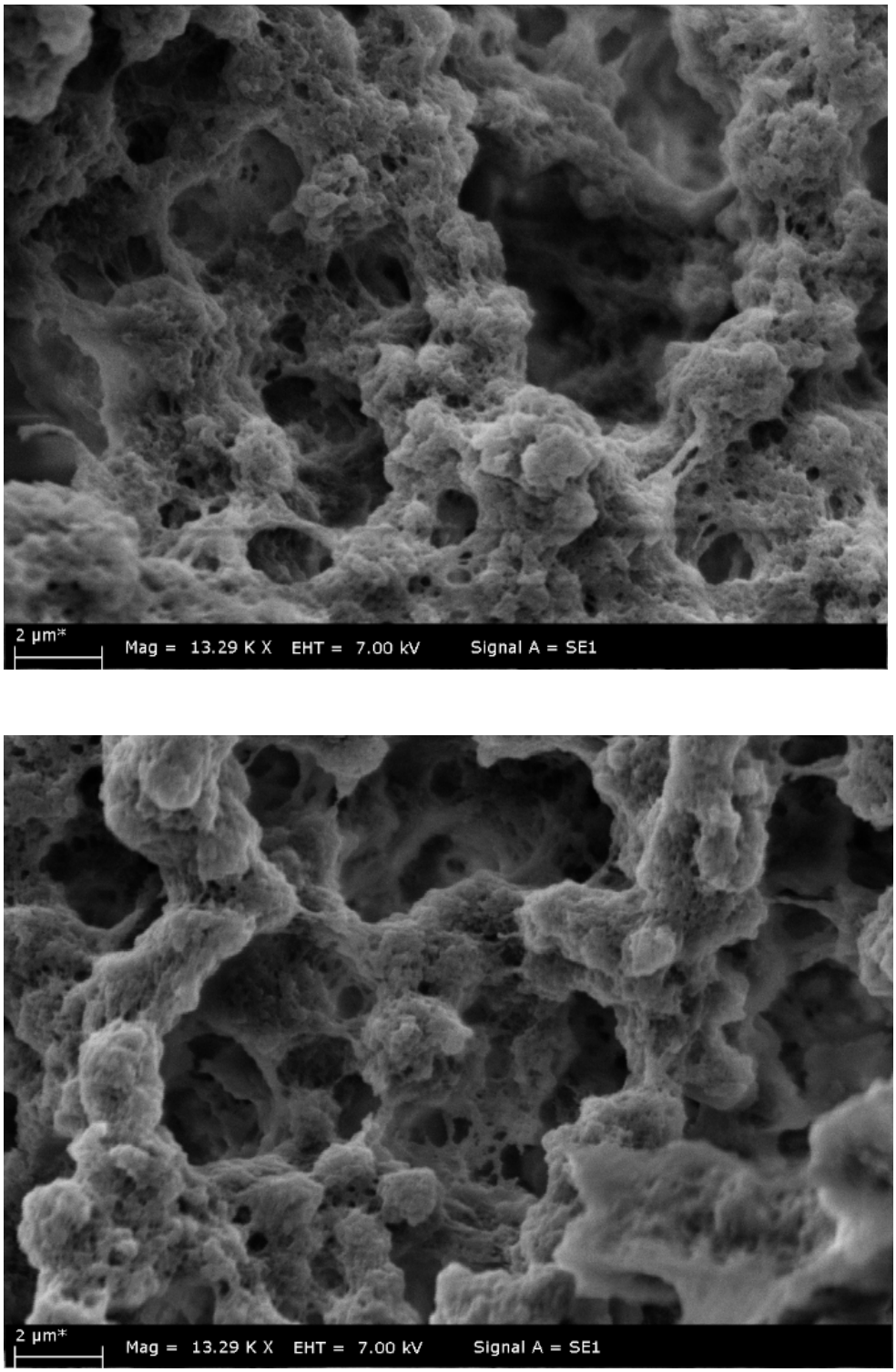


Figure 4.5. SEM photographs of PHEMAC-Fab cryogel.

