CHROMATOGRAPHY, AFFINITY

1. Introduction

Affinity chromatography is a liquid chromatographic technique that uses a biologically related agent as the stationary phase (1–7). This makes use of the selective interactions that are common in biological systems, such as the binding of an enzyme with a substrate or the binding of an antibody with a foreign substance that has invaded the body. These interactions are used in affinity chromatography by immobilizing one of a pair of interacting molecules onto a solid support. This support is then placed into a column or onto a planar surface. The immobilized molecule is referred to as the *affinity ligand* and it represents the stationary phase of the chromatographic system.

The earliest known use of affinity chromatography was in 1910, when Emil Starkenstein used insoluble starch to purify the enzyme α-amylase (8). Over the course of the next 50 years there were a few other reports that described additional solid-phase ligands for biological purification. However, it was not until the development of beaded agarose supports (9) and the cyanogen bromide immobilization method (10) in the 1960's that affinity chromatography came into common use (11).

Figure 1 shows the most common scheme for performing affinity chromatography (6,7). In this approach, a sample containing the compound of interest is first injected onto an affinity column in the presence of a mobile phase that has the right pH, ionic strength, and solvent composition for solute–ligand binding. This solvent, which represents the weak mobile phase of an affinity column, is

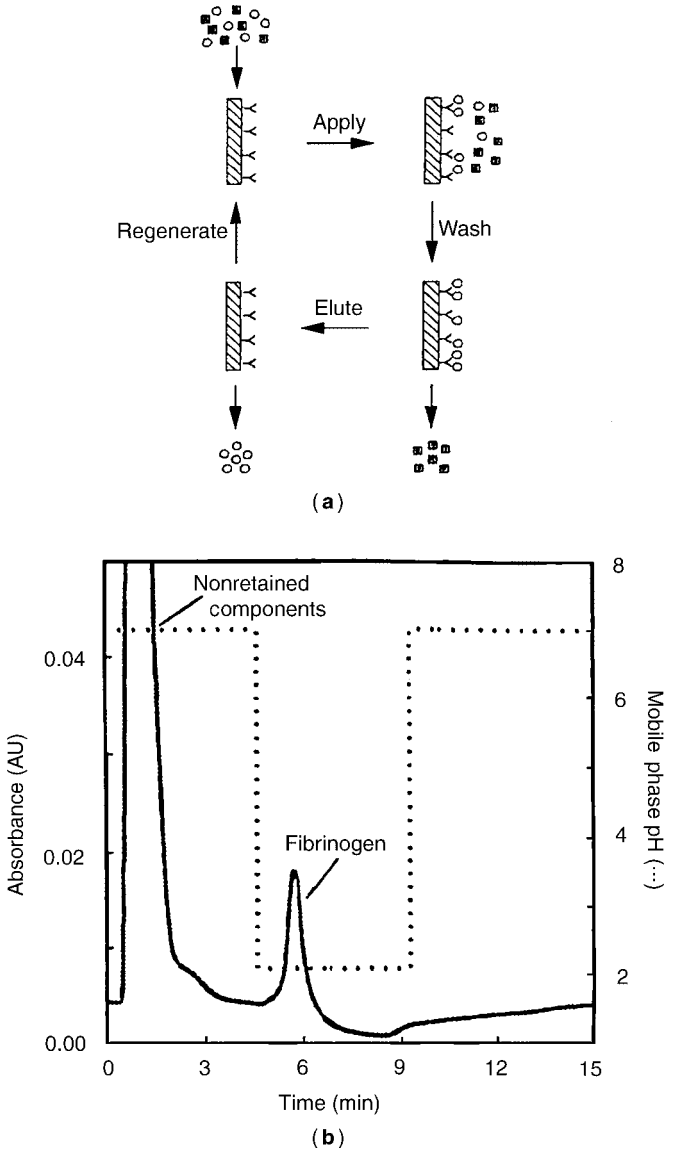

Fig. 1. A typical separation scheme for affinity chromatography. (**a**) Shows the general format that is used to apply and elute samples. The example given in (**b**) shows the use of this approach in the isolation of fibrinogen from a plasma sample, with antifibrinogen antibodies being employed as the affinity ligands and a low pH buffer being used for elution. Part **b** of this figure is reproduced with permission from Ref. 12.

referred to as the *application buffer*. As the sample passes through the column under these conditions, any compounds in the sample that are able to bind to the affinity ligand will be retained. However, due to the high selectivity of most such interactions, other substances in the sample tend to elute from the column as a nonretained peak.

After all nonretained or weakly retained substances have been washed from the column, the retained solutes are eluted by applying a solvent that displaces them from the column or promotes dissociation of the complex between each solute and affinity ligand. This second solvent, which acts as the strong mobile phase for the column, is usually referred to as the *elution buffer*. As the retained solutes are eluted from the column, they can either be collected for later use, as would be performed in a preparative application, or monitored by an on-line detector for analysis. After all of these substances have been removed from the system, the original application buffer is again applied and the affinity ligand is allowed to regenerate back to its original state prior to the application of another sample.

The wide range of ligands available for affinity chromatography makes this method a valuable tool for the purification and analysis of compounds present in complex samples. Examples that will be examined in this article include the use of both biological ligands (eg, antibodies, lectins and nucleic acids), as well as alternative ligands (eg, boronic acids, metal chelates, and biomimetic dyes) (3–7). Although most past reports on affinity chromatography have focused on its use in the isolation of biological compounds (3,4), more recent work has also used this method for chemical analysis in biochemistry, pharmaceutical sciences, clinical chemistry, and environmental testing (5–7,13–16). In addition, affinity chromatography has been used as a tool in measurements of the strength and rates of biological interactions (17,18).

