PREPARATION OF POLY(GLYCIDYL METHACRYLATE) BASED CRYOGELS FOR IMMUNOAFFINITY CHROMATOGRAPHY

İMMÜNOAFİNİTE KROMATOGRAFİSİ İÇİN POLİ(GLİSİDİL METAKRİLAT) TEMELLİ KRİYOJELLERLERİN HAZIRLANMASI

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Submitted to Institute of Science of Hacettepe University
as a Partial Fulfillment to the Requirements
for the Award Degree of Master of Science
in Chemistry
2014

This work titled "**Preparation of Poly(Glycidyl Methacrylate) Based Cryogels for Immunoaffinity Chromatography"** by **TURKAN MAMMADOVA** has been approved as a thesis for the Degree of Master of Science **IN CHEMISTRY** by the below mentioned Examining Committe Members.

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TURKAN MAMMADOVA

ABSTRACT

PREPARATION OF POLY(GLYCIDYL METHACRYLATE) BASED CRYOGELS FOR IMMUNOAFFINITY CHROMATOGRAPHY

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Degree of Master of Science, Department of Chemistry

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July 2014, 65 pages

Immunoaffinity chromatography (IAC) is a special type of bioaffinity chromatography that uses antibodies or antibody-related molecules as the stationary phase. This technique is used by many disciplines, particularly prefering in biochemistry on one-step separation and purification of functional protein. Also, immunoaffinity chromatography is used in antibody enrichment and separation of cells. IAC columns typically have strong and selective binding between antibodies and their target antigens. Antigen or antibody immobilized to the solid support and target antibody or cell is purified. Specific bioligands can be immobilized directly on polymeric material with simple acid base catalyst.

In our study, we prepared poly(glycidyl methacrylate) based cryogels and immobilized anti-insulin antibodies on porous surface of cryogels. We characterized cryogels with swelling test, Fourier transform infrared (FTIR) and Raman spectroscopy, and scanning electron microscopy. Also, effects of pH, initial insulin concentration, flow rate, and temperature on adsorption capacity were examined to optimize separation conditions.

Keywords: Immunoaffinity chromatography, anti-insulin antibody, polyglycidyl methacrylate, cryogels.

ÖZET

İMMÜNOAFİNİTE KROMATOGRAFİSİ İÇİN POLİ(GLİSİDİL METAKRİLAT) TEMELLİ KRİYOJELLERLERİN HAZIRLANMASI

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Danışman: Doç. Dr. Lokman Uzun

Teemmuz 2014, 65 pages

İmmünoafinite kromatografisi (IAK), biyoafinite kromatografisinin antikor veya antikor-temelli moleküllerin katı faz olarak kullanıldığı özel bir türüdür. Bu teknik, bir çok disiplin tarafından kullanılmakta olup özellikle biyokimyada fonksiyonel proteinlerin tek basamaklı ayrılması ve saflaştırılmasında tercih edilmektedir. Aynı zamanda immünoafinite kromatografisi, antikor zenginleştirilmesi ve hücre ayrılmasında da kullanılmaktadır. IAK kolonları antikor ve onların hedef molekülleri arasında kuvvetli ve seçici bağlama yapmaktadırlar. Antijen veya antikor bağlı katı destek malzemesi ve hedef antikor veya hücre saflaştırılır. Spesifik biyoligandlar polimerik malzeme üzerine basit asit/baz katalizörlüğünde bağlanabilir.

Çalışma kapsamında, poli(glisidil metakrilat) temelli kriyojeller hazırlanmış ve kriyojellerin gözenekli yüzeylerine anti-insulin antikorlarını immobilize edilmiştir. Kriyojeller şişme testi, Fourier dönüşümlü infrared (FTIR) ve Raman spektroskopisi ve taramalı elektron mikroskopu ile karakterize edilmiştir. Ayrıca; ayırma koşullarını optimize etmek için adsorpsiyon kapasitesine pH, başlangıç insülin derişimi, akış hızı ve sıcaklığın etkisi incelenmiştir.

Anahtar kelimeler: İmmünoafinite kromatografisi, anti-insülin antikoru, poliglisidil metakrilat, kriyojel.

ACKNOWLEDGEMENT

I am very greatly obliged and indebted to Prof. Dr. Adil Denizli, for his valuable guidance, professional advice, constrictive criticism and suggestion during my research.

Especially, I would like to thank Assoc. Prof. Dr. Lokman Uzun to for all contributions, supportings and helps in every steps of M.Sc. studies.

I wish to thank all of the members of BIOREG group for their help in laboratory, for their collaborating, assisting and providing me a pleasant atmosphere to work in. And consider it my duty to thank President of Ajerbaijan Republic for scholarship provides ("State Scholarship Program on Education of Azerbaijan Youth Abroad").

Finally, thanks for financial and moral support throughout my life to my dear mother Mehriban Mammadova, dear father Rasim Mammadov who made any sacrifice which is the largest shareholder.
Infinite thanks...

Turkan Mammadova
August 2014

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FIGURE LEGENDS

1. INTRODUCTION

The development of new techniques and methods for the separation and purification of proteins has been essential for many of the recent advancements in biotechnology and biomedicine. A wide variety of purification techniques are available today. Affinity chromatography is one of the most diverse and powerful chromatographic methods for purification of a specific molecule or a group of molecules from complex mixtures. Affinity chromatography based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. Specifically, immunoaffinity chromatography (IAC) relies on a solid stationary phase consisting of an antibody coupled to a chromatographic matrix or to magnetic beads, and harnesses the selective and strong binding of antibodies to their targets. Although the technique includes the separation of antibodies using immobilized antigens, it is more commonly performed for the identification, quantification, or purification of antigens. The power of immunoaffinity chromatography lies in the ability to separate a target biomolecule from a crude mixture such as a cell extract or culture medium with high degree of specifity and in high yield. IAC has also been extended for analytical purposes which include analysis of hormones and drugs in body fluids, analysis of toxins in food as well as analysis of herbicides and plant hormones in plant and food extracts.

Cryogels are interconnected supermacroporous gels prepared at sub-zero temperatures having applications in various research fields. Polymeric cryogels are efficient carriers for the immobilization of biomolecules and cells. 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, in combination with their osmotic, chemical and mechanical stability, makes them attractive matrices for chromatography of biological nanoparticles (plasmids, viruses, cell organelles) and even whole cells.

Insulin is one of the important polypeptide hormon produced by beta cells in the pancreas. The key functions of insulin are to stimulate anabolic reactions for

carbohydrates, proteins and fats. All controls the level of the sugar (glucose) in the blood. In addition cells in our body could not utilize glucose without insulin. All defects in both insulin production and resistance to it is action underline all types of *Diabetes mellitus*.

In this study, we prepared polyglycidyl methacrylate based cryogels and immobilized anti-insulin antibodies on their surface by a reaction between epoxy ring and amino groups. Then, the prepared cryogels were characterized by swelling test, scanning electron microscopy, Fourier transform infrared and Raman spectroscopy. After that, insulin separation conditions were optimized while considering effecting factors such as insulin concentration, flowrate, temperature, and pH.

2. GENERAL INFORMATION

2.1. Immunological Basis

The products of the adaptive immune response include a type of serum protein - immunoglobulin (Ig) or antibodies - and a type of blood cell - lymphocytes.

2.2. Immunoglobulins/Antibodies

An antibody, or immunoglobulin (Ig), is a serum glycoprotein. The immunoglobulins derived their name from the finding that they migrate with globular proteins when antibody-containing serum is placed in an electrical field. Immunoglobulins produced by plasma cells. Plasma cells mature from lymphocytes with called B lymphocytes (B cells), in response to an antigen. While B cells develop in the bone marrow of humans, a discrete organ (bursa of Fabricius) for B cell mellowing was firstly discovered in chickens, resulting in the term B cell. The term immunoglobulin (Ig) is generally used for all antibodies. But the term antibody is mostly used to show one particular set of immunoglobulins known to have specificity for a particular antigen. There are five classes of immunoglobulins (IgG, IgA, IgM, IgE, and IgD). All classes are characterized by differences in structure and function. Within two of the immunoglobulin classes are several subclasses: four subclasses of IgG and two subclasses of IgA [1].

IgG is the most common class of immunoglobulins, IgG constituting 80% to 85% of the immunoglobulins in the blood. Main function is accounting for most of the protective activity against infections. As a result of selective transport across the placenta, maternal IgG is the major class of antibody found in blood of the fetus and newborn. There are four subclasses of IgG: IgG1, IgG2, IgG3, and IgG4 [2].

IgA has two subclasses: IgA1 and IgA2. IgA1 is found mostly in the blood. In addition IgA2 is the dominant class, which is found in body secretions (secretory IgA). Secretory IgA is a dimer of two IgA molecules. Those identical molecules areheld together through a J chain and secretory piece. The secretory piece is

attached to IgA inside mucosal epithelial cells to protect these immunoglobulins against degradation by enzymes, which are in secretions [2].

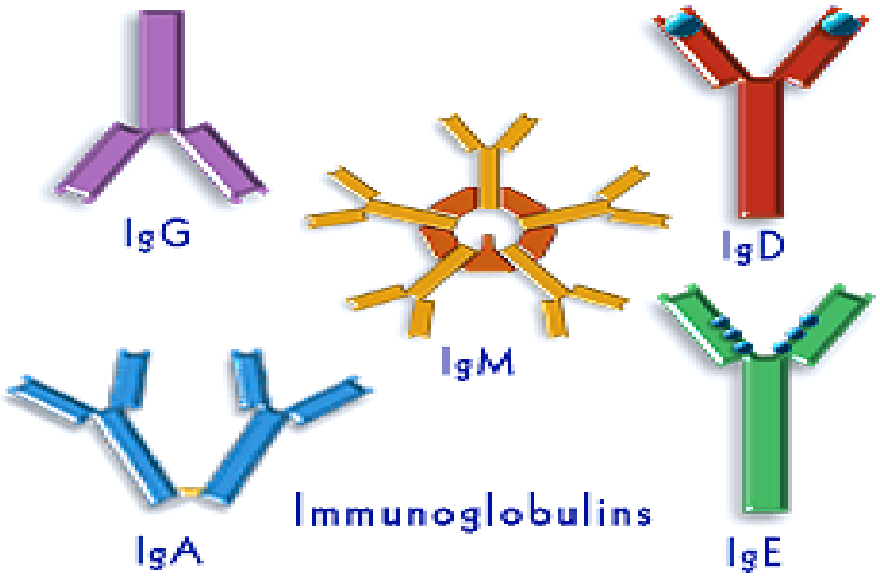


Figure 2.1. Five classes of immunoglobulins (adapted from [3]).

IgM is the largest immunoglobulin and usually presents as a pentamer (a molecule consisting of five identical smaller molecules) that is conjuctioned by a J chain. It is the first antibody produced during the primary response to antigens. IgM is synthesized early in neonatal life. Also its synthesis may be increased as a reaction to infection in utero [2].

IgD exists in low concentrations in the blood. Its first function is as an antigen receptor on the surface of early B cells [2].

IgE is normally at low concentrations in the circulation. It has very specialized functions as a mediator of many common allergic responses, also in the defense against parasitic infections [4].

2.2.1. Structure of immunoglobulins

All immunoglobulins have a four-chain structure as their basic unit. They are composed of two identical light chains (23kD) and two identical heavy chains (50-

70kD). They are separated functionally into variable domains. Main function is to bind antigens whereas constant domains define effector functions, such as activation of complement or binding to Fc receptors [5]. The variable domains are created by means of a complex series of gene rearrangement events. Then they can be subjected to somatic hypermutation after exposure to antigen to allow affinity maturation. Each variable domain can be separated into 3 regions of sequence variability termed the complementarity-determining regions (CDRs) and 4 regions of relatively constant sequence termed the framework regions. The 3 CDRs of the heavy chain are paired with the 3 CDRs of the light chain to form the antigen-binding site, as classically defined. Specifically the constant domains of the heavy chain can be enabled to allow changed effector function while maintaining antigen specificity. Here are 5 main classes of heavy chain constant domains. Also each class defines the IgM, IgG, IgA, IgD, and IgE isotypes. IgG can be split into 4 subclasses, IgG1, IgG2, IgG3, and IgG4, each with its own biologic properties. In addition IgA can also be divided into IgA1 and IgA2 [4].

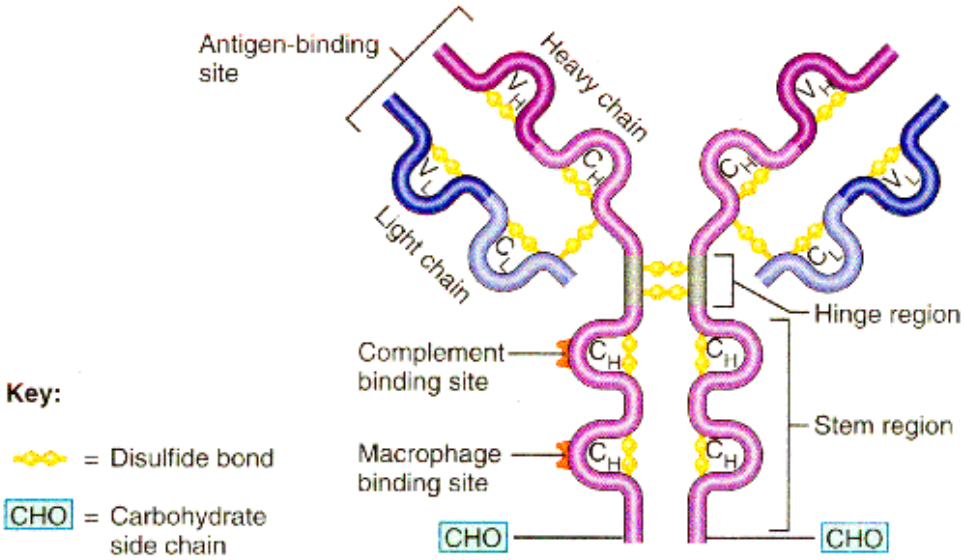


Figure 2.2. The structure of immunoglobulins (adapted from [6]).

2.2.2. Affinity and avidity of antibody binding

Antigen binding by antibodies is the primary function of antibodies and can result in protection of the host. Immunologists determine the *affinity* of an antibody for its antigen as the strength of the non-covalent association between one antigen-binding site (thus, one Fab arm of an antibody molecule) and one antigenic

epitope. Each immunoglobulin actually binds to a specific antigenic determinant. In opposite, the *avidity* of an antibody for its antigen is a measure of the total strength of all the Fab–epitope associations possible between the two molecules. Avidity relates to the fact that all antibodies are *polyvalent*; that is, all have more than one antigen-binding site and those that exist in polymeric forms have more than two. Moreover, most natural antigens are whole pathogens that are naturally polyvalent (although some of their component individual proteins are not). In the vast majority of cases, more than one contact is established between the antigen and an antibody binding to it [7].

2.2.2.1. Antibody affinity

The affinity of an antibody for an antigen is a measure of how many antibodies in a pool of identical Ig molecules have antigen bound to an antigen-binding site at any given moment (assuming that antigen is not limiting). The better the affinity, the more complexes will exist, and the longer complexes will endure. Affinity is determined mathematically according to the precepts of the Law of Mass Action as applied to a reversible chemical reaction.