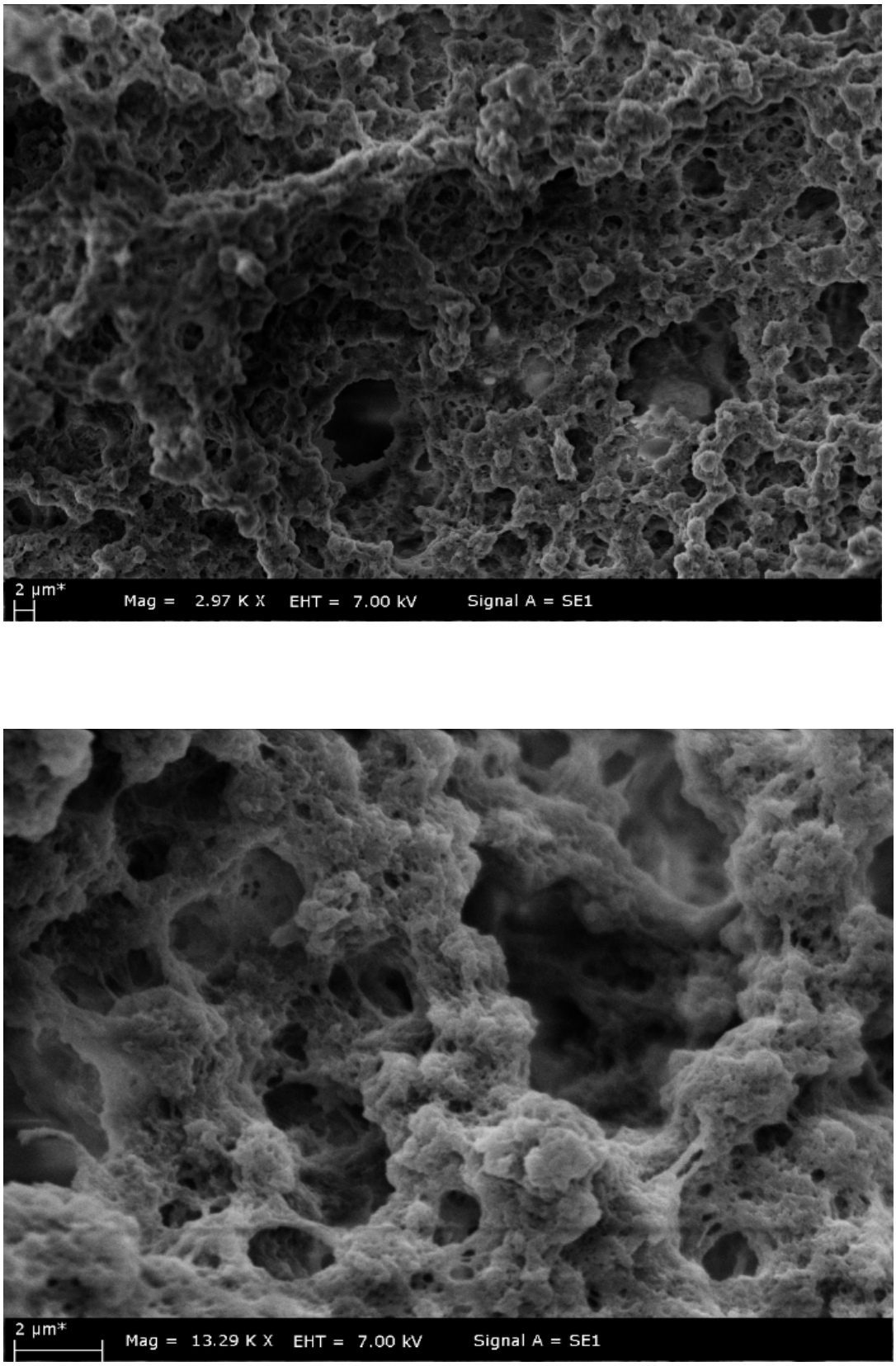


Figure 4.6. SEM photographs of PHEMAC cryogel.

4.1.5. Swelling Tests

The swelling ratio of the cryogels is increased with the increasing amount of the immobilized fragments. Maximum swelling ratio was observed for the immobilized column, which has fragments in its structure, and it absorbed water more than the other columns. Macroporosity of the columns were increased with increasing the immobilized fragments. The swelling rate of the cryogels results from the crosslinks. The two cryogels swelling ratios differ. The PHEMAC cryogel swelling grade is 143.3%. The swelling grade of PHEMAC-Fab cryogel is 265.8%. The swelling of the PHEMAC cryogels has many causes. One of these reasons is that the carboxyl group in the MAC polymer chain is hydrophilic. With the influence of the MAC monomer introduced into the structure, it is also important that the hydrophilic groups in the support material increase in number and interact more with the water molecules. The incorporation of the MAC monomer, eventually results in a smooth transition of the water molecules to the complex polymer chain and an increase in swelling rate. As the amount of non-immobilization increased, the macroporosity decreased and the maximum macroporosity ratio (41.6%) occurred in the non-immobilized column. Macroporosity in the immobilized probe is 54.3%. The cryogels prepared in the scope of the thesis is white, opaque, spongy and elastic.

4.2. HSA Adsorption from Aqueous Solutions

The effects of media pH, flow rate, concentration and other parameters on adsorption for adsorption experiments of aqueous solution albumin molecules were investigated, respectively. The obtained data were applied to Langmuir and Freundlich adsorption isotherm models and a suitable adsorption isotherm model was determined. Pseudo first and pseudo second-order kinetic equations were applied to determine the mechanisms on the adsorption of the albumin molecule, and a kinetic model suitable for adsorption was resoluted. In sequence to determine the reproducibility of the synthesized cryogel, the same cryogel was subjected to 10 adsorption-desorption cycles. At the last stage, the selectivity of the artificial blood sample solution against the albumin molecule was investigated using the same cryogel.

4.2.1. Effect of pH

Figure 4.7 shows the effect of pH on the immobilized PHEMAC-Fab. HSA adsorption was done at a pH of 4.0 to 8.0. The adsorbed HSA content showed the highest value at pH 5.0. On the other hand, the highest adsorption amount was 14.62 mg/g adsorbent. Since the isoelectric point of HSA is 5, the maximum adsorption was observed at pH 5.0. The adsorption capacity was reduced above and below pH 5.0. The capacity may be due to the electrostatic thrust among the same charged groups. At the isoelectric point, maximum protein adsorption is commonly monitored herein since proteins do not have net charges. Otherwise, the pH change can be due to the structural state of the HSA. The pH study with MAC monomers overlaps with previous studies in the literature.