2. Principles of Affinity Chromatography

2.1. Theory of Affinity Chromatography. A number of factors affect how a compound is retained or eluted by an affinity column. These factors include the type of mobile phase that is being applied to the column, the strength of the solute–ligand interaction in this solvent, the amount of immobilized ligand present in the column, and the kinetics of solute–ligand association and dissociation. Furthermore, the type of support material that is used in the column can also play a role in determining the speed and efficiency of this chromatographic process.

For a simple affinity ligand system, many of these factors can be described by using the following reaction that shows the binding between the applied (A) and immobilized ligand (L) within the chromatographic bed.

$$\mathrm{A} + \mathrm{L} \overset{K_a}{\rightleftharpoons} \mathrm{A{-\!\!-}L} \tag{1}$$

In this equation, K_a is the association equilibrium constant for the binding of A with L to form A—L, the solute–ligand complex. Based on this reaction, an expression for K_a can be written in the form shown in equation 2.

$$K_a = k_a/k_d = \{\mathrm{A{-\!\!-}L}\}/[\mathrm{A}]\{\mathrm{L}\} \tag{2}$$

In equation 2, [A] is the mobile phase concentration of A at equilibrium, while {L} and {A—L} represent the surface concentrations of the ligand and

solute–ligand complex, respectively. The term k_a is the second-order association rate constant for solute–ligand binding and k_d is the first-order dissociation rate constant for the solute–ligand complex.

The above reaction can be related to the retention of solute A on an affinity column by using the solute's retention factor (k). As in any other chromatographic method, this retention factor is related experimentally to the mean elution time of a solute from the column in a given mobile phase by using the expression $k = (t_R - t_M)/t_M$, where t_R is the average retention time for the solute under these conditions and t_M is the void time of the system (ie, the observed elution time for a totally nonretained solute). The retention factor can also be shown to be directly related to the association equilibrium constant for the solute with the affinity ligand, as is demonstrated in equation 3.

$$k = K_a m_L / V_M \tag{3}$$

In this equation, m_L represents the moles of active ligand in the column, V_M is the column void volume, and the combined term m_L/V_M represents the column's phase ratio (ie, the relative amount of stationary phase versus mobile phase) (7,17,18).

According to equation 3, the retention factor for a solute on an affinity column (as well as its retention time) in the presence of a given mobile phase will depend on both the strength of the solute–ligand binding under these conditions (as represented by the association equilibrium constant, K_a) and the amount of ligand that is available for binding within the column (as represented by the binding capacity or phase ratio, m_L/V_M). Since many biologically related ligands have moderate or large equilibrium constants for their target solutes (ie, $K_a > 10^6\ M^{-1}$), this gives these solutes strong retention and long elution times on their corresponding affinity columns under normal sample application conditions. It is for this reason that a step change to other conditions that promote lower binding is often used for the elution of solutes from affinity columns. However, for weaker binding systems ($K_a < 10^6\ M^{-1}$), work under isocratic conditions is also possible (19–21).

2.2. General Types of Affinity Ligands. The most important item that determines the selectivity and retention of an affinity chromatographic system is the type of ligand used as the stationary phase. There are many biological agents and biological mimics that have been used for this purpose. However, all of these ligands can be placed into one of two categories: (1) high specificity ligands and (2) general, or group-specific ligands (6).

The term *high specificity ligands* refers to compounds that bind only to one or a few closely related molecules, which is used in chromatographic systems when the goal is to analyze or purify a specific solute. Typical high specificity ligands include antibodies (for binding antigens), substrates or inhibitors (for separating enzymes), and single-stranded nucleic acids (for the retention of a complementary sequence). As this suggests, most high specificity ligands tend to be biological compounds. In addition, most of these ligands have large association equilibrium constants for their solutes and high solute retention.

General, or group-specific, ligands are compounds that bind to a family or class of related molecules. These ligands are used in methods where the goal is to

isolate a class of structurally similar solutes. General ligands can be of either biological or nonbiological origin. Examples include bacterial cell wall proteins (eg, proteins A and G), lectins, boronates, triazine dyes, and immobilized metal chelates. Many of these ligands have weaker binding for solutes than that observed for high specificity ligands. However, there are exceptions. For example, proteins A and G have association equilibrium constants of $10^7\ M^{-1}$ for some types of antibodies, their target solutes. Also, some molecules that are usually considered to be high specificity ligands, such as antibodies, can be used to retain an entire class of solutes if they recognize a feature which is common to a group of closely related agents (7).

2.3. Support Materials. Another factor to consider in affinity chromatography is the material used to hold the ligand within the column. Ideally, this support should have low nonspecific binding for sample components but should be easy to modify for ligand attachment. This material should also be stable under the flow-rate, pressure, sample, and solvent conditions to be used. In addition, the support should have sufficient surface area for immobilization, be readily available, and be simple to use in method development (22).

A wide variety of materials have been employed as supports in affinity chromatography. For instance, many carbohydrate-based materials have been used for this purpose, including starch, agarose, cross-linked agarose, cellulose, and various modified forms of cellulose. Inorganic materials like silica and glass have also been used in affinity chromatography; this first requires the modification of these substances to give them low nonspecific binding and functional groups that can be used for ligand attachment. Many types of synthetic organic-based supports have been used as well, including agarose–acrylamide copolymers, azalactone beads, dextran–acrylamide copolymers, hydroxylated polystyrene, polyacrylamide derivatives, polyethersulfone, and polymethacrylate derivatives. Most of these supports are available commercially and all can be prepared within the laboratory according to published procedures (4–7,22).