When our hypothetical antibody and antigen solutions are mixed, then antibody and antigen begin to find each other and to form complexes at a rate dependent both on their relative concentrations, and on how easily the non-covalent forces holding them together form. The rate of complex formation is known as the constant. Because the binding of antigen to antibody is fully reversible, the complexes will also separate at a rate that depends on how resistant the non-covalent binding forces are to being disrupted. The rate of complex dissociation is noted as the k_{off} constant. After a definite period of time, chemical equilibrium will be established. Those complexes will be formed at the same rate as they are dissociated, so that the amount of unbound antigen remains constant. The relation of the concentrations of free and bound antibody at equilibrium is a measure of the affinity of the antibody for the antigen. So, when the rate of association between antigen and antibody (k_{on}) is divided by the rate of their dissociation (k_{off}), we get a measure of the affinity of an antibody for antigen represented by the equilibrium constant K [8]

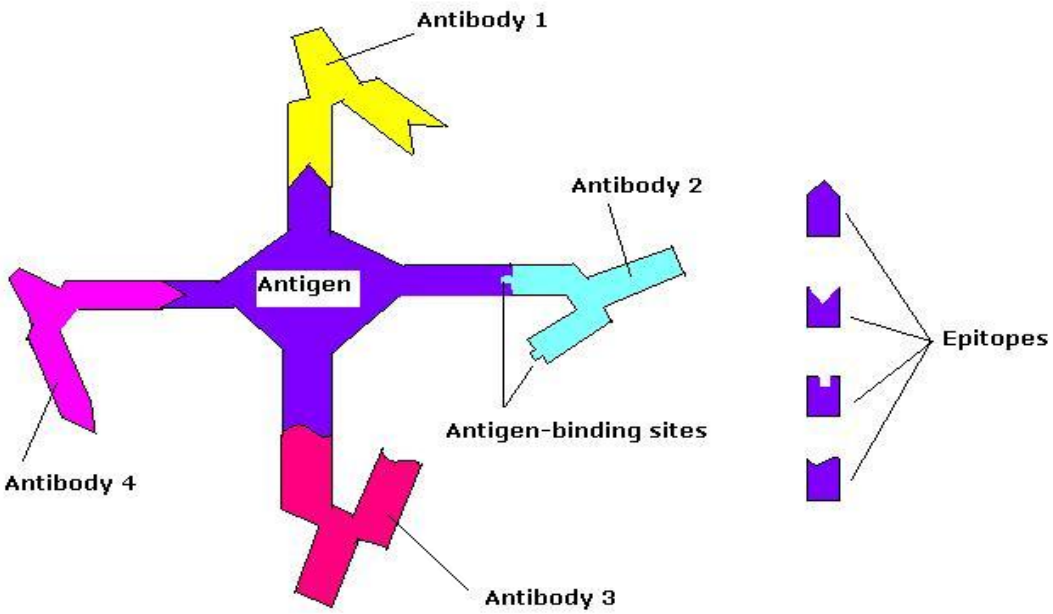


Figure 2.3. Antibodies fit precisely and bind to specific epitopes (adapted from [9]).

2.2.2.2. Antigen-antibody interaction

The general process of binding between an immobilized antibody (Ab) and a solution-phase antigen (Ag) in an immunoaffinity column (IAC) can be described by the following equations:

$$Ab + Ag \underset{kb}{\overset{ka}{\leftrightarrow}} Ab\text{-}Ag \quad (1.1)$$

$$Ka = ka / kb \quad (1.2)$$

Where k_a is the association equilibrium constant for the binding of Ab with Ag, Ab-Ag is the resulting antibody-antigen complex, and represents the molar concentration or surface concentration of each species at equilibrium. The term k_a is the second-order association rate constant for antibody-antigen binding and k_b is the first-order dissociation rate constant for the antibody-antigen complex [10].

The value of the association equilibrium constant for a typical antibody-antigen interaction under physiological conditions is in the range of 10^8 to 10^{12} M^{-1}. This results in extremely strong binding between analytes and immunoaffinity supports

under standard sample application conditions (i.e. a neutral pH application buffer with low-to-moderate ionic strength). For example, in a typical high performances immunoaffinity column (HPIAC) such values for K_a would lead to solute capacity factors of 10^3-10^7 and mean retention times of 1 day to several decades when using the sample application buffer for isocratic elution. The result is a chromatographic system that essentially has irreversible binding to the analyte under common sample injection conditions.

Although it is possible in IAC to perform isocratic elution on a reasonable time scale by using a competing agent in the mobile phase and low affinity antibodies, this does not work for the high or moderate affinity antibodies that are used in the vast majority of IAC columns. The only way that solutes can usually be eluted from these antibodies is to change the column conditions in order to lower the effective value of K_a (i.e. increase the relative value of k_b versus k_a) for antibody-analyte binding. The use of an acidic buffer (i.e. pH 1-3) and a step elution scheme is often used for this purpose. Another common approach is to perform gradient elution by gradually increasing the amount of a chaotropic agent (e.g., sodium thiocyanate or sodium iodide) that is present in the elution buffer. In some cases an organic modifier or denaturing agent (e.g., urea) might also be employed in step or gradient elution schemes. The proper choice of an elution buffer is important in analytical applications of IAC since it is usually desirable to elute the analyte as quickly as possible while avoiding any irreversible damage to the immobilized antibody support. This currently needs to be addressed on a case-by-case basis and is essential to consider if the same IAC column is to be reused for a large number of samples [7].

2.3. Affinity Chromatography

Affinity chromatography is a type of liquid chromatography in which a biologically related agent is used in a column as a stationary phase to purify or analyze the components of a sample. The ability of this method to selectively bind and purify its target compounds is based on the specific and reversible interactions present in many biological systems, such as the binding of a hormone to a receptor or an antibody to its antigen [11].

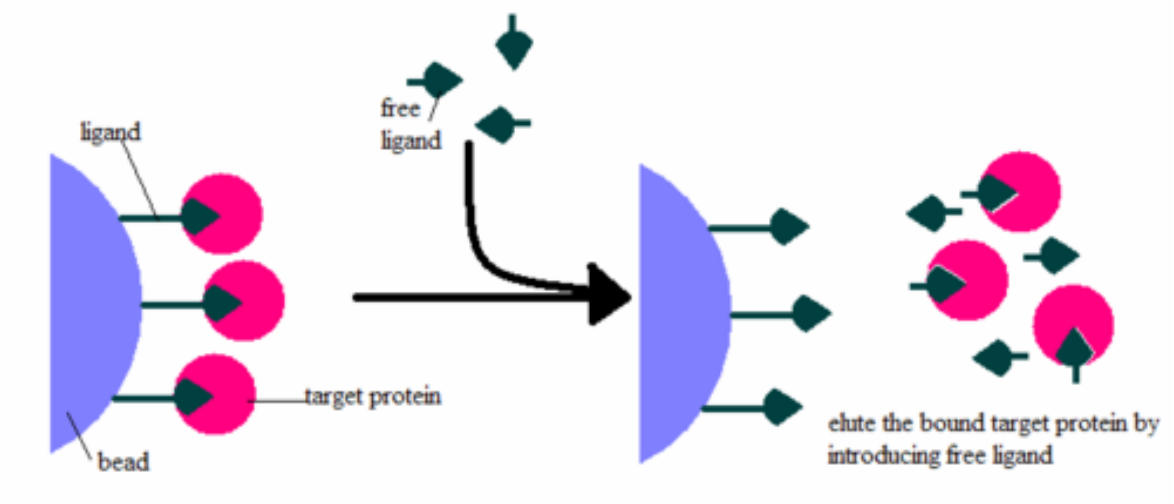


Figure 2.4. One way to elute the bound protein is to introduce free ligand that will bind to the target molecule (adapted from [12]).

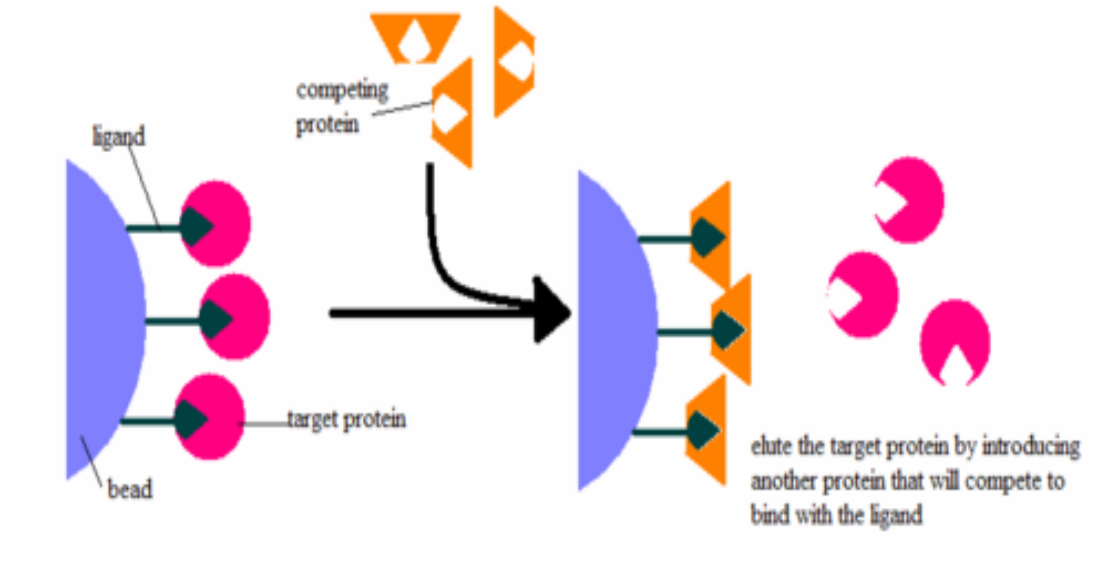


Figure 2.5. The bound protein can be eluted by introducing another protein that will outcompete the target protein and bind to the ligand (adapted from [13]).

To develop a method based on affinity chromatography, one of the pair of interacting species is first immobilized to a solid support, such as agarose beads or silica particles. The immobilized agent, called the affinity ligand, acts as the stationary phase for the affinity column. The other interacting compound is then injected onto the affinity column or applied in the presence of an application buffer, which allows the desired target to bind to the immobilized ligand [5]. After nonretained sample components have been washed from the column, the retained

target analyte is typically released in the presence of an elution buffer. If the retained compound has only weak or moderate binding to the immobilized ligand, it is also possible to use the application buffer to elute this target under isocratic conditions; this approach is known as weak-affinity chromatography (WAC). As the target elutes, it is collected for further use or analyzed by an on-line or off-line detector. The column is regenerated by reapplying the application buffer before the next sample injection [14].

2.3.1. Basic components of affinity chromatography

The effectiveness of any affinity separation depends mostly on the selection of the ligand immobilized in the column. Majority of the ligands which used in this technique are obtained from a biological source, as the antibodies, serum proteins, and lectins. Boronic acid, metal chelates, and triazine dyes, which are synthetic agents or inorganic molecules that can be employed as ligands in affinity chromatography [15].

The affinity ligands and amino acids can be divided into two main categories, such as highly specific ligands and general group specific ligands. Highly specific ligands are binding agents that recognize only one or a few closely related targets. These ligands are used when the goal is to isolate or separate a specific solute. This type of ligands include antibodies for the binding of antigens, substrates or inhibitors for separating or binding enzymes, and single-strand nucleic acids for the retention of complementary sequences of DNA or RNA. General group specific ligands are binding agents that retain a class of related molecules or structurally similar targets. This type of ligands include lectins, amino acids, dyes and boronates for binding carbohydrate-containing agents, some types of dyes for the retention of enzymes and proteins, and protein A or protein G for the binding of immunoglobulins [1].

One more important aspect to consider is the selection of the support material utilized for ligand attachment. This support material should have low nonspecific binding for sample components but be easy to modify for chemical activation and ligand attachment. Also, the support should be able to withstand the pressures

and flow rates used in the separation. In traditional affinity columns agarose is often useful support, which material consists of polymeric chains comprised of D-galactose and 3,6-anhydro-L-galactose [16]. Second common type of polysaccharide support is cellulose. Work has also been conducted in the use of affinity ligands with supports for high-performance liquid chromatography (HPLC), resulting in a method known as high-performance affinity chromatography (HPAC) or high-performance liquid affinity chromatography (HPLAC) [1]. Supports utilized in this latter method include silica particles, modified polystyrene supports, silica monoliths, and organic-based monoliths [17].

Some techniques are accessible for attaching a ligand to a chromatographic support. These techniques include both covalent and nonspecific immobilization methods [18]. Nonspecific immobilization techniques involve the physical adsorption of a ligand to a support [19]. One form of noncovalent immobilization is a biospecific adsorption, that uses the binding between two ligands, one of which has been previously bound to the support and the second of which is adsorbed to the first ligand and used to bind the final desired target. Covalent immobilization includes the chemical attachment of a ligand to a chromatographic support. In this technique, the support must first be activated for ligand attachment. These methods may involve several functional groups, such as amine, carboxyl, sulfhydryl, hydroxy, or aldehyde groups.

The feeding buffer used with an affinity column should have an appropriate pH and ionic strength to promote binding between the immobilized ligand and target [5]. The elution buffer is a mobile phase that disrupts the binding of the target with the ligand. This elution may be accomplished by changing the pH, ionic strength, or amount of organic solvent in the mobile phase, such an approach, referred to as nonspecific elution. That method is often used in analytical methods for the quick removal of a target from an affinity column. Respectively, a competing agent may be placed into the mobile phase to displace the target from the column by binding with either the target or ligand, thus preventing their further interaction, which is called biospecific elution. In addition biospecific elution is often slower than nonspecific elution. This method is useful when gentle removal and affinity purification of an active target are desired [20].

2.3.2. Affinity interactions

A good affinity ligand should possess the following characteristics:

1. The ligand must be able to form reversible complexes with the protein to be isolated or separated.
2. The specificity must be appropriate for the planned application.
3. The stability of the complex should be high enough for the formation of stable complexes at least sufficient retardation in the chromatographic procedure.
4. It should be easy to dissociate the complex by a simple change in the medium, without irreversibly affecting the protein to be isolated or the ligand.
5. It should have chemical properties that allow easy immobilization on to a matrix [21].

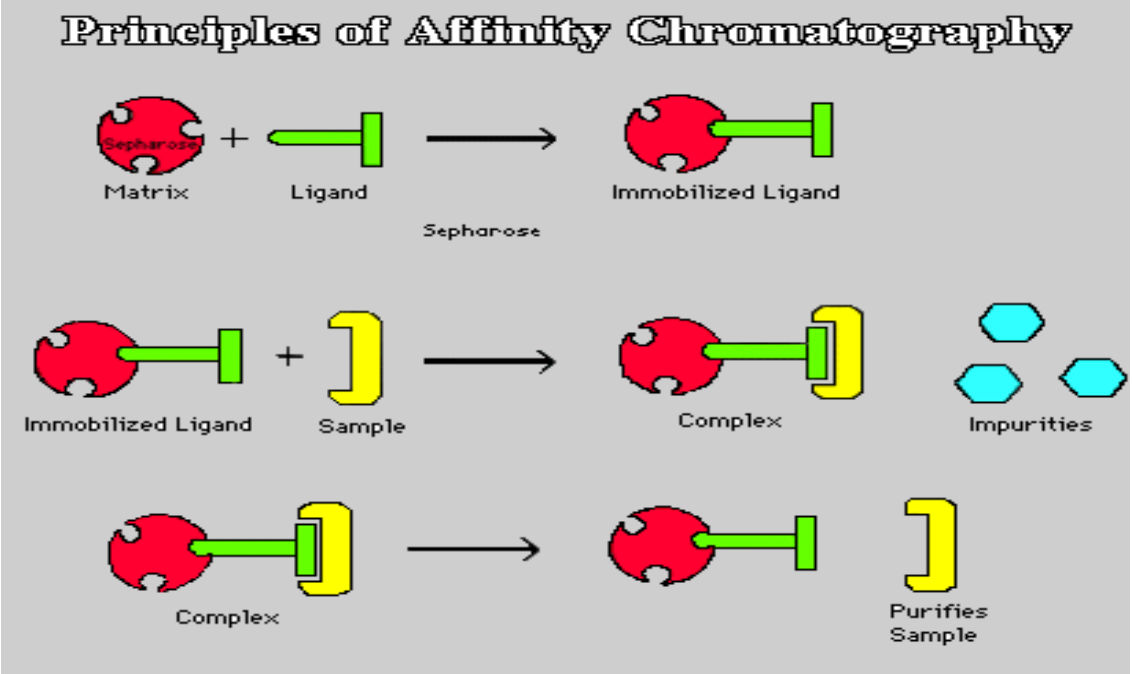


Figure 2.6. Steps for affinity chromatography. Top panel: bonding of ligand to Sepharose matrix results in immobilized ligand-matrix. Middle panel: Sample mixture is added to gel column. Complimentary proteins bind to ligand-matrix complex, while the non-binding impurities are washed out. Bottom panel: In order to remove the bound proteins and retrieve them from the gel, a stronger second wash is run through (adapted from [22]).

