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Chapter 3

Affinity Chromatography and Importance in Drug Discovery

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Additional information is available at the end of the chapter

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1. Introduction

Affinity chromatography which is known as a liquid chromatographic technique for separation and analysis of biomolecules based on their biological functions or individual structures has become increasingly important and useful separation method in pharmaceutical science, biochemistry, biotechnology and environmental science in recent years [1]. This technique is especially known as the most specific and effective technique for protein purification [2]. Separation of the biomolecules is based on highly specific biological interactions between two molecules, such as enzyme and substrate. These interactions, which are typically reversible, are used for purification by placing one of the interacting molecules, referred to as affinity ligand, onto a solid matrix to create a stationary phase while the target molecule is in the mobile phase [3]. Any component can be used as a ligand to purify its respective binding partner. Some typical biological interactions, frequently used in affinity chromatography, can be given as;

- Enzyme. ↔ substrate analogue, inhibitor, cofactor.
- Antibody ↔ . antigen, virus, cell.
- Lectin ↔ . polysaccharide, glycoprotein, cell surface receptor, cell.
- Nucleic acid ↔ . complementary base sequence, histones, nucleic acid polymerase, nucleic acid binding protein.
- Hormone, vitamin ↔ . receptor, carrier protein.
- Glutathione ↔ . glutathione-S-transferase or GST fusion proteins.

- Metal ions ↔ . Poly (His) fusion proteins, native proteins with histidine, cysteine and/or tryptophan residues on their surface [4-5].

In case a ligand is immobilized on a polymeric carrier, usually by covalent coupling, and filled in a column, it is possible to separate the substances which have affinity to the ligand and the other substances. As the solution containing the biologically active substance applied to the column, the compounds which have no affinity to the insoluble ligand will pass through the column and the biologically active compound will be captured on the column, in favorable conditions. The sorbed compounds can then easily be dissociated by changing the external conditions, such as ionic strength, pH, solvent, temperature etc. or alternatively by using dissociating agents [6-7]. As a result, it is possible to isolate and purify the analyte or make quantitative analysis with a suitable, immobilized ligand by means of molecular recognition [1-2].

Macromolecules such as proteins, polysaccharides, nucleic acids differ only in their physico-chemical properties within the individual groups and their isolation on the basis of these differences is therefore difficult and time consuming. Considerable decreases may occur during their isolation procedure due to denaturation, cleavage, enzymatic hydrolysis, etc. The ability to bind other molecules reversibly is one of the most important properties of these molecules. The formation of specific and reversible complexes of biological macromolecules can serve as basis of their separation, purification and analysis by the affinity chromatography [6].

Affinity chromatography is one of the oldest forms of liquid chromatography method [8]. The first use of the idea of affinity chromatography may be considered as the isolation of α-amylase by using an insoluble substrate, starch, in 1910 by Starkenstein [6,9]. Similar studies with starch and amylase were carried out in the 1920s through 1940s by other investigators. In another study polygalacturonase was used as a support and ligand for the adsorption of alginic acid, the purification of pepsin through the use of edestin, a crystalline protein and the isolation of porcine elastase with powdered elastin were also performed. Afterwards Willstatter et al. enriched lipase by selective adsorption onto powdered stearic aicd [10]. The majority of the previous studies related purification of the enzymes. However the selective purification of antibodies with biological ligands was also being conducted. In 1920, it was reported that antibodies can recognize and bind substances with a specific structure, "antigens" [8]. This principle is firstly used in order to isolate rabbit anti-bovine serum albumin antibodies on a specific immunoadsorbent column consisting bovine serum albumin coupled to diazotized *p-aminobenzyl*-cellulose [10]. According to this approach, antibodies were isolated using urease and exhibited that these antibodies were proteins [8].

Separation procedure in affinity chromatography can be simply illustrated as shown in Figure 1. A sample containing the compound of interest is applied to the affinity column in the presence of mobile phase which was prepared in suitable pH, ionic strength and solvent composition for solute-ligand binding. This solvent which is referred as the application buffer presents the weak mobile phase of an affinity chromatography. While the sample is passing through the column compounds which are complementary to the affinity ligand will bind. However other solutes in the sample will tend to be washed off or eluted from the column as

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nonretained compounds. After all nonretained components are washed off the column, binding solute or together with ligand as solute-ligand complex are eluted by applying a solvent. This solvent which is referred as elution buffer represents the strong mobile phase for the column. Later all the interested solutes are eluted from the column, then application buffer is applied and the column is allowed to regenerate prior to the next sample application [4,8].

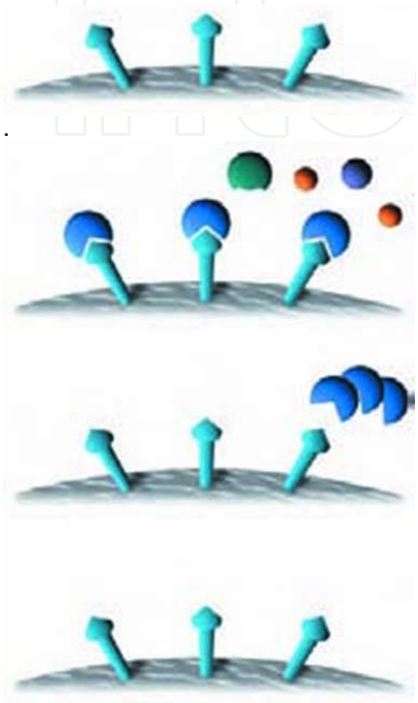

Figure 1. Separation procedure in affinity chromatography

The conditions in which the sample is applied to the column are chosen considering the conditions which the interaction between analyte and ligand is strong, mostly resembling the natural conditions of the analyte and ligand. The content apart from the analyte passes through the column without or with weak binding to the ligand while the analyte is retarded. After the analyte is obtained generally by using an elution buffer, the column is regenerated by washing with the application buffer in order to prepare the column for the next injection [1]. In the Figure 2, a typical scheme of an affinity chromatography application is shown.

As it is defined above; this technique is based on the interactions between specific bioactive substances, so the ligands are supposed to be originally biological substances, nevertheless columns with nonbiological ligands are also available and the same term "affinity chromatography" is used for the techniques performed by using these ligands. In order to distinguish the techniques according to the origin of the ligand, affinity chromatography with biological ligands may be termed as "bioaffinity chromatography" or "biospesific adsorbtion" [1]. The wide application potential of affinity chromatography leaded to the development of derived techniques some of which are listed below [7].

- Immunoaffinity chromatography

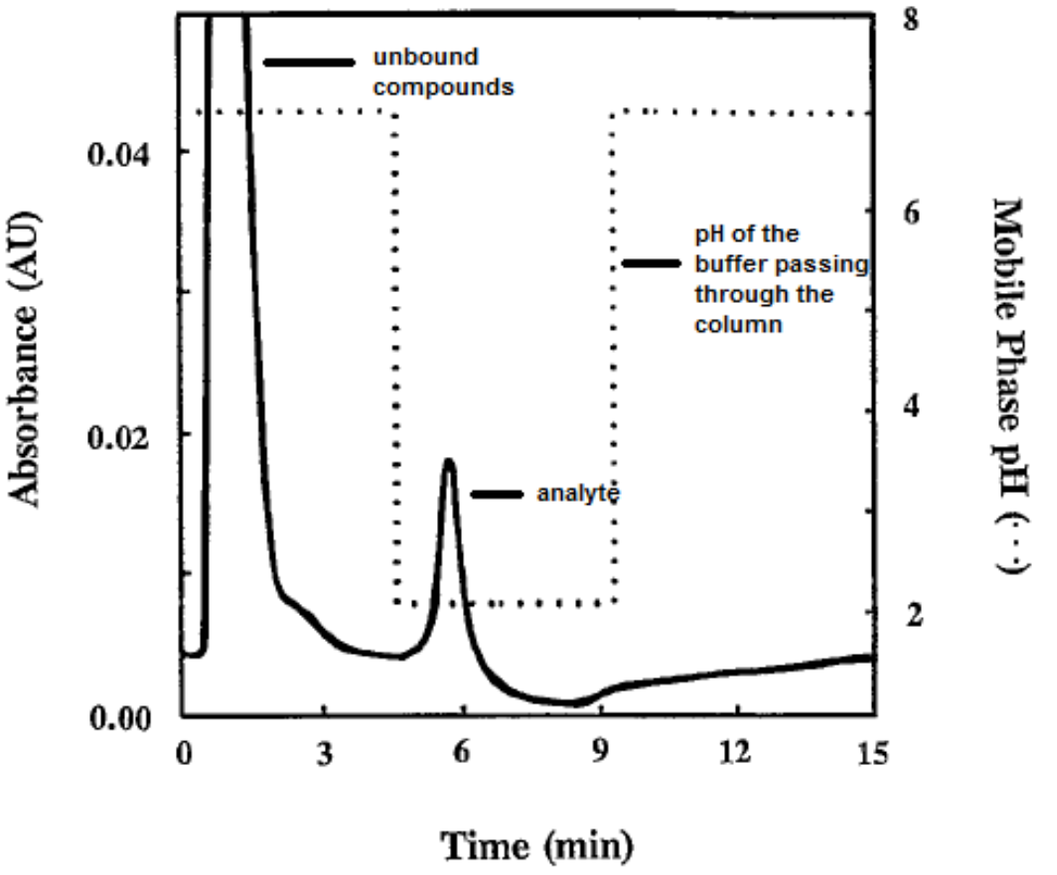

Figure 2. An example of a typical scheme of an affinity chromatography application [1].

- High performance affinity chromatography
- Affinity density perturbation
- Library-derived affinity ligands
- Lectin affinity chromatography
- Affinity partitioning
- Dye-ligand affinity chromatography
- Affinity electrophoresis
- Affinity capillay electrophoresis
- Centrifuged affinity chromatography
- Filter affinity transfer chromatography
- Affinity precipitation

- Avidin-biotin immobilized system
- Affinity tails chromatography
- Affinity repulsion chromatography
- Perfusion affinity chromatography
- Theophilic chromatography
- Weak affinity chromatography
- Receptor affinity chromatography
- Membrane-based affinity chromatography
- Molecular imprinting affinity
- Metal-chelate affinity chromatography
- Covalent affinity chromatography
- Hydrophobic chromatography

Affinity chromatography utilizes specific and irreversible biological interactions between a ligand covalently coupled to a support material and its complementary target. The solid support and ligand covalently attached on it, selectively adsorbs the complementary substance from the sample. The unbound part of the sample is removed and the purified substance can easily be recovered [11]. Selectivity of the ligand, recovery process, throughput, reproducibility, stability and economical criteria are some of the factors that influence the success of affinity chromatography process [9]. Successful affinity purification requires a certain degree of knowledge and understanding of the nature of interactions between the target molecule and the ligand to help determine the selection of an appropriate affinity ligand and purification procedure [3]. Therefore prior to start the process, materials and specifications listed below need to be selected [11]:

- Support material
- Activation method
- Ligand
- Immobilization method
- Conditions for adsorption and desorption

1.1. Support material

For successful separation in affinity chromatography, the important parameter is that solute of interest should be bound firmly and specifically while leaving all other molecules. This requires that the support within the column contain an affinity ligand that is capable of forming a suitably strong complex with the solute of interest [8]. The other important property is that the, support material must be biologically and chemically inert to avoid

the unspecific bindings [8,11] which requires that the support has a chemical character that is very similar to that of the medium in which it is operating. Since almost all affinity separations occur in aqueous solutions, the support should thus be as hydrophilic as possible. As a rule, the mobile phase used in affinity separations has a low ionic strength. The support should therefore contain as few charges as possible to prevent ionic interactions. Many supports are available which have desired properties or they gain such characteristics by hydrophilic coating [8]. Generally solid materials are used as support material though some soluble macromolecular materials are sometimes preferred for two-phase aqueous affinity partition processes. Uniformity in particle size and ease of the activation process are also required for support material that is used in affinity chromatography applications [11]. For the affinity chromatography at low pressure, nonrigid gels with large particle size are generally used as support materials while materials with small, rigid particles or synthetic polymers which are stable under high pressure and flow rates are used in high performance affinity applications [8,12].

There are many commercially available support materials for affinity chromatography can be divided into three groups as; natural (agarose, dextrose, cellulose); synthetic (acrylamide, polystyrene, polymethylacrylate) and inorganic (silica, glass) materials [7,13]. The most popular support material is agarose [13]. Agarose was used in the first modern application of affinity chromatography and still the most commonly preferred one [8]. Agarose consists of alternatively linked 1,3 bound β-D- galactopyranose and 1,4 bound 3,6-anhydro-*α*-*L*-galactopyranose, as shown in Figure 3 [8,14]. Agarose gels are stable to eluants with high concentrations of salt, urea, guanidine hydrochloride, detergents or water-miscible organic solvents but its stability is less beyond pH 4-9. To increase the thermal and chemical stability, cross-linked agarose is prepared. Cross-linked agarose is commercially available (Sepharose) and it can be used with many solvents, over pH 3-14 and at high temperatures up to 70°C [11]. However, strong acids, oxidizers as well as some rare enzymes may be harmful to agarose due to their damaging effects. On the other hand mild acid hydrolysis increases the quantity of sterically available galactose residues and turns agarose into an excellent sorbent for galactose binding proteins [14]. Due to its large beads and macroporous, accessible pore structures, agarose is well designed for use with large molecules. High capacity, presence of functional groups, good chemical stability especially at high pH, low non-spesific binding and good reproducibility are the advantage of agarose. Some properties of agarose such as solubility in hot water and non-aqueous solutions, sensitivity to microbial degredation and lack of rigidity restrict the usage under low or medium pressure [15]. Furthermore agarose must not be frozen or air dried. It is sold under several trade names, including Sepharose Fast Flow or Affi-Gel.

