Affinity Chromatography: Principles and Applications

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1. Introduction

Since the inception of affinity chromatography 50 years ago (Cuatrecasas et al, 1968), traditional purification techniques based on pH, ionic strength, or temperature have been replaced by this sophisticated approach. It has been stated that over 60% of all purification techniques involve affinity chromatography (Lowe, 1996). The wide applicability of this method is based on the fact that any given biomolecule that one wishes to purify usually has an inherent recognition site through which it can be bound by a natural or artificial molecule. Thus, we can say that affinity chromatography is principally based on the molecular recognition of a target molecule by a molecule bound to a column.

Affinity purification involves 3 main steps:

a. Incubation of a crude sample with the affinity support to allow the target molecule in the sample to bind to the immobilized ligand.
b. Washing away non-bound sample components from the support.
c. Elution (dissociation and recovery) of the target molecule from the immobilized ligand by altering the buffer conditions so that the binding interaction no longer occurs.

Since the beginning of this technique, the term affinity chromatography has raised many controversies among researchers. Some say it would be more accurate if termed bioaffinity chromatography (O'Carra et al, 1974) or hydrophobic affinity (Shaltiel, 1974). Nonetheless, the term affinity chromatography has been expanded to describe a potential method of separating biomolecule mixtures on the basis of specific biological interactions. Recently, a modern form of liquid chromatography referred to as "flash chromatography" was introduced.

2. History of affinity chromatography

In 1910, the German scientist, Emil Starkenstein published an article which described the concept of resolving macromolecule complexes via their interactions with an immobilized

substrate. This manuscript discussed the influence of chloride on the enzymatic activity of liver α-amylase and opened the door for the early beginnings of this approach by several researchers (Arsenis & McCormick, 1966; Bautz & Hall, 1962; Campbell et al, 1951; Sander et al, 1966). Later on, the term affinity chromatography introduced in 1968 by Pedro Cuatecasas, Chris Anfinsen and Meir Wilchek in an article that briefly described the technique of enzyme purification via immobilized substrates and inhibitors (Cuatrecasas et al, 1968). Other early articles described the activation of a Sepharose matrix using a cyanogen bromide (CNBr) reaction (Axen et al, 1967) and the use of a spacer arm to alleviate steric hindrance (Cuatrecasas et al, 1968).

Affinity chromatography is still developing. It has played a central role in many "Omics" technologies, such as genomics, proteomics and metabolomics. The breakthrough development of affinity liquid chromatography has enabled researchers to explore fields such as protein–protein interactions, post translational modifications and protein degradation that were not possible to be examined previously. Finally, the coupling of reversed phase affinity chromatography with mass spectrometry has ultimately aided in discovery of protein biomarkers.

3. Fundamental principles of affinity chromatography

Separation of a desired protein using affinity chromatography relies on the reversible interactions between the protein to be purified and the affinity ligand coupled to chromatographic matrix. As stated earlier, most of the proteins have an inherent recognition site that can be used to select the appropriate affinity ligand. The binding between the protein of interest and the chosen ligand must be both specific and reversible.
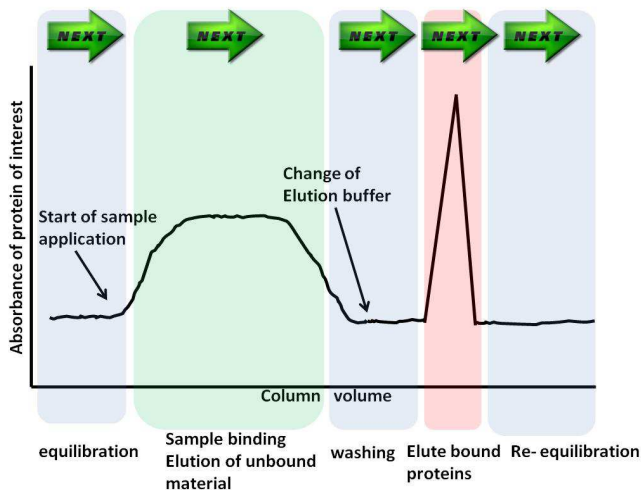

Fig. 1. Typical affinity chromatography purification

A typical affinity purification is shown in Figure 1 and involves several steps. First, samples are applied under conditions that favor maximum binding with the affinity ligand. After sample application, a washing step is applied to remove unbound sustances, leaving the desired (bound) molecule still attached to the affinity support. To release and elute the

bound molecules, a desorption step is usually performed either 1) specifically using a competitive ligand or 2) non-specifically by changing the media atmosphere (e.g. changing the ionic strength, pH or polarity) (Zachariou, 2008). As the elution is perfomed, the purified protein can be collected in a concentrated form.

3.1 Biomolecules purified by affinity chromatography

Antibodies were first purified using affinity chromatography in 1951 when Campbell et al. used affinity chromatography to isolate rabbit anti-bovine serum albumin antibodies (Campbell et al, 1951). For their purification, bovine serum albumin was used as the affinity ligand on a cellulose support. Two years later, this technique was expanded to purify mushroom tyrosinase using an immobilized inhibitor of the enzyme (azophenol) (Lerman, 1953). Since then, affinity chromatography is commonly used to purify biomolecules such as enzymes, recombinant proteins, antibodies, and other biomolecules.

Affinity chromatography is often chosen to purify biomolecules due to its excellent specificity, ease of operation, yield and throughput. In addition, affinity chromatography has the ability to remove pathogens, which is necessary if the purified biomolecules are to be used in clinical applications. The purity and recovery of target biomolecules is controlled by the specificity and binding constant of the affinity ligand. In general, the association constants of affinity ligands used for biomolecule purification range from $10^3 - 10^8$ M^{-1} (Janson, J-C, 1984). A common affinity ligand used in these purifications is an antibody, but other affinity ligands such as biomimetic dye-ligands, DNA, proteins and small peptides have been used as well. Figure 2 shows a wide variety of molecules that can be purified by affinity chromatography based on their polarity and volatility.
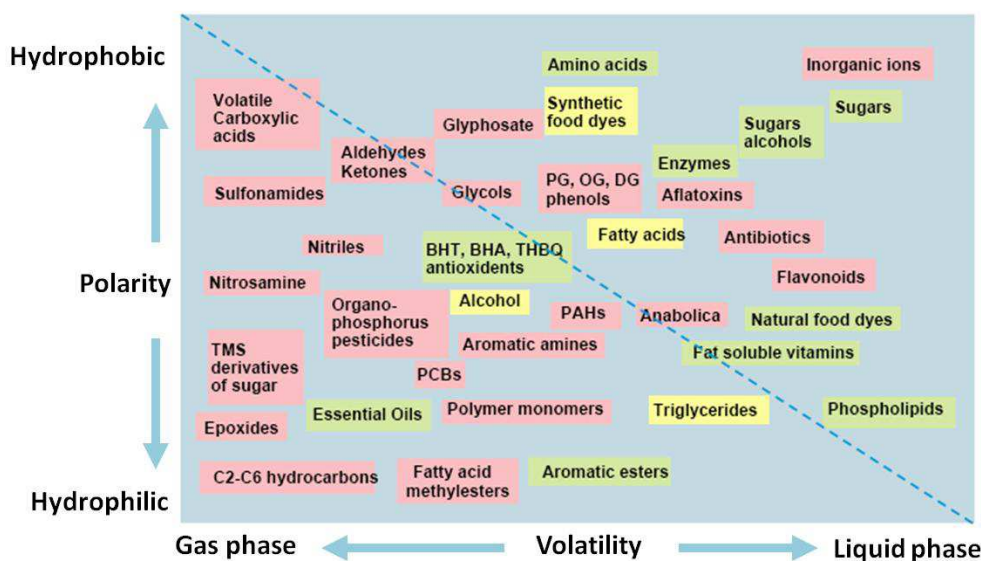

Fig. 2. Illustration showing different molecules that can be purified using affinity chromatography.

