5
Affinity Chromatography

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5.1
Introduction

Affinity chromatography is a form of liquid chromatography that utilizes a biologically-related binding agent as the stationary phase, producing a column that often results in highly selective separations [1–4]. There are a variety of binding agents that can be immobilized and used as the stationary phase in affinity chromatography. Examples include antibodies, antigens, enzymes, and enzyme inhibitors, as well as lectins, dyes, and transport proteins [1–10]. The immobilized binding agent is known as the *affinity ligand*. This agent provides the selectivity of affinity chromatography through the ability of the affinity ligand to bind a complementary target (e.g., the binding of an antigen with an immobilized antibody) [1–3].

The history of affinity chromatography dates back to 1910, when this method was first employed by Emil Starkenstein to purify α-amylase on a column containing starch [11,12]. This method slowly developed over the next five decades as a tool for the isolation of enzymes and antibodies [6–8,12,13]. Two key developments that occurred in the 1960s included the development of beaded agarose as a support material for liquid chromatography and the creation of the cyanogen bromide immobilization method [14,15]. The combination of these two techniques for enzyme purification was reported in 1968 by Cuatrecasas, Anfinsen and Wilchek [16]. The result of this work was renewed interest in this separation method and the beginning of the modern era of affinity chromatography [2–4,12].

Figure 5.1 shows a typical separation scheme that is used in affinity chromatography. This "on/off" elution method begins with the application of a sample, containing the target of interest, onto an affinity column that has an immobilized and complementary binding agent for the target [2–7,17]. Sample application is carried out by using a buffer that has the correct pH and composition to promote binding by the target to the immobilized affinity ligand. As the sample travels through the column, the target will tend to be strongly and selectively

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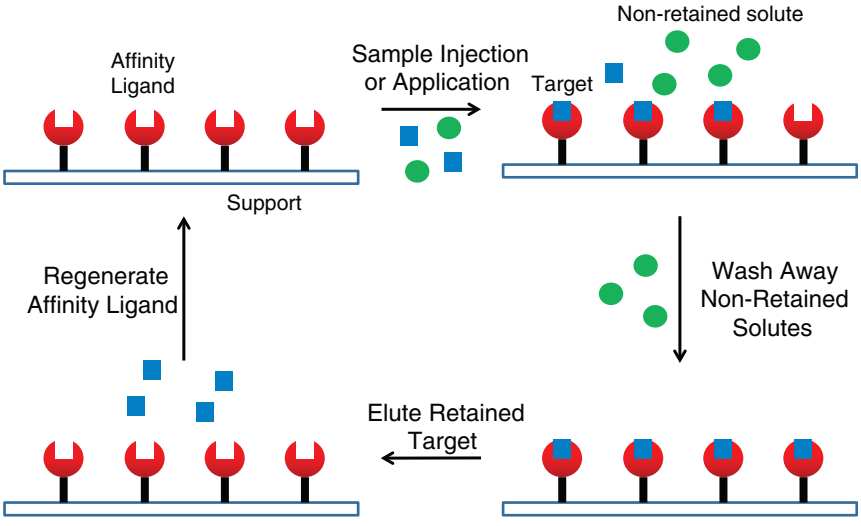

Figure 5.1 A typical on/off elution scheme for affinity chromatography.

retained by the affinity ligand, while most other sample components will be washed away from the column. The target is later released from the column by applying an elution buffer, with the target then being passed through a detector or collected for later use or analysis by another method. The affinity column can then be placed back into its original application buffer and the affinity ligand allowed to regenerate before another sample is passed through the column [3,17].

Since its development, affinity chromatography has had numerous applications. These applications have ranged from biomolecule purification to sample pretreatment, chemical analysis, and studies of biological interactions [1–10,18–30]. This method has also been utilized with many types of binding agents and with a variety of support materials [1–10,22–26,31–37]. This review will discuss the basic principles of affinity chromatography and the factors that need to be considered in the development or use of this separation technique. A summary of the applications for this method will also be provided, including recent examples of work in this field.

5.2 Principles of Affinity Chromatography

Separations in affinity chromatography are based on the reversible interactions that can occur between a target compound that is applied to the affinity column and a biologically related agent, or affinity ligand, that is immobilized within the column. Many of the binding agents that are used in affinity columns, such as antibodies or receptors, have recognition sites that can undergo highly specific

interactions with the target, while giving little or no interactions with other sample components that may be present [1–5,10,17].

There are several parameters to consider in the use of affinity chromatography. Some of these parameters include the amount of the immobilized affinity ligand that is present in the column, the strength of the interaction between the affinity ligand and the target, and the kinetics of this interaction [3,17,28–30]. The following equations can be used to describe the binding between an applied target (A) and an immobilized affinity ligand (L) in a column for a system in which there is a single type of reversible binding site for A with L.

$$\mathrm{A} + \mathrm{L} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \mathrm{A} - \mathrm{L} \tag{5.1}$$

$$K_a = \frac{k_a}{k_d} = \frac{\{\mathrm{A} - \mathrm{L}\}}{[\mathrm{A}]\{\mathrm{L}\}} \tag{5.2}$$

The term K_a in this system is the association equilibrium constant for the binding of A with L to form the complex A – L, while k_a and k_d are the association and dissociation rate constants for the formation and dissociation of this complex. The term [A] represents the molar concentration of A in the mobile phase at equilibrium, and {L} or {A – L} represents the surface concentrations of L and A – L at equilibrium [17].