Depending on the type of support material used, affinity chromatography can be characterized as being either a low or high performance technique. In *low performance (or column) affinity chromatography*, the support is usually a large diameter, nonrigid gel. Many of the carbohydrate-based supports and synthetic organic materials fall within this category. The low back pressure of these supports means that they can be operated under gravity flow or with a peristaltic pump. This makes these gels relatively simple and inexpensive to use for affinity purification. Disadvantages of these materials include their slow mass transfer properties and their limited stability at high flow rates and pressures. These factors limit the usefulness of these supports in analytical applications, where both rapid and efficient separations are desired.

In *high performance affinity chromatography (HPAC)*, the support consists of small, rigid particles capable of withstanding the high flow rates and/or pressures that are characteristic of high performance liquid chromatography (HPLC). Supports that can be used for this include modified silica or glass, azalactone beads, and hydroxylated polystyrene media. The mechanical stability and efficiency of these supports allows them to be used with standard HPLC equipment. Although the need for more sophisticated instrumentation does make HPAC more expensive to perform than low performance affinity chromato-

graphy, the better speed and precision of this technique makes it the method of choice for analytical applications (6,7,23).

Another way affinity supports can be categorized is based on the physical form of these supports. The most common type used in affinity chromatography is the porous particulate support, which is packed within an affinity column. Examples of such supports include silica, glass beads, agarose or cellulose beads, and azalactone particles. In addition, several alternative types of affinity supports have begun to see greater interest in recent years. Examples include nonporous particulate supports, fibers and membranes, flow through particles (eg, perfusion media), expanded bed particles, and continuous bed supports (ie, monolithic media).

2.4. Immobilization Methods. A third item to consider in using affinity chromatography is the way in which the ligand is attached to the solid support, or the *immobilization method*. There are several strategies used for this purpose, as illustrated in Figure 2. The most common way for placing the ligand within the affinity column is *covalent immobilization*. This is performed by reacting functional groups on the ligand with reactive sites on the surface of the support. Several techniques are available for this, as summarized in Table 1 (24,25). For a protein or peptide, covalent immobilization generally involves coupling these molecules through free amine, carboxylic acid, or sulfhydryl residues in their structures. Immobilization of a ligand through other functional sites (eg, aldehyde groups produced by carbohydrate oxidation) is also possible.

All covalent immobilization methods involve at least two steps: (*1*) an *activation step*, in which the support is converted to a form that can be chemically

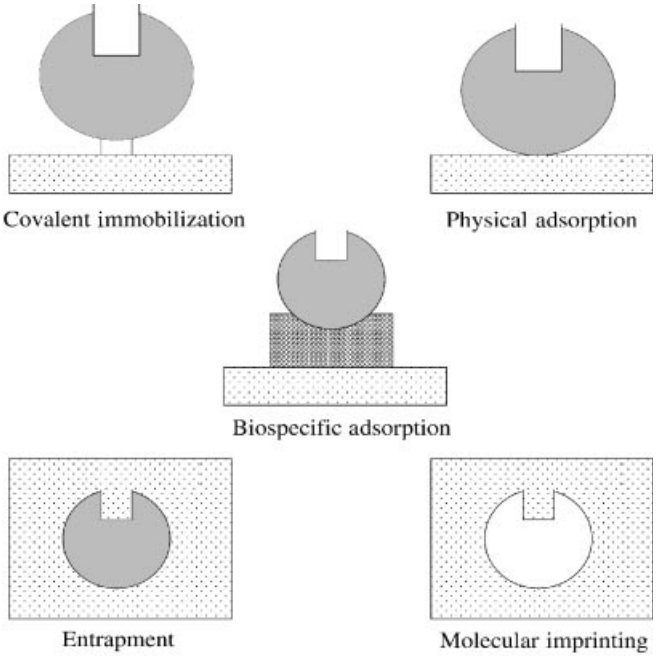

Fig. 2. General strategies that can be used to place a ligand within a column or chromatographic bed for affinity chromatography.

Table 1. **Methods for Covalent Ligand Immobilization**

Group/Compound	Immobilization technique
amines	azalactone method (for Emphaze supports)
	cyanogen bromide (CNBr) method
	N,*N*'-carbonyl diimidazole (CDI) method
	divinylsulfone method
	epoxy (bisoxirane) method
	ethyl dimethylaminopropyl carbodiimide (EDC) method
	fluoro methylpyridinium toluenesulfonate (FMP) method
	N-hydroxysuccinimide ester (NHS) method
	Schiff base (reductive amination) method
	tresyl chloride/tosyl chloride method
sulfhydryls	azalactone method (for Emphaze supports)
	divinylsulfone method
	epoxy (bisoxirane) method
	iodoacetyl/bromoacetyl methods
	maleimide method
	pyridyl disulfide method
	TNB–thiol method
	tresyl chloride/tosyl chloride method
carboxylates	ethyldimethylaminopropyl carbodiimide (EDC) method
hydroxyls	cyanuric chloride method
	divinylsulfone method
	epoxy (bisoxirane) method
aldehydes	hydrazide method
	Schiff base (reductive amination) method
nucleic acids	carbodiimide (CDI) method
	carboxymethyl method (for cellulose)
	cyanogen bromide (CNBr) method
	cyanuric chloride method
	diazonium (Azo) method
	epoxy (bisoxirane) method
	hydrazide method
	Schiff base (reductive amination) method
	ultraviolet (uv) irradiation (for cellulose)

attached to the ligand, and (*2*) a *coupling step*, in which the affinity ligand is attached to the activated support. With some techniques a third step, in which remaining activated groups are removed, is also required. The methods listed in Table 2 can be performed either in-house or by using preactivated supports available from commercial suppliers.