Cellulose is another example of polysaccharides which is used as support in affinity chromatography. Cellulose has a historical significance. Phospo- and DNA-cellulose are used especially for DNA related separations [14]. Antibody and enzyme purifications have also been carried out. However its fibrous and non-uniform character limits its use since cellulose detains macromolecules [11].

Dextran which is a linear *α*-1,6-linked glucose polymer produced by *Leuconostoc mesenteroides* is also used in affinity chromatography. Sephadex (cross-linked by glyceryl bridges) and

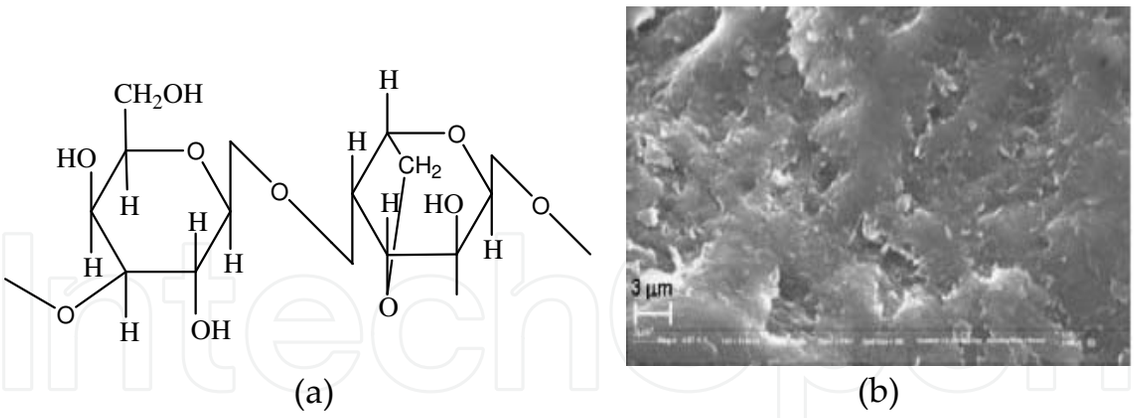

Figure 3. (a). Structure of agarose **(b).** SEM image of agarose [16]

Sephacryl (allyldextran cross-linked by *N,*N′-methylenebisacrylamide) are the two types of dextran gel used in separation. Sephadex is mainly used as a glucose polymer and employed for purification of many molecules such as lectins from *Helix pomatia* and *Vicia faba*, exoamylase from *Pseudomonas stutzeri* [14].

Polystyrene (Figure 4), which is a polymeric support is also unsuitable in its original form for affinity separations due to the highly hydrophobic character. Native polystyrene, which is often used as a reversed-phase material, must be first rendered hydrophilic by one of various surface-coating techniques before used in other chromatographic methods [8].

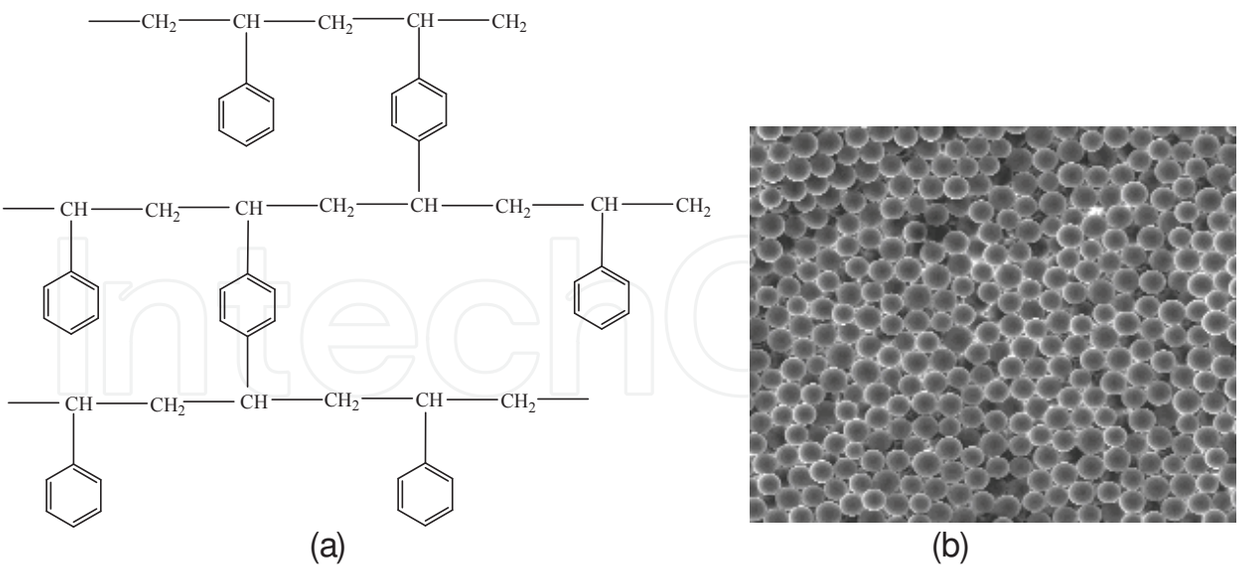

Figure 4. (a) Structure of polystyrene divinilybenzene **(b)** SEM image of polystyrene beads(x500) [17]

Polymeric supports based on polyacrylamide are synthesized by copolymerization of acrylamide and a cross-linking reagent and can be used directly in affinity chromatography due to its more hydrophilic properties than polystyrene supports. Polyacrylamide gels are either soft

or have small pores. However this gel is resistant against enzymatic attacks and does not absorb biomolecules, thus it is used widely despite its mechanic disadvantages [8,14].

Inorganic materials such as porous glass and silica are used when the extreme rigidity of the support material is needed [14]. Silica is especially used in the high-performance liquid affinity chromatography (HPLAC) or high-performance affinity chromatography (HPAC). Silica-based materials (Figure 5) are basically hydrophilic and they are suitable for affinity chromatography after they are modified at their surface. The native surface of silica is primarily covered with silanol groups which are weak acids and give strong negative charge to silica's surface at neutral pH. Irreversible adsorption of solutes (protein) can occur due to these charges and in combination with other binding forces. However, several methods can be used to render this surface inert toward such solutes, including polymer coating techniques and reactions between silica and alcohols or trialkoxysilanes [8]. They are also soluble at pH above 8 thus pH is an important parameter that limits the usage of silica [14].

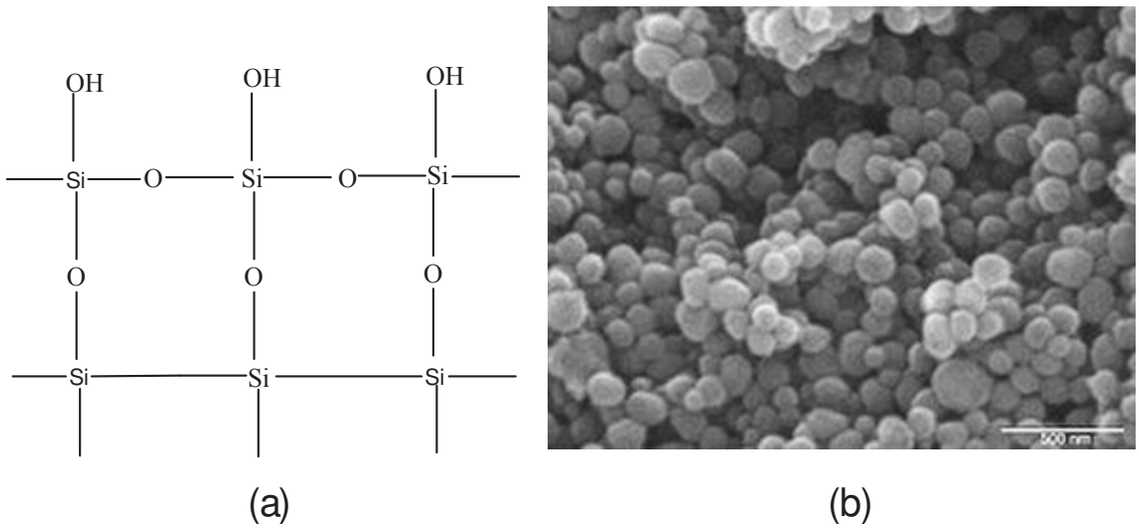

Figure 5. (a) Structure of silica **(b)** SEM picture of typical silicagel [18]

A support material should be inert toward solutes. On the other hand easy coupling with ligand is also desired. Support materials are rich in hydroxyl groups, therefore attachment of ligands have been focused mainly on using these regions as anchoring points. Ideal affinity support should allow unhindered access of a solute to the immobilized ligand. For a macromolecular solute, this requires a support that has large pores. Renkin equation can explain these pore sizes to be that allows one to estimate the effective diffusion coefficient (D_{eff}) of a solute in a porous material.

$$D_{eff} = D \ K_D \varepsilon_p [1 - 2.10(R_s / R_p) + 2.09(R_s / R_p)^3 - 0.095(R_s / R_p)^5] / t$$

In this equation, R_s/R_p is the ratio of the solute's radius (R_s) to the pore radius (R_p), ε_p is the particle porosity, τ is the tortuosity factor, K_D is the distribution coefficient for the solute, and D is the diffusion coefficient for the solute in free solution. By inserting different values for the

ratio R_s/R_p, one finds that the pore diameter should be at least five times the diameter of the solute to avoid severely restricted rates of diffusion.

For a protein in normal size (i.e., a diameter around 60 Å), a ratio of five for R_p/R_s means that the support pores should be in the range of 300 Å. Several common supports are available with such pore sizes. Support materials with very large pores give essentially unhindered diffusion for most solutes, but they also have a smaller surface area per milliliter of bed volume than supports with smaller pores. This reduced surface area leads to a diminished binding capacity. As a rule, a pore size of 300 to 700 Å is usually a good compromise in most situations encountered in affinity chromatography, since this gives fairly unrestricted diffusion for most biomolecules while also providing a relatively large surface area for retention [8].

Particle diameters of the affinity supports are available in a wide variety. These range from HPLC-type materials with diameters of 10 μm or less to large particles for preparative work that have diameters of 400 μm. The purpose of the separation, mechanical properties of the support and the characteristics of the sample are important factors on the selection of particle size of the support. From a theoretical viewpoint, it is always advantageous to have a small particle size, since this will promote fast mass transfer of a solute between the outer flow stream and interior of a support particle. Sample molecules are transported down through the column by the flow of the mobile phase in the spaces between the support particles. To reach the affinity ligands, these molecules must diffuse through the stagnant mobile-phase layer surrounding the particles (i.e., the film model) and proceed to the inside pore network (Figure 6). It is where the sample molecules will finally bind to the affinity ligand. When the retained molecules are eluted, the same steps occur but in a reversed order. Smaller support particles mean shorter diffusion distances, since they have shorter pores and a thinner stagnant mobile phase layer around and in the support. This results in shorter times needed for diffusion.

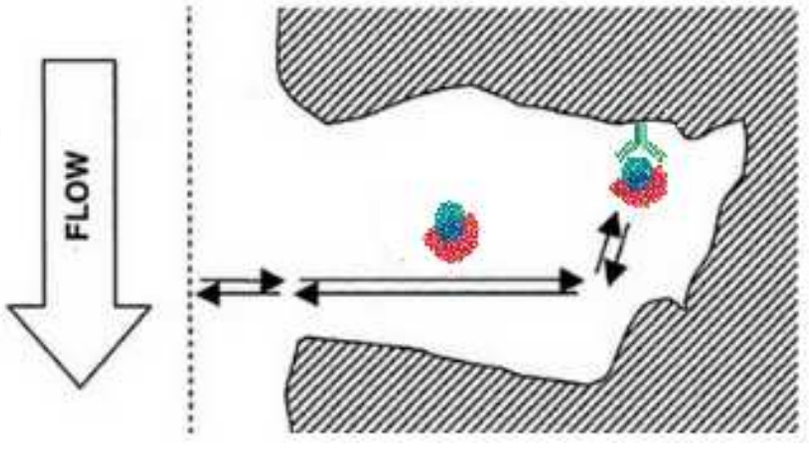

Figure 6. Transport processes that occur in a chromatographic column.