The association equilibrium constant in Equation 5.2 can be related to the retention of the applied target A through the retention factor (k) for this target. The value of k can be obtained from the observed retention time (t_R) for A by using the following relationship,

$$k = \frac{(t_R - t_M)}{t_M} \tag{5.3}$$

where t_M is the column void time. The retention factor can also be related to the equilibrium constant K_a through Equation 5.4 if a small amount of the target is being injected onto the column (i.e., linear elution conditions are present) and if the flow rate is sufficiently slow to allow a local equilibrium to be established at the center of the peak for A [17,29].

$$k = K_a m_L / V_M \tag{5.4}$$

In this equation, m_L denotes the moles of active affinity ligand that are present in the column, and V_M is the column void volume.

Equation 5.4 indicates that the retention for a target when it is applied to an affinity column will depend on the binding strength of this target with the affinity ligand, as represented by K_a. The retention of the target will also be affected by the amount of binding sites that are present in the column (m_L), or the amount of stationary phase versus mobile phase that is present, as given by the phase ratio m_L/V_M. The same factors will also affect the retention of a target in more complex systems that may involve multiple types of binding sites [17,28–30].

Many binding agents that are used in affinity chromatography have relatively strong interactions with their targets. This can lead to strong retention under mobile phase conditions that mimic those of the target and the binding agent in their natural environment. For example, an affinity column that contains the equivalent of 10 μM of immobilized polyclonal antibodies (which often have K_a values in the range of 10^8–10^{10} M^{-1}) will produce retention factors of 1000 to 100 000 for their targets in the presence of an application buffer [17,30]. To overcome this strong retention and to release the bound target, a change in the mobile phase is usually made for systems with K_a values of 10^5 to 10^6 M^{-1} or greater. This is often accomplished by using the on–off, or step gradient, elution format that was shown in Figure 5.1. Other types of gradient elution can also be used in this situation [17]. Alternatively, targets with lower affinities can sometimes be eluted under isocratic conditions and/or in the presence of the application buffer. This latter situation gives a method known as *weak affinity chromatography* (WAC) or *dynamic affinity chromatography* [17,30,38–40].

5.3 General Types of Affinity Ligands

In affinity chromatography, the main factor that determines the selectivity and retention of this method is the type of affinity ligand that is present [3,17,41]. Biological agents, mimics of these agents, and synthetic compounds have all been used as affinity ligands [1–10]. However, regardless of their type, all of these binding agents can be placed into two categories: *high-specificity ligands* and *general ligands* [3,12].

High-specificity ligands are those binding agents that tend to bind only one target or a group of closely related targets [3,12]. Typical examples of high-specificity ligands are biological agents such as antibodies and antigens, enzymes and their substrates or inhibitors, and single-stranded nucleic acids [1–8]. These ligands often have large association equilibrium constants for their targets, which results in affinity columns with high retention under their application conditions. The selectivity and strong retention of these affinity ligands are their key advantages. However, the costs of using these ligands often outweigh these benefits and tend to result in such ligands being used for mostly small-scale purifications or chemical analysis [21–24].

General, or group-specific, ligands are used when the goal is to isolate a group of targets with a common structural feature [3,12]. These general ligands can be either of biological or of nonbiological origin. Examples of general ligands that are of biological origin are immunoglobulin-binding proteins (e.g., protein A and protein G) and lectins [3,5,8,42]. General ligands that have a nonbiological origin include boronates, biomimetic dyes, and metal-ion chelates [3,5,22–24]. Affinity ligands such as biomimetic dyes are particularly common in large-scale purifications of biological products (e.g., biopharmaceuticals) because of their low cost, their ability to be modified for a specific target, and their good

stability under conditions that are commonly used for column elution and sterilization [21–24].

Many general ligands have weaker binding to their targets than high-specificity ligands. However, there are exceptions. For example, protein A and protein G have association equilibrium constants of $10^7\ M^{-1}$ or greater for some immunoglobulins, which are their targets [42]. Also, a few binding agents that are normally considered to be high-specificity ligands can sometimes be used as general ligands. An example is the use of monoclonal antibodies that are able to bind to a general feature that is common to a group of targets [17], as has been used in multidimensional separations and weak affinity chromatography [39,40,43–46].

5.4
Support Materials for Affinity Chromatography

There are many types of supports that can be used in affinity chromatography [1–10,31–37]. The support acts to contain the affinity ligand within the column, while allowing flow of the mobile phase and contact between the applied target and the affinity ligand for binding and retention to occur. An ideal support for affinity chromatography should be stable under the flow rate, mobile phase, and pressure conditions that are to be used during the separation. In addition, the support should allow for immobilization of the affinity ligand and yet have little or no nonspecific binding for components of the applied samples [31]. The support should also have fast mass transfer for the target to allow a separation with good efficiency to be obtained [3,9,31].

The most popular supports for preparative and industrial applications of affinity chromatography are agarose or cross-linked agarose, which are polymers of agarobiose (see Figure 5.2) [19,31]. Other carbohydrate-based supports that have been utilized in affinity chromatography include starch, cellulose, and modified forms of cellulose [31]. Silica and glass beads can also be used as supports, but they first must be modified (e.g., through conversion into a diol-bonded form) to reduce their nonspecific binding and provide functional groups that can later be used for the immobilization of an affinity ligand [3,9,31,37]. A variety of synthetic organic supports have been used as well, ranging from hydroxylated polystyrene, polymethacrylates, and polyacrylamide derivatives to azalactone beads, polyethersulfones, and agarose–acrylamide or dextran–acrylamide copolymers [1,8,31,33–36].