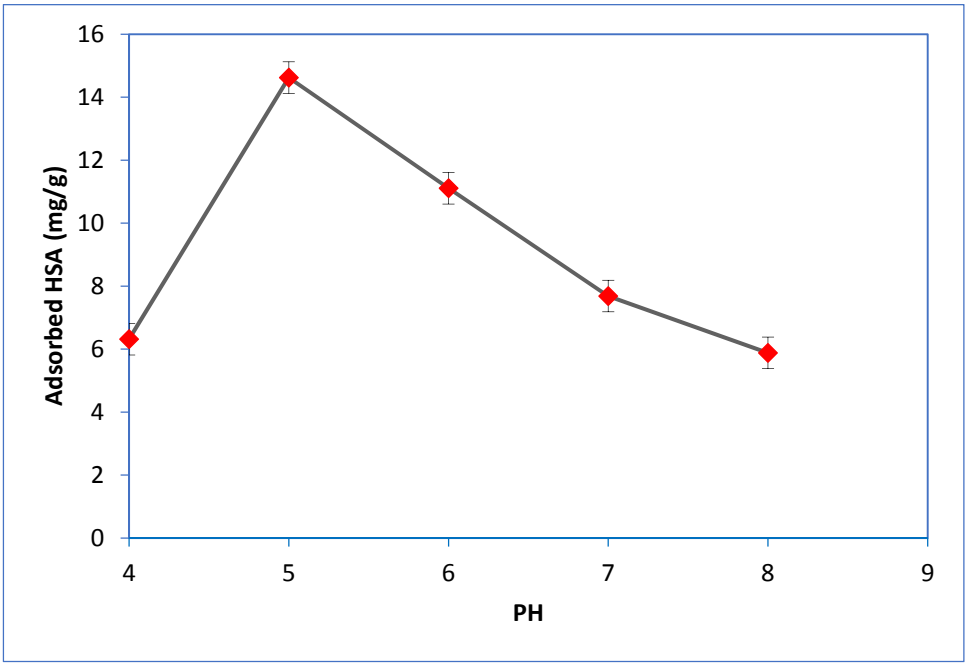


Figure 4.7. Effect of pH on HSA adsorption (HSA concentration 0.1 mg/ml, T:25°C).

4.2.2. Effect of Initial Concentration

Figure 4.8 shows the effect of the initial concentration on HSA adsorption. The concentration was in the range of 0.1-2 mg/mL. The highest adsorption amount of PHEMAC-Fab cryogel consists of 40 mg/g adsorbent. The amount of HSA adsorbed per unit mass of PHEMAC-Fab cryogel was first increased with the initial concentration of HSA and then reached a plateau at 40 mg/mL. Capacity increasing may be due to the interaction of Fab domain and the groups on the side chains of amino acids in HSA. The sharp slope observed at the initial stage of the adsorption isotherm of the PHEMAC-Fab cryogel column can be explained by the specific Fab cavities buried.

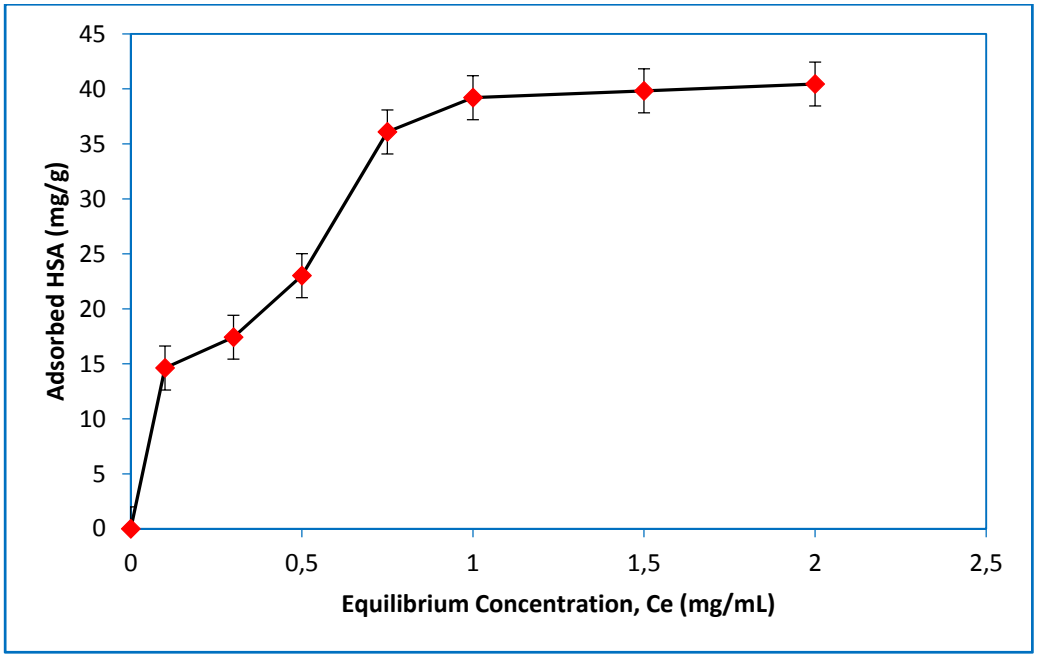


Figure 4.8. Effect of Initial concentration on HSA adsorption (pH: 5, T:25°C).

4.2.3. Effect of Flow Rate

Flow rate was controlled at 0.5-2.5 ml/min. However, the other experimental parameters are not changed. The effect is shown in Figure 4.9. At the lowest flow rate, the adsorption amount is about 17.43 mg/g adsorbent. The adsorption amount at the high flow rate is about 10.04 mg/g adsorbent level. A decrease in adsorption level is observed. This can be explained as; First, with increasing flow rate, the retention time of HSA in the cryogel of the molecule is diminished. Secondly, the increasing can be explained by the removal of the HSA molecules retained by the cryogel columns from

the attachment media. For this reason, HSA molecules require more time to disperse properly in the cryogel and give the highest peak at a flow rate of 0.5 mL/min.