2.4. Immunoaffinity Chromatography

Immunoaffinity chromatography (IAC) is a special type of bioaffinity chromatography. This specific technique uses antibodies or antibody-related molecules as the stationary phase. The main characteristic of IAC columns having strong and selective binding between antibodies and their target antigens. This speciality, as well as the ability to produce antibodies against a wide range of targets, has made IAC popular for use in both the purification and analysis of chemicals in a variety of complex samples [23]. The most prevalent technique used in IAC is the one elution model [24]. In this method, the sample injected onto the IAC column under conditions in which the target has a strong affinity to antibodies immobilized within the column.

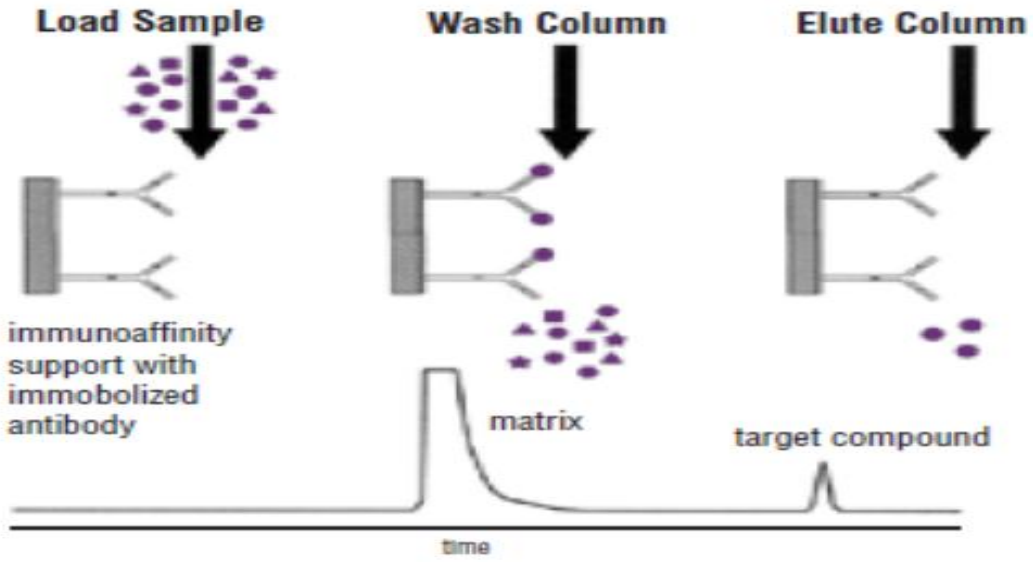


Figure 2.7. Schematic diagram of the stages involved in a typical immunoaffinity analysis: loading the sample, washing the column to remove matrix components and impurities and eluting the target compound (adapted from [25]).

Only the target and closely related compounds are retained on the IAC column. The other samples are not retained. After this process an elution buffer is applied to the column to dissociate the bound target from the column. In addition, it is often easy to obtain baseline resolution between the nonretained and retained peaks by changing the time at which the elution buffer is applied. Proteins, glycoproteins, carbohydrates, lipids, bacteria, viral particles, drugs, and environmental agents have all been isolated by IAC through the use of this format [26]. The main

characteristic of this approach is its speed and simplicity, especially when it is carried out as part of an HPLC system. Also, the use of antibodies or related binding agents with HPLC supports is referred to as high-performance immunoaffinity chromatography (HPIAC) [27].

Chromatographic immunoassays represent another significant mode of IAC. This technique uses immobilized antibodies, or antigens in a column to perform various competitive or noncompetitive immunoassays. Discovery in these methods involves the use of a labeled antibody or labeled analyte analog to indirectly measure the amount of a target in a sample. The detection format may be established on absorbance, fluorescence, chemiluminescence, electrochemical activity, radioactivity, or thermal measurements. The label may consist of an enzyme, fluorescent tag, chemiluminescent agent, liposome, or radioisotope, among other possibilities [27].

Competitive binding immunoassays include competition between a target in a sample and a labeled analyte analog (for a limited amount of antibodies). Then they have been allowed to compete for binding sites on the antibodies. So the bound and free parts of the target and labeled analog are separated. The amount of labeled analog in the bound or free fraction is then measured. The absence of any target results in the maximum amount of labeled analog in the bound fraction. An increase in the amount of target results in a decrease in the level of this bound labeled analog, which provides a signal that is indirectly related to the amount of target in the original sample [14].

Some formats are accessible for a competitive binding immunoassay in IAC. In the last injection format, the sample is injected first, after followed by injection of the labeled analog. A displacement immunoassay involves the adsorption of a large amount of labeled analog to an immobilized antibody column, followed by the displacement of some of this labeled analog upon sample injection. Simultaneous injection immunoassays have been used in the analysis of human serum albumin (HSA), IgG, theophylline, caffeine, and atrazine. Sequential injection immunoassays have also been used to measure HSA, IgG, and atrazine. When

displacement immunoassays have been employed for the analysis of cocaine, benzoylecgonine, di- and trinitrotoluene, and cortisol [1].

Noncompetitive immunoassays can be accomplished using IAC or HPIAC. There are two main types of noncompetitive immunoassays. One of the one-site immunometric assay and other the sandwich immunoassay, or two-site immunometric assay [1]. A one-site immunometric assay is carried out by incubating a sample with a known excess of labeled antibodies that are specific for the target. This mixture is then applied to a column containing an immobilized analog of the target. The column is used to capture the nonbound portion of the labeled antibodies while allowing antibodies that are bound to the target to pass through unretained. Measurement of the target is performed indirectly by monitoring either the change in response for the unretained labeled antibodies or by monitoring the amount of retained antibodies captured by the column and released during the elution step. This type of analysis has been utilized in the measurement of digoxin, thyroxine, and 17b-estradiol [1]. Another use of IAC columns is to monitor a target that is eluting from other types of columns. This approach is referred to as postcolumn immunodetection. The technique typically involves the use of a postcolumn reactor and an immobilized antibody or antigen column attached to the outlet of an analytical HPLC column. As the target elutes from the analytical column, it is mixed with an excess of labeled antibodies that can bind to the target. The remaining, unbound labeled antibodies are removed from this mixture by using a column that contains an immobilized analog of the target. The unretained labeled antibodies that are already bound to the target pass through the analog column and give a signal proportional to the target’s concentration. This type of detection has been used for various applications including the measurement of growth-hormone-releasing factor, digoxin, digoxigenin, and human granulocyte colony-stimulating factor [28].

2.5. Insulin

Insulin is one of the most known hormones. This hormone is the main remedy for the treatment of *Diabetus mellitus*. Insulin is a polypeptide hormone that is essential for the supply of energy to the cells of the body. Insulin's name is derived

from the Latin *insula* for "island" [29]. Insulin's structure varies slightly between species of animals. Insulin from animal sources differs somewhat in "strength" (in carbohydrate metabolism control effects) from that in humans because of those variations. Porcine insulin is especially close to the human version [30]. The main functions of insulin are to stimulate anabolic reactions for carbohydrates, proteins, and fats, all of which have the metabolic consequences of producing a lowered blood glucose level [31].

Insulin is secreted by group of beta cells in the pancreas, and is central to regulating carbohydrate and fat metabolism in the body [29]. Insulin produced by the pancreas in response to the detected increase in blood sugar. It causes cells in the skeletal muscles, and fat tissue to absorb glucose from the blood. Most cells of the body have insulin receptors, which bind the insulin that is in the circulation.

The formation of native insulin from proinsulin follows two steps, the first step involves folding and the formation of correct disulfide bridges and the second step involves proteolytic cleavage with subsequent release of the C-peptide. It was first proposed that the C-peptide is essential for the correct assembly of the A- and B-chains, but there is now sufficient evidence that the insulin A- and B-chains alone contain enough structural information to form native insulin (*Wang and Tsou, 1991*) [29].

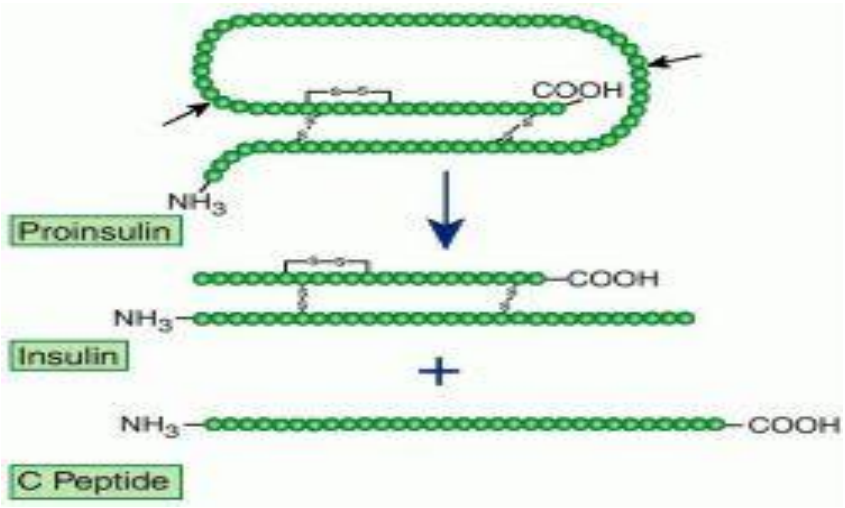


Figure 2.8. Insulin. The precursor proinsulin is cleaved internally at two sides *(arrows)* to yield insulin and C peptide (adapted from [32]).

The molecular origins of insulin go at least as far back as the simplest unicellular eukaryotes. Apart from animals, insulin-like proteins are also known to exist in Fungi and Protista kingdoms [33]. The human insulin protein is composed of 51 amino acids, and has a molecular weight of 5808 Da. It is a dimer of an A-chain and a B-chain, which are linked together by disulfide bonds.

2.5.1. Recombinant insulin market

Diabetus mellitus, which is caused by disorders in the secretion and synthesis of insulin, has been estimated to be the third-largest cause of death in industrialized countries [34]. Insulin is a polypeptide hormone secreted by pancreatic cells. The most widely produced form of recombinant insulin is recombinant human insulin, designed for use in people with insulin deficiencies. The development of recombinant DNA technology allowed for rapid, high-level production of insulin, and this technology has been widely used to treat diabetes. Human recombinant insulin (HRI) is the first hormone that was produced in a large scale. It is manufacturing takes about 25 000 kg per year world wide nowadays. Production of human insulin by recombinant *Escherichia coli* was first accomplished and commercialized in 1982 by the collaborative work of Genentech (San Francisco, USA) and Eli Lilly (Indianapolis, USA), but the big demand for affordable human insulin still requires the development of more cost efficient production processes [29]. Ninety years after the discovery of insulin, the therapy remains a staple of treatment for both type 1 and type 2 diabetes. Fueled by the growing prevalence of diabetes and the progressive nature of the disease, the insulin market has grown at a healthy 7% annual rate by volume during the past decade. Insulin reagents have shared almost half of the biomedical markets. Though the markets fluctuate, the sales still upswing. According to statistics, the top 3 insulin drugs are recombinant human insulin, insulin aspart and insulin glargine, whose market scale has exceeded 0.2 billion. Meanwhile, global insulin sales reached $16.7 billion in 2011, up 12.5% since 2010. U.S. insulin sales in 2011 totaled $8.3 billion, a 14.9% increase compared to 2010 [35].

2.5.2. Discovery of insulin

Insulin was the first hormone identified and its discoverers had been awarded with Nobel Prize. When they examined the pancreases of the dogs several weeks later, all of the pancreas digestive cells were gone and the only thing left was thousands of pancreatic islets. Then they isolated the protein from these islets and behold, they discovered insulin.

On 11th January 1922, clinicians at Toronto General Hospital injected a 14 year old, severely diabetic boy (*Leonard Thompson*) with 15 mL of pancreatic extract made by Banting and Best. This clinical test was a failure. The injection caused only slight reductions of glycemia and glycosuria. This method had no effect on ketoacidosis or the patient's subjective presentation, and resulted in the formation of a sterile abscess. These results were not as encouraging as those obtained by Zuelzer in 1908. Later Banting wrote a treatment, which was immediately discontinued. On January a new series of injections began. Thompson responded immediately. After injection his glycosuria almost disappeared, his ketonuria did disappear, his blood glucose dropped to normal. Later he became brighter and stronger. For the first time in history there was clear unambiguous evidence that scientists were able to replace the function impaired in diabetes. This was the demonstration of the isolation of the internal secretion of the pancreas.

The first successful insulin preparation came from cows and later pigs. From the 1922 to 1972 the only available insulin was purified from the pancreases of pigs and cows. The bovine and porcine insulin were purified, bottled and sold. But some patient's could develop an allergy or other types of reactions to the foreing protein. This insulin was quite valuable in prolonging the lives of diabetics who otherwise would have slowly died because glucose was unavailable to the human body cells. In the 198-'s protein technology had advanced to the point where we could make human insulin. The technology, which made this approach possible, was the development of DNA techniques. In simple terms, the human gene which codes for the insulin protein was cloned and then put inside of bacteria. Big vats of bacteria now make tons of insulin. And from this, pharmaceutical companies can isolate pure human insulin. Since the discovery of the fact that insulin produced by the pancreas and is responsible for lowering blood sugar levels [1] until the

present time has already passed more than 80 years. Nevertheless, to this day, this hormone induces great interest. *Diabetus mellitus* take the third place after cardiovascular diseases and cancer [36].

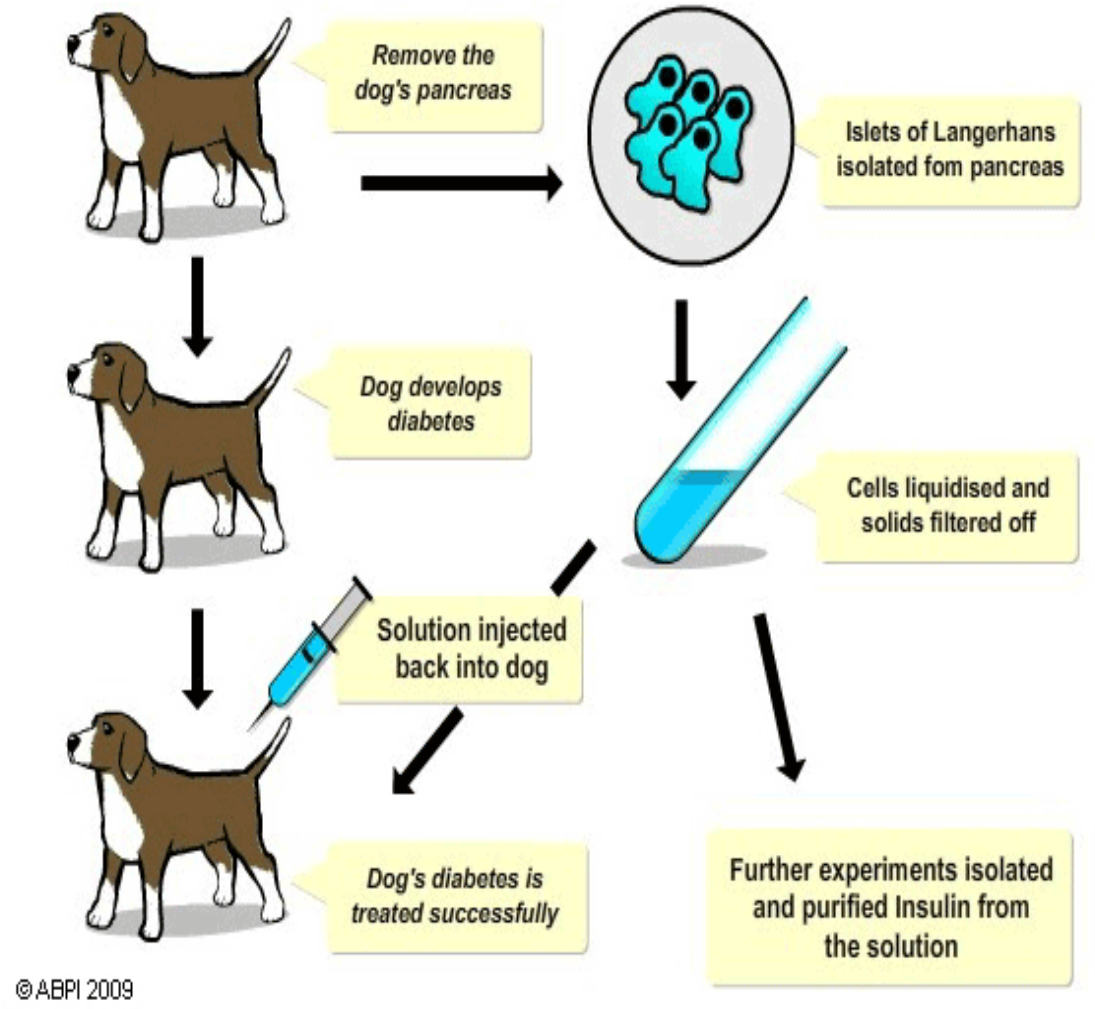


Figure 2.9. Banting and Best's discovery of insulin (adapted from [37]).