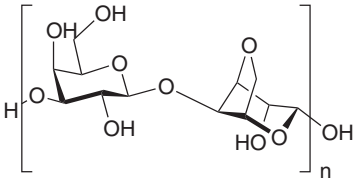

Figure 5.2 The structure of agarobiose, the basic structural unit of agarose.

The pore size of the support is another factor to consider. This pore size should be large enough to allow immobilization of the affinity ligand and access of the target to this ligand as samples are applied to the column. A support with a small pore size will tend to have a larger surface area for ligand attachment, but pores that are close to or smaller than the ligand in size may not be available for this immobilization process [31,47,48]. Particulate supports such as agarose and silica are available in a wide range of pore sizes that are suitable for use with affinity ligands as large as proteins or even larger binding agents (e.g., lipoproteins) [31]. In the case of some supports, such as organic monoliths, it is possible to vary the pore size and surface area of the support by changing the conditions that are used to prepare this material [47,49–51].

Other properties of the support are also important to consider. For instance, as the diameter of a particulate support is decreased, there will be an increase in the pressure across the column. However, small particle sizes allow for faster mass transfer for the targets as they move into and out of the support, resulting in more efficient columns, sharper peaks and separations with higher resolution [31]. If a large diameter, and often nonrigid, particulate support is present in the column, the resulting method is sometimes known as *low-performance, or column, affinity chromatography*. If small and more rigid particles are employed that can withstand high flow rates and pressures (e.g., as are found in high-performance liquid chromatography, or HPLC), the method is instead called *high-performance affinity chromatography* (HPAC) or high-performance liquid affinity chromatography [3,9,31,37].

Low-performance affinity chromatography typically uses carbohydrate-based supports (e.g., agarose) or synthetic organic materials. These supports are relatively inexpensive and simple to use for systems with low back pressures and that require only moderate resolution. These properties make such materials valuable for both small- and large-scale affinity purification methods. HPAC tends to use supports such as modified silica or glass, azalactone beads, and hydroxylated polystyrene media. The good mechanical stability and efficiency of these supports make them more suitable for separations that are to be carried out using HPLC instrumentation and in analytical applications of affinity chromatography [3,9,31,37].

Other supports besides porous particulate materials can be used in affinity columns [31]. Small nonporous particles, with typical diameters of 1–3 μm, can provide good efficiencies and have been used in affinity chromatography for analytical applications. Affinity membranes, fibers, and perfusion media (or flow-through particles) can be used at high flow rates while providing relatively low back pressures. These features make these materials valuable in large-scale purification methods for biopharmaceuticals, proteins, and other compounds. Expanded bed particles that contain a high-density core have also been employed for the large-scale purification of biopharmaceuticals and proteins. A unique feature of this latter support is its ability to form a fluidized bed with a low back pressure when used with upward flow during sample application, thereby helping to minimize column clogging even when used with samples that

contain material such as cell debris [31]. Monolithic columns, or continuous bed supports, have also seen growing use in affinity chromatography over the past decade [32]. These supports have been made from polymethacrylate, other organic polymers, silica, agarose, and cryogels, and have been used in both preparative and analytical applications of affinity chromatography [32–36].

5.5 Immobilization Methods for Affinity Ligands

Immobilization of the affinity ligand onto the support can be performed in many ways. The immobilization method should ideally be performed under conditions that are similar to the natural environment of the affinity ligand or that at least do not permanently affect the ligand's final activity. This method should also provide an orientation and spacing of the affinity ligand that do not block this ligand's binding sites for the target and that help provide the optimum retention of the target by this ligand [3,41]. Furthermore, this method should avoid the introduction of nonspecific interactions between the target and the support, and should be easy and safe to use in a routine laboratory setting. In some cases (e.g., for small affinity ligands), a spacer arm may be needed to place the ligand at a sufficient distance from the support's surface to allow binding by the target to occur [3,41]. Although numerous immobilization methods are available for use within laboratories, there are now many supports that are sold in a preactivated form to simplify the immobilization process [8,41].

The most common way for immobilizing a ligand is to covalently attach it to the support. *Covalent immobilization* requires a reaction between functional groups on the affinity ligand and reactive sites on the support. This process has two main steps: activation of the support and coupling of the affinity ligand onto the active support (see examples in Figure 5.3). This form of immobilization can be used with many types of ligands [3,41]. For example, proteins and peptides can be immobilized through amine groups, carboxylic acids, or sulfhydryl residues. Glycoproteins and other carbohydrate-containing ligands can also be immobilized through residues such as aldehyde groups that are produced through mild oxidation [8,41].

Noncovalent immobilization can also be used with affinity ligands. In this method, the ligand is held through adsorption and noncovalent interactions with the support's surface (e.g., through ionic interactions with a charged surface). This method is often easy to perform, as long as the support has appropriate sites for adsorption of the affinity ligand. The main drawback to this method is that the same support may have nonspecific interactions with other sample components, and the affinity ligand can bleed from the column over time (i.e., resulting in changes in the column's binding capacity) [41].