In preparative affinity chromatography, relatively large support particles are often used, making intraparticle diffusion the main factor limiting efficiency. In this case, diminishing the

particle size will increase the rate of movement of solutes between the support and surrounding flow stream, giving an improved column performance. It is this effect that was the original driving force behind the use of smaller supports in affinity columns, thus giving rise to the technique of HPLAC. Under such conditions, a decrease in particle size by a factor of five can make it possible to increase the flow rate by up to 25-fold and still retain good chromatographic performance. This results in a dramatic improvement in the productivity of the system. However, a point is eventually reached when a decrease in particle diameter no longer gives a proportional improvement in an affinity column's performance. This has been observed in many analytical-scale systems that use HPLC-type supports with particle sizes less than 10 μm in diameter. Under these conditions, diffusion in the particle is now relatively fast, and it is the adsorption/desorption of sample molecules to and from the affinity ligand that becomes the limiting factor in speed and efficiency. Although better efficiency is always obtained with small support particles, using a small particle size tengenders some difficulties. One problem is the much higher flow resistance of these smaller particles. This increased flow resistance may lead to bed collapse when using soft gels such as agarose. And, although supports like silica can tolerate the higher pressures, that results, these will require the use of more expensive pumps to work at such pressure, as is generally done in HPLC. Another route that could be taken with small affinity supports is to use a short and wide column instead of a long and narrow one. The advantages of this are that the shorter, wider column can be run at higher flow rates without creating high-pressure drops. Another drawback with small particle sizes, especially in preparative work, is the increased danger of fouling that exists when particulate contaminants are in the feed stream or sample. This occurs because the interstitial spaces in a bed of small particles can be too narrow for such agents to pass through. Such fouling will increase the flow resistance and may lead to bed collapse if the support material does not have sufficient mechanical strength.

As a result of these various requirements, the particle size to pick when designing a new affinity adsorbent will be a compromise between the desired chromatographic performances, properties of the feed stream, and the mechanical strength of the support. Some common selections made in specific cases will be described in the next few sections.

Porous supports like agarose, polymethacrylate, or silica beads are generally used in current applications of affinity chromatography. However, in the past several years other types of supports have also become available commercially. Many of these newer materials have properties that give them superior performance in certain applications. Materials that fall in this category include; nonporous supports, membranes, flow-through beads, continuous beds and expanded-bed particles.

Nonporous beads with diameters of 1 to 3 μm can be an optimum choice for fast analytical or micropreparative separations, since the limiting factor of pore diffusion is virtually eliminated in these materials. Such beads may also be the best choice for fundamental or quantitative studies of affinity interactions, since the binding and dissociation behavior observed in these materials should be more directly linked with the interactions occurring between solutes and the affinity ligand. However, there is a substantial loss of surface area and binding capacity.

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Membranes have been used for affinity chromatography in various formats, such as stacked sheets, in rolled geometries, or as hollow fibers. Materials that are commonly used for these membranes are cellulose, polysulfone, and polyamide. Because of their lack of diffusion pores, the surface area in these materials is as low as it is in nonporous beads. However, the flat geometry and shallow bed depth of membranes keep the pressure drop across them to a minimum degree. This means that high flow rates can be used, which makes these membranes especially well-suited for capturing proteins from dilute feed streams.

As stated earlier, porous supports with a larger diameter facilitate low column backpressures and allow easy passage of contaminants through the column. But it is also necessary to keep the diameter of these supports as small as possible to diminish diffusion distances and thereby improve their chromatographic performance. One solution of these contradictory requirements is to use particles that allow the flow of mobile phase directly through some of the pores. This is done in materials known as *perfusion media* or *through-pore particles*. Flow-through particles were initially developed in the early 1990s for ion-exchange chromatography and were later adapted for use in affinity chromatography.

Flow-through particles have a bimodal pore configuration, in which both small diffusion pores and large flow-through pores are present. Substances applied to a bed of this support are transported by mobile phase flow to the interior of each particle, leaving only short distances to be covered by diffusion to the support's surface (Figure 7). This combination leads to a dramatic improvement in performance compared with standard porous particles of the same size. This improvement is most pronounced in situations where slow diffusion is a limiting factor, such as in the chromatography of large molecules (e.g., proteins) at high flow rates.

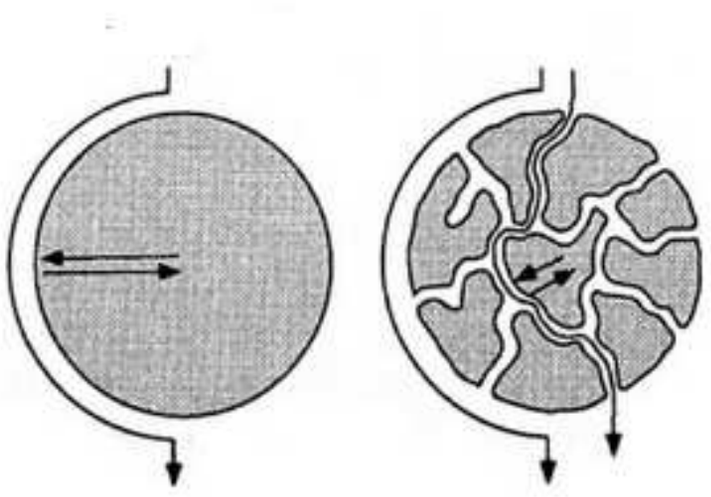

Figure 7. Comparison of a particle with normal porosity versus a particle that contains flow-through pores. The normal particle has long diffusion distances, whereas the flow-through particle has short diffusion distances.

Another format developed in 1990s was the *continuous bed* or *monolithic support*. Continuous bed supports consist of a single piece of material intersected by pores large enough to support chromatographic flow through the bed. Continuous beds have been developed using many well-known chromatographic materials, such as polyacrylamide, silica, polystyrene/polymethacrylates, cellulose, and agarose. Most of these continuous beds have two types of pores: large flow-carrying pores and smaller diffusion pores.

The preparation of a continuous bed is usually straightforward. These beds can often be prepared directly in a chromatographic column, thereby avoiding the time-consuming steps of size classification and column packing that are normally needed with particle-based supports. Reports using continuous beds in affinity chromatography have shown that the efficiency of these materials is as good as that for particle-based supports.

In order to avoid column clogging, various pretreatment methods like filtration and centrifugation are often necessary to remove particulate matter from samples. To cut down on the need for such methods, a new class of adsorbents has recently been developed to handle viscous and particle-containing feed streams. These materials are known as *expanded-bed adsorbents*. In expanded-bed chromatography the direction of mobile phase flow is upward through the column and is fast enough to fluidize the support particles in the column. This causes the column bed to expand. This expansion makes the interstitial spaces in the column bed larger so that solid contaminants like cells and cell debris can pass through, thereby avoiding column clogging.

Another new type of expanded-bed adsorbent uses a thin layer of active material (i.e., derivatized agarose) that surrounds a heavy core. These adsorbents have small diffusion distances for biomolecules along with a higher density than other expanded-bed particles. The advantage of this combination is that it allows better chromatographic efficiencies to be obtained at higher flow rates [8].

1.2. Ligand

Ligands are the molecules that bind reversibly to a specific molecule or group of molecules, enabling purification by affinity chromatography [4]. These molecules which play a major role in the specificity and stability of the system are essential for affinity chromatography [13]. The selected ligand must be capable of selectively and reversibly binding to the substance to be isolated and have some groups which are available for modifications in order to be attached to the support material. It is very important to ensure that the modifications do not reduce the specific binding affinity of the ligands. There are general ligands such as dyes, amino acids, Protein A and G, lectin, coenzyme, methal chelates as well as specific ligands such as enzymes and substrates, antibodies and antigens [19].

Affinity ligands are classified as synthetic and biological. Biological ligands are obtained from natural sources such as RNA and DNA fragments, nucleotides, coenzymes, vitamins, lectins, antibodies, binding or receptor proteins, or in vitro from biological and genetic packages, employing display techniques including oligonucleotides, peptides, protein domains and proteins. Synthetic affinity ligands are generated either by de novo synthesis or modification

of existing molecular structures (triaznyl nucleotide-mimetics, purine and pirimidine derivatives, non-natural peptides, triazinyl dyes, other triazine-based ligands, oligosaccharide and boronic acid analogues). These can be generated by rational design or selected from ligand libraries. Synthetic ligands are generated using three methods;

- The rational method features the functional approach and structural template approach.
- The combinatorial method relies on the selection of ligands from a library of synthetic ligands synthesized randomly.
- The combined method employs both methods the ligand is selected from an intentionally prepared library based on a rationally designed ligand.

Many parameters have to be taken into account in order to select appropriate ligand. Table 1 exhibits the advantages and disadvantages of synthetic and biological ligands. Selectivity and affinity are the main advantages of biological ligands. Such ligands can be generated by *in vitro* evolution approaches and selecting from large combinatorial ligand libraries based on biological/genetic packages. Protein ligands display special advantages for example; higher affinities, higher proteolytic stability, preservation of their biological activity when produced by fusion to a different protein or domain. However these ligands can be expensive and unstable to the sterilization and cleanin conditions used in manufacturing process of biologics because of their biological origin, chemical nature and production methods. There is high contamination risk of the end-product with potentially dangerous leaches, in addition to possible contaminants originated from the biological source [20].

	Synthetic ligands	**Biological ligands**
Capacity	High	Low to medium
Cost	Low to medium	Medium to high
Selectivity	Medium to high	Very high
Stability	High	Low to medium
Toxicity	Medium	Low

Table 1. Comparison of biological and synthetic ligands [20]

Despite the advantages of the affinity chromatography technique, its use is limited due to high cost of affinity ligands and their biological and chemical instability. The development of methods for production of stable synthetic ligands has enabled "utilization of these materials in large scale. For the design of synthetic ligands, information about structure of the target protein and a potential binding site are required, thus a structure-based design can be achieved, in case correct prediction of the ligand's comformation and the binding affinity of the designed ligand. Function-based design can be applied when the structure of the target is not known [9]. Substantially, selection and design of ligands may be performed by using a template which is a part of a natural protein-ligand couple, model-

ling a molecule which complements the binding sites of the target or directly resembling the natural interactions [2].

High selectivity of the biological ligands is a benefit; however these ligands have some handicaps, such as their low binding capacity, cost-efficacy issues, some problems in scale-up and purification process. Hence, synthetic ligands may offer a solution for these issues and enable to provide selectivity, efficacy and inexpensiveness in a body. Biomimetic textile dyes which are developed in 1970s are the most known synthetic ligands. The use of these dyes in biopharmaceutical field is limited due to some issues such as selectivity, purity and toxicity. These complications have led to new researches and developments about biomimetic dyes and new ligand design techniques [2].

The selection of the ligand may be done according to the specific binding site of a target, but this manner of selection may fail owing to the fact that immobilization process may change binding affinity. It is known that the affinity of the target to the ligand is dependent on the features of the target as well as support material, activation and coupling chemistry. Some other techniques other than using free ligand solution in order to predict the conditions of three-dimensional matrix. On the purpose of ligand selection, a great number of alternatives may be tested for binding the target or work with more accurate options by employing ligand design techniques. Therefore the idea to combine chemistry with computational tools has accelerated the developments on this field [2]. Along the development of affinity chromatography techniques, different laboratories are established with the purpose of collection of several ligands for affinity chromatography [9].

Protein-structure-based design of the ligands depends on the correct prediction of the structure of the target protein and the binding site. Apart from this, protein-function-based design is applicable in case the conformation of the target protein is not known. This method is based on the integration of some known properties of the ligand such as an essential molecular structure, a functional group or a derivative of some parts of the structure [9]. The design of a ligand requires several steps to fulfil [2]:

1. Determination of the binding site or possible biological interactions to use as a template for the modelling,
2. Initial design of the ligand using this template,
3. Preparation of a ligand library and chromatographic evaluation,
4. Selection of the ligand of interest,
5. Optimisation and chromatographic evaluotion of the adsorbent following the immobilization.

Beyond these design methods, some combinatorial approaches have been developed on the purpose of ligand selection. Synthetic peptide libraries which include all sequences for a length of a protein structure are one of these approaches. By means of these libraries, *in vitro* prediction of the action of the library mixture as it passes through the surface where the protein of concern is immobilized is possible. The ligands which possess affinity to the immobilized protein are

suggested as ligands for the affinity chromatography application. It is also of choice to use phage libraries and a screening method known as biopanning. Phage display method allows determining suitable ligands not only for peptides and proteins but also for nonpeptide structures (Figure 8).