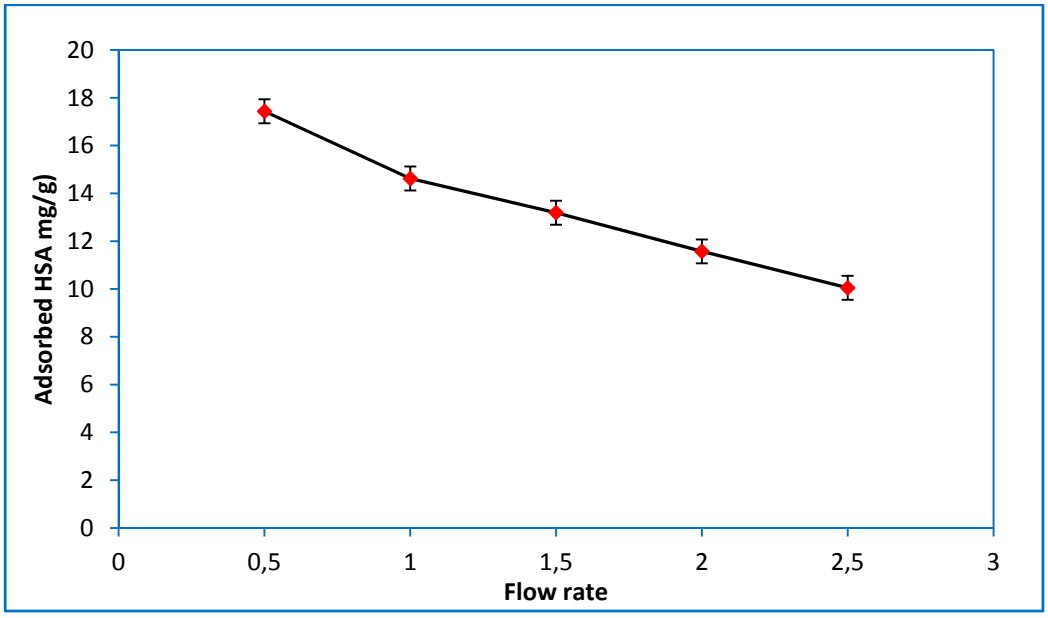


Figure 4.9. Effect of flow rate on HSA adsorption (pH: 5, initial concentration 0.1 mg/ml, T:25°C).

4.2.4. Effect of Adsorption Time

In figure 4.10 the time adsorption relation is shown. In sequence to regulate the adsorption stage of the prepared cryogel column solution to the adsorbent, the solution was agitated with adsorbent at a constant concentration and the times of equilibration were determined at certain time intervals. Adsorption-time graph was drawn from the values found. At first the adsorption is fast and the plateau is reached by the effect of this speed. Time study was done in 5-120 minutes. The amount of adsorption at 60 minutes is highest. It is 39.19 mg. As time progresses, more adsorption does not occur. The shape shows the effect of time on adsorption.

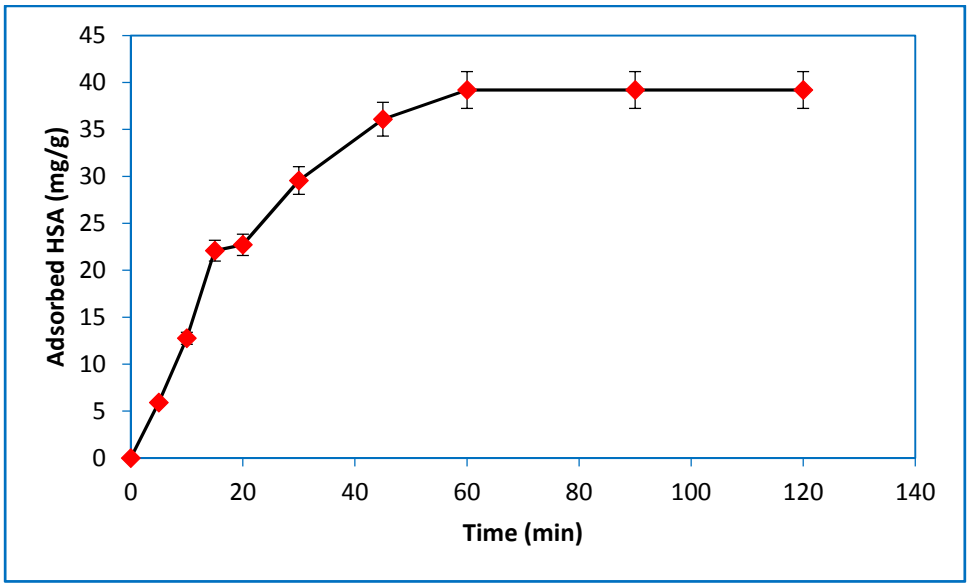


Figure 4.10. Effect of adsorption time on HSA adsorption (pH: 5, initial concentration 1 mg/ml, T:25°C).