A special form of noncovalent immobilization is *biospecific adsorption*. In this approach, the immobilization process is based on the adsorption of the affinity ligand to a secondary binding agent that is already attached to the support [41].

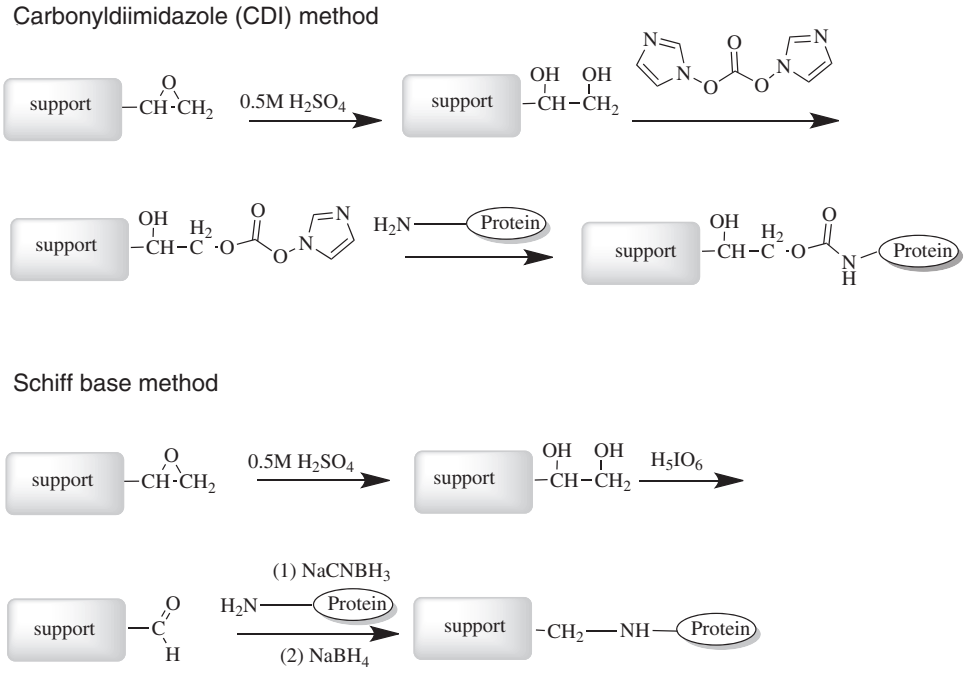

Figure 5.3 Examples of two common covalent immobilization methods for use with proteins or other amine-containing affinity ligands.

This method is frequently used in immunoaffinity chromatography by adsorbing antibodies to immunoglobulin-binding proteins such as protein A and protein G [8,42,43]. The use of immobilized avidin or streptavidin to bind to biotin-labeled agents is another common form of biospecific adsorption [42].

Entrapment, or encapsulation, is another method of immobilization that is sometimes utilized. This approach has been used with liposomes, cells, and proteins [41]. A common way of carrying out the entrapment of a protein is to place this binding agent into a sol–gel during the formation of this support. Recently, proteins have also been entrapped in porous supports by partially blocking the pores or surface of this material with large capping agents [52,53]. Liposomes, cells and membrane-based particles have been entrapped by altering their size through freeze drying, with these agents then being placed into a support with large pores (e.g., agarose) and allowed to expand in size (i.e., preventing them from later exiting the pores) [41,54,55].

Another approach for preparing an immobilized affinity ligand is *molecular imprinting* [41,54,55]. In this approach, the affinity ligand is made during the support's preparation. To do this, the target of interest is placed into the polymerization mixture that is to be used to form the affinity support. This mixture includes an initiation reagent, a cross-linking agent, and monomers with functional groups that can interact with the target. The product of this reaction is a

polymer that contains pockets that are formed directly about the target, and which complement the shape and arrangement of functional groups on the target. After the polymerization has been completed, the target is removed. The resulting unoccupied sites can then be used as affinity ligands to isolate or retain the same target from samples [41,56–59].

5.6 Application and Elution in Affinity Chromatography

The samples and target in affinity chromatography are typically injected or applied in the presence of a mobile phase that has the correct pH and composition to promote binding by the target to the affinity ligand. This solution is called the *application buffer* and acts as the weak mobile phase for the affinity column [3,17]. This mobile phase is also used to wash away sample components that are nonretained or only weakly retained by the affinity ligand.

The composition of the application buffer will depend on the nature of the affinity ligand and the target. The binding of these two components often involves multiple types of interactions (e.g., hydrogen bonds, ionic interactions, dipole–dipole interactions, van der Waals forces, and steric effects) [43]. The application conditions are often selected to maximize these forces and promote binding by providing a pH, ionic strength and solution polarity that mimic the conditions experienced by the affinity ligand and target in their native environment. The temperature of the system and the possible need for cofactors or other additives, such as metal ions, that are required for target-ligand binding should also be considered when optimizing retention by the target [17]. Nonspecific interactions of the target, or other sample components, within the chromatographic system can be minimized through the use of additives in the application buffer. For instance, additives such as surfactants (e.g., Triton X-100 and Tween 20) or proteins (e.g., bovine serum albumin) are sometimes included in this solution to block nonspecific binding [17].