3.2 Components of affinity medium

When affinity chromatography is used for the purification and separation of large biomolecules from complex mixtures, the support (matrix), spacer arms, and ligand must be considered.

3.2.1 Affinity supports (matrix)

Traditionally, affinity chromatography support materials have consisted of porous support materials such as agarose, polymethacrylate, polyacrylamide, cellulose, and silica. All of these support materials are commercially available and come in a range of particle and pore sizes. Some supports may be available with common affinity ligands already immobilized (e.g. protein A, Cibacron Blue, heparin). Other types of support materials are being developed including nonporous supports, membranes, flow-through beads (perfusion media), monolithic supports, and expanded-bed adsorbents.

Nonporous support materials consist of nonporous beads with diameters of 1- 3 µm. These supports allow for fast purifications, but suffer from low surface areas when compared to traditional porous supports. Membranes used in affinity chromatography also lack diffusion pores which limits surface area, but like the nonporous beads allow for fast separations. Flow-through beads or perfusion media (originally developed for ion-exchange chromatography) have both small and large pores present. The addition of the large flow-through pores allows substances to be directly transported to the interior of the particle which means only short distances are required for diffusion. Monolithic supports are based on the same principle as perfusion media – they contain both large flow-through pores and small diffusion pores. Expanded-bed adsorbents were designed to prevent column clogging and utilize a reverse in flow to allow for the expansion of the column bed which allows for particulates to flow freely through the column and prevent column fouling. See Figure 3. More information about expanded-bed chromatography can be found in (Mattiasson, 1999).
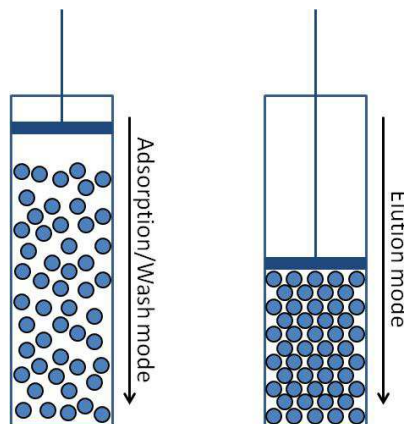

Fig. 3. Expanded-bed chromatography. In this type of chromatography, elution is performed in a normal packed-bed, but during the adsorption-wash step, the flow is reversed and the column bed expanded. This allows for particulate contaminates to pass freely through the column and prevent column clogging.

Regardless of the type of support used in the affinity purification, several factors must be considered when choosing a support material. These include chemical inertness, chemical stability, mechanical stability, pore size, and particle size.

Chemical inertness of the support material requires that the affinity support bind only the molecule of interest and have little or no nonspecific binding. While the specificity is related to the affinity ligand immobilized onto the support, the properties of the support must be chosen to limit the nonspecific binding of other molecules. Supports which have little or no nonspecific binding mimic the properties of the aqueous mobile phase. Therefore, chemically inert support materials are hydrophilic. In addition, most separations are performed in low ionic strength media. As a result, the number of charges on the support should be minimized to prevent nonspecific ionic interactions.

In addition, a support material must be chemically stable under normal operating conditions. This includes resistance to degradation by all enzymes and microbes, elution buffers, regenerating solvents, and cleaning agents that will be used within the column. These stability considerations must also be expanded to the stability of the affinity ligand-matrix linkage. Agarose-based support materials meet all of these requirements as they can be used between pH 3 and 12, are not attacked by enzymes, and are not affected by most aqueous eluants. However, ligand attachment in agarose support materials is often not as stable, depending on the type of linkage used.

Mechanical stability is another consideration when choosing a chromatographic support material for affinity chromatography. Support materials must be able to withstand the backpressures encountered during normal separations without compressing. While most commercial packing materials meet this requirement, the build-up of particulate contaminants may restrict column flow and lead to high backpressures. Under these pressures, soft porous gel supports such as agarose beads will compress and increase the pressure even further causing collapse of the support structure. More mechanically stable supports (e.g. silica and heavily cross-linked polymers) are able to withstand these high pressures, but the build-up of particulate contaminants should be avoided if at all possible.

Particle size is an additional consideration when choosing a support material. Ideally, small particle sizes are desired to limit mass transfer effects and limit band broadening. In addition, smaller particle sizes tend to offer greater surface area of the support material and allow for a larger number of affinity ligands to be immobilized on the surface of the support. Unfortunately, as particle size is decreased, backpressures are increased. In addition, when using smaller particles, the potential for the build-up of particulate contaminants and column fouling is increased. For this reason, in preparative applications large particles (30 – 100 μm) are often used. An alternative method to avoid the potential build-up of particulates is to use an expanded-bed support material as discussed earlier and seen in Figure 3.

Pore size is another item that must be considered when using affinity chromatography since the biomolecules of interest must be able to not only pass through the column but also be able to fully interact with the affinity ligand. Based on the Renkin equation which allows the estimation of the effective diffusion coefficient (Renkin, 1954), the pore diameter should be at least 5 times the diameter of the biomolecule being purified (Gustavsson & Larsson, 2006). Therefore, a typical protein with a 60 Å diameter would need a support with at least a 300 Å

pore size. Often, the optimal pore size takes into account the ability of the affinity ligand to interact with the biomolecule as well as the surface area of the column since increasing pore size leads to a decrease in the surface area which limits the number of affinity ligands which can be immobilized to the support material.

3.2.2 Spacer arms

Due to the fact that binding sites of the target molecule are sometimes deeply located and difficult to access due to steric hindrance, a spacer arm is often incorporated between the matrix and ligand to facilitate efficient binding and create a more effective and better binding environment. See Figure 4.
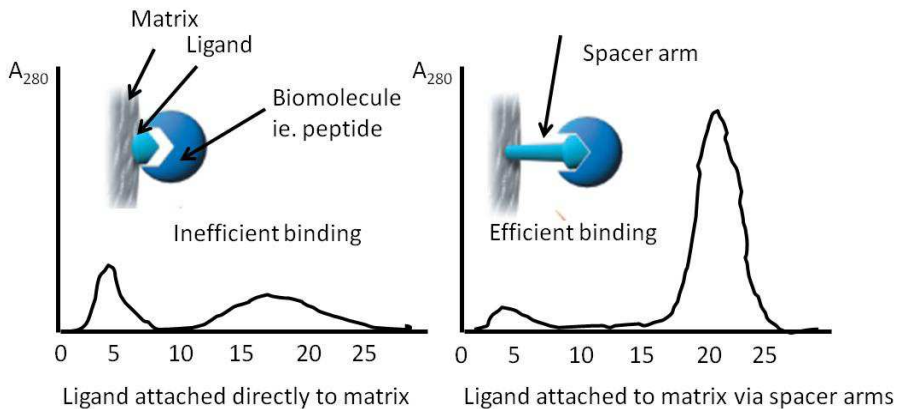

Fig. 4. Chromatogram showing better ligation and elution when spacer arms are introduced between the ligand and matrix

The length of these spacer arms is critical. Too short or too long arms may lead to failure of binding or even non-specific binding. In general, the spacer arms are used when coupling molecules less than 1000 Da.