2.5.3. Structure of insulin

Like most of the other hormones, insulin is a protein comprising of 2 polypeptide chains A (with 21 amino acid residues) and B (with 30 amino acid residues), which are linked by disulphide bridges. In addition, A-chain contains an intra-chain disulphide bridge-linking residue 6 and 11. C-chain, which connects A and B chains is liberated along with insulin after breakdown of proinsulin. Insulin monomers aggregate to form dimers and hexamers [38].

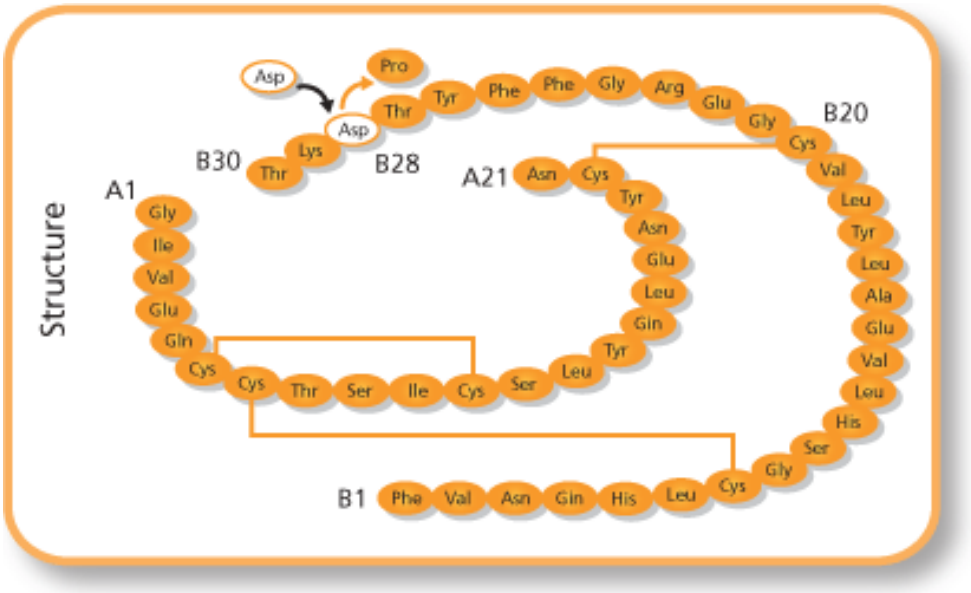


Figure 2.10. Structure of insulin (adapted from [39]).

2.5.4. Biosynthesis of insulin

Insulin is synthesized in the beta cells of pancreas in the form of preproinsulin which is the ultimate precursor and gene for the same is located on chromosome 11 close to that for insulin like growth factor-2 (IGF-2). Within a minute after synthesis it is discharged into cisternal space of rough endoplasmic reticulum where it is cleaved into proinsulin by proteolytic enzymes. Proinsulin with a C (connecting) chain linking A and B chains is then transported by microvesicles to the Golgi apparatus. Proinsulin is released in vesicles. Conversion of proinsulin to insulin continues in maturing granules through the action of prohormone convertase 2 and 3 and carboxy peptidase H4 maturing granules are translocated with the help of microtubules and microfilaments [40].

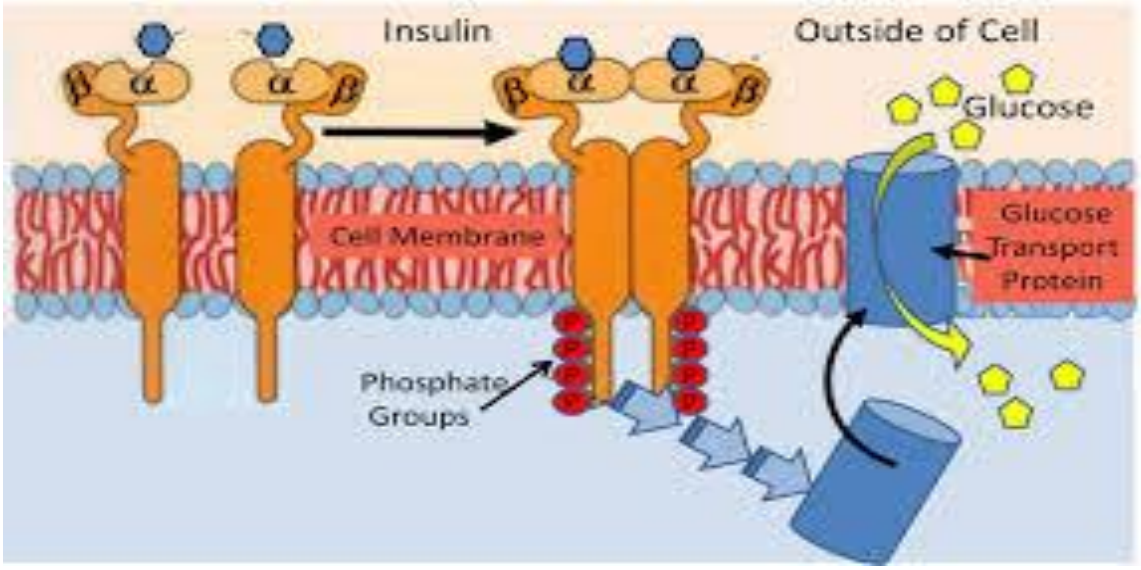


Figure 2.11. Insulin action (adapted from [41]).

2.5.5. Insulin secretion

Insulin is secreted from the beta cells in response to various stimuli like glucose, arginine, sulphonylureas though physiologically glucose is the major determinant. Various neural, endocrine and pharmacological agents can also exert stimulatory effect. Glucose is taken up by beta cells through GLUT-2 receptors. After entering the beta cell, glucose is oxidized by glucokinase, which acts as a glucose sensor. Glucose concentration below 90 mg/dl does not cause any insulin release. At such substimulatory glucose concentrations, K+efflux through open KATP channels keeps the beta cell membrane at a negative potential at which voltage-gated Ca^{2+} channels are closed. As there is increase in plasma glucose, glucose uptake and metabolism by the beta and non-sulphonylurea drugs act as insulin secretagogues by closing K ATP channels bypassing the beta cell metabolism. Diazoxide is a K channel opener and inhibits insulin secretion, independent of blood glucose levels [42].

Despite the fact that all humans lead different lifestyle, concentration of blood glucose in each of supported maintained within a quite narrow range, and varies little during the day. Normal glucose concentrations in blood plasma is from 3 to 8 mm. 15-30 minutes after taking food sugar level in human blood increases. Increasing the concentration of insulin in blood is the response of the organism to

increase the level of glucagone, a hormone, in turn, reduces the content of glucose to basal levels approximately in 2 hours after taking food [43].

Historically, the first method of producing insulin for therapeutic goals is the allocation of this hormone analog from natural sources (the Langerhans islands of the pancreas of cattle and pigs). In the 20th of the last century, it was found that bovine and porcine insulin (which are closest to human insulin in structure and amino acid sequence) in human organism show activity comparable to human insulin. After that, a long time for the treatment of patient first type of diabetes used cattle or pigs insulin. However, in course time, in some cases of human body begins to accumulate antibodies to bovine and porcine insulin causing unwanted allergic reactions. Especially that needs of insulin can not be covered by insulin of animal, because of limited resource base [44].

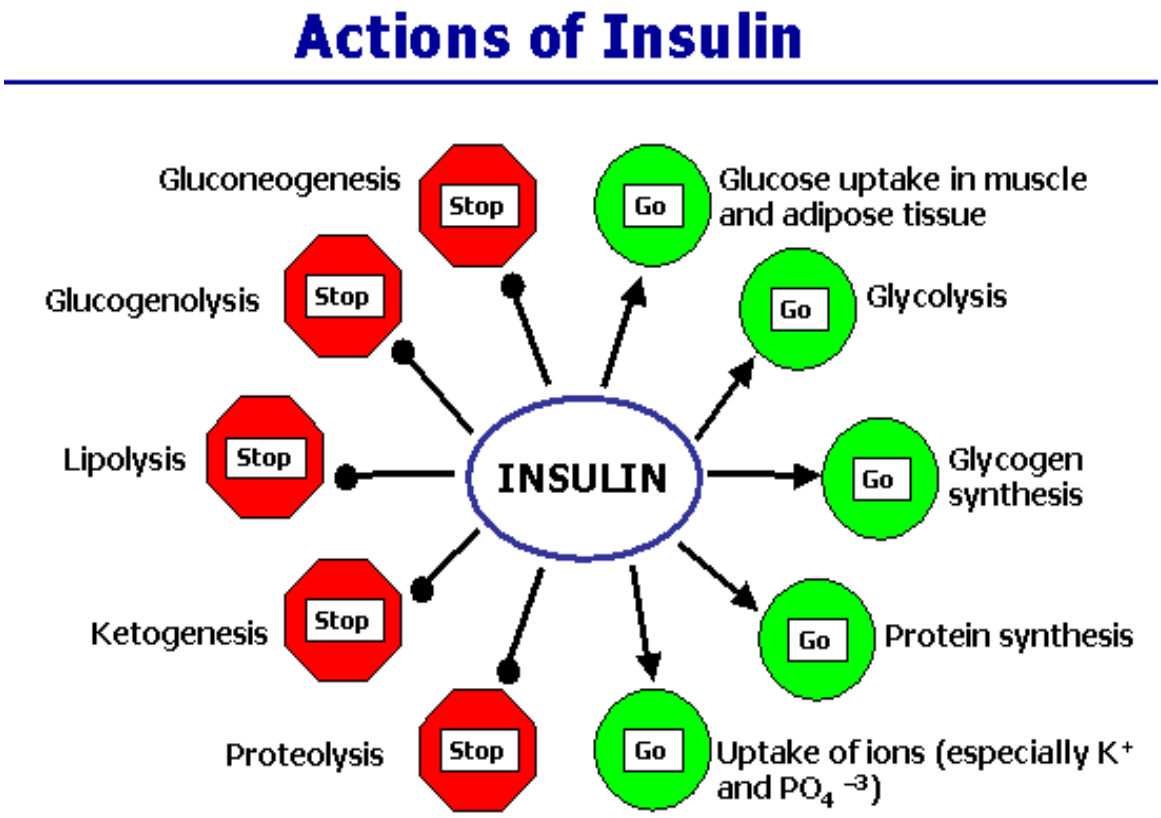


Figure 2.12. The bases of metabolic effects of insulin (adapted from [45]).

Insulin is a polypeptide hormone that plays a key role in metabolic processes. The main function of insulin is activating anabolic and inhibiting catabolic processes in muscle, liver and adipose. In particular, insulin increases the rate of glycogen

synthesis, fatty acids, proteins, and also stimulates glycolysis. Essential value is the penetration stimulation of glucose, some other sugars and amino acids into muscle cells and adipose. Contributing to transport glucose into the specified cells, it reduces the hormone levels in the blood (known as the hypoglycemic effect). Insulin inhibits catabolic processes such as glycogen decay [46].

Type 2 diabetes is result of progressive β-cell dysfunction in a chronic state of insulin insensitivity. Such β-cell disturbance is characterized by kinetic abnormalities, including abolition of first-phase, and reduction and delay of late-phase insulin secretion, and reduction of the rapid secretory spikes characteristic of a normal insulin secretory pattern [34]. Qualitative disturbances of insulin secretion are characterized by alteration of the ratio of insulin/proinsulin secretion by β-cells at the cost of inactive proinsulin. These abnormalities worsen with time in the natural history of β-cell dysfunction in type 2 diabetes, and explain the need for exogenous insulin administration in patients who fail to respond to oral hypoglycaemic agents (OHA) [47].

2.5.6. Diseases and syndromes

There are several conditions in which insulin disturbance is pathologic:

Diabetes mellitus – general term referring to all states characterized by hyperglycemia

Type 1 – autoimmune-mediated destruction of insulin-producing β-cells in the pancreas, resulting in absolute insulin deficiency

Type 2 – multifactoral syndrome with combined influence of genetic susceptibility and influence of environmental factors, the best known being obesity, age, and physical inactivity, resulting in insulin resistance in cells requiring insulin for glucose absorption.

Insulinoma - a tumor of pancreatic β-cells producing excess insulin or reactive hypoglycemia.

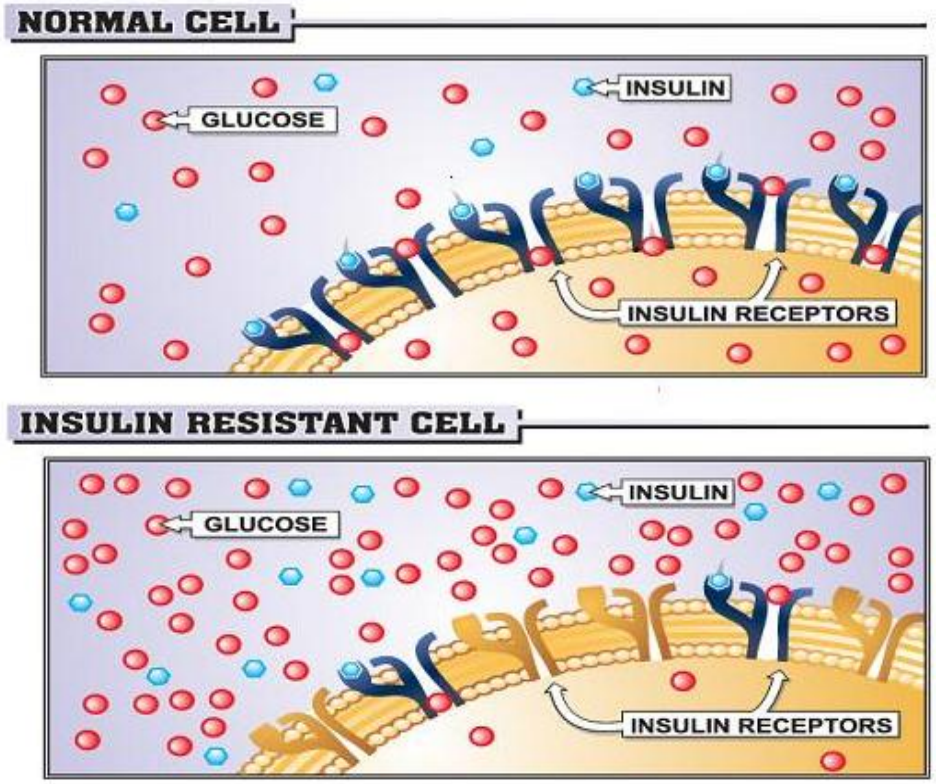


Figure 2.13. Normal cell and insulin resistant cell (adapted from [48]).

Metabolic syndrome – a poorly understood condition first called Syndrome X by Gerald Reaven, Reaven's Syndrome after Reaven, CHAOS in Australia (from the signs that seem to travel together). It is currently not clear whether these signs have a single, treatable cause, or are the result of body changes leading to type 2 diabetes. It is characterized by elevated blood pressure, dyslipidemia (disturbances in blood cholesterol forms and other blood lipids), and increased waist circumference (at least in populations in much of the developed world). The basic underlying cause may be the insulin resistance that precedes type 2 diabetes, which is a diminished capacity for insulin response in some tissues (e.g., muscle, fat). It is common that morbidities, such as essential hypertension, obesity, type 2 diabetes, and cardiovascular disease (CVD) develop.