There are some cases in which the target may be retained with a sufficiently low affinity (i.e., $K_a < 10^5–10^6\,M^{-1}$) to allow its elution in a reasonable time under isocratic conditions and using the application buffer. Such a situation occurs in the method of weak affinity chromatography [17,38–40]. However, when the association equilibrium constant is greater than $10^5–10^6\,M^{-1}$, it is necessary to change the mobile phase to a solution that dissociates the retained target from the affinity ligand [3,17]. An example of this process is the "on/off" elution format that was shown in Figure 5.1. The second mobile phase that is employed in this format is known as the *elution buffer*, and it acts as the strong mobile phase for the system. After the target has been eluted, the application buffer can then be reapplied and the column allowed to regenerate prior to the next application of a sample [3,17].

In *nonspecific elution*, an elution buffer is selected to promote fast removal of the target from the column. This can be achieved either by changing the pH,

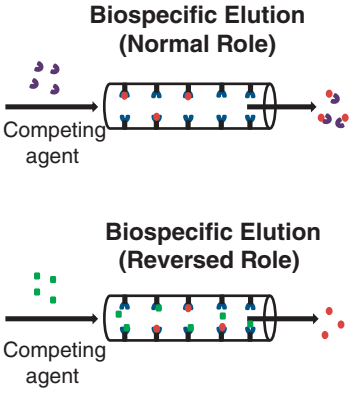

Figure 5.4 The "normal role" and "reversed role" modes of biospecific elution in affinity chromatography.

ionic strength, or polarity of the mobile phase or by adding a denaturing agent or chaotropic agent to the mobile phase [3,17]. These changes can result in an alteration in the structure of the target and/or affinity ligand, thereby leading to a lower association equilibrium constant for their interaction and a decrease in target retention. Nonspecific elution is commonly used in analytical applications of affinity chromatography because it tends to result in rapid elution and a sharp peak for the target, thus also helping to provide good limits of detection. The main disadvantage of this method is that there is some risk of irreversibly damaging the affinity ligand if the selected elution conditions are too harsh [3,17].

In *biospecific elution*, a competing agent is added to the mobile phase to displace the target from the column (see Figure 5.4) [3,17,28–30]. Biospecific elution involves gentler conditions than nonspecific elution because it is carried out under essentially the same solution conditions that are used for sample application, only with a competing agent now being present. This approach is widely used for the affinity purification of substances such as proteins and biopharmaceutical agents, and in situations where a high recovery of active target is desired. Biospecific elution may be performed either by adding an agent to the elution buffer that competes with the affinity ligand for the target (i.e., *normal role elution*) or by adding an agent that competes with the target for binding sites on the affinity ligand (i.e., *reversed role elution*). In both cases, the retained target is eluted from the column by displacement and mass action. The main disadvantages of this method are its slower elution times and broader peaks than are seen in nonspecific elution, and the frequent need to later remove the competing agent from the eluted target [3,17].

The sample size that is applied to the affinity column is another factor that should be considered. In preparative applications, it is usually desirable to maximize the sample throughput to minimize the overall separation time and obtain the highest possible recovery of the target from the affinity column. In analytical applications, it is usually more important to use linear elution conditions, in

which the amount of applied sample is sufficiently small to avoid any significant change in the retention time of the target as the sample size is varied. For the use of affinity chromatography in the study of biological interactions, as will be discussed in Section 5.9, a sample that saturates the column (e.g., in frontal analysis) or that is small compared to the total column binding capacity (e.g., in zonal elution) might be used, depending on the nature of the experiment [17].

When determining the appropriate amount of sample that can be applied to an affinity column, it is helpful to first estimate the total moles of binding sites that are present in the column. One approach is to use an assay to determine the total moles of ligand that have been immobilized to the support. An example would be the use of a protein assay to determine how much of an antibody has been immobilized. However, not all of the immobilized ligand may be active. Thus, a better estimate for the amount of active ligand can be obtained by performing a binding assay on the support, either before this support has been placed in the column or after it is in the column [17].

The flow rate that is used for sample application can also affect the amount of target that will bind to an affinity column [17]. Slow kinetics of adsorption or mass transfer within the column may decrease the amount of target that is retained during the application step through a phenomenon known as the split-peak effect [17,60–62]. In such systems, it is sometimes possible to see some nonretained target, even when using small amounts of sample and a column that has a sufficient binding capacity for this target. Ways in which this effect can be minimized include reducing the flow rate for sample injection, increasing the column size or binding capacity, increasing the efficiency of the support, or changing the immobilization method to obtain an affinity ligand with the ability to undergo faster binding to the target [17,60].

5.7
Bioaffinity and Immunoaffinity Chromatography

Bioaffinity chromatography is a type of affinity chromatography in which the affinity ligand is a biological agent [7,42]. This was the first type of affinity chromatography that was reported, and its earliest use was in enzyme purification [11,12], which is still a common application for this method [1–8,12,42]. There are several affinity ligands that can be utilized for enzyme purification, including enzyme inhibitors, substrates, cofactors, and coenzymes [2–4,6,63,64]. One example is the use of immobilized RNA or DNA for the purification and isolation of polymerases and nucleases [2,64]. Other examples include the use of NAD to purify dehydrogenases and the isolation of kinases by using immobilized nucleotide mono-, di-, or triphosphates [2,4,42,64].