An alterative approach to covalent attachment is that of *physical adsorption*. In this method, the ligand is held in place through noncovalent interactions (eg, Coulombic interactions with a charged surface). This approach is relatively easy to perform but can result in a stationary phase which is unstable and uses a support that may have nonspecific interactions with sample components.

Another, more selective approach for immobilization is *biospecific adsorption*. This involves the noncovalent coupling of the ligand of interest to another secondary ligand that, in turn, is coupled to the affinity support. An example of this would be the adsorption of antibodies to an immobilized protein A or protein

G affinity column, where proteins A and G are used to capture antibodies for use in immunoaffinity chromatography.

A fourth possible approach is to use *encapsulation* or *entrapment* of the ligand within the support. One way this can be accomplished is by combining the ligand with the material used to prepare the affinity support. An example would be the entrapment of a protein by a sol–gel by preparing the sol–gel in the presence of the protein. Another example would be the trapping of large particles in the pores of the support by altering the size of the ligand particles during the entrapment process. This latter approach has been used in combination with freeze-drying to place liposomes or membrane-based particles with associated proteins into large pore-size supports such as agarose (26,27).

One final method for ligand immobilization is to make the ligand during the support's preparation. In this approach, the ligand is actually formed by binding pockets located within the support with a well-defined arrangement of functional groups. This process is known as *molecular imprinting*. In this procedure, the solute of interest is combined with a polymerization mixture that is to be used to form the affinity support. This mixture contains a cross-linking agent, monomers with functional groups that can interact with the solute, and an initiation reagent for the polymerization reaction. The resulting polymer contains pockets that were formed directly within the support and that complement the shape and arrangement of functional groups on the target solute. After the polymerization has reached completion, the imprinted solute is washed away and the now unoccupied sites can be used to isolate or retain the same solute from samples (28–30).

The correct choice of immobilization method is important in affinity chromatography since it can have a great affect on the properties of the ligand and affinity separation. For instance, if the correct immobilization procedure is not used, a decrease in ligand activity can result from multisite attachment, improper orientation and/or steric hindrance (6). *Multisite attachment* refers to the coupling of a ligand to the support through more than one functional group, which can lead to distortion and denaturation of the ligand's active region. *Improper orientation* can lead to a similar loss in ligand activity, but can be avoided by coupling the ligand through groups that are distant from its active region. *Steric hindrance* refers to the loss of ligand activity due to the presence of a nearby support or neighboring ligand molecules. Steric hindrance between neighboring ligands can be minimized by using a low ligand coverage, while steric hindrance due to the support can be reduced by adding a *spacer arm*, or tether, between the ligand and supporting material. Spacer arms are particularly important to use with small ligands that are meant to retain large solutes. Examples of spacer arms used in affinity chromatography are 6-aminocaproic acid, diaminodipropylamine, 1,6-diaminohexane, ethylenediamine, and succinic acid anhydride (24,25).

2.5. Application and Elution Conditions. Most application buffers in affinity chromatography are solvents that mimic the pH, ionic strength, and polarity experienced by the solute and ligand in their natural environment. Any cofactors or metal ions required for solute–ligand binding should also be present in this solvent. Under these conditions, the solute will probably have its highest association constant for the ligand and, therefore, its highest degree

of retention on the column. The proper choice of an application buffer can also help to minimize any nonspecific binding due to undesired sample components. For example, Coulombic interactions between solutes and the support can often be decreased by altering the ionic strength and pH of the application buffer. In addition, surfactants and blocking agents [eg, Triton X-100, Tween-20, bovine serum albumin (BSA), gelatin, etc] may be added to the buffer to prevent nonspecific retention of solutes on the support or affinity ligand.

The activity of the immobilized ligand should be considered in determining how much sample can be applied to the affinity column with each use. A rough indication of the maximum possible column binding capacity can be made by assaying the total amount of ligand present. However, a better approach is to actually measure the ligand's activity. This measurement can be done by continuously applying a known concentration of solute to the affinity column (ie, breakthrough curves) or by combining the immobilized ligand with a known excess of solute and measuring the amount of free solute that remains after binding has occurred.

With some affinity systems, it is possible to see a significant fraction of nonretained solute during the application step even when the amount of injected sample is less than the known column binding capacity. This phenomenon is known as the *split-peak effect* and is caused by the presence of slow adsorption and/or mass transfer kinetics within the column (31–33). Ways in which this effect can be minimized include reducing the flow rate used for sample injection, increasing the column size, or placing a more efficient support within the column. In some cases, changing to a different immobilization method may also help by providing a ligand with more rapid binding kinetics.

The conditions used for removal of retained solutes is another item that should be considered in the design of an effective affinity separation. Even though the use of step gradient is commonly used for solute elution in affinity chromatography (as shown in Fig. 1), it is also possible to use isocratic or linear gradient methods in certain circumstances. For example, isocratic elution can be used if the association equilibrium constant for a solute with the ligand is sufficiently small to allow the solute to pass through the column in a reasonable amount of time under the application conditions. This is often used in the case of chiral separations that are performed on affinity columns and with ligands that have been selected to have weak binding affinities. This approach is referred to as *weak affinity chromatography* or *dynamic affinity chromatography* (7,19–21).