3.2.3 Ligands used in affinity chromatography

Antibodies have several advantages including their high specificity and relatively large binding constants. Antibodies or immunoglobulins are a type of glycoprotein produced when a body's immune system responds to a foreign agent or antigen. Due to the variability of the amino acid sequence in the antibody binding sites (F_{ab} regions shown in Figure 5), it has been estimated that antibodies can be produced for millions or even billions of different foreign agents.

Antibodies which are produced by separate cell lines are referred to as polyclonal antibodies. Monoclonal antibodies are produced when a single antibody producing cell is combined with a carcinoma cell to create a hybridoma which can be grown in a cell culture. Monoclonal antibodies are often more desirable than polyclonal antibodies in affinity chromatography due to their lack of variability which allows for the creation of a more uniform affinity support.
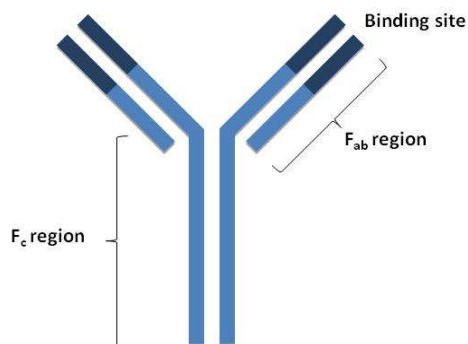
Fig. 5. Typical structure of an antibody. The amino acids in the F_c region generally have the same sequence, whereas the amino acids in the F_{ab} region have variable amino acid sequences which allows for the specificity of the binding interaction against a wide range of antigens.

Another type of affinity ligand which can be used to purify biomolecules from complex mixtures is a dye-ligand. Dye ligand chromatography originated in 1968 when Haeckel et al. were purifying pyruvate kinase using gel filtration chromatography and found that Blue Dextran (a small dye molecule) co-eluted with the protein (Haeckel et al, 1968). After further investigation, it was determined that binding between the dye and enzyme caused this co-elution. The dye-enzyme binding was later utilized in the purification of pyruvate kinase using a Blue Dextran column in 1971 (Staal et al, 1971).

Biomimetic dye-ligand chromatography takes dye-ligand chromatography one step further and utilizes modified dyes which mimic the natural receptor of the target protein. In addition to offering better binding affinities, these modified dyes were initially developed as a result of the concerns over purity, leakage, and toxicity of the original commercial dyes (Lowe et al, 1992). Cibacron Blue 3GA is one of the most common modified triazine dyes that has been used for protein purification. Its structure can be seen in Figure 6. Covalent attachment of the dye can be achieved through nucleophilic displacement of the dye's chlorine atom by hydroxyl groups on the support's surface (Labrou, 2000; Labrou et al, 1995; Labrou, 2002).
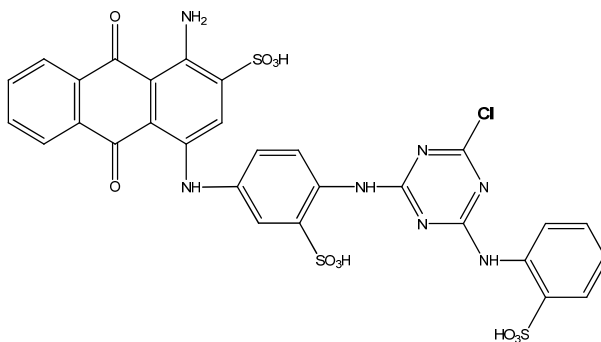

Fig. 6. Chemical structure of blue sepharose dye-ligand (Cibacron Blue 3GA) commonly used for purification of albumin as well as enzymes (NAD^+ and $NADP^+$).

Chlorotriazine polysulfonated aromatic molecules (triazine dyes) have been used for the purification of albumin, oxidoreductases, decarboxylases, glycolytic enzymes, nucleases, hydroloases, lyases, synthetases, and transferases (Labrou, 2000; Labrou et al, 1995). The main advantages of using dye-ligands and biomimetic dye-ligands are their low cost and resistance to chemical and biological degradation. The main disadvantage of these synthetic ligands is that the selection process for a particular biomolecule is empirical and requires extensive screening processes during method development. More information on biomimetic dyes can be found in reference (Clonis et al, 2000).

DNA can also be used as an affinity ligand. It can be used to purify DNA-binding proteins, DNA repair proteins, primases, helicases, polymerases, and restriction enzymes. The scope of biomolecules which can be purified using DNA is expanded when aptamers are utilized. Aptamers are single-stranded oligonucleotides which have a high affinity for a target molecule. SELEX (Systematic Evolution of Ligands by Exponential Enrichment) allows for the isolation of these oligonucleotide sequences and allows for a wide range of potential targets including biomolecules which typically have no affinity for DNA or RNA. The SELEX process for DNA is shown in Figure 7.
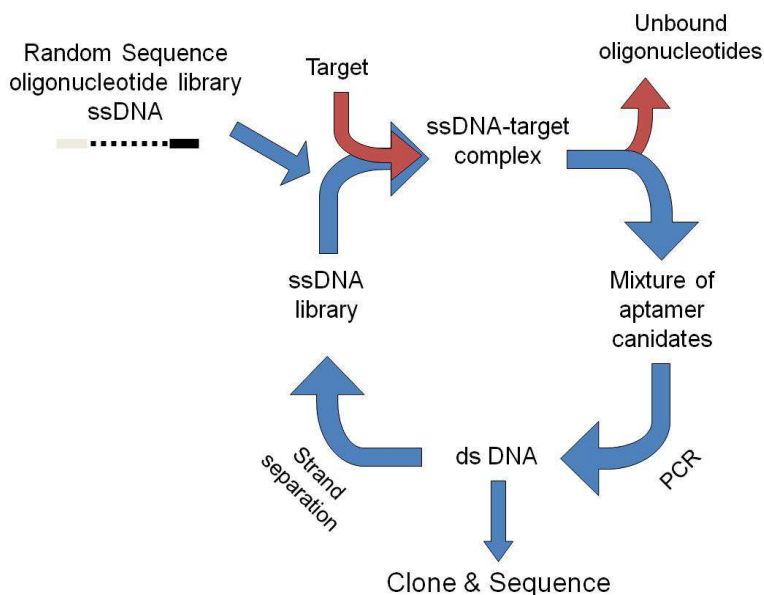

Fig. 7. Diagram depicting the SELEX process for the selection of aptamers against a target. First a random short stranded ssDNA (or RNA) library (10^{14} sequences) is exposed to the target compound and allowed to bind. The unbound oligonucleotides are then separated from the ssDNA-target complexes and removed. The remaining complexes are then disrupted leaving a mixture of aptamer candidates and the target compound. The ssDNA aptamer candidates are then amplified using PCR, the strands separated and the cycle repeated. After multiple cycles (typically 5 – 15), the initial DNA library will have been condensed down to a few sequences which tightly bind the target. These candidates can then be cloned, sequenced and used for affinity chromatography.

A similar process can be used to develop RNA affinity ligands. Once a potential aptamer sequence is identified, it can synthesized *in vitro* and used as the affinity ligand on a chromatographic support. An example of aptamers usage as in purification of L-selectin (Romig et al, 1999) and RNA binding proteins (Dangerfield et al, 2006; Windbichler & Schroeder, 2006).