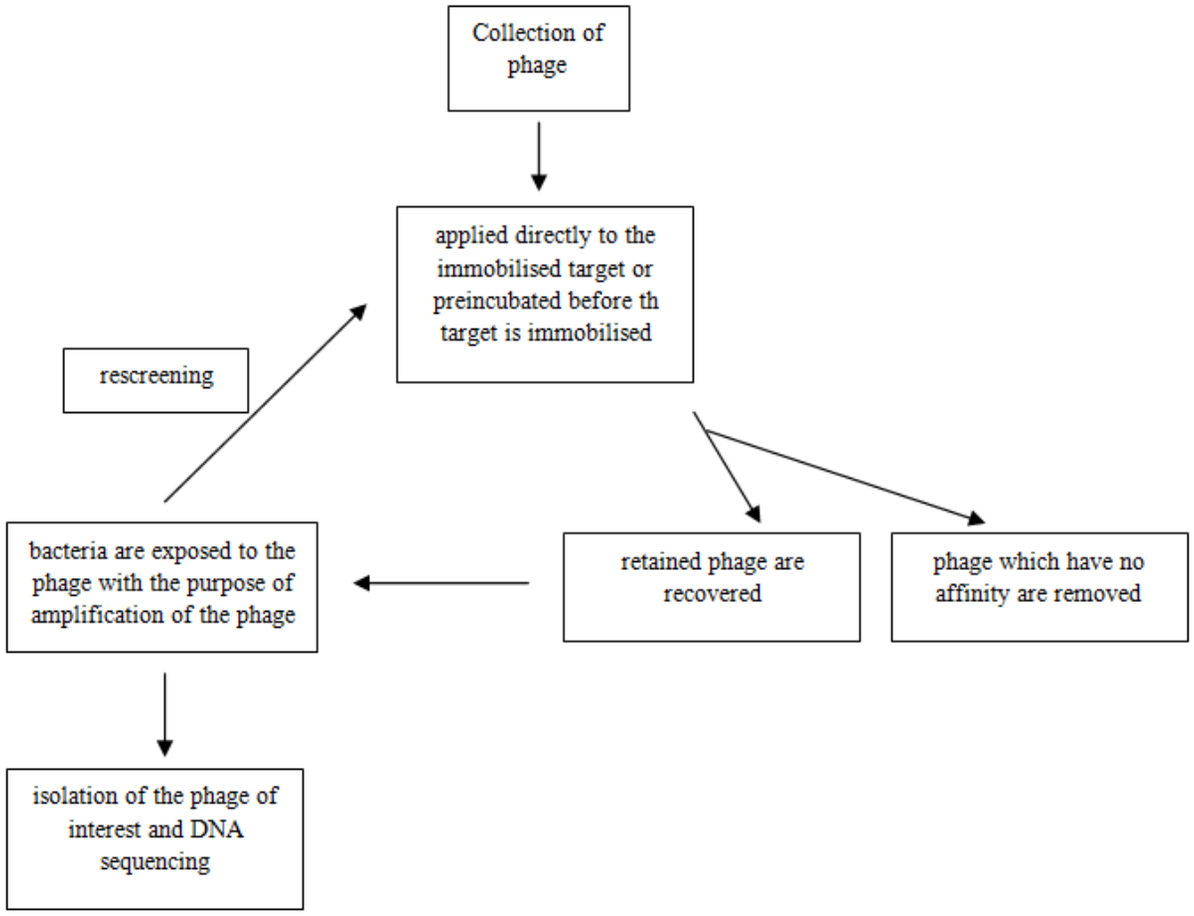

Figure 8. Selection of the phage from the phage-displayed combinatorial peptide library [9]

A collection of phage is incubated with the target and retained ligands are considered to be candidates for ligand [9]. At the end of the cycle, the process is repeated in order to increase the amount of the protein which has required binding features. The sequence of the protein is provided from the viral DNA. Phage display libraries are indisputably useful especially for epitope mapping, vaccine development, bioactive peptides and some non-peptide structures and the ligands determined using this method are appropriate for chromatographic analyses, nevertheless there are some cases that limit the use of this technique, such as some optimization problems and some issues as working in large scale as well as the limitation of the application since peptides does not work unless it is a part of the phage, not in free solution [2].

Although its use in ligand selection for large scale of affinity chromatography is not wide, ribosome display and systematic evolution of ligands by exponential enrichment (SELEX) may

be mentioned as another approach and a potential ligand design and selection method due to its versatility and rapidity [9]. Ribosome display method enables to select and develop a protein library *in vitro* [2]. The principle of ribosome display is depicted in Figure 9. A collection of DNA encoding the selected peptide is exposed to an *in vitro* transcription and translation process, then in favourable condition, the complex of mRNA, peptide and ribosome since the stop codon does not exist. Thereafter the complex is passed thtough the immobilised target and the peptides which possess high affinity to the target are retained. At the end of this process, it is possible to seperate mRNA usually by EDTA, then by means of reverse transcription, DNA are attained and amplified [9].

SELEX is a widely used technique for screening of aptamers which are nucleic acid ligands. According to this method, a pool of DNA with a random sequence region attached to a constant chain is constituted by amplification then transcribed to RNA. RNA pool is separated according to the affinity of RNA molecules to a target protein. DNA molecules obtained by reverse transcription from retarded RNA molecules are amplified and the cycle is repeated.

The selection of the ligand may be designed according to the structure of the target protein as well. Under the favor of combination of the structure-based design and combinatorial chemistry, the efforts to synthesis a convenient structure are minimized [9].

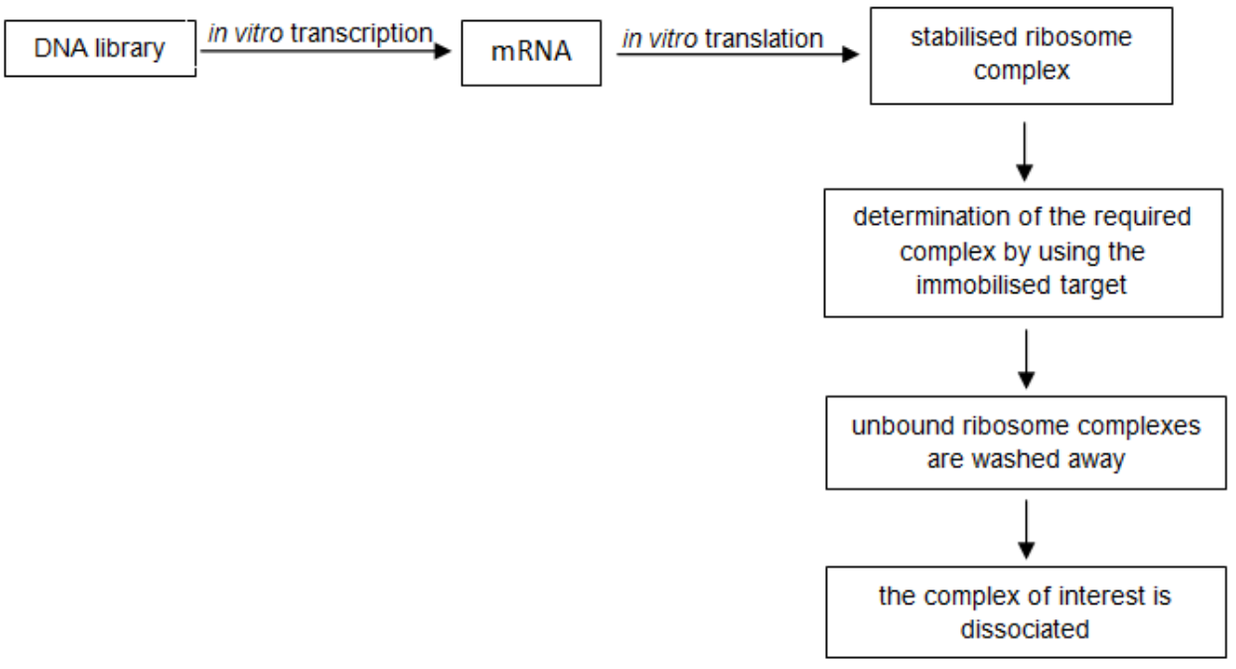

Figure 9. Ribosome display method [9]

1.3. Immobilization

The immobilized ligand is an essential factor that determines the success of an affinity chromatographic method [12]. The method which is used for affinity ligand immobilization is important because actual or apparent activity of the final column can be affected. Decrease in

ligand activity which result in multisite attachment, inappropriate orientation or steric hindrance can be observed if the correct procedure is not pursued [21]. Several methods are available to couple a ligand to a pre-activated matrix. The correct selection of coupling method depends on the ligand characteristics [4].

Before ligands are coupled matrix is activated. Among several methods used for activation, the cyanogen bromide activation is the most frequently preferred. Activation using this method produces a highly reactive cyanate ester [7]. The ligands are attached to the support via primary aromatic or aliphatic amino groups. High toxicity of cyanogen bromide is the disadvantage of this method [13,19]. Subsequent coupling of ligands to the activated matrix results in an isourea linkage. Despite the popularity of this method, the isourea linkage of the ligands causes several problems during the purification procedure, including nonspecific binding due to charge and leakage of the ligand because of instability of the isourea bond. *N*-hydroxysuccinimide (NHS) esters have also been used for immobilizing ligands. The preparation of active esters requires a matrix that contains carboxylic groups. Such matrices can be easily obtained from agarose by activation of the hydroxyl groups with different reagents, including cyanogen bromide, activated carbonates, etc. and successive reaction with ω-amino acids of different sizes depending on the length of the spacer arm required. The NHS ester is then prepared by mixing the carboxylic matrix with dicyclohexylcarbodiimide and NHS. Due to the stability problem a different method based on *N*,*N*,*N*′,*N*′-Tetramethyl (succinimido) uronium tetrafluoroborate can be also used. The covalent attachment of ligands to such activated carriers provides the production of stable amide bonds. Another method for activating polysaccharides is the use of *N*′*N*-disuccinimidyl carbonate (DSC), which forms highly reactive carbonate derivatives with polymers containing hydroxyl groups. These derivatives react with nucleophiles under mild, physiological conditions (pH 7.4), and the procedure results in a stable carbamate linkage of the ligand coupled to the carrier. The immobilization of different ligands (e.g., enzymes, enzyme inhibitors, antigens and antibodies) on activated carbonate carriers has been achieved, together with excellent maintenance of biological activity of the proteins [7]. Pre-activated commercial matrices are also available (Table 2) to avoid many steps and problems of chemical activation process. A wide range of coupling chemistries, involving primary amines, sulfhydryls, aldehydes, hydroxyls and carboxylic acids are available for covalently attaching ligands to the matrices [12]. The use of commercially available, pre-activated media is recommended to save time and avoid the use of the potentially hazardous reagents that are required in some cases [4].

After the activation of the support material, it is ready for the immobilization process of the ligand. In case the ligand is a small molecule, steric hindrance will occur between the immobilized support and the compound of interest (Figure 10). This may reduce or totally block specific binding of the substance. Use of the supports having a spacer arm attached or attachment of a spacer molecule to the support before immobilization of the ligand generally solves this problem. Spacer arm keeps ligand at a suitable distance from the surface of the support (Figure 9), thus the substance of interest will not be prevented to attach to the immobilized ligand. It is possible to bind spacer arms directly to the support prior to the imobilization of the ligand. Then a secondary reaction provides the attachment of ligand to

Product name	Functional group specifity
UltraLink Iodoacetyl resin	-SH
CarboLink Coupling resin	-CHO, C=O
Profinity™ Epoxide resin	-NH_2, -OH, -SH
Affi-Gel 10 and 15	-NH_2
Pierce CDI-activated resin	-NH_2
Epoxy-activated Sepharose™ 6B	-NH_2, -OH, -SH
CNBr-activated Sepharose 4 Fast Flow	-NH_2
EAH Sepharose™ 4B	-COOH, -CHO
Thiopropyl Sepharose™ 6B	-SH
Tresyl chloride-activated agarose	-NH_2, -SH

Table 2. Activated commercially available resins of affinity chromatography

the spacer. The substance of interest doesn't be able to bind the ligand unless the spacer arm is long enough, but it is also possible to shorten the spacer arm in salt buffer [19].

Figure 10. Spacer arm, keeping ligand at a suitable distance from the surface of the support

Properties of an ideal spacer arm are listed below:

1. It should be long enough (at least 3 atoms) to keep the substance at an appropriate distance.
2. It should be inactive not to cause a non-specific binding.
3. It should have bifunctional group for the reaction with both support and the sample [9].

Compounds which have diamine groups such as hexanediamine, propanediamine and ethylenediamine are the most preferred spacer arms used in affinity cromatography. Some other examples of spacer arms are shown in Table 3 [19].

The following step is the immobilization of ligands on the activated matrix by isourea bonds. Immobilization through isourea linkage has some disadvantages including nonspesific binding of the ligand because of the instability of the bonds. Another method for immobilization is to use active esters such as *N*-hydroxy-succimide (NHS) esters. The carboxyl groups required for preparation of active esters can be prepared by activation of hydroxyl groups of

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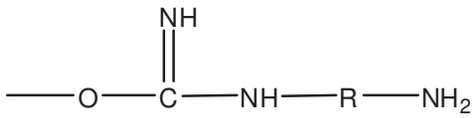
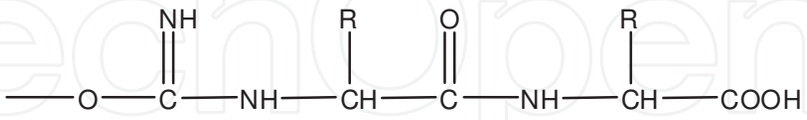
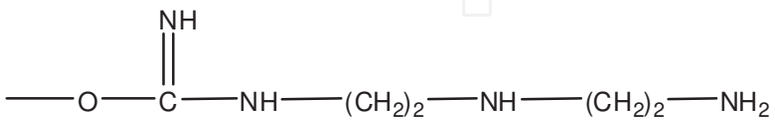
Name	Structure
Alkylamine	
Diamine	
Polypeptides	
Polyamine	
Polyether	-0$(CH_2)_2O(CH_2)_2O(CH_2)_2OH$
Amino acid	NH_2RCOOH

Table 3. Some examples for spacer arms and their structures

agarose. The ligands attach to this type of matrix via amide bonds. It is also possible to activate polysacharides by formation of highly reactive carbonate derivatives. In this case the polymer which contains hydroxyl groups is activated by the use of *N'N*-disuccinimidyl carbonate (DSC). The resultant carbonate derivatives create stable carbamate bonds with nucleophiles under mild, physiological conditions. Immobilization methods can be categorized as follow (Figure 11) [7].