Just as the application conditions are selected to maximize specific solute–ligand interactions, the elution conditions are chosen to promote fast or gentle removal of solute from the column. The elution buffer used in affinity chromatography can be either a solvent which produces weak solute–ligand binding (ie, a small association constant) or a solvent that decreases the extent of this binding by using a competing agent that displaces solute from the column. These two approaches are known as *nonspecific elution* and *biospecific elution* (6).

Biospecific elution is the gentler of these two elution methods and is carried out under essentially the same solvent conditions as used for sample application. This makes this approach attractive for purification work, where a high recovery of active solute is desired. Biospecific elution may be performed either by adding

an agent to the eluting solvent that competes with the ligand for solutes (ie, normal role elution) or by adding an agent that competes with the solute for ligand-binding sites (ie, reversed-role elution). In both cases, retained solutes are eventually eluted from the column by displacement and mass action. The main advantage of biospecific elution is its ability to gently remove analyte from the column. The main disadvantages of this method include its slow elution times, broad solute peaks, and frequent need to remove the competing agent from the eluted solute.

In *nonspecific elution* the column conditions are changed in a more drastic fashion to weaken the interactions between retained solutes and the immobilized ligand. This can be done by changing the pH, ionic strength, or polarity of the mobile phase or by adding denaturing or chaotropic agents. This results in an alteration in the structure of the solute or ligand, leading to a lower association constant and lower solute retention. Nonspecific elution tends to be much faster than biospecific elution in removing analytes from affinity columns, resulting in sharper peaks that in turn produce lower limits of detection and shorter analysis times. For these reasons, nonspecific elution is commonly used in analytical applications. This elution method can also be used in purifying solutes, but there is a greater risk of solute denaturation with this approach than there is with biospecific elution. Also, care must be taken in nonspecific elution to avoid conditions that are too harsh for the column.

3. Specific Types of Affinity Chromatography

3.1. Bioaffinity Chromatography. *Bioaffinity chromatography*, or *biospecific adsorption*, is the oldest and most common type of affinity chromatography (3–8). This refers to affinity methods that use a biological molecule as the affinity ligand. This was the first type of affinity chromatography developed and represents the most diverse category of this technique.

The earliest application of bioaffinity chromatography involved its use in enzyme purification (8), which has continued to be a major use of this technique. Some ligands used for this purpose are enzyme inhibitors, coenzymes, substrates, and cofactors. Examples include methods that use nucleotide mono-, di- and triphosphates for the purification of various kinases, the use of NAD for collecting dehydrogenases, the use of pyridoxal phosphate for the isolation of tyrosine and aspartate aminotransferases, and the use of RNA or DNA for the purification of polymerases and nucleases (3,4,34,35).

Lectins represent a class of general ligands that are common in bioaffinity chromatography. This field is sometimes referred to as *lectin affinity chromatography*. The lectins are nonimmune system proteins that have the ability to recognize and bind certain types of carbohydrate residues (36). Two lectins often used in affinity chromatography are concanavalin A, which binds to α-D-mannose and α-D-glucose residues, and wheat germ agglutinin, which binds to D-*N*-acetylglucosamines. Other lectins that can be employed are jackalin and lectins found in peas, peanuts, or soybeans. These ligands are used in the separation of many carbohydrate-containing compounds, such as polysaccharides, glycoproteins (eg, immunoglobulins or cell membrane proteins) and glycolipids (3–6,24).

Another useful class of bioaffinity ligands are bacterial cell wall proteins, such as protein A from *Staphylococcus aureus* and protein G from group G *streptococci* (6,24,37–39). These ligands have the ability to bind to the constant region of many types of immunoglobulins. This makes them useful in antibody purification. Protein A and protein G have their strongest binding to immunoglobulins at or near neutral pH but readily dissociate from these solutes when placed into a lower pH buffer. These two ligands differ in their ability to bind to antibodies from different species and classes. However, recombinant proteins that blend the activities of these compounds are also available.

Nucleic acids and polynucleotides can act as either general or specific ligands in bioaffinity chromatography. For instance, as high-specificity ligands they can be used to purify DNA/RNA-binding enzymes and proteins or to isolate nucleic acids that contain a sequence that is complementary to the ligand (40,41). As a group-specific ligand, an immobilized nucleic acid can be used to purify solutes that share a common nucleotide sequence. An example is the use of immobilized oligo(dT) for the isolation of nucleic acids containing poly(A) sequences.

3.2. Immunoaffinity Chromatography. The most common type of bioaffinity chromatography is that which uses an antibody or antibody-related agent as the affinity ligand. This set of methods is often referred to as *immunoaffinity chromatography (IAC)* (42–44). The high selectivity of antibody–antigen interactions and the ability to produce antibodies against a wide range of solutes has made immunoaffinity chromatography a popular tool for biological purification and analysis. Examples include methods developed for the isolation of antibodies, hormones, peptides, enzymes, recombinant proteins, receptors, viruses, and subcellular components. The strong binding constants of many antibodies requires non-specific elution for most immunoaffinity columns. However, isocratic elution methods can also be used with low affinity antibody systems (44).