Peptide affinity chromatography is another method which can be used for purifying biomolecules. Peptide affinity ligands are typically identified using one of two techniques (Wang et al, 2004); biological combinatorial peptide libraries (e.g phage-displayed libraries) (Cwirla et al, 1990; Devlin et al, 1990; Smith & Scott, 1993) or solid-phase combinatorial libraries (e.g. one-bead-one-peptide libraries) (Lam et al, 1991). Since then, peptide sequences have been isolated for a wide range of targets (Casey et al, 2008) and have been used to purify staphylococcal enterotoxin B (Wang et al, 2004), β-tryptase (Schaschke et al, 2005), and α-cobratoxin (Byeon & Weisblum, 2004). The main advantages of using peptides as affinity ligands are their low cost and stability.

Other ligands can be used in affinity chromatography for biomolecule purification. For more information on all types of affinity ligands see references (Clonis, 2006; Hage, 2006).

3.2.4 Immobilization of affinity ligands

Immobilization of the affinity ligand is also very important when designing an affinity chromatography method for biomolecule purification. When immobilizing an affinity ligand, care must be taken to ensure that the affinity ligand can actively bind the desired target after the immobilization procedure. Activity of the affinity ligand can be affected by multi-site attachment, orientation of the affinity ligand, and steric hindrance. See Figure 8.

Multi-site attachment occurs when an affinity ligand is attached through more than one functional group on a single ligand molecule. If these multiple attachment sites cause the affinity ligand to become denatured or distorted, multisite attachment can lead to reduced binding affinity. However, in some instances, the additional attachment sites can result in more stable ligand attachment. In general, it is best to try for site-specific attachment of the affinity ligands to limit the potential for multi-site attachment. For example, when immobilizing antibodies, covalent attachment is often directed toward the carbohydrate moieties within the F_c region of the antibody. Not only does this limit the number of attachment sites, but it can also help direct the binding and, thus, help orientate the antibody so the binding regions (F_{ab}) are exposed. Another way to prevent multi-site attachment is to use a support that has a limited number of reactive sites. By limiting the reactive sites, the potential for multiple attachments from a single affinity ligand is greatly reduced. A general rule of thumb is the larger the affinity ligand to be immobilized, the fewer number of reactive sites on the support needed.

Obviously, when performing affinity purifications, it is important to ensure the affinity ligands are immobilized so that the binding regions are exposed and free to interact and bind with the target molecule(s). Ideally, immobilization methods which specifically avoid attaching the affinity ligand via functional groups within the binding site(s) are used. One way to achieve this when immobilizing proteins is to use site-directed mutagenesis to introduce a single cysteine residue at a site known to be far away from the binding site(s) (Huang et al, 1997).

Once the cysteine residue is introduced, the protein can be immobilized using a cysteine specific coupling reagent such as N-γ-maleimidobutyryl-oxysuccimide ester.
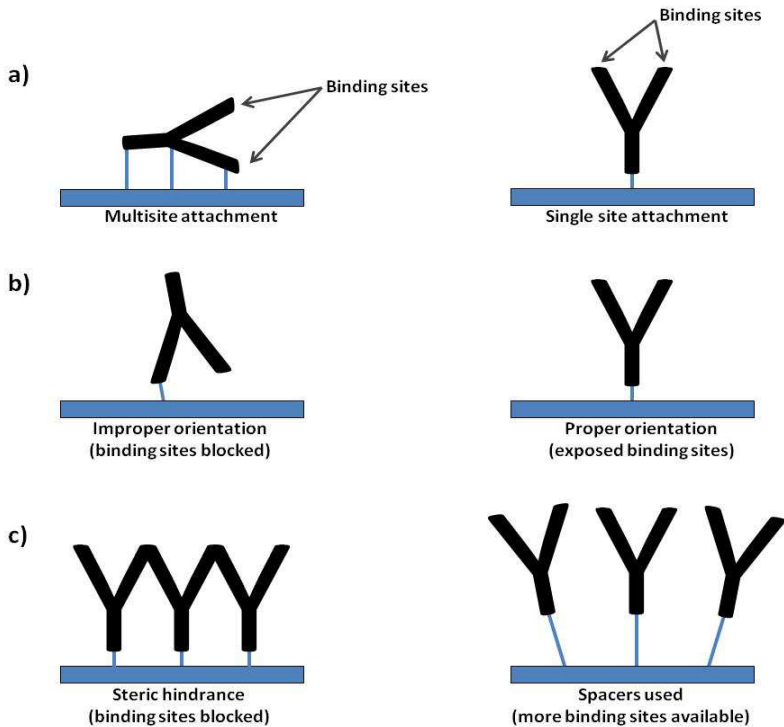

Fig. 8. Potential immobilization problems which can affect affinity ligand activity by a) multi-site attachment, (b) improper orientation, and (c) steric hindrance.

Affinity ligands can be covalently immobilized, adsorbed onto a surface via nonspecific or biospecific interactions, entrapped within a pore, or coordinated with a metal ion as in metal-ion affinity chromatography (IMAC). Each of these methods has advantages and disadvantages and is briefly discussed below.

Covalent immobilization is one of the most common ways of attaching an affinity ligand to a solid support material. There is a wide range of coupling chemistries available when considering covalent immobilization methods. Amine, sulfhydryl, hydroxyl, aldehyde, and carboxyl groups have been used to link affinity ligands onto support materials. More information about these specific reactions can be found in reference (Kim & Hage, 2006). Although covalent attachment methods are more selective than other immobilization methods, they generally require more steps and chemical reagents. While this may lead to a greater initial cost of preparation, the stability of these supports typically is greater and the support does not need to be periodically regenerated with additional affinity ligands as is typically the case when using adsorption techniques. As a result, covalent immobilization may be more economical in the long-term for the immobilization of costly affinity ligands.

Adsorption of affinity ligands may also be used to immobilize affinity ligands onto support materials. The adsorption can be either nonspecific or specific. In nonspecific adsorption the affinity ligand simply adsorbs to the surface of the support material and is a result of Coulombic interactions, hydrogen bonding, and/or hydrophobic interactions. Biospecific adsorption is commonly performed by using avidin or streptavidin for the adsorption of biotin containing affinity ligands or protein A or protein G for the adsorption of antibodies. Both of these immobilization methods allow for site-specific attachment of the affinity ligand which minimizes binding site blockages. When biospecific adsorption is used for immobilization, the primary ligand (i.e. avidin, streptavidin, protein A or protein G) must first be immobilized onto the support material. Avidin, streptavidin, protein A and protein G can be immobilized using amine-reactive methods. Avidin is glycosylated and can also be immobilized through its carbohydrate residues.

Entrapment of affinity ligands was demonstrated by Jackson et al. when human serum albumin (HSA) was entrapped using hydrazide-activated supports and oxidized glycogen as a capping agent (Jackson et al, 2010). Their method can be used with other affinity ligands ranging from 5.8 to 150 kDa. This type of immobilization method is generally less harsh than other immobilization methods and does not require the use of recombinant proteins. In addition, no linkage exists between the affinity ligand and the support which eliminates the potential immobilization problems seen in Figure 8.

Sol-gel entrapment is another method of encapsulation of affinity ligands (Avnir et al, 2006; Jin & Brennan, 2002; Pierre, 2004). The sol-gel entrapment process is as follows: First, the sol is formed from a silica precursor (e.g. alkoxysilane or glycerated silane). Once the sol has been formed, the buffered protein solution is added and the gelation reaction initiated. This is followed by an aging process in which the sol-gel is dried and further crosslinking of the silica occurs leaving the protein physically trapped within the cross-linked silica gel.

4. Current techniques involving affinity chromatography

Affinity chromatography is currently being used for a wide variety of applications ranging from the study of drug-protein binding interactions to the depletion of high abundance proteins to enhance the detection/quantification of dilute proteins.