A second group of biological ligands that are used in affinity chromatography are lectins, creating a method known as *lectin affinity chromatography* [42]. Lectins are nonimmune system proteins with the ability to recognize and bind certain carbohydrate residues [65]. Concanavalin A is a lectin that is often used in

bioaffinity chromatography, and which has the ability to bind targets that contain α-D-mannose or α-D-glucose residues. Another lectin that is utilized in bioaffinity chromatography is wheat germ agglutinin (WGA), which binds to D-*N*-acetylglucosamine. Other examples of lectins are jacalin, which binds to α-D-galactosyl groups, and *Sambucus nigra* agglutinin (SNA), which binds to sialylated carbohydrate groups [42,65]. These lectins have been used in the isolation and separation of many carbohydrate compounds, such as glycopeptides, glycoproteins, and glycolipids [8,42].

A third group of important biological ligands are bacterial cell wall proteins that bind to various immunoglobulins. Two common examples are protein A, which is produced by *Staphylococcus aureus*, and protein G, which is produced by group G *Streptococci* [3,42,66–68]. Both of these proteins have the ability to bind to the constant region of many types of immunoglobulins. This feature makes these proteins useful for antibody purification and analysis or as secondary ligands for the biospecific adsorption of antibodies within affinity columns [3,42–44]. Protein A and protein G do have some differences in their selectivities for some species or classes of immunoglobulins, but a recombinant hybrid form of protein A and protein G is also available that combines the selectivity of these two binding agents [42].

Biological agents have been employed in other types of separations. For example, many chiral separations are based on the binding of targets to stereoselective binding agents such as cyclodextrins, α-chymotrypsin, ovomucoid, serum albumins, and cellobiohydrolase I [42,69,70]. A recent example is shown in Figure 5.5 [51]. In addition, polynucleotides and nucleic acids can be used to isolate DNA/RNA-binding proteins and enzymes or to bind nucleic acids that are complementary in sequence to the affinity ligand [71].

Immunoaffinity chromatography (IAC) is a special type of bioaffinity chromatography that employs antibodies or antibody-related substances as the stationary phase. Antibodies, or immunoglobulins, are glycoproteins that are produced by the immune system to bind and recognize foreign agents. Many methods

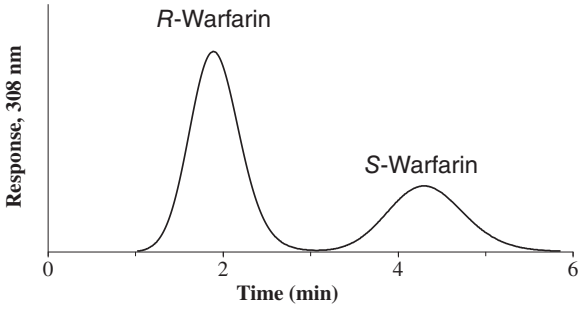

Figure 5.5 Chiral separation obtained at 2.0 ml/min for *R*- and *S*-warfarin on a 4.6 mm i.d. × 10 mm affinity column containing immobilized human serum albumin. The mobile phase was pH 7.4, 0.067 M phosphate buffer containing 0.5% 1-propanol. (Reproduced with permission from Ref. [51].)

utilizing IAC have been developed for the isolation and purification of antibodies, enzymes, hormones, peptides, viruses, and other biologically relevant agents [43,44,72,73]. These purification and pretreatment methods often make use of low-performance supports such as agarose or cellulose, although other materials can be employed. The ability of antibodies to recognize and bind specific targets with high affinities is an attractive feature of these methods, as it provides them with good selectivity and the ability to work with trace amounts of a target in even complex biological samples [43,44].

Another application of IAC is a technique known as *high-performance immunoaffinity chromatography* (HPIAC) [43,44]. This is a type of IAC that makes use of immobilized antibodies, or antibody-related agents, that are placed onto a chromatographic support (e.g., silica particles or a monolith) and that can be used at the flow rates and pressures encountered in HPLC. This combination makes it possible to combine the strong and specific interactions of antibodies with the precision, speed, and wide range of detection formats that are possible when using HPLC [43,44,72,73]. HPIAC can be used alone for the direct analysis of targets in samples or in combination with other techniques (e.g., reversed phase chromatography or mass spectrometry) in multidimensional analytical methods [43,44,72].

A *chromatographic immunoassay*, or flow-injection immunoanalysis, can be carried out in various formats by using either HPIAC or IAC [43,44,72]. Figure 5.6 shows one possible format for this type of application (i.e., a one-site immunometric assay). Competitive binding immunoassays and two-site immunometric assays are also possible, and these formats can be combined with various detection methods (e.g., absorbance, fluorescence, chemiluminescence, and electrochemical detection). Chromatographic immunoassays can be used alone or for detecting targets that have been previously separated by some other