Immunoaffinity chromatography was first reported by Campbell and coworkers in 1951, who used an antigen immobilized to *p*-aminobenzyl cellulose for antibody purification (45). Many current applications are still based on the use of low performance supports, particularly agarose. However, during the past decade much work has also been performed using derivatized silica, glass and perfusion media in immunoaffinity columns. The use of these supports along with an antibody or antigen ligand is referred to as *high performance immunoaffinity chromatography (HPIAC)* (43,44).

Several unique applications of immunoaffinity chromatography have appeared in recent years. One of these involves the use of affinity columns to perform immunoassays, giving rise to a technique known as a *chromatographic immunoassay* or *flow-injection immunoanalysis* (44,46). An example of a chromatographic immunoassay is given in Figure 3. Various immunoassay formats have been performed with affinity columns, including direct detection assays, immunometric assays, and competitive binding immunoassays. These assays can either be performed as stand-alone methods or as a means for detecting substances after they have been resolved by a separate chromatographic column. In this latter case, these chromatographic-based immunoassays are also referred to as *postcolumn immunodetection* (44).

Another growing use of immunoaffinity chromatography has been as a means for sample isolation and pretreatment prior to analysis by other analytical

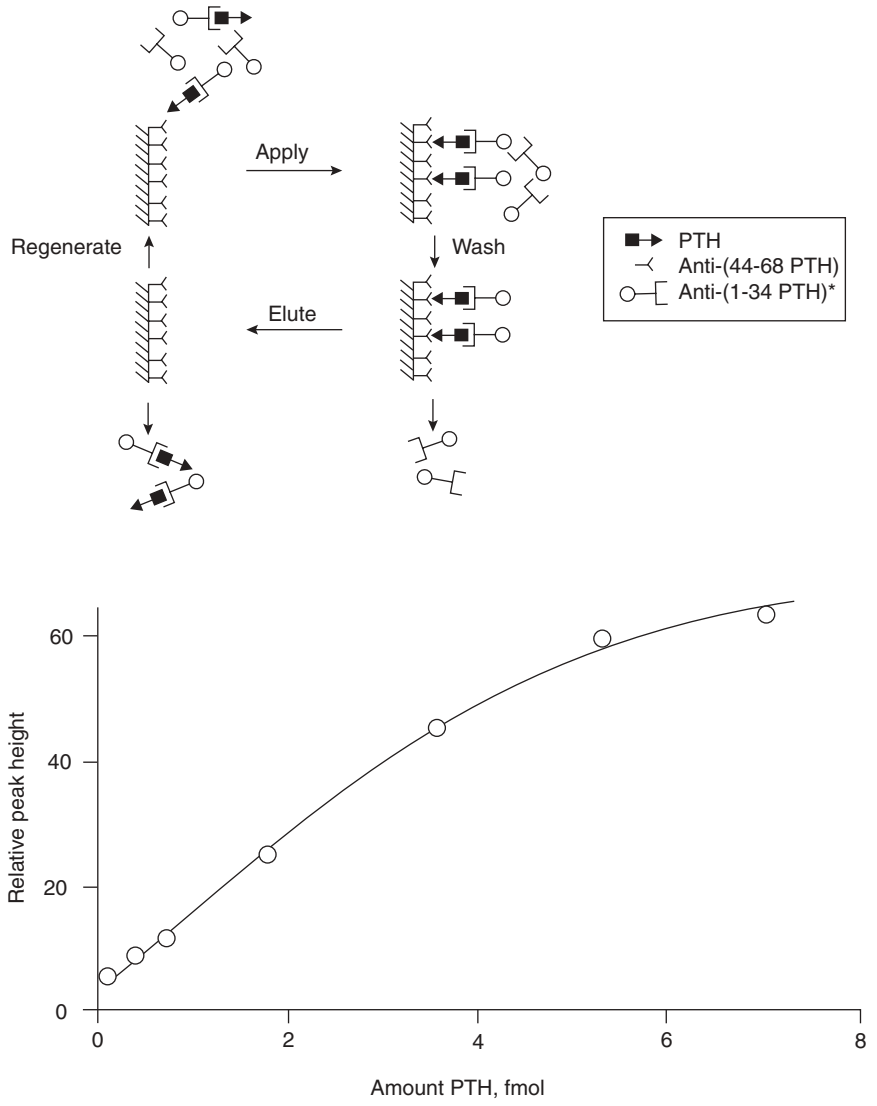

Fig. 3. An example of a chromatographic immunoassay. This particular example shows a sandwich immunoassay for measuring the hormone parathyrin (PTH) in plasma samples. The affinity column contained anti-(44-68 PTH) antibodies, while labeled anti-(1-34 PTH) antibodies were added to the sample for placing a detectable tag on the analyte. Reproduced with permission from Ref. 47.

methods, which is often referred to as *immunoextraction*. Immunoextraction can be performed as an off-line technique, in which the isolated sample components are collected from the immunoaffinity column and later transferred to a second method for measurement. But immunoextraction can also be used directly (or on-line) with some analytical methods. For instance, antibody based columns have been coupled directly with systems used in reversed-phase liquid chromatography, ion-exchange chromatography, size-exclusion chromatography, gas

chromatography, capillary electrophoresis, and liquid chromatography–mass spectrometry. This produces multidimensional methods that combine the selectivity of immunoaffinity chromatography with the ability to separate structurally related compounds (as might be performed by reversed-phase liquid chromatography) or to provide structural information (as is obtained by mass spectrometry) (44).