Polycystic ovary syndrome – a complex syndrome in women in the reproductive years where anovulation and androgen excess are commonly displayed as hirsutism. In many cases of PCOS, insulin resistance is present [47].

2.6. Cryogels

Cryogels are gel matrices that are formed in moderately frozen solutions of monomeric or polymeric precursors [49]. 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, in combination 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 [50].

2.6.1. Brief history

The first mention about flowing reactions in frozen systems belong to the 1930s, but the real research in this area of chemistry began only in the 1960s. A classical concept about frozen solutions as polycrystalline solids at temperatures below the eutectic does not explain the flow of different mechanism of chemical reactions in such systems. The first who suggested that freezing solution in the systems along with the solid phase remain liquid region between the crystals of the solvent, where concentrated solutes were Butler and Bruce [51]. Later in the study of kinetics reaction of low molecular weight substances in crystallized solvents (aqueous and organic) and Pinkok Kiovski also concluded that these reactions in the containing high concentrations of reagents liquid inclusions located between the crystalline solid [52]. The first cryogel, poly(vinyl alcohol) (PVA) was first synthesized in Japan and probably independently in the USSR in early 1970s.

Polymer cryogels is an emerging class of biomaterials, which have recently started attracting attention as potential tissue scaffolds for regenerative medicine. The word ‘cryogel’ is comprised of two parts – 'cryo' from the Greek κριοσ (cryos) meaning cold or ice, and 'gel' meaning a colloid semisoft material. Currently, the word cryogel has at least three different meanings in scientific literature and related to three distinctively different groups of substances: (1) gelatinous precipitate forming during cryoprecipitation – blood plasma treatment upon cooling to c. 4°C; (2) polymeric and inorganic cryogels produced by sol–gel technology

with subsequent freeze drying; thus, a number of individual and mixed inorganic oxides, polymer, and carbon (by carbonization of polymer) cryogels have been obtained; and (3) synthetic and natural polymer cryogels produced in a frozen solvent, usually water [53].

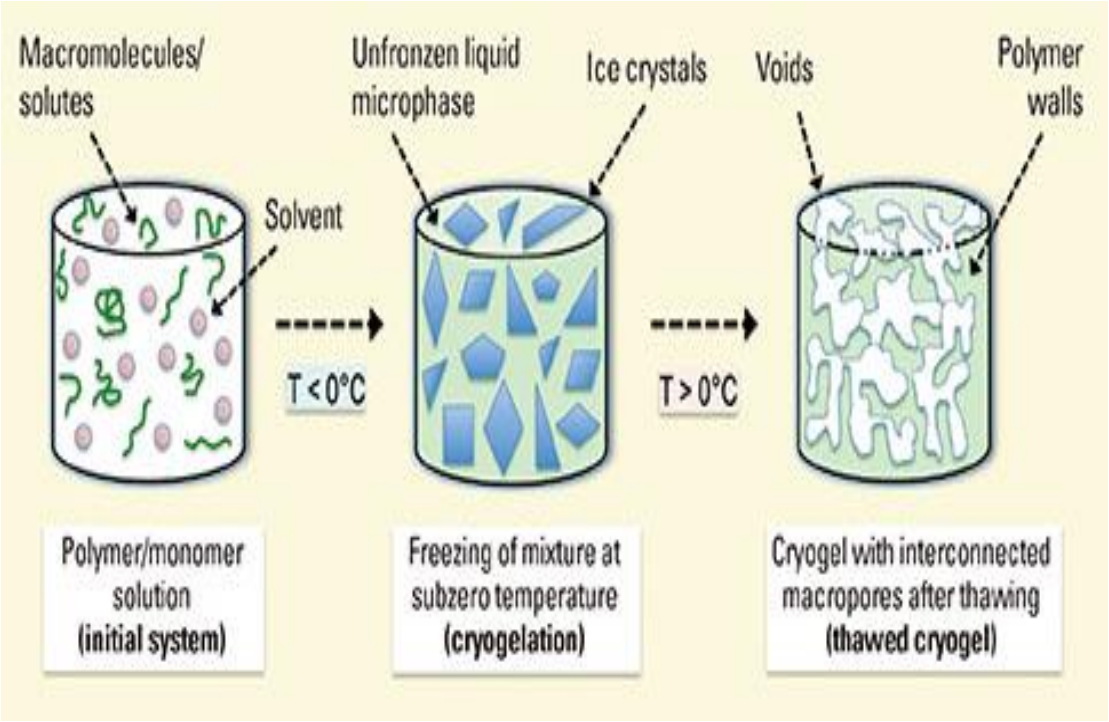


Figure 2.14. Cryogelation process (adapted from [54]).

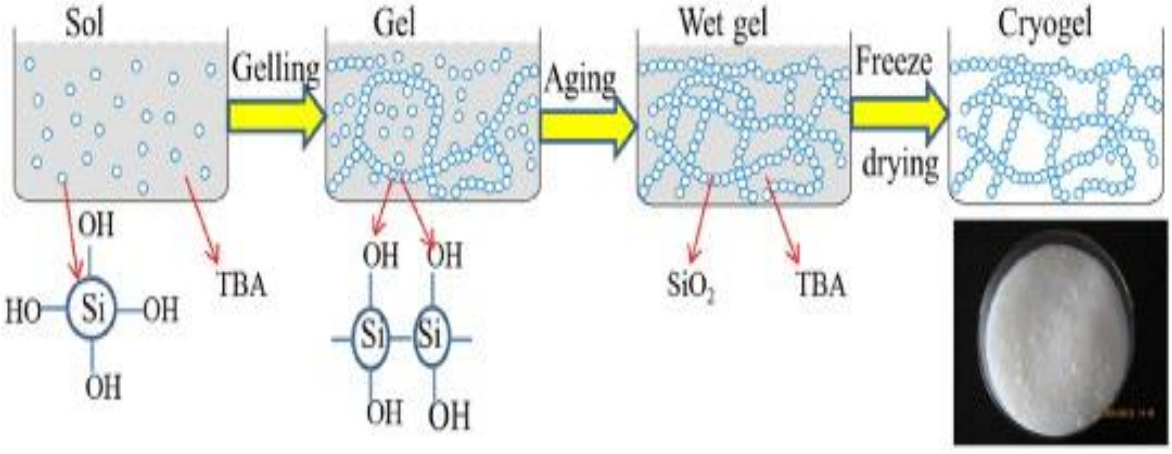


Figure 2.15. Production scheme of silica cryogels using TBA as a reaction solvent (adapted from [55]).

2.6.2. Preparation and properties of macroporous cryogels

Cryogels are generally prepared at temperatures between -5 and -20°C. The solution containing gel precursors (monomers, cross-linkers, initiators) is frozen. While the reaction mixture is freezing, the mixture consists of two main parts the ice crystals and a non-frozen liquid microphase between the ice crystals. The chemical reaction takes place in this non-frozen liquid microphase. The ice crystals continue to grow during freezing and they merge with other crystals until a complete frozen frame of interconnected ice crystals is formed. At the end of a proper gelation period, the cryogel is brought to room temperature. After thawing of the ice crystals, a matrix containing continuous interconnected macroporous polymer network is obtained. The shape and the size of the ice crystals determine the shape and the size of the pores formed. These highly porous polymeric materials can be synthesized from any gel-forming agents with different morphologies and porosities. The pores in the range of 10–200 μm are sometimes referred to in the cryogel literature as supermacropores. Chemistry (IUPAC) defines macropores as pores with diameter larger than 50 nm. The IUPAC distinction between pores of different size – micro-, meso-, and macropores – is based on different mechanisms of gas adsorption, which clearly has very little, if any, relevance to surface interaction with biomolecules and larger biological objects in aqueous media.

A prefix approach to the pore-size classification, which defines pores as nanopores (micropores) if their size is within the nanometer (micrometer) range, does not clarify this matter. The prefix ‘super’ underlines the fact that such pores are suitable for cell accommodation, which are biological objects with the average size from 4 to 10 μm in humans. Such pore size and interconnectivity are difficult to achieve in three- dimensional (3D) porous materials prepared by other methods. One of the principal differences between cryogels and other macroporous materials (with pores of similar sizes) is that the cryogels possess a tissue-like elasticity and with stand extensive deformations without being destroyed [56].

2.6.3. Cryogel matrices for immobilization of biomolecules

Cryogels with immobilized biomolecules (enzymes, polysaccharides, nucleic acids, antibodies, peptides) as well as synthetic ligands are suitable affinity adsorbents. Generally, there are two main approaches for immobilization; covalent coupling of the ligand to a preformed gel or physical entrapment of the ligand during gelation. It should be noted that there are many different approaches represented, e.g. very specific interactions, group specific affinity and also relatively non-specific interactions [53].

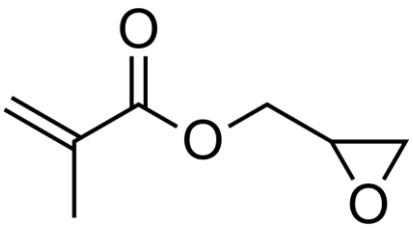
3. EXPERIMENTAL

3.1. Chemicals

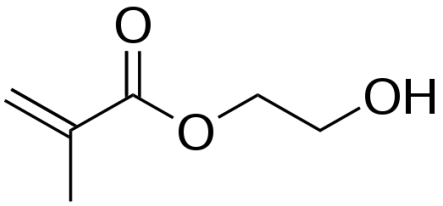
2-Hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (GMA), ethyleneglycol dimethacrylate (EDMA) were supplied from Sigma-Aldrich (St. Louis, USA). Ammonium persulfate (APS), N,N,N',N'-tetramethylene diamine (TEMED), sodium lauryl sulfate (SLS) were obtained from Fluka A.G. (Buchs, Switzerland). Anti-insulin antibody (Rb pAb to insulin, 1.00 mL (2 mg/mL), ab 115951) was supplied from AbCam (Cambridge, MT, USA). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany) unless otherwise noted. All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system. Laboratory glassware was kept overnight in a 5% nitric acid solution. Before use the glassware was rinsed with deionised water and dried in a dust-free environment.

3.2. Preparation of Cryogel

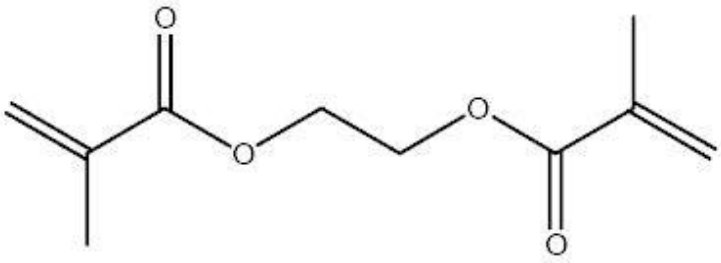
The supermacroporous cryogel based on HEMA and GMA was synthesized by copolymerization of the monomers in presence of EDMA as crosslinker under partially frozen conditions. For this aim, HEMA (2.25 mL) and GMA (0.50 mL) were dissolved in deionized water (3.25 mL) in order to form phase I. Meanwhile, EDMA (1.2 mL) and SLS (0.5 g) were dissolved in 12.8 mL of water to form phase II. Then, both phases were mixed and cooled in ice-bath after addition of 10 mg of APS for 20 min. After that, 50 μL of TEMED (1% (w/v) of the total monomers) was added in order to initiate the radical formation and the reaction mixture was stirred for 1 extra min. Then, the reaction mixture was poured into plastic syringe (4 ml, id. 0.8 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 extensive washing with water (200 mL) and ethanol (100 mL), the cryogels were stored in buffer containing 0.02% sodium azide at 4°C until use.



Glycidyl methacrylate (GMA)



2-Hydroxyethyl methacrylate (HEMA)



Ethylene glycol dimethacrylate (EDMA)

Figure 3.1. Molecular structures of basic functional monomers used.

3.3. Anti-insulin Antibody Immobilization

In order to gain the biospecificity to the cryogels, the bioligand anti-insulin antibody was immobilized onto the cryogels through the reaction between epoxy groups of GMA monomer and amino functionalities of antibody molecules under alkaline condition. For this step, the cryogels were previously interacted with sodium hydroxide solution (1 M, 50 mL) for 6 h. Then, the cryogel was reequilibrated with phosphate buffer (pH 7.4, 50 mL) for 1 h. The immobilization solution was also prepared in phosphate buffer (pH 7.4, 10 mL) having final antibody concentration as 100 μg/mL. The incubation of the cryogel with immobilization solution was performed by using a peristaltic pump (Watson Marlow, 400B Series, Falmouth, UK) for 6 h. The amount of antibody immobilized on the cryogel was calculated from the absorbance measurements before and after immobilization period at 280 nm by using UV-Vis spectrophotometer (Shimadzu UV-1601, Tokyo, Japan). After

immobilization, the cryogel was extensively washed with equilibration buffer to remove non-specifically bound antibody molecules. Here, we have noted that the washout solution was also conducted to UV-vis spectrophotometrical measurements to ensure whether it contained any protein molecules or not. The epoxide groups of cryogels without anti-insulin antibody were deactivated by performing ethanolamine (10 M) treatment after sodium hydroxide activation step.

3.4. Characterization of Cryogel

3.4.1. Swelling test

Water uptake ratio of cryogel was determined in distilled water. The experiment was conducted as follows cryogel sample initially dried to constant weight was carefully weighed (W_{dried}) before being placed in a proper beaker containing 10 mL of distilled water. The beaker was put into an isothermal water bath with a fixed temperature (25 ± 0.5°C) for 1 h. The sample was taken out from the water, wiped using a filter paper to remove water molecules bound onto cryogel surface, and reweighed ($W_{swollen}$). The water content of the cryogel was calculated by using the following expression;

$$\text{Water uptake ratio, \%} = [(W_{swollen} - W_{dried})] \times 100\% \quad (3.1)$$

W_{dried} and $W_{swollen}$ are the weights of cryogel before and after uptake of water (g), respectively.

3.4.2. Surface morphology

The surface morphology of the cryogels was examined by using scanning electron microscopy (SEM; Jeol, JSM-6060 LW, USA). The samples were firstly dried in a frige and solvent was removed by using freeze-dryer system (Christ Freeze Dryer-Alpha 1-2LD, Maryland, USA) and treated at 10.0 μbar and −52°C for 24h. Finally, it was coated with gold-palladium (40: 60) and examined using a JEOL JSM 6060 scanning electron microscope (Tokyo, Japan).

3.4.3. FTIR and Raman studies

The characteristic functional groups of cryogels before and after immobilization of the were analyzed by using a Fourier transform infrared spectrophotometer (PerkinElmer, Spectrum One, USA). The FTIR samples were prepared by mixing approximately 2 mg of cryogel samples dried and crushed into fine particles with 98 mg of water-free powdered KBr (IR-Grade, Merck, Germany), and pressed into a pellet form. The FTIR spectra were then recorded in the wavenumber range of 400-4000 cm^{-1}. Raman spectra of cryogel samples were also obtained by using DXR™ Raman microscope (Thermoscientific, Waltham, MA, USA). For this aim, after drying and crushing the cryogel samples into fine particles, they were examined by to Raman microscope to observe whether antibody molecules had been immobilized onto cryogel or not. Amide bands in the wavenumber range of 1200-1700 cm^{-1} were especially noticed to discuss immobilization process.