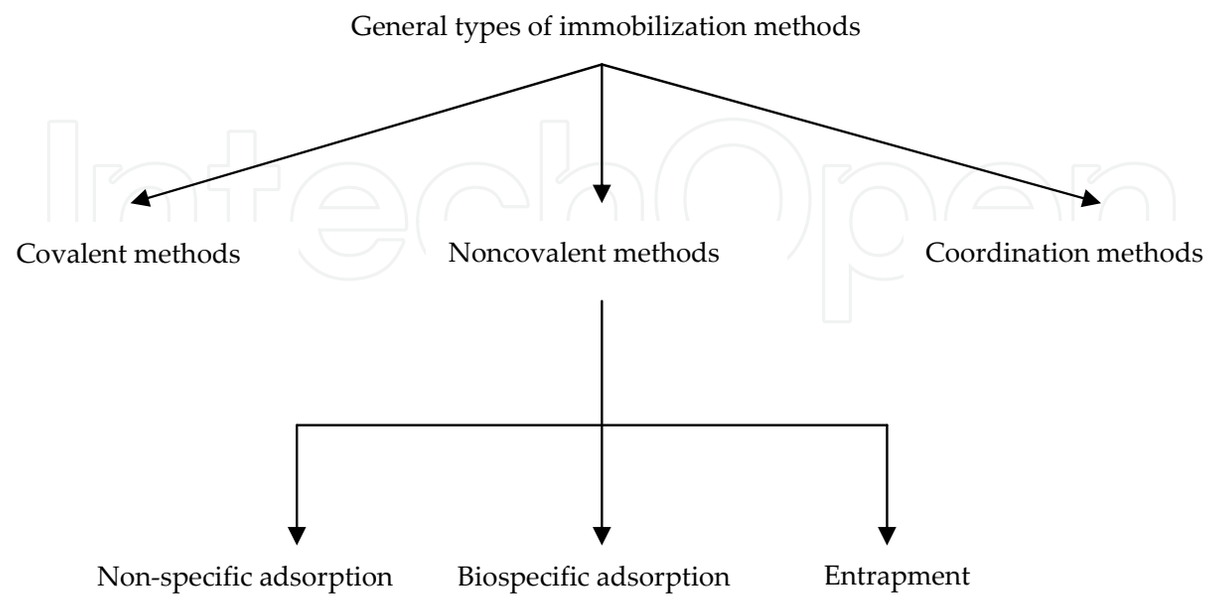


Figure 11. Immobilization methods used in affinity chromatography

1.3.1. Noncovalent immobilization technique

The simple adsorption of ligand to surface, binding to a secondary ligand, or ligand immobilization through a coordination complex can be classified as this type of immobilization. This technique can be subdivided as follow;

a. Nonspecific Adsorption; It is based on the attachment of ligand to support that has not been specifically functionalized for covalent attachment. Adsorption of the ligand to a support depends on the chemical characteristics of both the ligand and support. Columbic interactions, hydrogen bonding, and hydrophobic interactions involve in this type of immobilization.

b. Biospecific Adsorption; In this type of noncovalent immobilization method the ligand of interest bind to a secondary ligand attached to the support. Although a variety of secondary ligands can be used for this purpose, two of the most common are avidin and streptavidin for the adsorption of biotin-containing compounds and protein A or protein G for the adsorption of antibodies.

c. Coordination Complexes; A coordination complex can be used to prepare an immobilized ligand in some cases. This is used to place metal ions into columns for immobilized metal-ion affinity chromatography (IMAC) which is based on the formation of a complex between a metal ion and electron donor groups.

1.3.2. Covalent immobilization methods

Covalent immobilization is the most popular method in affinity chromatography. In this method, it is necessary to activate the ligand and/or the support first. Activation of the ligand can be conducted when it is desired to couple this ligand through a specific region. An example is the creation of aldehydes in the carbohydrate regions of an antibody for its attachment to a support that contains amines or hydrazide groups. The use of an activated support is more common for ligand immobilization but tends to be less specific in nature. Examples include the immobilization of proteins through their amine groups to supports activated with *N*-hydroxysuccinimide or carbonyldiimidazole. The support used for covalent immobilization must meet several requirements. First, sufficient number of groups for activation and ligand attachment should be. Hydroxyl groups on the support are employed in most covalent coupling methods. Depending on how its surface is activated, a support can be used to immobilize ligands through their amine, sulfhydryl, hydroxyl, or carbonyl groups, among others.

1. Amine-Reactive Methods; Amine groups is often used for the immobilization of proteins and peptides. Specific methods are cyanogen bromide method, reductive amination, *N*-hydroxysuccinimide technique, and carbonyldiimidazole method.

a. Cyanogen Bromide Method ; The cyanogen bromide (CNBr) method was the first technique used on a large scale for immobilizing amine-containing ligands and involves the derivatization of hydroxyl groups on the surface of a support to form an active cyanate ester or an imidocarbonate group. Both of these active groups can couple ligands through

primary amines, but the cyanate ester is more reactive than the imidocarbonate. The CNBr method utilizes relatively mild conditions for ligand attachment, making it suitable for many sensitive biomolecules. But one problem with this approach is that the isourea linkages obtained by the reaction of CNBr with the support are positively charged at a neutral pH. This means that these groups can act as anion exchangers and nonspecific binding can be occur. Other problems with this method include the toxicity of CNBr, requiring the use of adequate safety precautions during the activation process, and the leakage of ligands that can result from CNBr-activated supports (Figure 12).

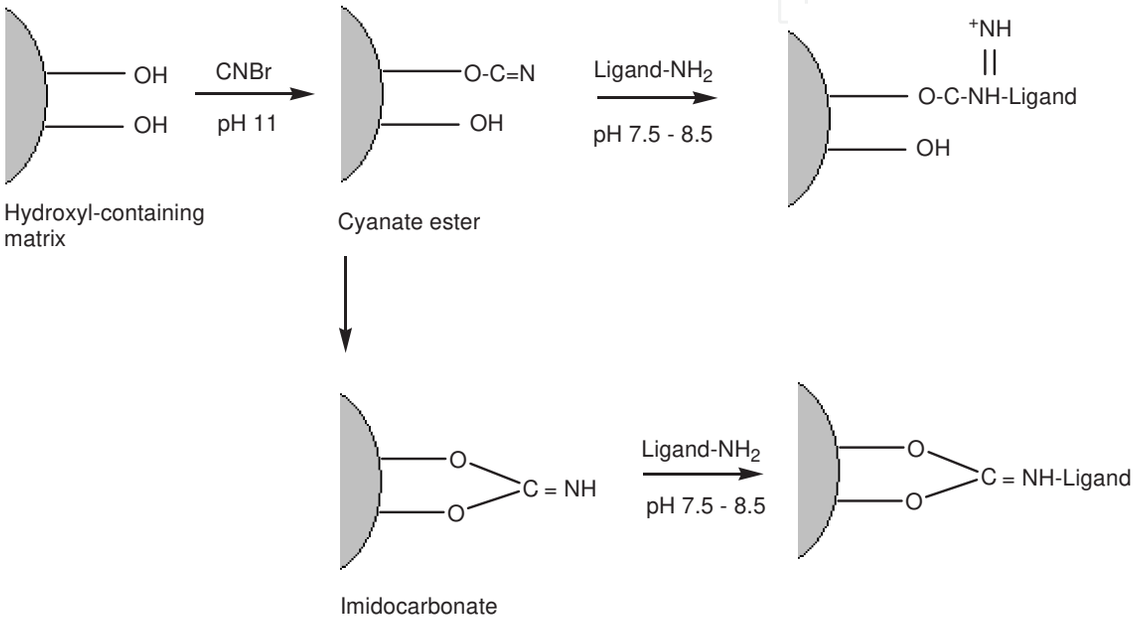


Figure 12. Cyanogen bromide immobilization method patway [8]

b. Reductive amination (also known as the Schiff base method); Reductive amination couples ligands with amine groups to activated periodate is used to oxidize diol groups on the support's surface to give aldehydes. This can be performed directly on carbohydrate-based supports like dextran or cellulose. However, materials like silica or glass must first be treated to place diols on their surface. This can be accomplished by reacting the silica or glass with γ-glycidoxypropyltrimethoxysilane, followed by acid hydrolysis. When an amine-containing ligand reacts with the aldehyde groups, the resulting product is known as a Schiff base. Since this is a reversible reaction, the Schiff base must be converted into a more stable form. This is achieved by including sodium cyanoborohydride in the reaction mixture. Cyanoborohydride is a weak reducing agent that converts the Schiff base into a secondary amine without affecting the aldehydes on the support. After the coupling reaction is completed, the remaining aldehyde groups can be removed by treating the support for a short period of time with a stronger reducing agent (i.e., sodium borohydride) or by reacting these groups with an excess of a small amine-containing agent (e.g., ethanolamine). The Schiff base method is relatively easy to perform

and often gives a higher ligand activity than other amine-based coupling methods. This also results in ligands that have stable linkages to the support and that can be used for long periods of time. However, there are some disadvantages of this method. One is the need to work with relatively hazardous agents such as sodium cyanoborohydride and sodium borohydride. Thus, care must be taken to perform this technique with proper ventilation and safety precautions. The use of sodium borohydride for the removal of excess aldehyde groups must also be carried out with caution, since the use of conditions that are too harsh may result in the loss of ligand activity.

c. *N*-Hydroxysuccinimide Method; The N-hydroxysuccinimide (NHS) method is another technique often employed when immobilizing biomolecules through amine groups. This gives rise to the formation of a stable amide bond. There are a number of ways a support can be activated with NHS. The relative ease with which activated supports can be prepared is one advantage of the NHS method. But the fast hydrolysis of NHS esters tends to compete with the immobilization of ligands. This rate of hydrolysis increases with pH and is particularly important when dealing with dilute protein solutions. The half-life of these NHS groups at pH 7 and 0°C is approximately 4 to 5 h and decreases to as little as 10 min at pH 8.6 and 4°C (Figure 13).

d. Carbonyldiimidazole Method; Carbonyldiimidazole (CDI) can also be used to activate supports for the immobilization of amine-containing ligands. This reagent can react with materials that contain hydroxyl groups to produce an acylimidazole, which forms an amide linkage as the result of the interaction with primary amines on a ligand. Supports with hydroxyl groups will react with CDI to produce an imidazolylcarbamate. The reaction of imidazolylcarbamata with primary amines proceeds at pH 8.5 to 10.0. The CDI method is relatively simple and easy to perform. In addition, supports that have been activated by production imidazolylcarbamate groups are more stable to hydrolysis than those activated by the NHS method. A CDI-activated support is stable when stored in dry dioxane, with a half-life of greater than 14 weeks. Another advantage of this method is that the amide linkages formed by this technique (as well as those created by the NHS method) are more stable than the isourea linkages obtained by CNBr immobilization. One disadvantage of the CDI method is that it tends to produce ligands with a lower activity than alternative techniques (e.g., reductive amination) (Figure 14).

e. Other methods; One example is the use of cyanuric chloride (or 2,4,6-trichlorotriazine) to activate hydroxyl- or amine-containing supports for ligand attachment. Cyanuric chloride has been widely employed as a cross-linking agent and as a reagent for protein modification. It has three reactive acyl-like chlorines, each of which has a different chemical reactivity. The first chlorine is reactive toward hydroxyls and amines at 4°C and pH 9. After the first chlorine reacts, the second requires a slightly higher temperature for its reaction (20°C), and the third chlorine needs an even higher temperature (80°C). Other techniques for amine-containing ligands include the azalactone, divinylsulfone, bisoxylane, ethyldimethylaminopropyl carbodiimide, and tresyl chloride-tosyl chloride methods. Some of these methods are specific for certain supports (e.g., the azalactone method),