Affinity chromatography can be used to study drug-protein binding interactions. Frontal analysis, zonal elution, and the Hummel-Dreyer method can be used to measure drug-protein binding constants, to quantify kinetic properties of the various interactions, to quantify allosteric interactions, and to identify drug binding sites. More information about the measurement of drug-protein binding constants can be found in two review articles (Hage, 2002; Hage et al, 2011). Information on quantifying kinetic properties of drug-protein interactions can be found in a review by (Schiel & Hage, 2009). A discussion on the quantification of allosteric interactions by affinity chromatography can be seen in an article by (Chen & Hage, 2004). Additional information on the identification of drug-binding sites can be found in a review article (Hage & Austin, 2000).

When trying to analyze low abundance proteins, it is often necessary to remove high abundance proteins prior to analysis. This removal effectively enriches low abundance proteins and allows more of them to be identified and quantified. Removal of the top 7 or

top 14 high-abundance proteins has been shown to result in a 25% increase in identified proteins (Tu et al, 2010). Moreover, affinity chromatography is widely used in many 'omics' studies (e.g. proteomics, metabolomics and genomics) and is currently used in tandem with other methods to develop high-throughput screening methods for potential drugs.

5. Biokinetics of affinity chromatography

The reaction between the ligand (L) and target compound (T) in an affinity atmosphere (either adsorption or desorption) is represented in Figure 9.
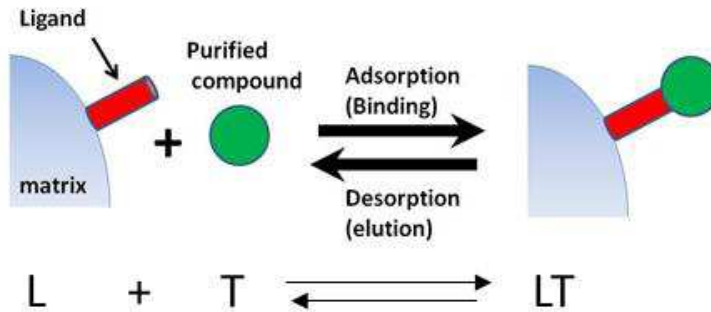

Fig. 9. Basic reaction between compound to be purified and ligand.

The standard definition of the term equilibrium dissociation constant [K_D] can be expressed in equation 1,

$$K_D = \frac{[L]*[T]}{[LT]} \tag{1}$$

where [L] is the free ligand, [T] is the target compound, and [LT] is the ligand-target complex.

According to the postulation of (Graves & Wu, 1974), the bound target-total target ratio can by represented as shown in equation 2,

$$\frac{\text{Bound target}}{\text{Total bound}} \approx \frac{L_0}{K_D} + L_0 \tag{2}$$

where L_0 is the concentration of the ligand (usually 10^{-4} – 10^{-2} M). To achieve successful binding, the ratio of bound to total target must be near 1. Therefore, K_D should be small compared to ligand concentration. K_D can be greatly affected by changing in pH, ionic strength, and temperature. Thus changing these parameters can be used to control the binding and elution efficiency of the reaction and can be expressed as seen in equations 3 and 4,

$$L + T \rightleftharpoons LT \tag{3}$$

$$LT \rightleftharpoons L + T \tag{4}$$

In equation 3, the K_D range is between 10^{-6} – 10^{-4} M which means there is more binding and less elution. In equation 4, the K_D is decreased to 10^{-1} – 10^{-2} M by the elution conditions which results in less binding and more elution of the target compound.

The interactions described in equations 3 and 4 apply under non-selective (noncompetitive) elution conditions. In case of selective elution or competitive elution, the interaction can be represent as shown in Figure 10.
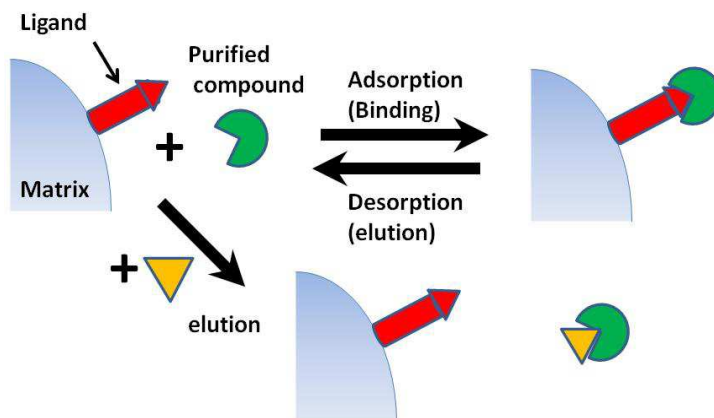

Fig. 10. Competitive elution of the target by adding a competitive free ligand (triangle).

When adding a competing binding substance or a free ligand (C) that binds to the purified compound of interest during elution, the interaction can be represented as shown in Equation 5.

$$C + T \rightleftharpoons CT \tag{5}$$

The equilibrium constant, K_D, is calculated according to equation 6

$$K_D = \frac{[C] * [T]}{[CT]} \tag{6}$$

where [C] and [T] are the concentration of the free competing ligand and target, respectively and [CT] is the concentration of the competing ligand-target complex.

(Graves & Wu, 1974) have shown that the eluted target to total target compound ratio can be represented by equation 7

$$\frac{\text{Eluted target}}{\text{Total bound target}} \approx \left(\frac{p}{p+1} \right) * \left[\frac{pC_0}{pC_0 + \frac{K_{DComp} * L0}{K_D}} \right] \tag{7}$$

where, p is the ratio between the volume of competitor added and the pore volume of the gel, K_D is the dissociation constant for coupled ligand, K_{Dcomp} is the dissociation constant for

the free competing ligand, C_0 is the concentration of the competing ligand (usually 10^{-2} – 10^{-1} M) and L_0 is the concentration of the coupled ligand, usually 10^{-4} – 10^{-2} M.

If both K_D and K_{Dcomp} are similar, then the concentrations of the competing and coupled ligand must be similar to achieve an efficient elution. On the other hand, if K_{Dcomp} is equal to $5*K_D$ we would expect that the concentration of the competing ligand will need to be 5x higher to achieve successful elution.

6. Applications and uses of affinity chromatography

6.1 Immunoglobulin purification (antibody immobilization)

Antibodies can be immobilized by both covalent and adsorption methods. Random covalent immobilization methods generally link antibodies to the solid support via their free amine groups using cyanogen bromide, N-hydroxysuccinimide, N,N'-carbonyldiimidazole, tresyl chloride, or tosyl chloride. Alternatively, free amine groups can react with aldehyde or free epoxy groups on an activated support. As these are random immobilization methods, the antibody binding sites may be blocked due to improper orientation, multi-site attachment or steric hindrance as shown in Figure 8.

Site-specific covalent immobilization of antibodies can be achieved by converting the carbohydrate residues located in the F_c region of the antibody to produce aldehyde residues which can react with amine or hydrazide supports (Ruhn et al, 1994). Another site-specific immobilization of antibodies can be accomplished by utilizing the free sulfhydryl groups of F_{ab} fragments. These groups can be used to couple the antibody fragments to an affinity support using a variety of established methods including epoxy, divinylsulfone, iodoacetyl, bromoacetyl, thiol, maleimide, TNB-thiol, tresyl chloride, or tosyl chloride methods (Hermanson et al, 1992).

Antibodies can also be immobilized by adsorbing them onto secondary ligands. For example, if an antibody is reacted with hydrazide biotin, the hydrazide can react with oxidized carbohydrate residues on the F_c region of the antibody. The resultant biotinylated antibody can then be adsorbed onto an avidin or streptavidin affinity support. This type of biotin immobilization allows for site-specific immobilization of the antibody and can be performed using commercially available biotinylation kits.