3.3. Dye-Ligand and Biomimetic Affinity Chromatography. Two other, related categories of affinity chromatography are the techniques of *dye-ligand affinity chromatography* and *biomimetic affinity chromatography* (48). In dye-ligand affinity chromatography, a synthetic substance like a triazine or triphenylmethane dye is used as the immobilized ligand. This approach was first reported in 1971, when Staal and co-workers used a column containing Blue Dextran to purify the enzyme pyruvate kinase (49).

Many dyes can be used as ligands in affinity chromatography. Examples are Cibacron Blue 3GA, Procion Red HE-3B, Procion Rubine MX-B, Procion Yellow H-A, and Turquoise MX-G. The structure of one of these dyes is given in Figure 4. In each case, a portion of the dye's structure interacts with a target protein by mimicking the binding of a native solute to that site. As an example, the dye Cibacron Blue 3GA binds to NAD(P)H:quinone reductase by mimicking the AMP portion of NADP+ and interacting with its associated site on the enzyme.

The selectivity of dye–ligand affinity chromatography and its use of synthetic ligands in place of natural ones has made this method an extremely popular tool for enzyme and protein purification. Well over 500 compounds have been isolated by this technique (3), including kinases, dehydrogenases, restriction endonucleases, polynucleotides, synthetases, Coenzyme A (CoA)-dependent enzymes, hydroxylases, glycolytic enzymes, phosphodiesterases, decarboxylases, clotting factors, serum lipoproteins, interferons, transferrin, and serum albumin.

Dye–ligand affinity chromatography is actually a subset of the more general technique known as biomimetic affinity chromatography. As the name of this latter method implies, it makes use of any ligand that acts as a mimic for a natural compound. This includes the use of synthetic dyes as ligands, as well as other types of agents. For instance, combinatorial chemistry and computer modeling have been used with peptide libraries to design biomimetic ligands for enzymes and other target compounds. Phage display libraries, aptamer libraries, and ribosome display have also been used for this purpose (50–52).

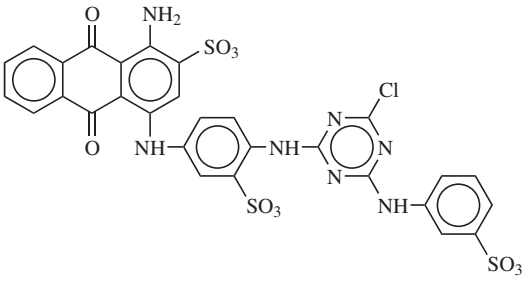

Fig. 4. Structure of Cibacron Blue G3A, a common ligand used in dye-ligand affinity chromatography.

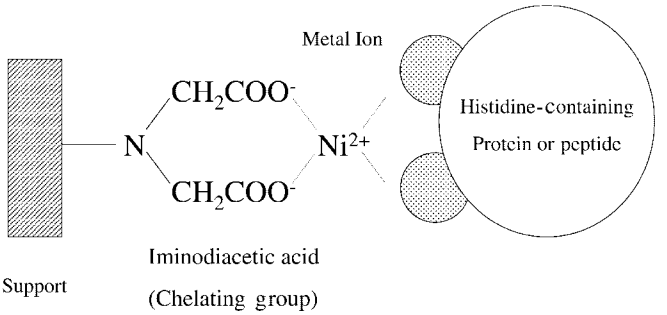

Fig. 5. Structure of Ni–IDA, a metal–chelate complex that is often employed in immobilized metal-ion affinity chromatography (IMAC) for the retention of histidine-containing proteins and peptides.

3.4. Immobilized Metal-Ion Affinity Chromatography. Another type of affinity chromatography that uses a ligand of nonbiological origin is *immobilized metal-ion affinity chromatography (IMAC)*. This method is also known as *metal chelate chromatography* or *metal ion interaction chromatography*. In this approach, the affinity ligand is a metal ion complexed with an immobilized chelating agent (53,54). One common example is the use of Ni^{2+} ions that are complexed to a support containing iminodiacetic acid (IDA) as the chelating agent (see Fig. 5), although other types of metal ions or chelating groups can also be employed.

This type of affinity chromatography was first described by J. Porath and co-workers in 1975 (55). It was initially used to separate proteins and peptides containing electron donor groups, such as histidine, tryptophan, or cysteine residues, which can interact with the immobilized metal chelate. In more recent years, IMAC has been used for other purposes, such as the isolation of recombinant histidine-tagged proteins, studies of protein surface topography, and the isolation of phosphorylated proteins for proteomic studies.

3.5. Boronate Affinity Chromatography. Boronic acid and its derivatives are another class of synthetic substances that have been used as affinity ligands. This makes use of the ability of such substances to form covalent bonds with compounds that contain cis-diol groups in their structure (see Fig. 6). Such a property has made boronate ligands useful for the purification and analysis of many compounds which contain sugar residues, such as polysaccharides, glycoproteins, ribonucleic acids, and catecholamines (56–58). One

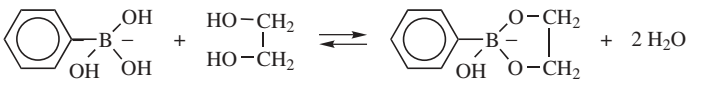

Fig. 6. Reaction of boronate with a cis-diol, illustrating the mechanism of retention in boronate affinity chromatography.

important clinical application of immobilized boronate ligands is their use in the analysis of glycosylated hemoglobin in diabetic patients (13).