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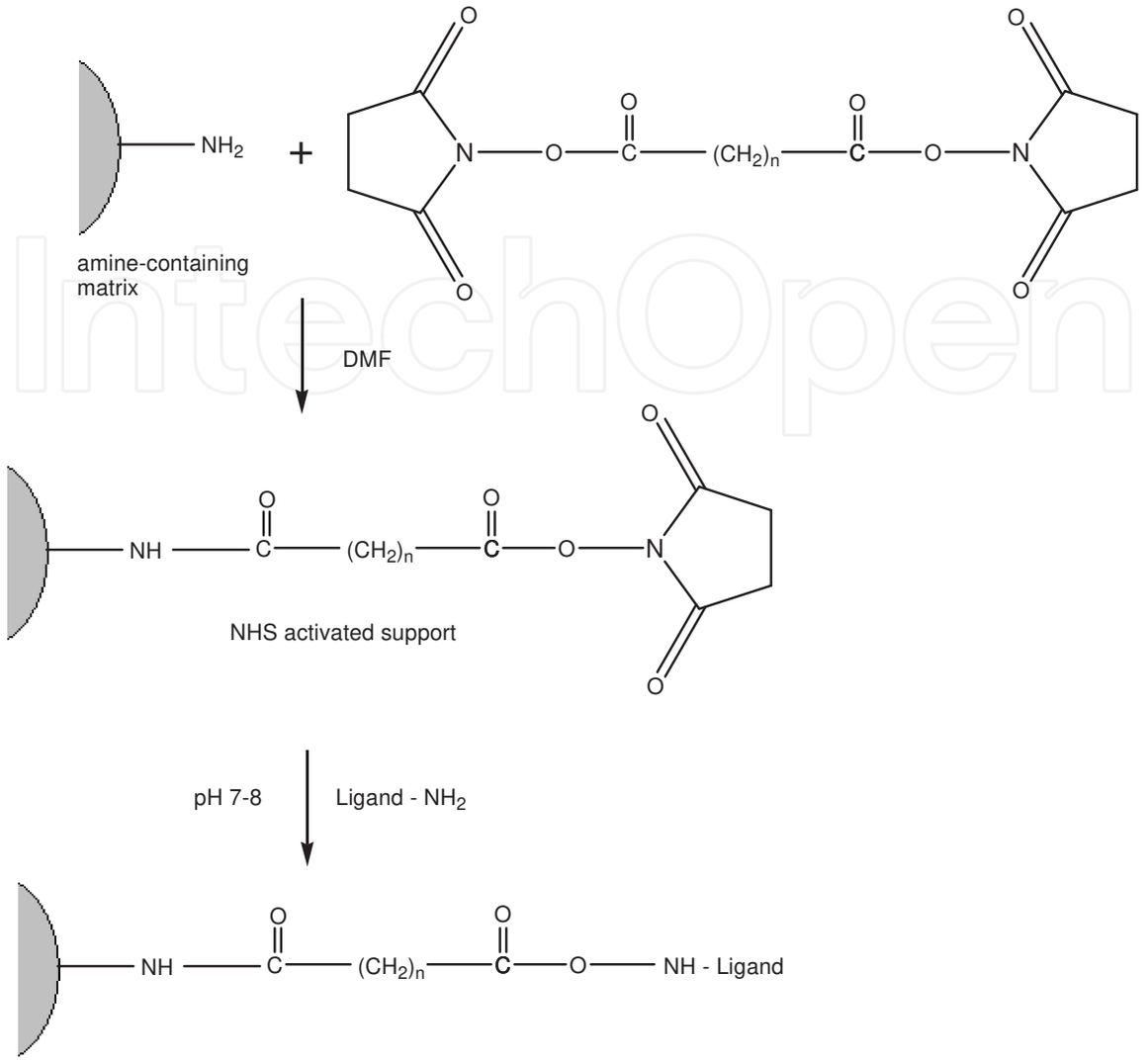

Figure 13. *N*-Hydroxysuccinimide immobilization method [8]

while others can be used with a variety of materials. The final selection among these approaches will often depend on the type of ligand being immobilized, the support desired for this ligand, and the conditions that can be tolerated by both the ligand and support during the immobilization process.

2. Sulfhydryl-Reactive Methods; The use of sulfhydryl groups on ligands is another approach for preparing affinity supports. If a ligand has a free sulfhydryl group on its surface, using this group is advantageous, since it often provides site-specific immobilization and a cleavable product. If the ligand is a protein or peptide that has no free sulfhydryl groups but that does have a disulfide bond, this bond can be reduced to allow ligand attachment. It is also possible to introduce sulfhydryl groups on a ligand by thiolating aminesor carboxyl groups. A support can be activated in several ways for the immobilization of ligands through sulfhydryl groups. Unlike amine-reactive methods,

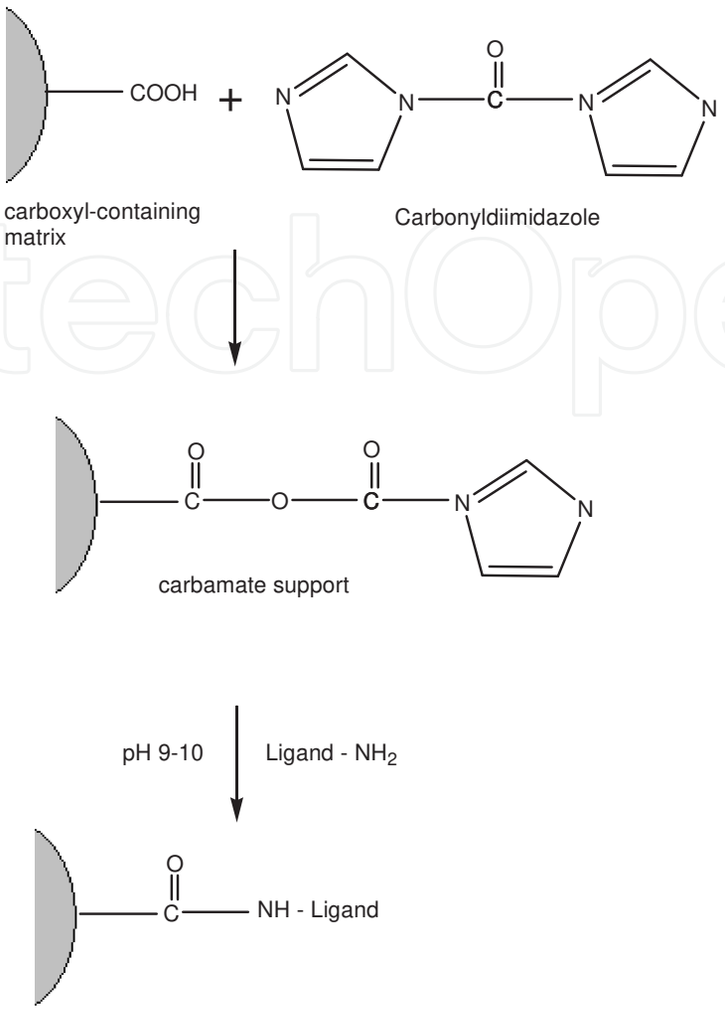

Figure 14. The carbonyldiimidazole immobilization method [8]

where hydroxyl groups on the support are generally used, most sulfhydryl-reactive methods require the introduction of an amine,carboxyl group, or some other intermediate site onto the support. For example, silica cannot be used directly with sulfhydryl-reactive methods but must be reacted with aminopropyltriethoxysilane or mercaptopropyltrimethoxysilane to convert it into a suitable form.There are various approaches that can be used to immobilize ligands with sulfhydryl groups. The following subsections examine some of these techniques, including the haloacetyl, maleimide, and pyridyl disulfide methods.

a. Haloacetyl Method; The haloacetyl method uses supports that contain iodoacetyl or bromoacetyl groups for the immobilization of ligands through sulfhydryl residues. These supports are usually prepared via the reaction of an amine-containing material with iodoacetic or bromoacetic acid in the presence of ethyldimethylaminopropyl carbodiimide (EDC) at pH 4 to 5. EDC reacts with the carboxylic acid in iodo- or bromoacetic acid

to form a reactive ester, which can react with primary amine groups on the support. The second part of this process involves combination of the haloacetyl-activated support with a ligand containing a sulfhydryl group. This reaction proceeds by nucleophilic substitution and produces a thioether. The resulting bond is comparable to an amide linkage in stability. Although the reactivity of haloacetyl-activated supports toward sulfhydryls is relatively selective, these can react with methionine, histidine, or tyrosine under appropriate conditions. If the immobilization is carried out above pH 8, amines can also react with these supports.

b. Maleimide Method; Maleimides are another group of reagents employed for the selective coupling of a ligand through sulfhydryl groups. These tend to be more selective than a haloacetyl for such a reaction. The activation of a support with a maleimide is accomplished by using a homobifunctional or heterobifunctional cross-linking agent. One agent employed for this purpose is bis-maleimidohexane (BMH), which is a homobifunctional cross-linker with a maleimide group on both ends. The first of these groups can react with a support that has a sulfhydryl group. After the excess BMH has been washed away, the maleimide at the other end can react with a sulfhydryl group on a ligand.

c. Pyridyl Disulfide Method ; Pyridyl disulfide (or 2,2′-dipyridyldisulfide) is a homobifunctional cross-linking agent used for immobilizing ligands with sulfhydryl groups to supports that contain sulfhydryls on their surface. Activation of the support is accomplished by disulfide exchange between the sulfhydryl groups on the support and pyridyl disulfide, giving rise to the release of pyridyl-2-thione.

d. Other Methods; Divinylsulfone (DVS) can be used to activate a hydroxylcontaining support by introducing a reactive vinylsulfonyl group on its surface at pH 10 to11. This support can then be reacted with ligands that contain sulfhydryl, amine, or hydroxyl groups, with the rate of this reaction following the order –SH > –NH > –OH. Although the resulting bond for a sulfhydryl group is labile, the linkage for amine-containing ligands is more stable.

3. Hydroxyl-Reactive Methods; A number of methods have been used to couple ligands through hydroxyl groups. However, unlike many amine- and sulfhydryl-reactive methods, techniques for hydroxyl-containing ligands are not that selective. For example, the divinylsulfone method can be used for coupling an amine-, sulfhydryl-, or hydroxyl-containing ligand. Many supports used in affinity chromatography already contain hydroxyl groups on their surface. One way for the activation of these groups is to introduce bisoxirane (epoxy) groups. The most frequently used oxirane for this purpose is 1,4-butanediol diglycidyl ether, which contains two epoxy groups. One of the epoxy groups can react with the hydroxyl groups on a support while the other is used for coupling ligands containing sulfhydryl, amine, the reactivity of the terminal epoxide to other groups follows the order –SH > –NH > –OH. Strong alkaline conditions (pH 11) allow coupling by this method through hydroxyl groups, while amines and sulfhydryl groups can react at a lower pH (pH 7 to 8). Cyanuric chloride is another agent used for attaching a hydroxyl-containing ligand to a support. In this method, this can only be used effectively in the absence of amine groups due to the higher reactivity of these groups. As before-

mentioned, divinylsulfone can be used for coupling hydroxyl-containing ligands. This, however, is not usually performed if the immobilized ligand is present at a pH higher than 9 to 10.

4. Carbonyl-Reactive Methods; Although most immobilization techniques involve coupling ligands through amine or sulfhydryl groups, the large number of such groups can create a problem with improper orientation or multipoint attachment. This can be avoided by using alternative groups that occur only in specific locations on the ligand. One example is the immobilization of antibodies through their carbohydrate residues. To use the carbohydrate groups of an antibody (or any other glycoprotein) for immobilization, these groups must first be oxidized to form reactive aldehyde groups. This can be accomplished by enzymatic treatment; however, it is usually performed through mild treatment with periodate. These aldehyde groups are then reacted with a support containing amine or hydrazide groups for ligand immobilization. This approach has been used not only for antibodies but also for glycoenzymes, RNA, and sugars. Supports with amine groups can be used for coupling aldehyde-containing ligands by reductive amination. Hydrazide-activated supports can also be employed for immobilizing ligands with aldehyde groups. Such supports can be prepared by formation aldehyde groups on the support and reaction of these with an excess of a dihydrazide (e.g., oxalic or adipic dihydrazide). An advantage of using a hydrazide-activated support is that no reducing agent is needed to stabilize the linkage between the ligand and support, as is required in reductive amination.

5. Carboxyl-Reactive Methods; There are currently no activated supports that react specifically with a ligand containing carboxyl groups. This is a result of the low nucleophilicity of carboxyl groups in an aqueous solution. However, there are reagents that will react with carboxylic acids and allow them to be activated for ligand attachment. 1-Ethyl-3-(dimethylaminopropyl) carbodiimide is an example of such a reagent. One problem of this process is that severe cross-linking is possible, since amine groups as well as carboxyl groups can react if excess EDC is present. In addition, the activated derivative formed, O-acylisourea, is not stable in an aqueous environment. This means that the activated ligand must be used immediately for immobilization without further purification.

6. Other Immobilization Techniques; Along with noncovalent and covalent immobilization methods, other techniques have been developed for the preperation of affinity supports. Such methods include entrapment, molecular imprinting, and the use of the ligands as both the support and stationary phase. Although these methods are not as common as the approaches already examined, they have important advantages in some applications [8].

Activation methods which are used in affinity chromatography can be summarized as follow:

Amine groups :

- Cyanogen bromide (CNBr) method
- Schiff base (reductive amination) method
- N-hydroxysuccinimide (NHS) method

- Carbonyldiimidazole (CDI) method
- Cyanuric chloride method
- Azalactone method (for Emphaze supports)
- Divinylsulfone (DVS)
- Epoxy (bisoxirane) method
- Ethyl dimethylaminopropyl carbodiimide (EDC)
- method
- Tresyl chloride/tosyl chloride method

Sulfhydryl groups:

- Azalactone method (for Emphaze supports)
- Divinylsulfone method
- Epoxy (bisoxirane) method
- Iodoacetyl/bromoacetyl method
- Maleimide method
- Pyridyl disulfide method
- TNB-thiol method
- Tresyl chloride/tosyl chloride method

Hydroxyl groups:

- Cyanuric chloride method
- Divinylsulfone method
- Epoxy (bisoxirane) method

Aldehyde groups

- Hydrazide method

Carboxyl groups

1.4. Elution

Elution is one of the critical step for successful separation. Sample application in affinity chromatography is performed usually by injection or application in the presence of mobile phase which is prepared in appropriate pH, ionic strength and solvent composition for solute-ligand binding. This solvent is referred as application buffer [8]. In the presence of application buffer, compounds which are complementary to the affinity ligand will bind while the other solutes in the sample will tend to pass through the column as nonretained compounds. After

$$L + T \rightleftharpoons LT$$

$$K_D = \frac{[L][T]}{[LT]}$$

all nonretained components are washed off the column, the retained solute or together with ligand as solute-ligand complex can be eluted by applying a solvent. This solvent which is referred as elution buffer represents the strong mobile phase for the column. Later all the interest solutes are eluted from the column, regeneration is performed by elution with application buffer and the column is allowed to regenerate prior to the next sample application [4,8,21]. Step gradient elution or in other word on/off elution method is the most common method employed for affinity chromatography. Figure 15 shows the typical separation in affinity using step gradient elution.