Alternatively, antibodies can be directly adsorbed onto a protein A or protein G support due to the specific interaction of antibodies with protein A and G. Immobilized antibodies on the protein A or G support can easily be replaced by using a strong eluent, regenerating the protein A/G, and re-applying fresh antibodies. Generally, this method is used when a high capacity/high activity support is needed. If a more permanent immobilization is desired, the adsorbed antibodies may be cross-linked to the support material using carbodiimide (Phillips et al, 1985) or dimethyl pimelimidate (Schneider et al, 1982; Sisson & Castor, 1990).

6.2 Recombinant tagged proteins

Purification of proteins can be easier and simpler if the protein of interest is tagged with a known sequence commonly referred to as a tag. This tag can range from a short sequence of

amino acids to entire domains or even whole proteins. Tags can act both as a marker for protein expression and to help facilitate protein purification.

The properties of fusion tags allow tagged proteins to be easily manipulated in the laboratory. Most significantly, the well-characterized tag-ligand chemistry enables single-step affinity purification of tagged molecules using immobilized versions of their corresponding affinity ligands. In addition, antibodies to fusion tags are also available and can be used for "universal" purification and detection of tagged proteins (i.e., without having to obtain or develop a probe for each specific recombinant protein).

In general, the most commonly used tags are glutathione-S-transferase (GST), histidine fusion (His or polyHis tag) and protein A fusion tags. Other types of fusion tags are also available including maltose-binding protein (di Guan et al, 1988), thioredoxin (LaVallie et al, 1993), NusA (Whetstone et al, 2004), GB1 domain for protein G (Davis et al, 1999), and others (Balbas, 2001; Thorn et al, 2000). The decision to use any of these tagging methods depends mainly on the needs of of the researcher. Table 1 compares GST and $(His)_6$ tags and may help when deciding which tag is appropriate for a particular purification.

GST tag	$(His)_6$ tag
Can be used in any expression system	
Purification procedure gives high yields of pure product	
Selection of purification products available for any scale	
Site-specific proteases enable cleavage of tag if required	Site-specific proteases enable cleavage of tag if required. N.B. enterokinase sites that enable tag cleavage without leaving behind extra amino acids are preferable
GST tag easily detected using an enzyme assay or an immunoassay	$(His)_6$ tag easily detected using an immunoassay
Simple purification. Very mild elution conditions minimize risk of damage to functionality and antigenicity of target proteins	Simple purification, but elution conditions are not as mild as for GST fusion proteins. Purification can be performed under denaturing conditions if required. Neutral pH but imidazole may cause precipitation. Desalting to remove imidazole may be necessary
GST tag can help stabilize folding of recombinant proteins	$(His)_6$ -dihydrofolate reductase tag stabilizes small peptides during expression
Fusion proteins form dimers	Small tag is less likely to interfere with structure and function of fusion partner
	Mass determination by mass spectrometry not always accurate for some $(His)_6$ fusion proteins

Table 1. Comparison of GST and His tags for protein purification. The information from this table is summarized fromthe Amersham recombinant protein handbook and (Geoghegan et al, 1999).

In the following sections, the most commonly used purification techniques and methods utilizing affinity chromatography will be discussed.

6.2.1 GST tagged purification

Glutathione S-transferase (GST) is a 26 kDa protein (211 amino acids) located in cytosole or mitochondria and present both in eukaryotes and prokaryotes (e.g. *Schistosoma japonicum*). The enzymes have various sources both native and recombinantly expressed by fusion to the N-terminus of target proteins (Allocati et al, 2009; Allocati et al, 2011; Sheehan et al, 2001; Udomsinprasert et al, 2005). GST-fusion proteins can also be produced in *Escherichia coli* as recombinant proteins. Separation and purifcation of GST-tagged proteins is possible since the GST tag is capable of binding its substrate, glutathione (tripeptide, Glu-Cys-Gly).

When glutathione is reduced (GSH), it can be immobilized onto a solid support through its sulfhydryl group. This property can be used to crosslink glutathione with agarose beads and, thus, can be used to capture pure GST or GST-tagged proteins via the enzyme-substrate binding reaction (Beckett & Hayes, 1993; Douglas, 1987). Binding is most efficient near neutral physiological conditions (pH 7.5) using Tris saline buffer and mild conditions to preserve the structure and enzymatic function of GST. As a result of the potential for permanent denaturation, denaturing elution conditions are not compatible with GST purification. In addition, upon denaturation or reduction, the structure of the GST fusion tag often degrades.

Following a washing step to remove unbound samples, the bound GST-fusion protein can be recovered by the addition of excess reduced glutathione since the affinity of GST for free glutathione is higher than the affinity for immobilized glutathione. The free glutathione replaces the immobilized glutathione and releases the GST-tagged protein from the matrix allowing its elution from the column.
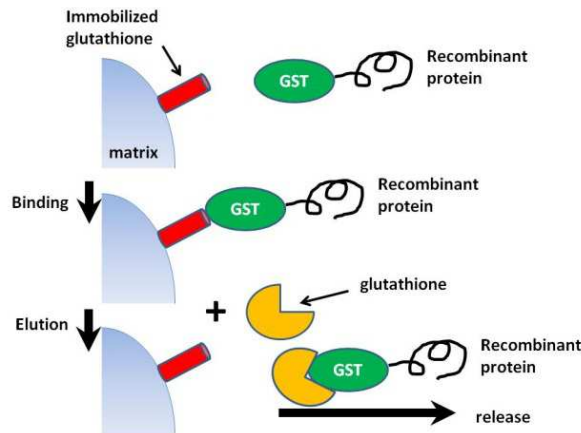

Fig. 11. GST- tagged protein immobilization.

6.2.2 His-tagged protein purification

Recombinant proteins which have histidine tags can be purified using immobilized metal ion chromatography (IMAC). The His-tag can be placed on either the N- or C-terminus. Optimal binding and, therefore, purification efficiency is achieved when the His-tag is freely accessible to metal ion support (Dong et al, 2010).

Histidine tags have strong affinity for metal ions (e.g. Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+}). One of the first support materials used immobilized iminodiacetic acid which can bind metal ions and allow for the coordination complex with the His-tagged protein. One difficulty with iminodiacetic acid supports is the potential for metal ion leaching leading to a decreased protein yield. Modern support materials, including nickel-nitrilotriacetic acid (Ni-NTA) and cobalt-carboxymethylasparate (Co-CMA), show limited leaching and, therefore, result in more efficient protein purifications. The coordination of a His-tag with a Ni-NTA support can be seen in Figure 12. Once the tagged protein is bound by the immobilized chelating agent, it can be eluted by introducing a competing agent for the chelating group (imidazole) or an additional metal chelating agent (EDTA).
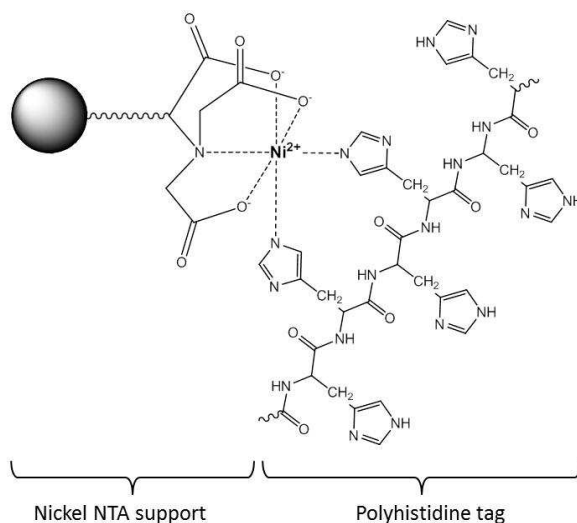

Fig. 12. showing the complex formed between the poly-histidine tag and a nickel NTA support.