4.2.5. Effect of Ionic Strength

The consequence of the salt concentration on the adsorption of HSA was explored using aqueous solutions of HSA containing NaCl salt at different ratios. Studies were conducted in the scope of 0.01-0.5 M NaCl. Significant increase in HSA adsorption was detected with increasing salt concentration, and the results are shown in 4.11. Since the PHEMAC-Fab cryogel structure has a hydrophilic surface, the interaction between cryogel and HSA molecules increases the adsorption capacity as the salt concentration increases. As the amount of salt increases, it causes an increase in electrostatic interaction. The maximum adsorption capacity was found to be 47.90 mg/g (0.05 M NaCl).

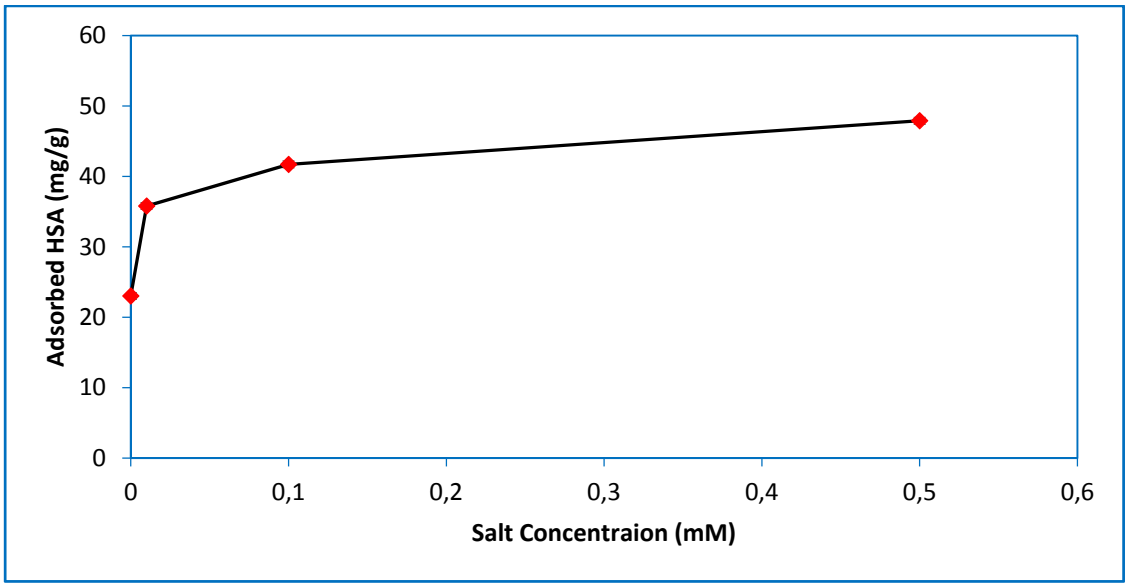


Figure 4.11. Effect of salt concentration on HSA adsorption (pH: 5, initial concentration 0.5 mg/ml, T:25°C).

4.2.6. Adsorption Isotherms

The adsorption process continues until the relationship between the concentration of the substance in the adsorbent surface and the concentration of the substance in the solution is achieved. Adsorption isotherm: kept constant without any temperature change to ensure the concentration in the solution. The task of adsorption isotherms is to define the function between the amount of ions adsorbed with the help of the concentration of dissolved ions in the equilibrium solution. The most commonly used isotherms are the Langmuir and Freundlich isotherms [98]. As the Freundlich and Langmuir models characterize adsorption from mathematically diluted solutions, it is known that adsorption data are compatible with these isoterms when studied at mean concentration intervals.

The application of Langmuir isotherm adsorption occurs on a homogeneous surface. The adsorbent surface has the same number of active areas as the same energy. The adsorption takes place only in the form of a layer and the lowest adsorption is the adsorption of molecules bound to the adsorbent surface in order to form a saturated layer. The simplest theoretical model for adsorption is the Langmuir model. The following equation

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

May be recovered as follows:

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

In this equation, Q represents the adsorbed amount of albumin (mg/g), C_{eq} is the equilibrium albumin concentration in the solution (mg/mL), b is the Langmuir constant (mL/mg) and Q_{max} is the adsorption capacity [99].

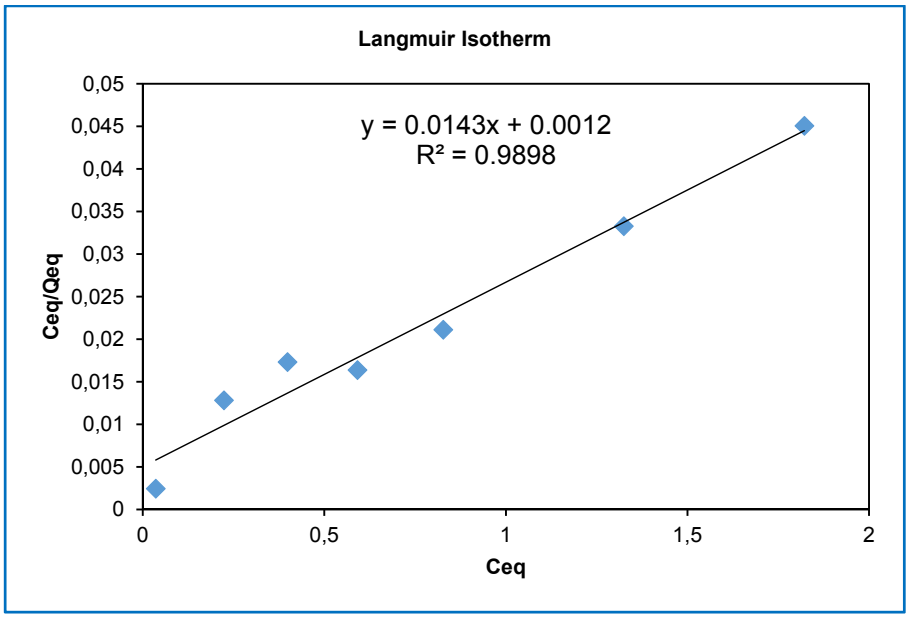


Figure 4.12. Langmuir adsorption isotherms of the PHEMAC-Fab cryogel.