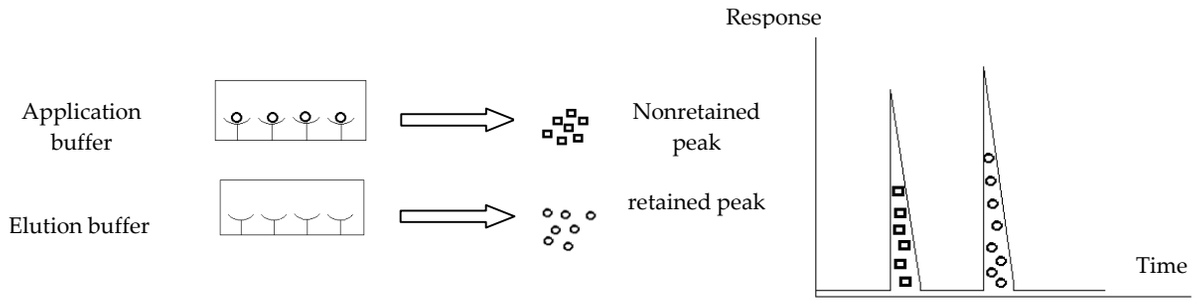

Figure 15. Typical separation in affinity using step gradient elution.

Step elution mode is employed if the ligans have high affinity for the target molecule. It is also possible to use isocratic elution in affinity chromatography. This elution mode generally selected if the target molecule and ligand have weak interaction. This approach is known as **Weak Affinity Chromatography** or **Dynamic Affinity Chromatography** [8,21].

In affinity chromatography there are many factors such as strength of solute-ligand interaction, the amount of immobilized ligand present and the kinetics of solute-ligand association and dissociation which have important influences on retention and elution of the compound. The reaction between the target protein (T) and ligand (L) on the other word binding (adsorption) and elution (desorption) process can be explain by following equation in case of a target protein has single site binding to a ligand [1,4,21].

K_D is the equilibrium dissociation constant

[L] is the concentration of free ligand

[T] is the concentration of free target

[LT] is the concentration of the ligand/target complex

The equation that is placed below explains the bound target-total target ratio. In order to achieve successful binding the ratio should be near 1 in this equation.

$$\frac{\text{Bound target}}{\text{Total target}} \approx \frac{L_0}{K_{D+L_0}}$$

L_0 is the concentration of ligand (usually 10^{-4} - 10^{-2}M)

K_D is the equilibrium dissociation constant

K_D can be changed by pH, ionic strength, temperature and other parameters. Therefore these parameters can be used to control the binding and elution efficiency of the reaction [1,4,22]

Obtaining stable biomolecules in high yield and purity is aimed for elution process. Elution is achieved by reducing the association constant of the ligand-solute interaction. Biospecific or non-specific elution can be utilized. Biospecific elution is based on solute displacement from the column by addition of molecule that acts as a competing agent. Two different types of biospecific elution can be applied for elution. In first method, normal role elution, molecule competes with the ligand for binding the desired solute. In second type of biospecific elution, reversed role elution, molecule competes with desired solute for binding the ligand [13]. The main advantage of biospecific elution is that a target can be gently removed from the column. However this elution is slow and generally results in broad solute peaks. Additionally competing agent needs to be removed from the eluted solvent therefore usage is limited. Another disadvantage especially in analytical application is need to use a competing agent that does not produce a large background signal under the conditions used for analyte detection [8]. Non-specific elution is performed by changing solvent conditions like pH, ionic strength and polarity. High concentration of chaotropic salts (NaCl, $MgCl_2$ or LiCl), denaturating agents and detergents (guanidine hydrochloride, sodium dodecyl sulfate and urea) can be used. Organic solvents can be used especially for the elution of low molecular weight compounds [13]. Alteration in structure of the solute or ligand which leads to a lower association constant and lower solute retention is provided by nonspecific elution [8]. Non-specific elution is faster than specific elution but there is a risk for denaturation of solute. The conditions which are applied for the elution may be too hard for column. If this is not considered it may result in long column regeneration times or irreversible loss of ligand activity [8].

For biospecific elution solvent is selected according to the type of target and ligand. The solvent usually has a pH and ionic composition similar to the application buffer but contains a competing agent. Reversed role elution is generally preferred when the target is a small compound while the normal elution is often used for isolation of macromolecules. Readily available in an inexpensive form and be soluble in the elution buffer are desired properties for competing agent in reverse role elution. In reversed-role elution it must be possible to remove the competing agent from the target when the affinity column is used for purification [8].

A wide range of mobile phase additives can be used in non-specific elution. In this elution nature of the target-ligand interaction is changed. This can be achieved by several ways such

as altering pH of the targets and ligands that interact by weakly acidic or basic groups. Changing pH can lead to the alteration in the conformation of the target or ligand. Either increasing or decreasing of pH value can be used for this purpose but decreasing of pH is commonly preferred. Irreversibly denaturation of target, ligand or support may occur in this step. Collection of the eluted target in a neutral pH buffer and regeneration the column as soon as possible after the elution step can overcome this problem [8]. Changes in ionic strength induced by high salt solutions are a second way for nonspecific elution. Disruption of ionic bonds can be achieved by this method but hydrophobic interactions are promoted. Chaotropic salts (NaCl, $MgCl_2$ or LiCl) are useful for altering retention of targets. They distrupt the stability of water and interfere with hydrophobic interactions [8,23]. The main advantage of using either chaotropic salt of a change in ionic strength is that this usually leads to gentle elution of the target in an active form [8]. Denaturating agents such as urea, guanidine hydrochloride and sodium dodecyl sulfate which dissociate hydrogen bonds can also be used for elution. Sodium dodecyl sulphate (SDS) contains both hydrophobic and strong ionogenic groups and binding to hydrophobic regions results in a layering of negative charges on the protein's surface, causing irreversible unfolding of the structure. The denaturating effect of these solutions limits their usage. They should be only used in analytical applications if the ligand is quite stable or in preparative applications if both the ligand and target are relatively stable and it is enable to recover their activity after such elution [8,23]. Organic solvents in the mobile phase are also used in some cases such as using of 1-propanol in chiral affinity separations in order to improve solute retention and produce narrow peaks for good resolution. Polyols like ethylene glycol are also utilized in affinity separations [8]. In order to select the elution buffer several approaches can be followed. However the best way is that the buffer should be selected based on information in the literature, structure of the ligand, target and past experiences with these substances [8].

2. Types of affinity chromatography

The type of ligand can be used to divide affinity techniques into various subcategories such as lectin, immunoaffinity, dye ligand etc. These techniques are placed as below [1].

2.1. Immunoaffinity chromatography

Immunoaffinity chromatography is one of the most popular techniques of affinity derivatived method and it enables to produce ligands in case the ligand required is not available [7]. In this technique, stationary phase comprises of an antibody or antibody-related agent [1]. It is possible to isolate variable subtances using this technique due to high specifity of antibodies [1]. It is reported that immunoaffinity chromatography may be used for natural food contaminants such as aflatoxins, fumonisins and ochratoxins [11].

On the purpose of purification using antibodies as ligands, antibodies initially are immobilized on a support. In order to,bind the ligand on the surface of the support properly, protein A and G are usually used as a bridge which provides enough space for the ligand-protein binding.

Columns, dialysis membranes, capillaries or beads may be used in immunoaffinity application which is a non-covalent, irreversible purification process based on highly specific interactions between analyte and antibody [11].

Initially, the antibodies should be purified prior to prepare the immunoaffinity column. Precipitation with ammonium sulfate, ion-exchange chromatography, gel filtration chraomatography or affinity chromatography may be employed with the aim of antibody purification. Activated beads which are coated with bacterial proteins A or G may be used as the support material. Some parameters may be changed for the elution of the sample solution for example the ionic conditions of mobile phase may be changed or chaotropic buffers may be used [11].

Both small and large analytes can be determined using direct detection in IAC. Additionally it is possible to use this technique either separately or in combination with other chromatographic techniques [1]. If this technique is performed as part of HPLC system the method can be referred as high performance immunoaffinity chromatography.

Immunoaffinity chromatography is probably the most highly specific of all forms of bioaffinity chromatography. However this technique has some disadvantages such as: this technique relatively high cost, leakage of ligands may accur from the column and sometimes the desorption procedure results in partial denaturation of the bound protein [24].

2.2. Protein A or protein G affinity chromatography

Protein A is produced by *Staphylococcus aureus* while protein G is of group G *Streptococci*. These ligands are capable of binding to many types of immunoglobulins at around neutral pH and they dissociate in a buffer with a lower pH [1]. Protein A binds to the immunoglobulin G (IgG) obtained by human and other mammalian species with high specificity and affinity. In some cases protein G may be used instead of protein A [24]. These two ligands differ in their ability to bind to antibodies from different species and classes. Strong specifity and binding properties to immunoglobulins of protein A and protein G serve them as good ligands for the seperation of immunoglobulins. Protein A and protein G have use as secondary ligands for the adsorption of antibosies onto the support material in immunoaffinity applications. This method may also be employed in case high antibody activity or replacement of the antibodies in the affinity chromatography is needed [1].

2.3. Lectin affinity chromatography

Lectins which are non-immun proteins are produced by plants, vertebrates and invertebrates. Especially various plant seeds synthesize high levels of lectins [24]. Certain types of carbohydrate residues may be seperated via this method due all lectins have the ability to recognize and bind these types of compounds. Mostly used lectins for affinity columns are concanavalin A, soybean lectin and wheat germ agglutinin [1, 24]. Concanavalin A is specific for α-D-mannose and α-D-glucose residues while wheat germ agglutinin binds to D-*N*-acetyl-glucosamine. Lectins which are commonly used for the isolation of compunds containing carbohydrates such as polysaccharides, glycoproteins and glycolipids in affinity chromatography are given in Table 4. [1].

Lectin	Source	Sugar specificity	Eluting sugar
Con A	Jack bean seeds	α- $_D$-mannose, α- $_D$ -glucose	α - $_D$ -methyl mannose
WG A	Wheat germ	*N*-acetyl-β- $_D$ -glucosamine	*N*-acetyl-β- $_D$ -glucosamine
PSA	Peas	α- $_D$ -mannose	α- $_D$ -methyl mannose
LEL	Tomato	*N*-acetyl-β- $_D$ -glucosamine	*N*-acetyl-β- $_D$ -glucosamine
STL	Potato tubers	*N*-acetyl-β- $_D$ -glucosamine	*N*-acetyl-β- $_D$ -glucosamine
PHA	Red kidney bean	*N*-acetyl-β- $_D$ -glucosamine	*N*-acetyl-β- $_D$ -glucosamine
ELB	Elderberry bark	Sialic acid or *N*-acetyl-β- $_D$ -glucosamine	Lactose
GNL	Snowdrop bulbs	α -1→ 3 mannose	α -methyl mannose
AAA	Freshwater eel	α – $_L$-fucose	$_L$ -fucose

Table 4. Some lectins which are commonly used in affinity chromatography

Enzymes, inhibitors, cofactors, nucleic acids, hormones or cell chromatography can also be utilized as ligands in bioaffinity chromatography types. Examples of these methods include Receptor Affinity Chromatography and DNA Affinity Chromatography [21].

2.4. Dye-ligand affinity chromatography

Development of the dye-ligand affinity chromatography could be attributed to observation of some proteins irregular elution characteristics during fractionation on gel filtration column in presence of blue dextran. Blue dextran consists of a triazine dye (cibacron blue F3G-A) covalently linked to high molecular mass dextran. Some proteins bind triazine dye and this allows to its use as an affinity adsorbent by immobilization [24]. This method is especially popular tool for enzyme and protein purification [21]. Dye-ligand adsorbents are of interest due to inexpensiveness, ease of availablity and immobilization process. These adsorbents may be used in analytical, preparative analysis and large scale studies. Although dye-ligand affinity technique for pharmaceuticals may be preferred owing to these advantages, concerns about leakage and toxicity has stopped its use. Therefore proteins purified using this technique is convenient for analytical or technical uses. Procion Red HE3b, Red A, Cibacron Blue F3G-A are some examples of dye-ligands which are used for purification [9].

2.5. Metal-chelate affinity chromatography (Immobilized-metal (Ion) affinity chromatography)

In 1970s, first application of metal-chelate affinity chromatography which is later named as "immobilized-metal (ion) affinity chromatography (IMAC) was perfomed. Metal-chelate chromatography technique exploits selective interactions and affinity between transition metal immobilized on a solid support (resin) via a metal chelator and amino acid residues which act as electron donors in the protein of interest [25-26]. As well as aromatic and heterocyclic compounds, proteins such as histidine, tyrosine, tyriptophane and phenylalanine posses affinity to transition metals which form complexes with compounds rich in electrons [25,27].

Among these amino acids histidine is the most commonly used one. Attachment of histidine tags to the recombinant proteins polypeptides is the most known development in the field of IMAC. Histidine and other metal affinity tags are widely used for protein purification [26]. Adsorbents may be prepared by binding chelators onto the surface and metals to the chelators. Free coordination sites of the metal ions are needed for the analyte to bind to metal ions [25].