3.5. Adsorption-Desorption Studies

The insulin adsorption studies were performed via continuous experimental setup by using a recirculating system (Watson Marlow, 400B Series, Falmouth, UK). The experimental setup applied in the study was shown in Figure 3.2. In general experimental cycle was performed as follows: the cryogel was washed with 15 mL of deionized water and then equilibrated with proper buffer system (feeding solution). Acetate and phosphate buffers (50 mM) were used for pH ranges as 4-6 and 7-8, respectively. After equilibration step, feeding buffer solution containing target molecules (insulin) was interacted with column for 2 h. The adsorption capacity of the cryogel was monitored by measuring the decrease in UV-vis absorbance at 280 nm by using spectrophotometer (Shimadzu 1601, Tokyo, Japan). In this step, factors effecting the adsorption capacity, such as pH, insulin concentration, flowrate, contact time, temperature, and salt concentration were examined. The pH values were varied in the range of 4.0-8.0 by using appropriate buffer system. The initial insulin concentration and salt (sodium chloride) concentration were varied between 0.0-1.0 mg/mL and 0.01-1.0 M, respectively. Contact time was varied in 2h whereas flowrate was varied in the range of 0.5-2.0 mL/min. The effect of temperature was determined for different temperatures (5ºC,

25ºC, 37ºC, and 45ºC) in dependence to initial insulin concentration. The amount of adsorbed insulin was calculated as:

$$Q = (C_{initial} - C_{final}) x \frac{V}{m} \quad , \qquad \text{(Eq 3.1)}$$

Here, Q is insulin adsorption capacity meaning the amount of insulin adsorbed onto unit mass of cryogel (mg/g); $C_{initial}$ and C_{final} are the concentrations of insulin in the initial solution and in the aqueous phase after treatment for adsorption period, respectively (mg/mL); V is the volume of the aqueous phase (mL); and m is the mass of the cryogel used (g). Herein, it should be noted that each experiment was performed thrice at least for quality control and statistical calculations.

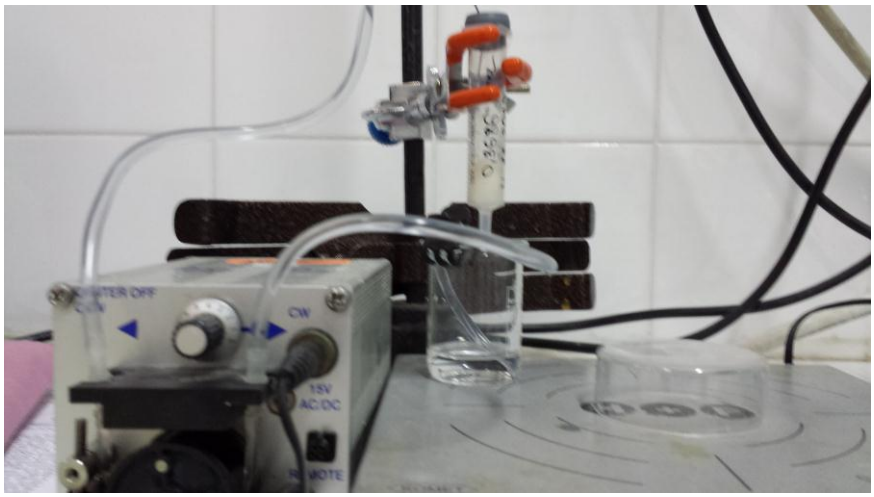


Figure 3.2. Continuous system used for adsorption experiments.

3.6. Desorption and Reusability

The desorption of insulin molecules adsorbed onto cryogel was also performed in continuous experimental setup. After adsorption period, insulin adsorbed cryogel were interacted with 0.5 M NaCl solution at room temperature for one hour. The final insulin concentration in the desorption medium was determined by absorbance measurements at 280 nm. The desorption efficiency and retained adsorption capability of the cryogel against insulin molecules were calculated by using following equation.

$$\text{Desorption Ratio (\%)} = \frac{C_{insulin} \text{ x } V_{desorp}}{Q \text{ x } m} \text{ x } 100\% \quad (3.4)$$

where, undefined parameters are that $C_{insulin}$ is insulin concentration in the desorption medium (mg/mL), V_{desorp} is volume of the desorption medium (mL).

In order to show the reusability of cryogel, adsorption-desorption cycle was repeated ten times while performing a regeneration step after each desorption procedure. For this aim, cryogel was washed out with 50 mM sodium hydroxide solution (10 mL).

3.6.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

In order to show whether desorption process cause any denaturation or not on the the insulin mollecules during adsorption-desorption cycle, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed while using separating gel containing 10% acrylamide dissolved in 1.5 M Tris-HCl (pH 8.3) and water. Meanwhile, the adsorption-desorption cycle was performed as given above by applying optimal adsorption-desorption conditions determined. Herein, samples were collected from feeding buffer before and after adsorption and from desorption solution. After that, these samples (15 µL) were mixed with bromophenol blue (2‰ in 50% aqueous glycerol solution). These samples and protein ladder which is a 3-color ladder with 10 proteins covering a molecular weight range from 10 to 170 kDa (PageRuler™ Prestained Protein Ladder, ThermoScientific, Waltham, USA) were fed into electrophoresis channelles and run against Tris-glycine buffer (pH 8.3) containing 0.1% SDS. Separation was performed at a current of 40 mA, 45 V, 2w for 8h. After completeness of electrophoretic separation, the gels were stained with 0.25% (w/v) Coomassie Brilliant R 250 in acetic acid-methanol-deionized water (1:5:5, v/v/v) to visualize the bands; subsequently, destaining was performed with ethanol-acetic acid-water (1:4:6, v/v/v).

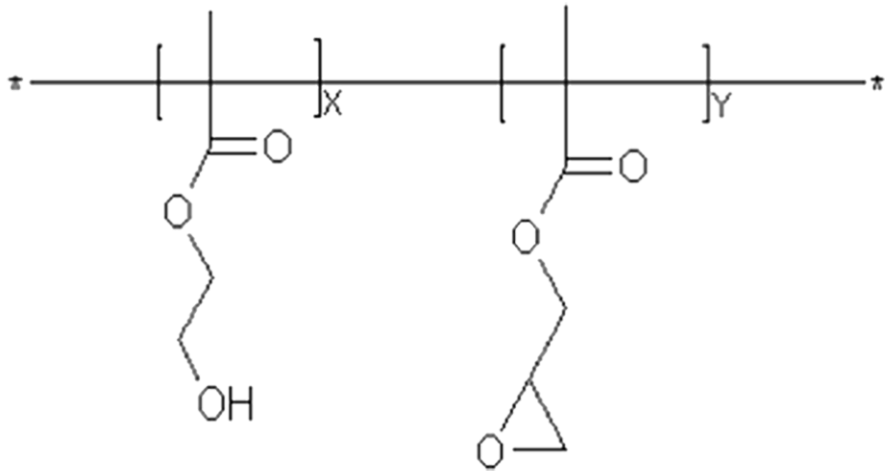
4. RESULTS AND DISCUSSION

4.1. Characterization of Cryogels

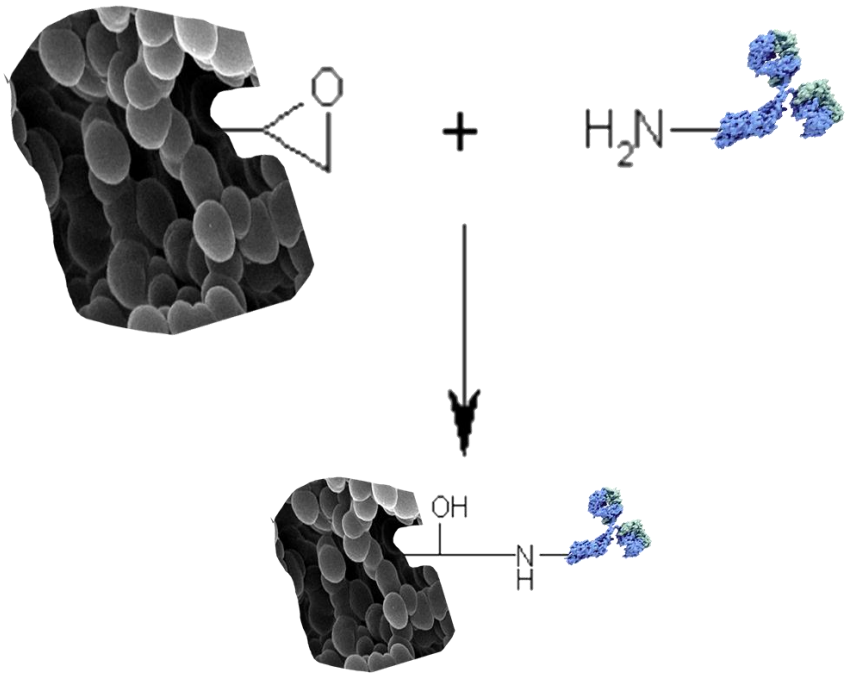
In this thesis, we have prepared glycidyl methacrylate containing methacrylate-based cryogel for insulin adsorption via immunoaffinity chromatography approach. By incorporating glycidyl groups into polymeric backbone, activation of polymeric adsorbents before ligand immobilization was eliminated. Anti-insulin antibody molecules were used as bioligand for immunoaffinity recognition of insulin molecules, which were immobilized onto cryogels through reaction between amine-group of antibody molecules and epoxy functionalities of cryogel (Figure 4.1). The amount of antibody molecules immobilized onto cryogels were determined via spectrophotometry as 0.348 mg/g. Antibody immunoaffinity cryogels or antibody immobilized and plain, were characterized by swelling test, scanning electron microscopy observation, and spectroscopy including Fourier transform infrared and Raman. The results were given below and discussed one-by-one.

4.1.1. Swelling Test

The equilibrium degree of swelling is an important property of an adsorbent, influencing the permeability and mechanical properties of cryogels. Cryogels are crosslinked hydrophilic polymers that can imbibe water or biological fluids. Their biomedical and pharmaceutical applications include a very wide range of systems and processes that utilize several molecular design characteristics. Plain and anti-insulin antibody immobilized cryogels were subjected to swelling test after drying them until constant weight (Figure 4.2). As seen in figure, both cryogels swelled fast in a few minutes. Swelling ratios of plain and immunoaffinity cryogels were calculated as 580 g H_2O/g cryogel and 598 g H_2O/g cryogel, respectively. The slight increase in swelling ratio of immunoaffinity cryogel depends on the water compatibility of biomolecules immobilized.



(a)



(b)

Figure 4.1. Molecular structure (a) and antibody immobilization strategy (b) of the cryogel.

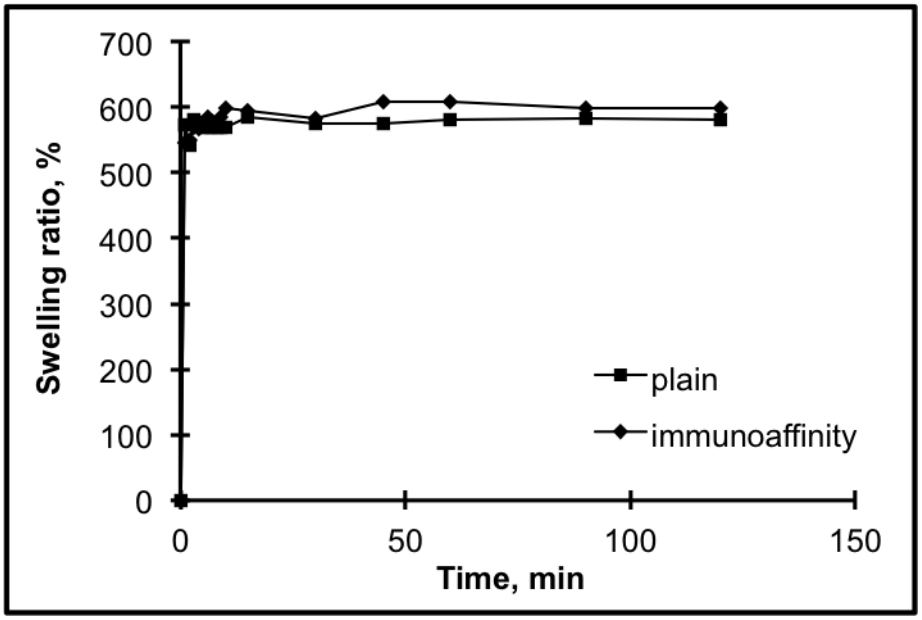


Figure 4.2. Swelling behavior of the cryogels.

4.1.2. Surface morphology

In order to examine the surface morphology of the cryogels, we have used scanning electron microscopy (SEM) after drying and gold-coating of cryogel surfaces (Figure 4.3). As seen in figure, cryogels have rough surfaces with interconnected macropores. The pores diameters indicated by SEM images were so wide and in the range of 2-100 μm. The SEM images of the cryogels synthesized in this thesis differs from the classical images of cryogels because of including sodium lauryl sulfate as a surfactant to reduce surface tension of water and to increase solubility of the crosslinker, used as ethylene glycol dimethacrylate. Therefore, these cryogels have rough surfaces including small beads formations instead of smooth surfaces of traditional acrylic based cryogels. By this way, the specific surface area of the cryogels was increased while retaining supermacroporous structure and interconnected flow channels of the cryogels.

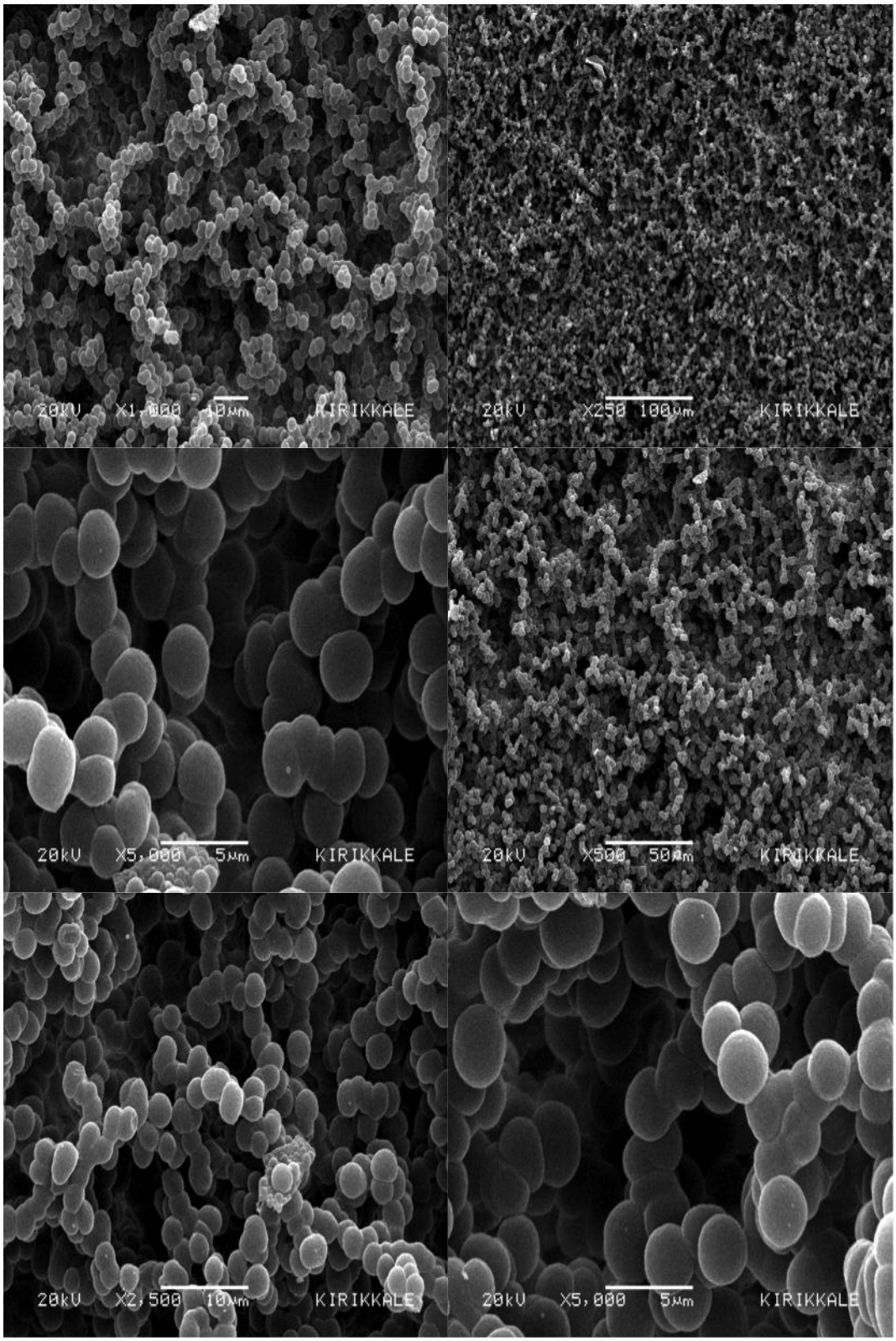


Figure 4.2. SEM images of the cryogel.