One advantage of using His-tags for protein purification includes the small size of the affinity ligand. Due to the small size, it has minimal effects on the folding of the protein. In addition, if the His-tag is placed on the N-terminal end of the protein, it can easily be removed using an endoprotease. Another advantage of using His-tag purification methods is that polyhistidine tags can bind proteins under both native and denaturing conditions. The use of denaturing conditions becomes important when proteins are found in inclusion bodies and must be denatured so they can be solubilized.

Disadvantages of using His-tag protein purification include potential degradation of the His-tag, dimer and tetramer formation, and coelution of other histidine-containing proteins. First, when a few histidine residues are proteolytically degraded, the affinity of the tagged protein is greatly reduced leading to a decrease in the protein yield. Second, once a protein has a His-tag added to its structure, it has the potential to form dimers and tetramers in the presence of metal ions. While this is often not a large problem, it can lead to inaccurate molecular mass estimates of the tagged protein. A third disadvantage of protein purification using His-tags is coelution of proteins that naturally have two or more adjacent histidine residues.

6.3 Protein A, G, and L purification

Proteins A, G, and L are native or recombinant proteins of microbial origin which bind specifically to immunoglobulins including immunoglobulin G (IgG). IgG represents 80% of serum immunoglobulins. Native and recombinant protein A can be cloned in *Staphylococcus aureus*. Recombinant protein G (cell surface protein) is cloned in *Streptococcus* while recombinant protein L is cloned from *Peptostreptococcus magnus*. Both protein A and G specifically bind the F_c region of IgG while protein L binds to the kappa light chains of IgG.

The most popular matrixes or supports for affinity applications which utilize protein A, G, or L is beaded agarose (e.g. Sepharose CL-4B; agarose crosslinked with 2,3-dibromopropanol and desulphated by alkaline hydrolysis under reductive conditions), polyacrylamide, and magnetic beads (Grodzki & Berenstein, 2010; Hober et al, 2007; Katoh et al, 2007; Tyutyulkova & Paul, 1995).

All three proteins bind extensively with the IgG subclass. In general, protein A is more suitable for cat, dog, rabbit and pig IgG whereas protein G is generally more preferable when purifying mouse or human IgG. A combination of protein A and G is also applicable for purifying a wide range of mammalian IgG samples. Since protein L binds to the kappa light chain of immunoglobulins and these light chains exist in other immunoglobulins (i.e IgG, IgM, IgA, and IgE), protein L is suitable for the purification of different classes of antibodies. The binding characteristics of antibody binding proteins (A, G and L) to a variety immunoglobulin species is summarized in Table 2. IgGs from most species bind to protein A and G near physiological pH and ionic strength. To elute purified immunoglobulins from protein G sepharose, the pH should be less than 2.7.

Species	Protein A	Protein G	Protein L*
Human	Strong	Strong	Strong
Mouse	Strong	Strong	Strong
Rat	Weak	Medium	Strong
Cow	Weak	Medium	Strong
Goat	Weak	Strong	No binding
Sheep	Weak	Strong	No binding
Horse	Weak	Strong	Unknown
Rabbit	Strong	Strong	Weak
Guinea pig	Strong	Weak	Unknown
Pig	Strong	Weak	Strong
Dog	Strong	Weak	Unknown
Cat	Strong	Weak	Unknown
Chicken	Unknown	Unknown	Unknown

*Binding affinity based on total IgG binding, L proteins binds to Kappa light chains while Proteins A and G bind to F_c region.

Table 2. Binding affinity for proteins A, G, and L with a variety of immunoglobulin species.

6.4 Biotin and biotinylated molecules purification

If a biotin tag can be incorporated into a biomolecule, it can be used to purify the biomolecule using a streptavidin or avidin affinity support. One way is to insert a

biotinylation sequence into a recombinant protein. Biotin protein ligase can then be used to add biotin in a post-translational modification step (Cronan & Reed, 2000). Biotin, also known as vitamin H or vitamin B_7, is a relatively small cofactor present in cells. In affinity chromatography it is often used an affinity tag due to its very strong interactions with avidin and streptavidin. One advantage of using biotin as an affinity tag is that it has a minimal effect on the activity of a large biomolecule due to its small size (244 Da).

Streptavidin is a large protein (60 kDa) that can be obtained from *Streptomyces avidinii* and bind biotin with an affinity constant of 10^{13} M^{-1}. Avidin is a slightly larger glycoprotein (66 kDa) with slightly stronger binding to biotin (10^{15} M^{-1}). Both avidin and streptavidin have four subunits that can each bind one biotin molecule. To purify biotinylated biomolecules, streptavidin is immobilized onto a support material and used to extract the biotinylated molecules out of solution. Both avidin and streptavidin may be immobilized using amine-reactive coupling chemistries. In addition, avidin can also be immobilized via its carbohydrate residues.

Due to the strong interaction between biotin and (strept)avidin, harsh elution conditions are required to disrupt the binding. For example, 6 M guanidine hydrochloride at pH 1.5 is commonly used to elute the bound biotinylated biomolecule. This prevents the recovery of most proteins in their active form. To overcome this difficulty, modified (strept)avidin or modified biotin may be used to create a lower affinity interaction. In one study chemically modified avidin had relatively strong binding (>10^9 M^{-1}), but was also able to completely release biotinylated molecules at pH 10 (Morag et al, 1996). In addition, at any pH between 4 and 10, a 0.6 mM biotin solution could be used to displace and elute the biotinylated molecules.

Biotin is also used in isotopically coded affinity tags (ICATs) which can be used to compare the protein content in two different samples (Bottari et al, 2004). The ICAT consists of two labels, one which contains deuterium (heavy) and one which contains only hydrogen (light). The two labels (light and heavy) are added separately to the cell lysates being compared. Since the reagent contains a thiol-specific reactive group, it will covalently bind free cysteines on proteins. The labeled lysates are combined, digested with trypsin, and then isolated on a streptavidin column. After a second separation step, the labeled proteins are analyzed using mass spectrometry. The change in protein expression between the two cell lysates can then be quantified and related to the different conditions applied to the two sets of cell lysates.

6.5 Affinity purification of albumin and macroglobulin contamination

Affinity purification is a helpful tool for cleaning up and removing excess albumin and α_2-macroglobulin contamination from samples since these components can mask or interfere with subsequent steps of analysis (e.g. mass spectrometry and immunoprecipitation). One purification method which can be used to remove these contaminants either before or after other purification steps is Blue sepharose affinity chromatography. In this method, the dye ligand is covalently coupled to sepharose via a chlorotriazine ring. Albumin binds in a non-specific manner by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand (Antoni et al, 1978; Peters et al, 1973; Travis & Pannell, 1973; Young & Webb, 1978). The most commonly used dye is Cibacron blue F-3-GA which can be immobilized onto

sepharose to create an affinity column. See Figure 6. This dye is capable of removing over 90% of albumin in the sample (Travis et al, 1976).