The Freundlich model is the oldest model applied to adsorption on heterogeneous surfaces [100].

$$Q_{eq} = K_f . C_{eq}^{1/n} \quad (4.3)$$

In this equation, Q_{eq} adsorption amount (mg/g) and C_{eq} solution analytical change (mg/L). K_f and 1/n are the Freundlich constants showing the adsorption capacity and the adsorption strength, respectively. Finally, if they are equal;

$$\ln Q_{eq} = \ln K_f + 1/n \times \ln C_{eq} \quad (4.4)$$

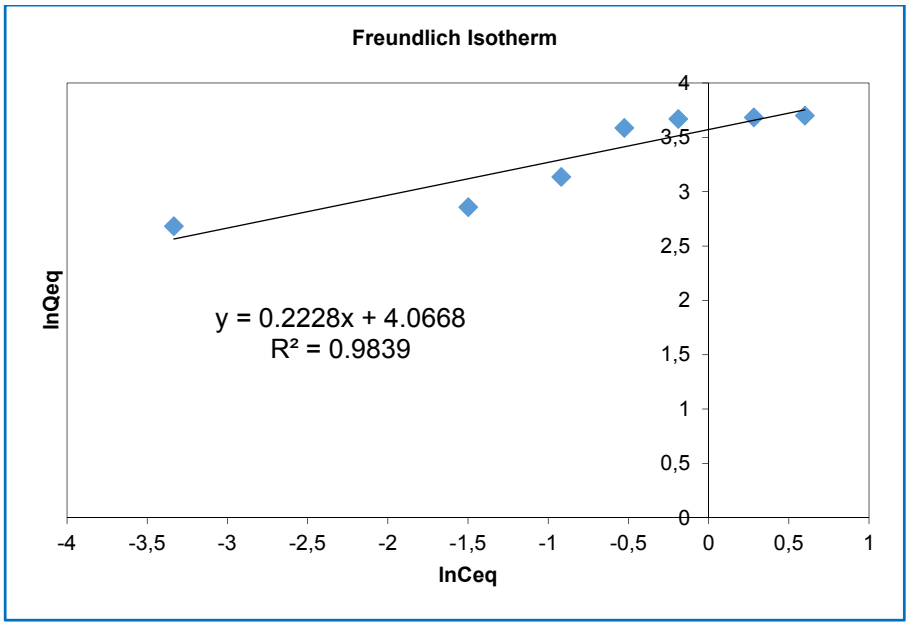


Figure 4.13. Freundlich adsorption isotherms of the PHEMAC-Fab cryogel.

Values are calculated and plotted for use in the Langmuir and Freundlich isotherms. This procedure with the aid of the correlation coefficients, can be explained by the Langmuir model. Q_{max}, the Langmuir persistent value, is based on empirical information. Another point is that albumin can be easily separated from the aqueous platform and preferential adsorption states.

4.2.7. Adsorption Kinetic

First and second order kinetic methods were applied to determine the factors that determine adsorption rate to empirical information [101].

Velocity equation is given as:

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

Where k_1 is the pseudo-first order adsorption rate constant (min^{-1}), q_{eq} and q_t represent the adsorbed amount (mg/g) at equilibrium time and at any time t, respectively. applying $q_t = 0$ at $t = 0$ and applying $q_t = q_t$ boundary conditions at $t = t$;

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

The final version of your equality can be confirmed as in thus;

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

Degree equality is below:

$$dq_t /d_t = k_2 (q_{eq}-q_t)^2 \quad (4.8)$$

Equally, we apply some conditions to equation x, $q_t = 0$ at $t = 0$, $q_t = q_t$ at $t = t$,

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

Linearity of equality;

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

In order for the quadratic kinetics to be applicable, the t graph against t/q_t must be

linear. The rate constant (k_2) and the equilibrium adsorption (q_{eq}) can be obtained from the cut point and inclination, seriatim.