4.1.3. FTIR and Raman studies

In order to characterize chemical structure of both cryogels, plain and immunoafinity, we used two spectroscopic methods, Fourier transform infrared (FTIR) and Raman (Figure 4.4 and Figure 4.5). As seen in FTIR spectra, both cryogels have almost same spectra. The common respective bands were –OH, C=O, C-H (aliphatic) assigned at around 3400 cm^{-1}, 1710 cm^{-1}, and 2980 cm^{-1}, respectively. In addition to these bands, immunoaffinity cryogel has extra bands around 1650 cm^{-1} that were stemmed from amide bonds found in insulin. The Raman spectra also proved these results because the amide vibrations were clearly seen in the spectra.

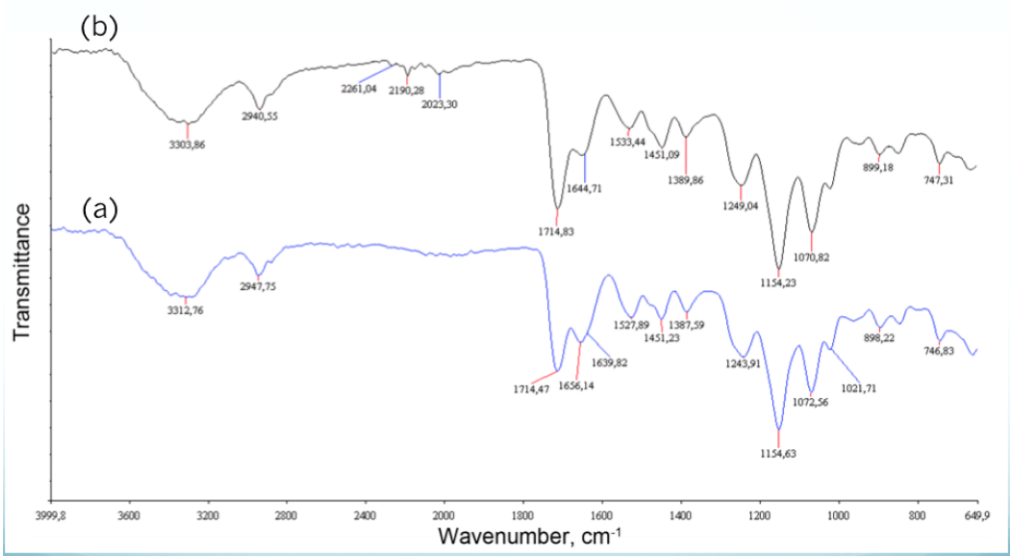


Figure 4.3. FTIR spectra of (a) immunoaffinity and (b) plain cryogels.

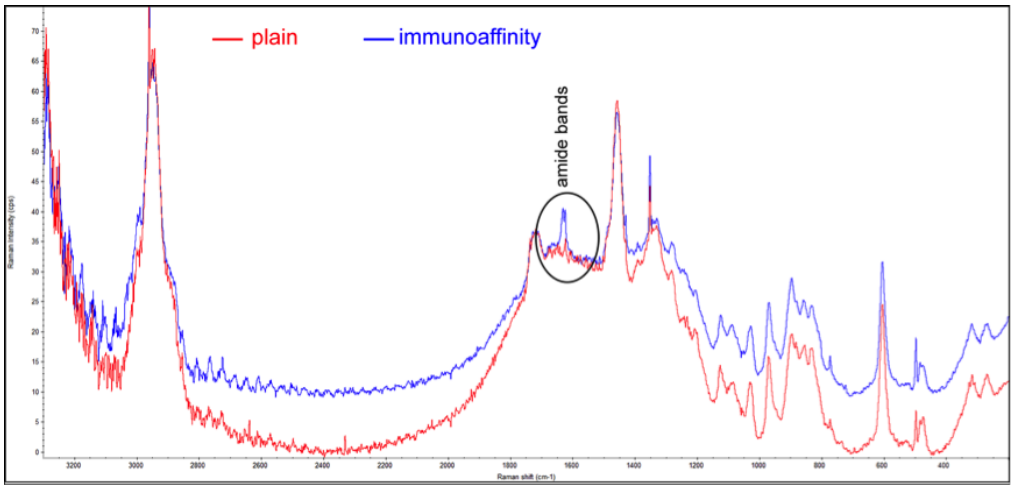


Figure 4.4. Raman spectra of (a) immunoaffinity and (b) plain cryogels.

4.2. Adsorption-Desorption Studies

In this thesis, we have developed immunoaffinity cryogel for insulin adsorption from aqueous solution. For this aim, we immobilized anti-insulin antibody molecules onto glycidyl methacrylate-based cryogels. After modification and characterization studies, we optimized the insulin adsorption conditions while considering effective factors including pH, insulin concentration, flow rate, contact time, salt concentration, and temperature. After that, the reusability of immunoaffinity cryogels was examined in light of the data obtained from desorption of insulin molecules from cryogels.

4.2.1. Effect of pH

The first parameter examined was the effect of pH on the insulin adsorption capacity of immunoaffinity cryogel. For this aim, pH value of feeding solution was varied from 4.0 to 8.0 by using proper acetate (4-5) and phosphate (6-8) buffer couples (Figure 4.6). As seen in the figure, the insulin adsorption capacity of immunoaffinity cryogel was directly related to pH variation in the feeding medium. The maximum adsorption capacity was achieved at pH 7.4. At lower and higher pH values, the adsorption capacity of immunoaffinity cryogel decreased significantly. The results may depend on the net charge density and conformational changes of bioligand, anti-insulin antibody, and target molecules, insulin, although insulin (pI 5.4) and antibody molecules (pI 6.5-7.2) have isoelectric points which are lower than 7.4. The results for plain cryogel supported this conclusion. Because, no significant effect on the adsorption capacity was observed in respect to pH variation in case of plain cryogel used. This means that non-specific insulin adsorption occurred through diffusion and physically adsorption into micropores found in the cryogel network; therefore this behavior wasn’t effected by pH-variation. The pH value (7.4) was accepted as optimal value and kept constant for further studies.

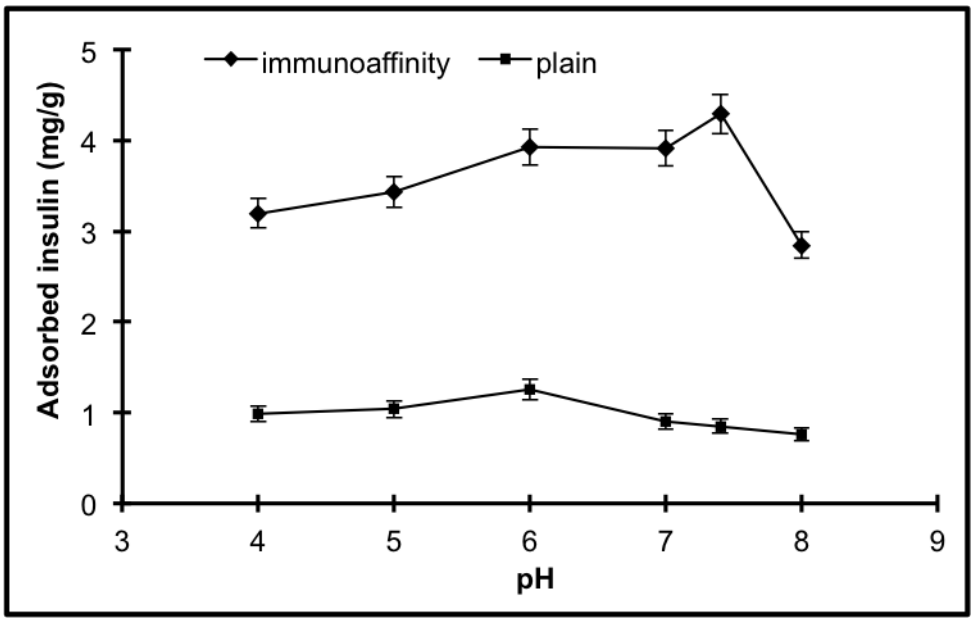


Figure 4.5. The effect of pH onto insulin adsorption capacity of immunoaffinity cryogel. Initial insulin concentration: 0.25 mg/mL; flowrate: 0.5 mL/min; contact time: 120 min; temperature: 25°C.

4.2.2. Effect of insulin concentration

The effect of initial insulin concentration onto the adsorption capacity of immunoaffinity cryogel was examined in the concentration range of 0.05-1.0 mg/mL (Figure 4.7). As seen in figure, the adsorption capacity values increased with increasing initial insulin concentration. Also, anti-insulin antibody immobilization caused high specificity for cryogels. Immunoaffinity cryogels showed an adsorption capacity (9.62 mg/g) which is 11-folds higher than plain cryogel had (0.88 mg/g), because, anti-insulin antibody immobilized immunoaffinity cryogel showed higher specificity against insulin molecules. At the beginning part of the curve, the increase/slope was so steep, then it started to decline because of occupation of active binding sites of immunoaffinity cryogel. The optimal initial insulin concentration was accepted as 0.25 mg/mL and kept constant for further studies.

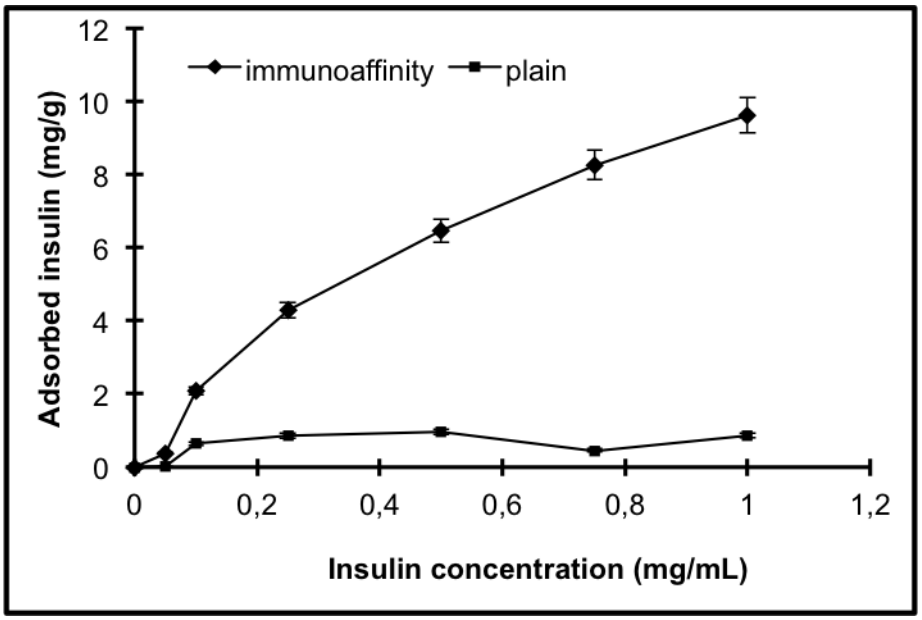


Figure 4.6. The effect of initial insulin concentration onto insulin adsorption capacity of immunoaffinity cryogel. pH: 7.4; flowrate: 0.5 mL/min; contact time: 120 min; temperature: 25°C.

4.2.3. Effect of flowrate

The flowrate of the feeding solution is one of the important parameters to design an efficient separation system in related to high adsorption capability and economic parameters. The possibility to study at higher flowrates reduces operation time and daily cost for separation system. Cryogels offer excellent flow-dynamics including structural properties such as highly interconnected flowchannels, super macroporous nature, good solution compatibility, high mass transfer ability and low pressure drop. These properties make them promising alternatives for traditional chromatographic adsorbents. The immunoaffinity cryogel developed in this study was also conducted to optimize the flowrate of the feeding buffer. The flowrate was varied between 0.5 mL/min and 2.0 mL/min meanwhile other parameters were kept constant (Figure 4.8). As seen in figure, the insulin adsorption capacity of immunoaffinity cryogel was significantly effected from increasing flowrate. The adsorption capacity decreased from 4.28 mg/g to 3.27 mg/g when flowrate increased from 0.5 mL/min to 2.0 mL/min. Similarly, non-specific adsorption onto plain cryogel also decreased with increasing flowrate because of dragging and shearing effect of feeding solution.

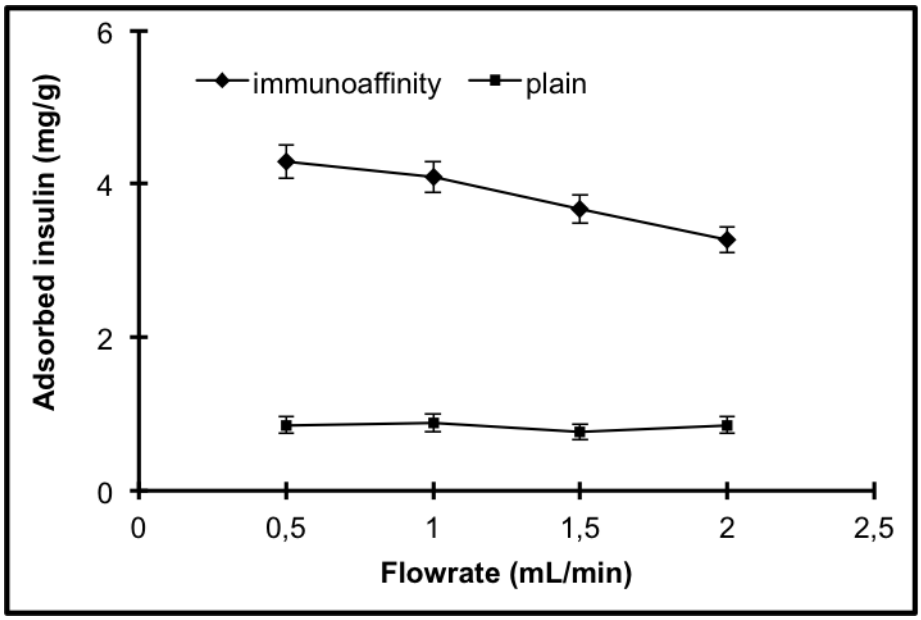


Figure 4.7. The effect of flowrate onto insulin adsorption capacity of immunoaffinity cryogel. pH: 7.4; initial insulin concentration: 0.25 mg/mL; contact time: 120 min; temperature: 25°C.

4.2.4. Effect of contact time

In term of operation duration, the contact time between adsorbent and target molecules should also be optimized. In this context, we varied the contact time in the range of 5-120 min while taking sample from feeding solution at desired time interval (Figure 4.9). As seen in figure, the adsorption capacity of immunoaffinity cryogel reached to equilibrium value very fast. These results also supported to structural advantages of the cryogels and high affinity between antibody and insulin molecules. In addition, the fast adsorption kinetic emphasized that there was no diffusion problem in the developed adsorption system and the mass transport mainly achieved through convection.

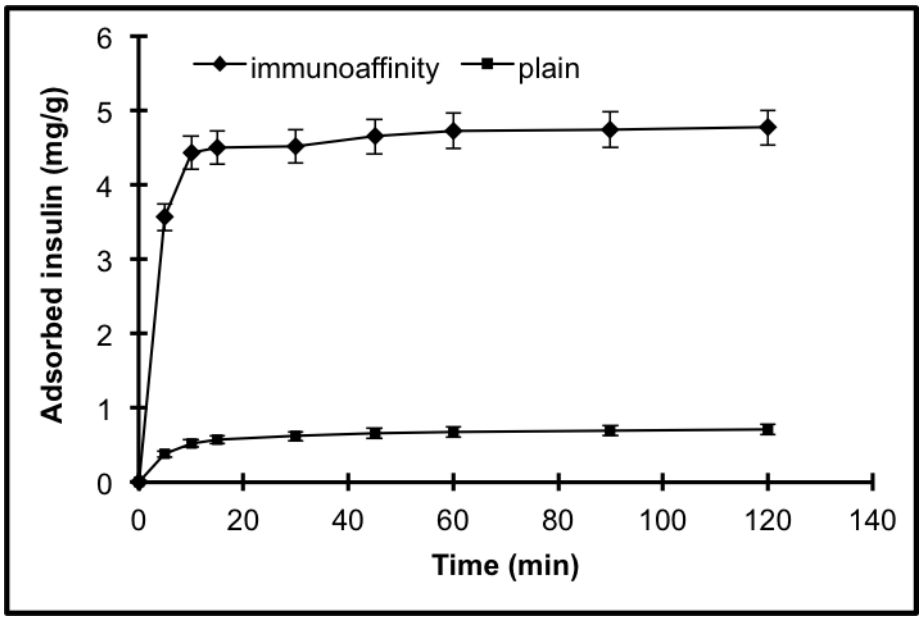


Figure 4.8. The effect of contact time onto insulin adsorption capacity of immunoaffinity cryogel. pH: 7.4; initial insulin concentration: 0.25 mg/mL; flowrate: 0.5 mL/min; temperature: 25°C.