6.6 Lectin affinity chromatography

Lectin affinity chromatography is one of the most powerful techniques for studying glycosylation as a protein post translational modification (Hirabayashi et al, 2002; Spiro, 2002). Lectins are carbohydrate binding proteins that contain two or more carbohydrate binding sites and can be classified into five groups according to their specificity to the monosaccharide. They exhibit the highest affinity for: mannose, galactose/N-acetylgalactosamine, N-acetylglucosamine, fucose, and N-acetylneuraminic acid (Sharon, 1998). In this affinity technique, protein is bound to an immobilized lectin through its sugar moeities (N-linked or O-linked). Once the glycosylated protein is bound to the affinity support, the unbound contaminants are washed away, and the purifed protein eluted.

Currently, many lectins are commercially available in an immobilized form. Among them, Concanavaline A (Con A) Sepharose and wheat germ agglutinin (WGA) are the most popular for glycoprotein purification. As shown in Table 3, several different types of lectin may be used in affinity chromatography.

Acronym, Organism and source	Metal ions required	Sugar specificity	Elution conditions	Useful for binding
Con A (*Canavalia ensiformis;* jack bean seeds)	Ca²⁺, Mn²⁺	α-Man > α-Glc	0.1–0.5 M α-MeMan	High-Man, hybrid, and biantennary N-linked chains
LCA or LCH (*Lens culinarus;* lentil seeds)	Ca²⁺, Mn²⁺	α-Man > α-Glc	0.1–0.5 M α-MeMan	Bi- and triantennary N-linked chains with Fuc α1-6 in core region
PSA (*Pisum sativum;*peas)	Ca²⁺, Mn²⁺	α-Man	0.1–0.5 M α-MeMan	Similar to LCA/LCH
WGA (*Triticum vulgaris;* wheat germ)	Ca²⁺, Mn²⁺	ß-GlcNAc	0.1–0.5 M GlcNAc	GlcNAc- and Sia- terminated chains, or clusters of *O*-GlcNAc; succinylated form selectively binds GlcNAc>Sia
HPA (*Helix promatia;* albumin gland of edible snail)	-	α-GalNAc	0.1–0.5 M GalNAc	Proteins with terminal α-GalNAc or GalNAcα-*O*-Ser/Thr (Tn antigen)
UEA-I (*Ulex europaeus;* furze gorse seeds)	-	α-L-Fuc	0.1–0.5 M L-Fuc or methyl-α-L-Fuc	Sugar chains with terminal α-Fuc, especially in α1-2 linkage, but much less with α1-3 or α1-6 linkages
LBA (*Phaseolus lunatus;* lima bean)	Mn²⁺, Ca²⁺	Terminal α-GalNAc	0.1–0.5 M GalNAc	Proteins with blood group A structure GalNAcα1-3(Fucα1-2)Gal–

Table 3. Some examples of lectins used for glycoprotein purification modified from current protocols in protein science.

Lectin affinity columns can be prepared by immobilizing lectins with different specificities toward oligosaccharides to a variety of matrices, including agarose (West & Goldring, 2004), silica (Geng et al, 2001), monolithic stationary phases (Okanda & El Rassi, 2006) and cellulose (Aniulyte et al, 2006). These immobilized lectins are invaluable tools for isolating and separating glycoproteins, glycolipids, polysaccharides, subcellular particles and cells. In addition, lectin affinity columns can be used to purify detergent-solubilized cell membrane components. They also are useful for assessing changes in levels or composition of surface glycoproteins during cell development and in malignant or virally transformed variants. In subsequent chapters, more detailed examples of lectin affinity purification can be found.

6.7 Reversed phase chromatography

Reversed phase chromatography is a kind of affinity interaction between a biomolecule dissolved in a solvent (mobile phase) that has some hydrophobicity (e.g. proteins, peptides, and nucleic acids) and an immobilized hydrophobic ligand (stationary phase) (Dorsey & Cooper, 1994). Reversed phase chromatography is generally more suitable for separating non-volatile molecules. The term "reversed phase" was adopted because the binding occurs between a hydrophobic ligand (octadecyl; C18) and molecules in a polar aqueous phase which is reversed from normal phase chromatography [where a hydrophilic polar ligand binds to molecules in a hydrophobic nonpolar mobile phase].

In general, the macromolecules (e.g. protein or peptides) are adsorbed onto the hydrophobic surface of the column. Elution is achieved using a mobile phase which is usually a combination of water and organic solvents (such as acetonitrile or methanol) applied to the column as a gradient (e.g. starting with 95:5 aqueous:organic and gradually increasing the organic phase until the elution buffer is 5:95 aqueuos:organic). The macromolecules bind the hydrophobic surface of the column and remain until the concentration of the organic phase is high enough to elute the macromolecules from the hydrophobic surface.

When using reversed phase chromatography, the most polar macromolecules are eluted first and the most nonpolar macromolecules are eluted last: the more polar (hydrophilic) a solute is, the faster the elution and vice versa. In summary, separations in reversed phase chromatography depend on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase.

As illustrated in Figure 13, the initial step of reversed phase separation involves equilibration of the column under suitable conditions (pH, ionic strength and polarity). The polarity of the solvent can be modified by adding a solvent such as methanol or acetonitrile and an ion pairing agent such as formic acid or trifluoroacetic acid may be added. Next, sample is applied and bound to the immobilized matrix. Following this step, desorption and elution of the biomolecules is achieved by decreasing the polarity of the mobile phase (by increasing the percentage of organic modifier in the mobile phase). At the end of the separation, the mobile phase should be nearly 100% organic to ensure complete removal of all bound substances. Once everything has eluted from the column, the initial mobile phase is reapplied to the column to reequilibrate the column for a subsequent sample application.
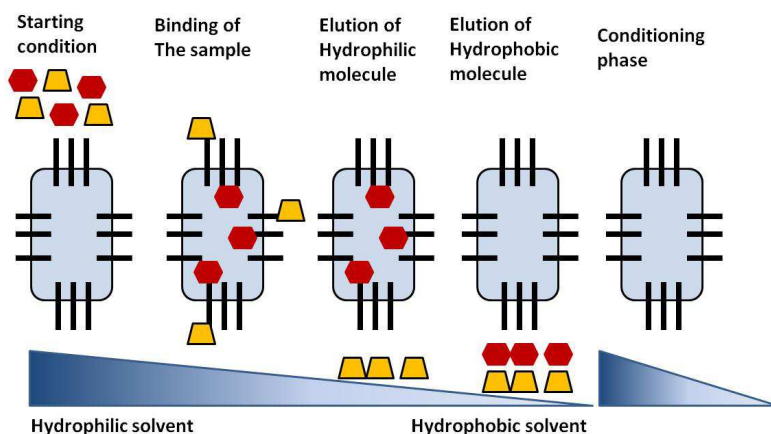
Fig. 13. Steps of a of reversed phase chromatography separation.

7. Funds

8. References

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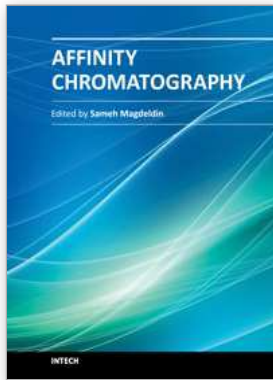
Affinity Chromatography

Edited by Dr. Sameh Magdeldin

Most will agree that one major achievement in the bio-separation techniques is affinity chromatography. This coined terminology covers a myriad of separation approaches that relies mainly on reversible adsorption of biomolecules through biospecific interactions on the ligand. Within this book, the authors tried to deliver for you simplified fundamentals of affinity chromatography together with exemplarily applications of this versatile technique. We have always been endeavor to keep the contents of the book crisp and easily comprehensive, hoping that this book will receive an overwhelming interest, deliver benefits and valuable information to the readers.

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