3.6. Analytical Affinity Chromatography. Besides its use in separating molecules, affinity chromatography can also be employed as a tool for studying solute–ligand interactions. This particular application of affinity chromatography is called *analytical affinity chromatography* or *quantitative affinity chromatography* (17,59,60). Using this technique, information can be acquired regarding the stoichiometry, thermodynamics, and kinetics of biological interactions.

Two experimental formats that are used in this field are *zonal elution* and *frontal analysis*. Both of these are illustrated in Figure 7. Zonal elution involves

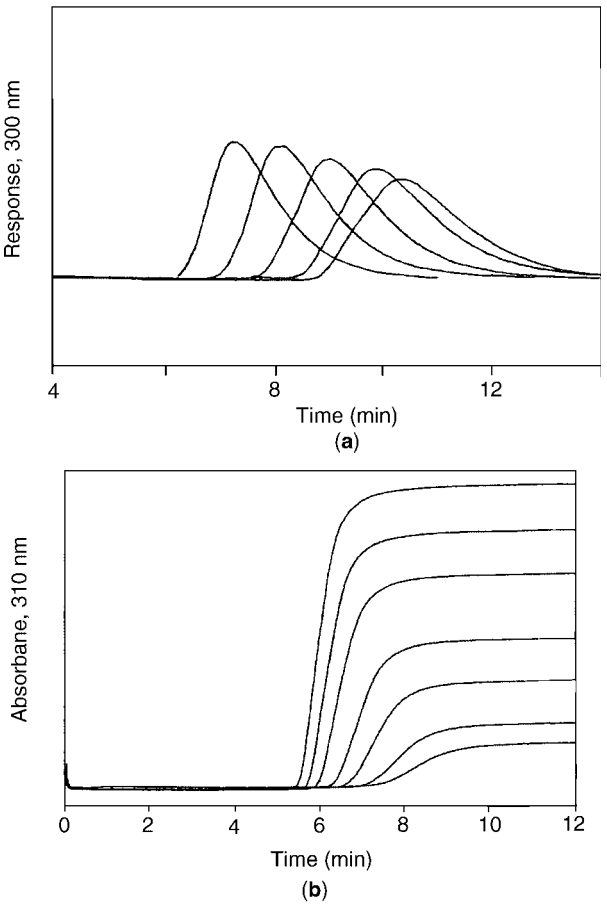

Fig. 7. Examples of studies based on (**a**) zonal elution and (**b**) frontal analysis for the examination of solute–ligand interactions by analytical affinity chromatography. The top figure shows peaks obtained for small injections of warfarin on an immobilized human serum albumin column in the presence of increasing (from right-to-left) amounts of a competing agent in the mobile phase. The bottom figure shows frontal analysis curves obtained on an immobilized human serum albumin column for increasing (from bottom-to-top) concentrations of warfarin in the mobile phase. Reproduced with permission from Ref. 60.

the injection of a small amount of solute onto an affinity column in the presence of a mobile phase that contains a known concentration of competing agent. The equilibrium constants for binding of the ligand with the solute (and competing agent) can then be obtained by examining how the solute's retention changes with competing agent concentration. This technique was first used to study biological interactions in 1973–1974 (61,62). Since that time it has been used to examine a number of systems, such as enzyme–inhibitor binding, protein–protein interactions, and drug–protein binding (17,18,59,60).

Frontal-analysis is performed by continuously applying a known concentration of solute to an affinity column at a fixed flow-rate. The moles of analyte required to reach the mean point of the resulting breakthrough curve is then measured and used to determine the equilibrium constant for solute-ligand binding. This method was used first in 1975 to examine the interactions of trypsin with various peptide ligands (63). One advantage of this approach over zonal elution is that it simultaneously provides information on both the equilibrium constants and number of active sites involved in analyte-ligand binding. The main disadvantage of this method is the need for a larger quantity of solute than is required by zonal elution.

Information on the kinetics of solute–ligand interactions can also be obtained using affinity chromatography. A number of methods have been developed for this, including techniques based on band-broadening measurements, the split-peak effect, and peak decay analysis (17,31,60). These methods are generally more difficult to perform than equilibrium constant measurements but represent a powerful means of examining the rates of biological interactions.

Recently, a new approach for such measurements has become possible through the availability of flow-through biosensors. *Surface plasmon resonance* is one detection scheme that has been used for this purpose. In these devices, an affinity ligand is immobilized or adsorbed at the sensor's surface, while the solute of interest is applied to the surface in a flow stream of the desired buffer. Changes in the optical properties of this surface are then monitored as the solute binds to the ligand. In this approach, the rate of solute–ligand association is measured as the solute-containing buffer is applied to the system, and rate of solute release is examined when only buffer is applied to wash the surface. These results are then analyzed to determine the association and dissociation rate constants for the system, which are then combined to give the equilibrium constant for solute–ligand binding (64,65).

3.7. Miscellaneous Methods. Other methods that are related to affinity chromatography include *hydrophobic interaction chromatography (HIC)* and *thiophilic adsorption*. Hydrophobic interaction chromatography is based on the interactions of proteins, peptides and nucleic acids with short nonpolar chains on a support. This was first described in 1972 (66,67) following work that examined the role of spacer arms on the nonspecific adsorption of affinity columns. It is commonly used as a method for the purification of proteins and peptides.

Thiophilic adsorption is also known as *covalent chromatography* or *chemisorption chromatography*. This makes use of immobilized thiol groups for solute retention. Applications of this method include the analysis of sulfhydryl-containing peptides or proteins and mercurated polynucleotides (68,69).

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