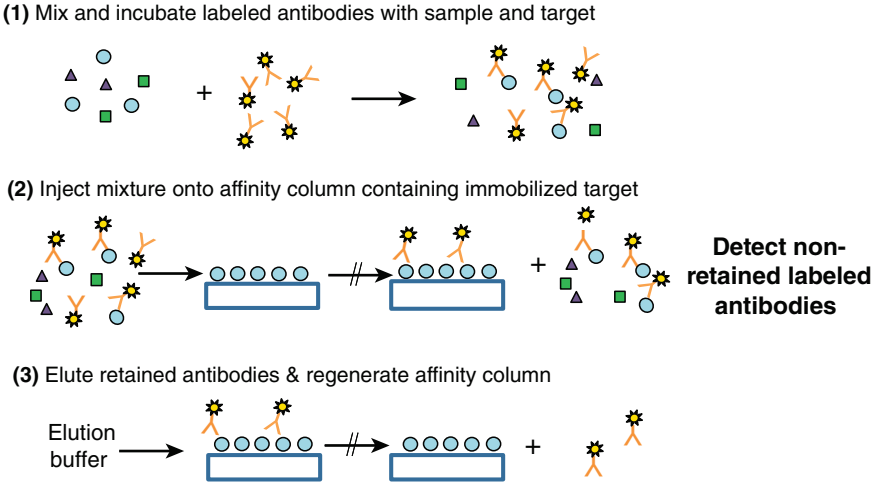

Figure 5.6 General scheme for a one-site immunometric assay.

column, with this latter method being known as *postcolumn immunodetection* [43,72].

Yet another application of IAC is in the pretreatment of a sample prior to its analysis by another technique. The use of IAC to isolate a target for later analysis is known as *immunoextraction*. This method can be combined offline or online with another technique [42,73]. Offline immunoextraction is often faster to carry out initially, but online extraction results in more efficient use of the sample and is easier to automate. Examples of methods that have been combined with immunoextraction are ion-exchange chromatography, reversed phase liquid chromatography, size-exclusion chromatography, liquid chromatography–mass spectrometry, gas chromatography, and capillary electrophoresis [43,72,73]. Another popular application of IAC is the utilization of this method to remove some sample components prior to the analysis of a target in the remaining matrix. This form of IAC is known as *immunodepletion*. This method is often employed in proteomics for the removal of major proteins from biological samples, making it easier to examine less abundant proteins in the same sample [73].

5.8 Affinity Chromatography Based on Nonbiological Ligands

A variety of nonbiological binding agents can be used as ligands in affinity chromatography. *Dye-ligand affinity chromatography* employs an immobilized synthetic dye as the affinity ligand, which is often used for protein purification [74,75]. Dyes that have been utilized for this purpose include Cibacron Blue 3GA, Procion Yellow H-A, Procion Rubine MX-B, and Procion Red HE-3B. A portion of such a dye is used as a mimic of a chemical that would normally interact with the target protein. For example, Cibacron Blue 3GA can interact with the enzyme NAD(P)H:quinone reductase by binding to the part of this target that normally binds to $NADP^+$ [75]. Hundreds of proteins have been purified by dye–ligand affinity chromatography. This method is particularly attractive for large-scale purifications because of the good stability and low cost of these dye ligands and the ability to modify the affinity ligand's structure to help it bind to a particular target [22,23,74,75].

The ability to generate and use an affinity ligand that mimics a natural binding agent, as occurs in dye–ligand affinity chromatography, is sometimes referred to as *biomimetic affinity chromatography* [75]. Other binding agents besides synthetic dyes can be used for this purpose. Affinity ligands that have been generated or produced by using ribosome display, phage display libraries, and libraries of aptamers are a few examples. In other cases, computer modeling or combinatorial chemistry might be used to select an affinity ligand, as has occurred in the use of peptide libraries to create biomimetic ligands [75].

Another set of nonbiological agents that are used as ligands in affinity chromatography are metal-ion chelates. This method is often known as *immobilized metal-ion affinity chromatography* (*IMAC*) and is also sometimes referred to as

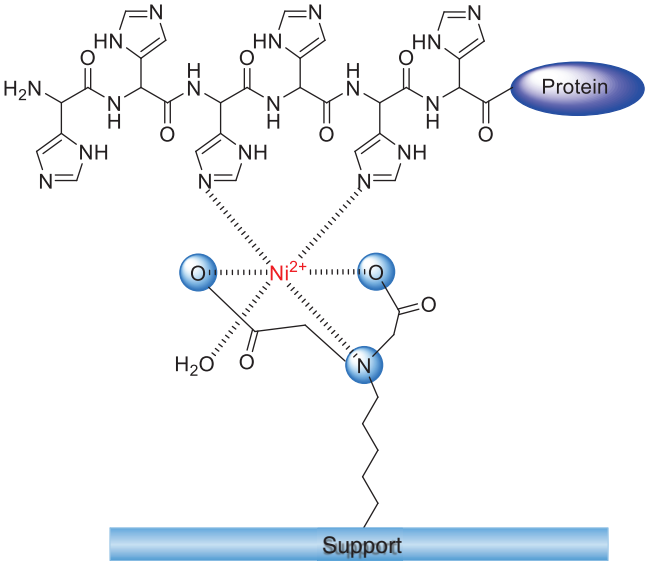

Figure 5.7 Interaction of a histidine-tagged protein with an immobilized metal ion chelate containing Ni^{2+} [78].

"metal ion interaction chromatography" or "metal chelate chromatography" [76–78]. In this technique, a chelating agent is placed onto the support and used to bind a metal ion. This metal ion complex is then used as the affinity ligand. For instance, Ni^{2+} is often bound to immobilized iminodiacetic acid (IDA) for use in IMAC as an affinity ligand to capture and isolate histidine-tagged recombinant proteins (see Figure 5.7). Other metal ions and chelating agents have been used as well to bind to peptides and proteins that contain groups such as tryptophan, cysteine, and histidine, or phosphorylated residues [76].