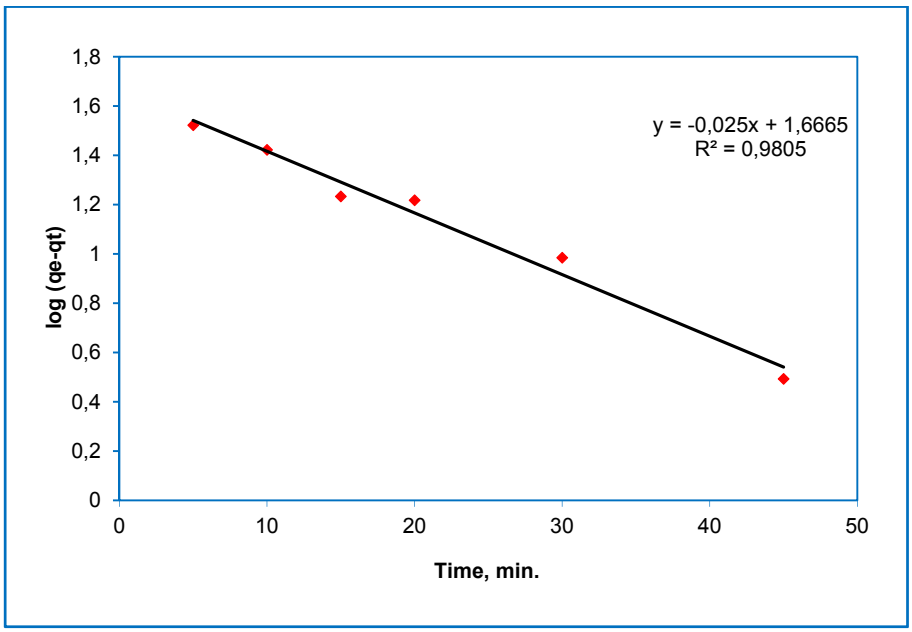


Figure 4.14. Pseudo-first order kinetic of the experimental data for the adsorbent.

From a calculational standpoint, the second-order kinetic method for albumin in aqueous solutions appears to be more suitable for cryogels attached to Fab particles. The theoretical Q_{eq} values employed are almost the same as for empirical values. The meaning is that chemical control of adsorption is provided. In other words, the equivalent adsorptive attitude in the so-called quadratic kinetic method is thought to be insignificant, and therefore the specific binding reaction between the chemical moiety and the albumin, the chemical adsorption, is supposed to control the kinetic behavior.

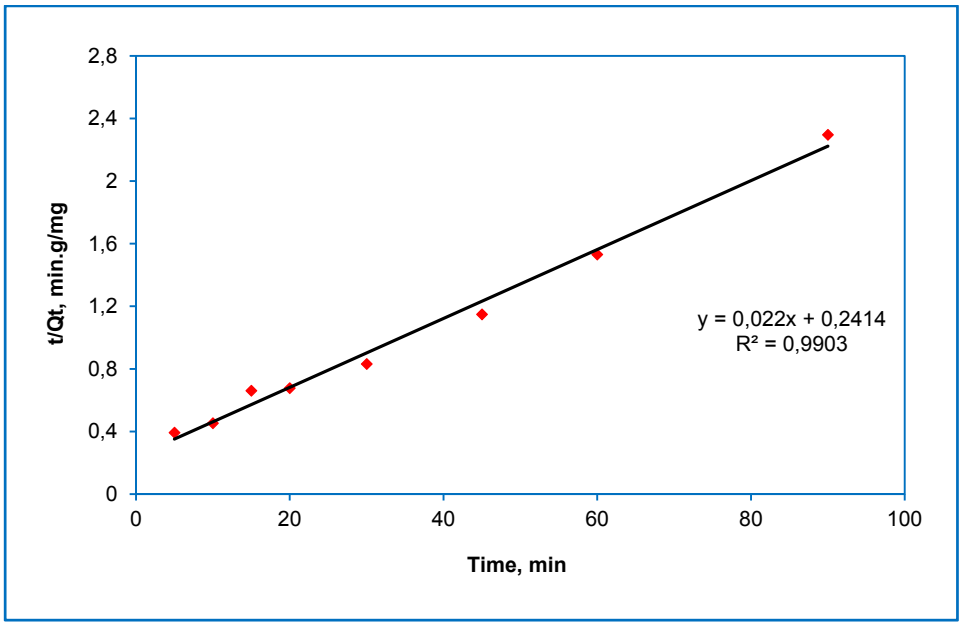


Figure 4.15. Pseudo-second order kinetic of the experimental data for the adsorbent.

4.3. Desorption Experiments

It is the last part of the desorption stage of the protein that has been adsorbed at the most appropriate time and at the highest concentration. Determination of the renatability of affinity adsorbents after each cycle is a requirement. As a result of many investigations and studies made, the desorption solution process completion of the 1M NaCl PHEMAC-Fab cryogel was provided at a flow rate of 0.5 mL/min to achieve the adsorption of the HSA in this work, and this treatment was applied for 2 hours. After that, 80% of adsorbed HSA almost all of which can be obtained at the end of 30 minutes of treatment with NaCl as a desorption agent. It was observed that the Fab fragment was not released during desorption processes in PHEMAC-Fab cryogel. It would have been correct to link randomly and properly directed antibody molecules as ligands to covalently immobilize PHEMAC cryogels.

4.3.1. Reusability

Cryogels, which have been used repeatedly, gradually deteriorate in pore structure and impurities accumulate in this structure. Thus, the adsorption ability of the adsorbent decreases. Because the pores are filled with impurities, the diffusion is reduced and the result is that it can not be regenerated. The physical power of the adsorbents must be large enough to withstand the recovery process. Scope of work, in order to show that there is no problem in the functioning of the cryogels, 10 cycles of adsorption/desorption are carried out by using the cryogels. The 10 cycles made have caused a loss of capacity of 8.49. Cryogel have not suffered any wear or tear in formal terms.

4.4. Albumin Adsorption from Artificial Plasma

The method we used was inspired in the way we had done before by SDS-PAGE to verify the purity of the eluted albumin (102). Prior to the experiment, artificial plasma was diluted 1/10. In Figure 4.16, HSA purified by 1/10 dilution before adsorption in lane 3, diluted HSA after adsorption on lane 2 and finally HSA purity of PHEMAC-Fab after elution on lane 1.