Zn^{2+}, Ni^{2+} and Cu^{2+} are the most commonly used metal ions. Basic groups on protein surfaces especially the side chain of hisitidine residues, are attracted to the metal ions to form a weak coordinate bonds [24].

The metal ions in the class of hard Lewis acids such as K^{+}, Ca^{2+}, Mg^{2+}, Fe^{3+}; soft Lewis acids such as Ag^{+}, Cu^{+} and transition metals classified as borderline acids (Co^{2+}, Cu^{2+}, Ni^{2+}) may be used in IMAC applications, especially Ni^{2+} which has six coordination sites and electrochemical stability. The affinity of the metals may be predicted according to the principles of soft acids and bases which is the theory explaining one of the two atoms attached acts as a Lewis acid and the other as a Lewis base. Ligands with oxygen (e.g. carboxylate), aliphatic nitrogen (e.g. asparagin, glutamine) and phosphor (phosphorylated amino acids) are hard Lewis bases, as the ones with sulfur (e.g. cysteine) are soft bases and those with aromatic nitrogen (e.g. histidine, tryptophane) are borderline bases. In case Cu(II), Ni(II), Co(II) or Zn(II) ions are the ions used in the IMAC, the target amino acids on the protein surface are imidazolyl, thiol and indolyl groups; as carboxyl and phosphate groups are of that in case of the use of Fe(III) and Mg(II). Histidine, tryptophane and cysteine are accepted to be the most important amino acids for IMAC due to their strong affinity to metal ions and the retention times. It is reported that histidine residues attached to the protein surface significantly change the retention time of the protein of interest [26].

Chelating Compound	Coordination	Metal Ions
Aminohydroxamic acid	bidentate	Fe(III)
Salicylaldehyde	bidentate	Cu(III)
8-Hydroxy-quinoline (8-HQ)	bidentate	Al(III), Fe(III), Yb(III)
Iminodiacetic acid (IDA)	tridentate	Cu(II), Zn(II), Ni(II), Co(II)
Dipicolylamine (DPA)	tridentate	Zn(II), Ni(II)
ortho-phosphoserine (OPS)	tridentate	Fe(III), Al(III), Ca(II), Yb(III)
N-(2-pyridylmethyl)aminoacetate	tridentate	Cu(II)
2,6-Diaminomethylpyridine	tridentate	Cu(II)
Nitrilotriacetic acid (NTA)	tetradentate	Ni(II)
Carboxymethylated aspartic acid (CM-Asp)	tetradentate	Ca(II), Co(II)
N,N,N'-tri(carboxymethyl)ethylenediamine (TED)	pentadentate	Cu(II), Zn(II)

Table 5. Some examples of chelating compouns used in IMAC [28]

Multidentate chelating compounds are widely used in order to strengthen the complex which is comprised of chelator, metal ion and protein. Different length of spacers is used to bind the chelator onto the surface of the support. Type of the chelator influences the strength of the chelation and retention power, for instance metal binds to the nitrogen atom and two carboxylate oxygens and reveals three free sites in case of tridentate iminodiacetic acid (IDA); tetradentate nitrilotriacetic acid (NTA) binds the metal by an additional carboxylate oxygen and this provides stronger chelation, but a weaker retention power. IDA is the chelator which is commonly used in the applications of IMAC. Although most of the chelators are carboxymethylated amines, there also some other compounds which are commonly used such as dye-resistant yellow 2KT, OPS and 8-HQ [26]. Some examples of chelating compounds are given in Table 5.

2.6. Boronate affinity chromatography

In case of use boronic acid or boronates as ligand of the affinity chromatography, this type of methods are called boronate affinity chromatography. Most of the boronate derivatives are known to bind compounds with cis-diol groups covalently at a pH above 8. Separation of glycoproteins from non glucoprotein structures is possible by boronate affinity method due to cis-diol groups of the sugars. For instance, this method may successfully performed to seperate glucohemoglabin and normal hemoglobin or to determine different types of glycoproteins in a sample [1].

There are many other chromatographic methods which are closely related to traditional affinity chromatography. For example **Analytical Affinity Chromatography** (**Quantitative Affinity Chromatography** or **Biointeraction Chromatography**) which is used as a tool for determination of solute-ligand interactions [21]. It is possible to investigate several biological systems, such as lectin/sugar, enzyme/inhibitor, protein/protein, DNA/protein interactions as well as binding of drugs or hormones to serum proteins with this technique. Thus competition of drugs with other drugs or endogenous compounds for protein binding sites may successfully be detected by this method. Either immobilized drugs or immobilized proteins may be used in the studies about drug-protein and hormone-protein binding, although protein-based columns which may be use for multiple experiments are more common [1,29]. The competition between two solutes for binding sites can also be examined by this method and this technique is known as **Frontal Affinity Chromatography** [21]. **Hydrophobic Interaction Chromatography** and **Thiophilic Adsorption** methods also related to affinity chromatography. Immobilized thiol groups are used as ligands in **Thiophilic Adsorption** (**Covalent/Chemisorption Chromatography**) in order to separation of sulfhydryl-containing peptides or proteins and mercurated polynucleotides. In **Hydrophobic Interaction Chromatography** short non-polar chain, such as those that were originally used as spacer arms on affinity supports provide binding with proteins, peptides and nucleic acids. **Chiral Liquid Chromatography** methods can be also considered as affinity based techniques [21]. These techniques are widely utilized in pharmaceutical industry and clinical chemistry for the separation of individual chiral forms of the drugs and the quantification of different chiral forms of drugs or their metabolites. Since most of the ligands used in affinity chromatography are chiral, they may be preffered as

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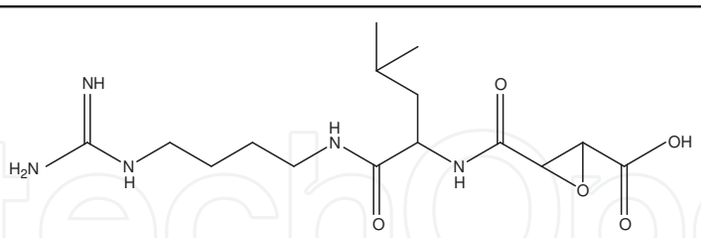
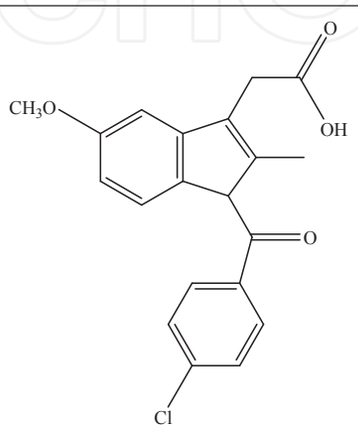
stationary phases for chiral seperations. Protein-based and carbohydrate-based ligands may be used as the stationary phases in the analysis of chiral compounds via HPLC [1]. Orosomucoid (α_1-acid glycoprotein), bovine serum albumine and ovomucoid (a glucoprotein of egg whites) are some examples of protein-based stationary phases, while cyclodextrins (especially β-cyclodextrin) are of carbohydrate-based stationary phases [1,29].

3. Affinity chromatography and drug discovery

There are a number of areas related to affinity chromatography that have also been of great interest in pharmaceutical and biomedical analysis. One such area is the use of affinity chromatography in drug discovery [12]. In drug discovery explaining the mechanism of action of bioactive compounds, which are used as pharmaceutical drugs and biologically active natural products, in the cells and the living body is important. For this pupose isolation and identification of target protein(s) for the bioactive compound are essential in understanding its function fully. Affinity chromatography is a useful method capable of isolating and identifying target molecules for a specific ligand, utilizing affinity between biomolecules such as antigen–antibody reactions, DNA hybridization, and enzyme–substrate interactions. Since the development of affinity chromatography in the early 1950s, various types of target proteins for bioactive compounds have been isolated and identified. Selected samples of the target proteins isolated by affinity chromatography are listed in Table 6. Since then, affinity chromatography has been gaining renewed attention as a widely applicable technique for the discovery the target proteins for bioactive compounds [30].

Bioactive compound	Molecular structure	Target protein
E-64 (cystein protease inhibitor)		Cathepsin B, H, L
Indomethacin (antitumor drug)		Glyoxalase 1

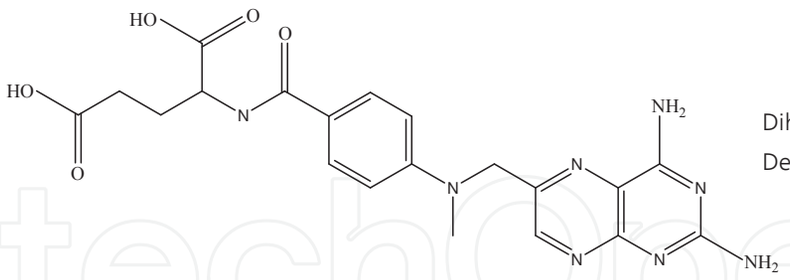
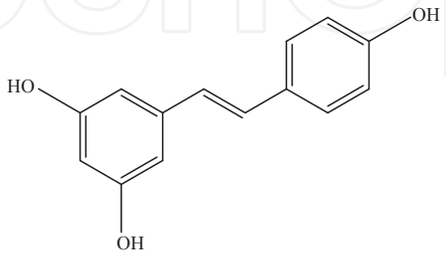
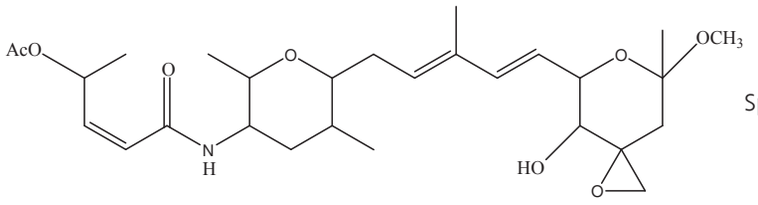
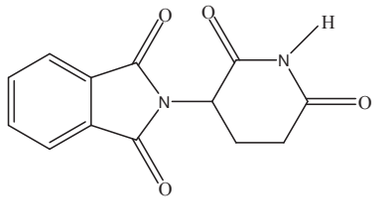
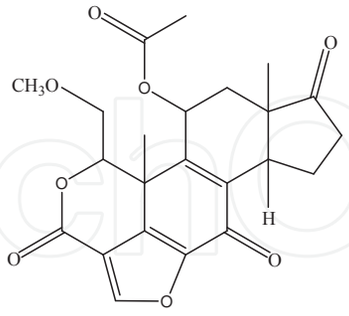
Bioactive compound	Molecular structure	Target protein
Methotrexate (anticancer drug)		Dihydrofolate reductase Deoxycytidine kinase
Resvertatrol (health benefits including life span)		Tif1 (yeast elF4A)
Spliceostatin A (splicing inhibitor)		Splicing factor 3b
Thalidomide (sedative, anticancer drug)		Cereblon
Wortmannin (PI 3-kinase inhibitor)		Polo-like kinase 1 Polo-like kinase 3

Table 6. Selected examples of the target protein(s) isolated and identified through affinity chromatography [30]

4. Conclusion

Liquid chromatographic techniques have been widely used in pharmaceutical and clinical laboratories. Reversed phase, ion exchange, size exclusion and normal phase chromatogra-

phy are commonly used types of these techniques. Affinity chromatography which provides analysis of bioactive molecules based on their biological functions or individual structures has become increasingly important as another liquid chromatography technique [1,12]. Affinity chromatography is based on the simple principle that every biomolecule recognize another natural or artificial molecule such as enzyme and substrate or antibody and antigen [2,7]. In fact this technique is one of the oldest forms of liquid chromatography method [8]. The first use may be considered as the isolation of α-amylase by using an insoluble substrate, starch, in 1910 just three years after the discovery of chromatography by Tsewett [6,9]. The modern applications have started since 1960s with the creation of beaded agarose supports and the use of cyanogens bromide immobilization method. Since then, affinity chromatography has been gaining attention as a widely applicable technique for discovering the target proteins for bioactive compounds [3]. Up to the present time many different types of target proteins for bioactive compounds have been isolated and identified by affinity chromatography [21]. Today affinity chromatography is utilized as a valuable technique for the separation, purification and analysis of compounds present in complex samples and used in biochemistry, pharmaceutical science, clinical chemistry and environmental sciences. Application of affinity chromatography has significant advantages. The important one is that affinity chromatography involves many types of interactions between ligand and target such as steric effects, hydrogen bonding, ionic interactions, van der Waals forces, dipol-dipol interactions and even covalent bonds while other chromatographic techniques involve just one or a few of them. The combination of these multiple interactions leads to separation with high selectivity and retention in affinity chromatography [8]. However there are also several drawbacks in affinity chromatography system, such as non-specific binding of irrelevant proteins during affinity purification and chemical modification of bioactive compounds of interest used as ligands. These drawbacks limit its extensive application. After the completion of the Human Genome Project drug discovery research has focused on an approach that includes identification and characterization of molecular and cellular functions of a wide variety of proteins encoded by genomes. Thus, bioactive compounds become more important not only as therapeutic agents to treat diseases and disorders but also as useful chemical tools to examine their complex biological processes in vitro and in vivo [30]. Affinity chromatography which allows explaining the mechanism of action of bioactive compounds that are used as pharmaceutical drugs and biologically active natural products, therefore, has also significant importance in modern drug discovery [30].

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