Boronates are another group of nonbiological ligands that have been used in affinity chromatography, resulting in a method called *boronate affinity chromatography* [79,80]. These ligands can form reversible bonds with targets that contain *cis*-diol groups. The most common boronate that is used as a stationary phase in affinity chromatography is 3-aminophenylboronic acid [79,80]. Boronate affinity columns have been utilized to isolate glycopeptides, glyoenzymes, and glycoproteins [80]. Boronate columns have also become popular tools in clinical testing for the separation and measurement of glycated hemoglobin in diabetic patients [5,25].

5.9
Biointeraction Analysis by Affinity Chromatography

Yet another application of affinity chromatography is as a tool to study biological interactions. This approach is known as *analytical affinity chromatography*,

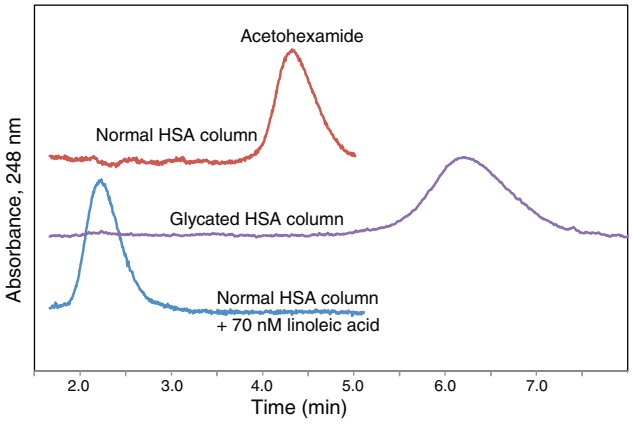

Figure 5.8 Chromatograms from zonal elution experiments examining the binding by acetohexamide to affinity columns containing normal human serum albumin (HSA) or glycated HSA in the absence or presence of linoleic acid as a mobile phase additive. (Reproduced with permission from Ref. [85].)

biointeraction chromatography, or *quantitative affinity chromatography* [28–30,81,82]. Information can be acquired through this method on various characteristics of a biological interaction, such as the stoichiometry of this reaction and its thermodynamics or kinetics. Data on the location of a binding site for a target on an affinity ligand can also be obtained, as well as information on the mechanism for this interaction [28–30,82].

Zonal elution is one technique that is often used in affinity chromatography for biological interaction studies [28–30,83,84]. Zonal elution, as illustrated in Figure 5.8, is carried out by injecting a small amount of a target onto an affinity column in the presence or absence of a mobile phase that contains a competing agent [85]. The target's retention can be used as a measure of the binding strength of this target with the affinity ligand. In addition, the change in this retention as the concentration of the competing agent is varied can provide information on the types of interactions that are occurring between the target and this competing agent and provide some of the equilibrium constants for these interactions. Zonal elution not only has been frequently used to examine drug–protein binding but it has been used to study protein–protein interactions and enzyme–inhibitor interactions [28–30,81,82].

Frontal analysis (or *frontal affinity chromatography*, FAC) is another method that can be employed in affinity chromatography for biological interaction studies [28–30,86]. This technique involves the steady application of a fixed concentration of the target to an affinity column. As the column begins to saturate, the amount of target that exits the column increases and forms a breakthrough curve. The amount of target that is required to reach the mean point of this

curve is then measured at various concentrations of the target. These data can then be fit to various interaction models to determine both the equilibrium constant(s) for target–ligand binding and the number of binding sites in the column [28–30]. One advantage of this approach over zonal elution is that it can simultaneously provide information on both equilibrium constants and the number of binding sites in a column; however, it also usually requires a larger amount of the target than zonal elution [29,30].

Information on the kinetics of target–ligand interactions can also be obtained by using affinity chromatography [28–30]. Several methods have been developed for this purpose, including techniques based on band-broadening measurements, the split-peak effect, peak decay analysis, and peak fitting methods [28–30,60–62,87–89]. In addition, ultrafast affinity extraction has recently been used for this purpose [90].

5.10 Future Directions

The historical development of affinity chromatography has often been linked to the availability of new or improved support materials [1–10,12,14,16,31]. In addition to particulate supports, it is expected that the use of materials such as perfusion media, monolithic columns, nonporous supports, membranes, and fibers will continue to expand in affinity separations [31–36]. The availability of HPLC media with enhanced efficiencies (e.g., supports for ultraperformance liquid chromatography) may also see growth in the coming years for use in affinity-based separations.

Continued research in either more site-selective or general immobilization methods (e.g., entrapment or molecular imprinting) should further help broaden the use and impact of affinity chromatography in routine separations [41,52–59]. Along with the wide variety of affinity ligands that are already available, it is expected that there will be greater use of biomimetic ligands (including peptide or aptamer libraries), synthetic binding agents, and recombinant proteins in future applications of affinity chromatography [74,75].

Another area of research and development that is expected to continue is in the creation of new formats and applications for affinity chromatography. One such area is in the combination of affinity columns with other methods for chemical analysis. A few examples include the growing use of affinity columns for sample pretreatment prior to mass spectrometry, liquid chromatography/mass spectrometry, and capillary electrophoresis in areas like proteomics, glycomics, biomarker analysis, and drug discovery [43,44,73,91–93]. The strong and selective binding of affinity columns has also made these tools of interest for use in microscale analytical methods for sample processing, chemical analysis, and biological interaction studies [91–94].

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