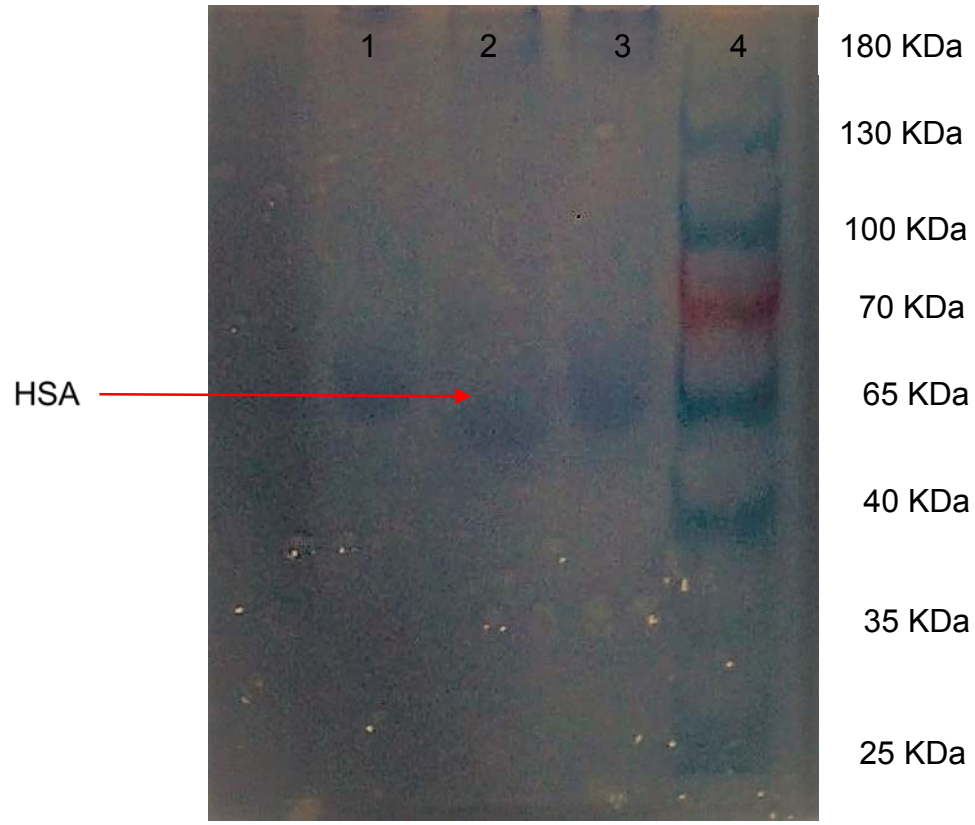


Figure 4.16. Sodium Dodecyl Sulfate Gel Electrophoresis (SDS-PAGE) images of the PHEMAC-Fab cryogel.

5. CONCLUSION

- The aim of this thesis to purify HSA by synthesizing immobilized cryogel columns with the Fab fragment.
- For this purpose firstly, MAC monomer (N-methacryloyl-(L)-cysteine methylester) was utilized in the resolution step of the albumin. The superiority of the MAC monomer synthesized from systeine and methacryloyl chloride is chemically consistent and biologically harmonious. The superiority of the MAC monomer is chemically consistent and biologically harmonious.
- PHEMAC-Fab and PHEMAC cryogels were made with an average diameter of 20-80 μm by weight.
- The specific surface area of the PHEMAC-Fab column is 38.6 m^2/g; The surface area of unimmobilized columns is calculated as 20.0 m^2/g.
- Structural examination with a scanning electron microscope revealed that PHEMAC-Fab had cryo-porous and interconnected porosities.
- The maximum rate of swelling was observed in immobilized columns and more water was absorbed from the other columns. The PHEMAC cryogel swelling grade was calculated to be 143.3%. The swelling grade of PHEMAC-Fab cryogel is 265.8%. One of these reasons is that the carboxyl group in the MAC polymer chain is hydrophilic. The macropore ratio in the immobilized column is 54.3% and macropore ratio of non-immobilized cryogel is 41.6%.
- Albumin selectivity depends on changes in the Fab region. Thus, instead of immobilizing the whole of the molecule, the functional Fab region responsible for antigen binding is immobilized. Thus, both the immobilization process is facilitated and the efficiency of the column is increased. PHEMA-based cryogels are biocompatible with proteins. In this study, HSA purification was carried out selectively from aqueous solution.

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CURRİCULUM VITAE

ID Information

Name Surname : Sabina Hüseynli

Place of Birth : : Baku/Azerbaijan

Year of Birth : 1988

Marital Status : : Single

E-mail : : sabinalarss@gmail.com

Address : Hacettepe University Biochemistry Research Lab 1.

06800 Beytepe, Çankaya/ANKARA

Education

High School : Baku School N19, Azerbaijan

B.Sc. : Azerbaijan State Pedagogical Univesity, Faculty of Science, Department of Chemistry, Azerbaijan

M.Sc. : Hacettepe University, Faculty of Science, Department of Chemistry

PhD : Hacettepe University, Faculty of Science, Department of Chemistry

Foreign Language and Level

Fields of Experience

Awards and Grades Received

Projects and Budget Produced from Thesis

Publications Produced From Thesis

Meetings that have been submitted with the thesis and / or poster presentation

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Student No:	N12240278
Department:	Chemistry
Program:	Ph.D.
Status:	☐ Masters ☒ Ph.D. ☐ Integrated Ph.D.

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APPROVED.

Prof. Dr. Adil Denizli