4.2.5. Effect of salt concentration

The effect of the salt (sodium chloride, NaCl) concentration onto the adsorption capacity of immunoaffinity cryogel was also examined. The increase in salt concentration significantly decreased the adsorption capacity. The presence of salt ions in the medium has direct effect on the orientation of water molecules around the biomolecules. Sometimes, the salt molecules disrupt highly ordered water molecules around apolar sites of the biomolecules, which enhances the interactions between apolar sites of biomolecules (hydrophobic interaction). In another case, salt ions coordinate the oppositely charged sites of biomolecules, which diminished the interaction between these sites and masking the sites to come together (electrostatic interaction). Herein, we determined an opposite relationship between adsorption capacity and salt concentration. The results showed that the interactions between antibody and insulin molecules had mainly electrostatic, hydrogen bonding, and electron/proton sharing nature. In accordance to these results, we decided that 0.5 M NaCl solution had enough strength to desorb insulin molecules from cryogels as well.

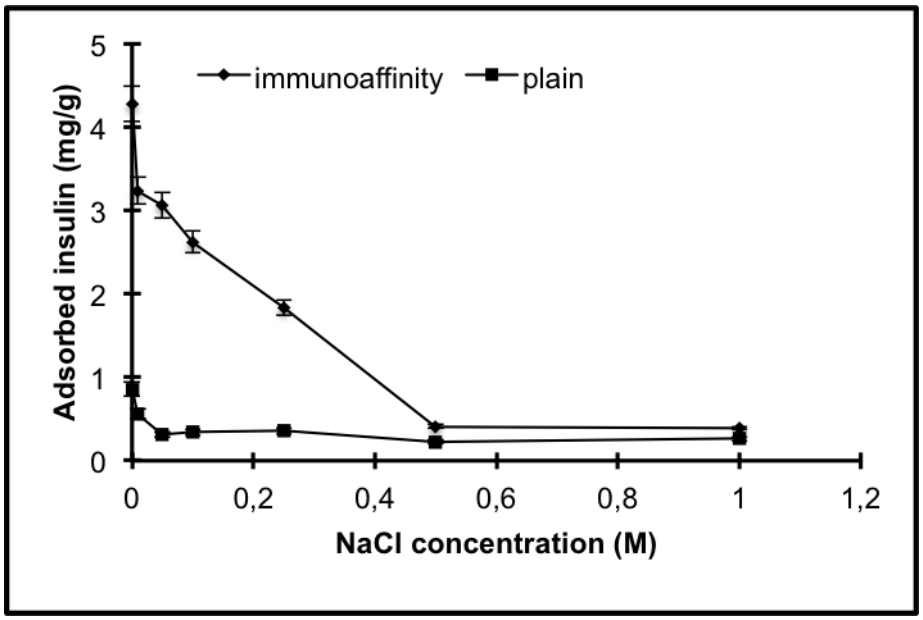


Figure 4.9. The effect of salt concentration onto insulin adsorption capacity of immunoaffinity cryogel. pH: 7.4; initial insulin concentration: 0.25 mg/mL; flowrate: 0.5 mL/min; contact time: 120 min; temperature: 25°C.

4.2.6. Effect of temperature

Another parameter to analyze the interaction between adsorbent and target molecule is temperature. Herein, we performed adsorption studies at four different temperatures (5ºC, 25ºC, 37ºC, and 45ºC) meanwhile varying initial insulin concentration also at four different values (0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, and 1.0 mg/mL) (Figure 4.11). As seen in figure, increase in temperature decreased the adsorption capacity of immunoaffinity cryogel. But, at all temperature values, the insulin concentration was parallel to adsorption capacity. Beside to the exothermic nature of adsorption process, the results also improved that the interaction between insulin and immunoaffinity cryogel mainly depend on the secondary forces including hydrogen bonding, electrostatic interaction, and electron/proton sharing.

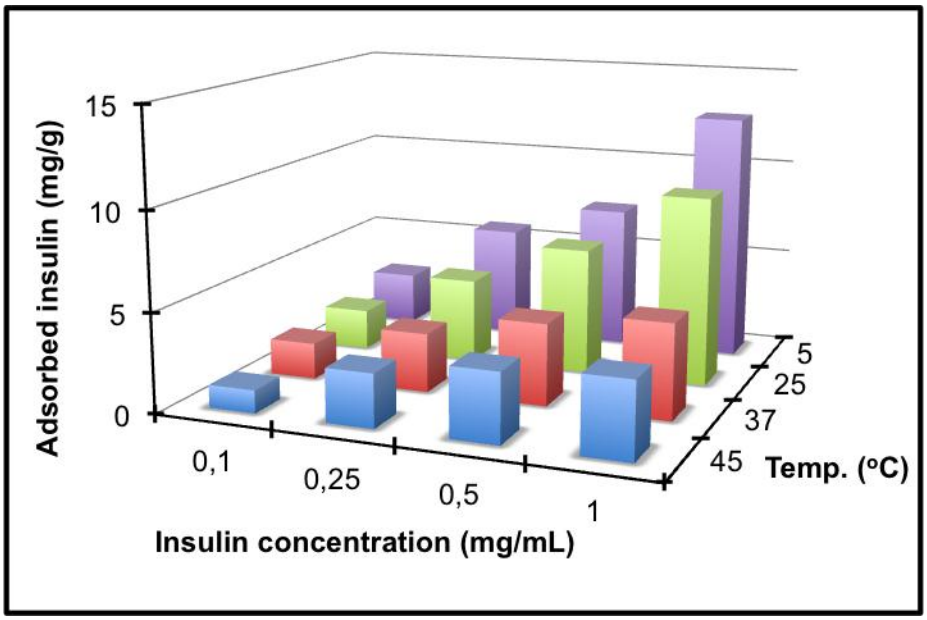


Figure 4.10. The effect of temperature onto insulin adsorption capacity of immunoaffinity cryogel as a function of insulin concentration. pH: 7.4; flowrate: 0.5 mL/min; contact time: 120 min.

4.3. Desorption and Reusability

Desorption efficiency, reusability, and retained adsorption capacity are key-points for adsorbent development strategy. Because, high desorption efficiency and retained adsorption capacity expose a possibility to reuse same adsorbent several times which decreased operational cost. Therefore, we examined whether immunoaffinity cryogels could be used several time or not. Herein, we applied adsorption-desorption-regeneration-equilibration cycle by using feeding solution (phosphate buffer, pH 7.4) with insulin (0.25 mg/mL), salt solution (NaCl, 0.5 M), alkaline solution (NaOH, 50 mM), and feeding regeneration solution without insulin. For each cycle, approximately 98.5% of insulin molecules were desorbed from immunoaffinity cryogels by using 0.5 M NaCl solution as desorbing agent. In addition, the adsorption capacity was not significantly effected/decreased after tenth cycles. After tenth cycle, 91.4% of first adsorption capacity was retained. It should be noted here all optimization experiments with their replications have been performed by using same immunoaffinity cryogel. In this section, we performed consecutive ten cycles to show reusability of the immunoaffinity cryogel. As a result, the developed immunoaffinity cryogel could be classified as good

alternative with prominent properties such as high reusability and cost-friendly adsorbent.

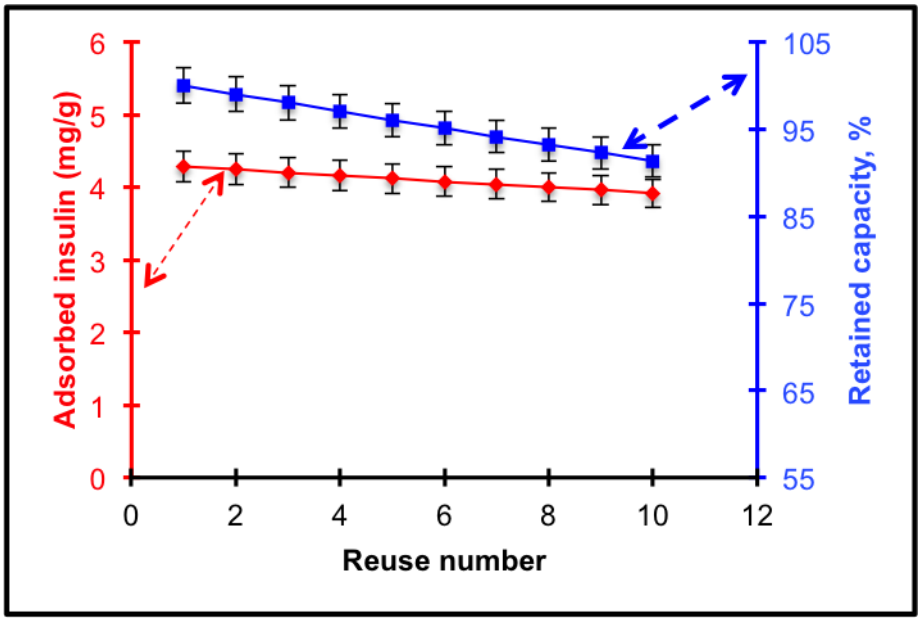


Figure 4.11. The reusability and retained adsorption capacity of immunoaffinity cryogel. pH: 7.4; initial insulin concentration: 0.25 mg/mL; flowrate: 0.5 mL/min; contact time: 120 min; temperature: 25°C; desorbing agent: 0.5 M NaCl, regenerating agent: 50 mM NaOH.

4.3.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

In order to show whether desorption condition caused any denaturation problem on insulin molecules or not, we performed SDS-PAGE analysis of four samples taken from different steps (from standard insulin solution, feeding solution before and after adsorption; desorption solution after elution) of process (Figure 4.13). As seen in figure, the insulin concentration of feeding solution (Lane 2) decreased in accordance to adsorption process (Lane 5). Because, the color of band shown with respect to insulin getting pale after adsorption process. Lane 2 included the standard insulin solution (3mg/mL) for control purpose. After desorption, the eluted insulin molecules also appeared at same level (Lane 6) which means that there was not any denaturation occurred during desorption and desorbing agent was proper for this aim. As a result, the all steps for the separation process developed here are appropriate for purification insulin molecules from aqueous media.

1 2 3 4 5 6

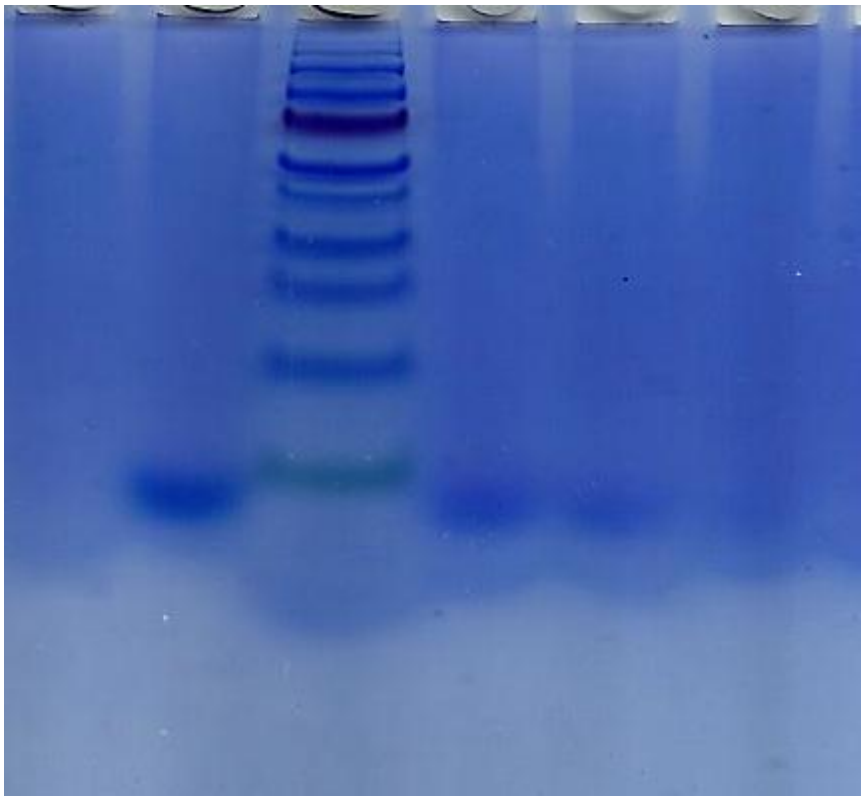


Figure 4.12. SDS-PAGE image of insulin adsorption cycle. Lane 2: standart insulin solution (3 mg/mL); Lane 3: protein ladder; Lane 4: insulin solution before adsorption (1 mg/mL); Lane 6: desorbed insulin solution.

5. CONCLUSION

Immunoaffinity chromatography (IAC) combines the use of LC with the specific binding of antibodies or related agents. The resulting method can be used in assays for a particular target or for purification and concentration of analytes prior to further examination by another technique. This technique represents a special sub category of affinity chromatography, in which a biologically related binding agent is used for the selective purification or analysis of a target compound. The selective and strong binding of antibodies for their given targets has made these agents of great interest for many years as immobilized ligands in affinity chromatography.

Cryogels are very good alternative adsorbents for protein purification with many advantages such as large pores, short diffusion path, low pressure, drop and very short residence time for both adsorption and elution. The unique structure of cryogels, in combination with their osmotic, chemical and mechanical stability, makes them attractive matrices for chromatography tecniques.

Recombinant human insulin is a form of insulin made from recombinant DNA that is identical to human insulin, which is used to treat diabetics. Currently, there are about 0.3 billion people who are suffering from diabetes. Among them, insulin is of vital importance for human whole life. Because of this reason insulin is a commercial protein.

The interactions between the epoxy groups of Poly(HEMA-GMA) cryogels and amino groups of antibody molecules had high result of immobilization capacity such as 0.348 mg/g, which was measured by spectrophotometry.

The swelling ratios of plain and immunoaffinity cryogels were 580% to 598%, respectively.

The cryogel columns were examined by scanning electron microscopy (SEM). The cryogel samples have rough surfaces including acrylic based cryogels.

Insulin adsorption onto cryogel seemed to depend on the pH. Buffer ranges are 4.0 to 8.0. In phosphate buffer, adsorption capacity is higher than other buffers. Maximum adsorpsion capacities are observed at pH 7.4.

The adsorption capacities at different flow rates are investigated. The adsorption capacity decreased from 4.18 mg/g to 3.27 mg/g for the immunoaffinity cryogel with the increase of the flow-rate from 0.5 mL/min to 2.0 mL/min.

The contact time between adsorbent and target molecule was varied in the range of 5-120 min. As a result, we found that the adsorption capacity immunoaffinity cryogel reached to equilibrium value quickly.

Insulin adsorption capacity decreased with the increasing salt concentration. When the salt concentration increased in the adsorption medium, this can lead to coordination of deprotonated amino groups of the insulin with cations of the salts, which leads to low protein adsorption.

The effect of insulin concentration on adsorption capacity was studied. Optimal insulin concentration value on immunoaffinity cryogel was accepted as 0.25 mg/mL.

The effect of temperature on insulin adsorption was studied at four different values (0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, and 1.0 mg/mL). The equilibrium adsorption of insulin onto immunoaffinity cryogels significantly decreased with increasing temperature.

More than 98% of the adsorbed insulin molecules was removed easily from the cryogel in all cases whith acetate buffer containing desorption agent 0.5 M NaCl.

In order to show reusability of the cryogels, the adsoption-desorption cycle was repated ten times using the same cryogel. It is observed that the adsorption behavior of insulin to the Poly (HEMA-GMA) cryogels little changed over ten cycles. These results demonstrated the stability of the present cryogel as an affinity adsorbent.

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