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Review

Pharmaceutical and biomedical applications of affinity chromatography: Recent trends and developments

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ABSTRACT

Affinity chromatography is a separation technique that has become increasingly important in work with biological samples and pharmaceutical agents. This method is based on the use of a biologically related agent as a stationary phase to selectively retain analytes or to study biological interactions. This review discusses the basic principles behind affinity chromatography and examines recent developments that have occurred in the use of this method for biomedical and pharmaceutical analysis. Techniques based on traditional affinity supports are discussed, but an emphasis is placed on methods in which affinity columns are used as part of HPLC systems or in combination with other analytical methods. General formats for affinity chromatography that are considered include step elution schemes, weak affinity chromatography, affinity extraction and affinity depletion. Specific separation techniques that are examined include lectin affinity chromatography, boronate affinity chromatography, immunoaffinity chromatography, and immobilized metal ion affinity chromatography. Approaches for the study of biological interactions by affinity chromatography are also presented, such as the measurement of equilibrium constants, rate constants, or competition and displacement effects. In addition, related developments in the use of immobilized enzyme reactors, molecularly imprinted polymers, dye ligands and aptamers are briefly considered.

Contents

1. Introduction

Liquid chromatography and high performance liquid chromatography have been extensively used for decades in clinical and pharmaceutical laboratories. Examples of liquid chromatographic methods that are commonly used in these fields include reversed-phase, ion-exchange, size-exclusion and normal-phase chromatography [1,2]. However, another liquid chromatographic technique that has become increasingly important in work with biological samples and pharmaceutical agents is affinity chromatography [3]. This review will discuss the basic principles behind affinity chromatography and will examine recent developments that have occurred in the use of this method for biomedical and pharmaceutical analysis.

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2. Overview of affinity chromatography

Affinity chromatography can be defined as a type of liquid chromatography that uses a biologically related agent, or "affinity ligand", as a stationary phase to selectively retain analytes or to study biological interactions [4–6]. The affinity ligand can consist of a wide variety of binding agents, ranging from a protein or enzyme to an antibody, an antigen, a sequence of DNA or RNA, a biomimetic dye, an enzyme substrate or inhibitor, or a low mass compound (e.g., a drug or hormone). The affinity ligand is immobilized within a column and used to selectively bind a given target or group of targets within a sample. Because of the highly selective nature of many affinity ligands, the result is a column that can be used to isolate, measure, or study specific targets even when they are present in complex biological samples [4–8].

The immobilized ligand is an important factor that determines the success of an affinity chromatographic method. Most affinity ligands that are used in this technique are obtained from a biological source, such as antibodies, enzymes, transport proteins, and carbohydrate-binding proteins [3–8]. However, affinity chromatography can also use synthetic ligands such as metal ion chelates, boronates and biomimetic dyes [3–5,7]. The type of ligand that is employed in this method can be used to divide affinity chromatography into several categories. This review will discuss developments in many of these categories, including lectin affinity chromatography, boronate affinity chromatography, immunoaffinity chromatography, and immobilized metal ion affinity chromatography [3,5,7].

Another critical factor in the design and use of an affinity column is the type of support to which the ligand is immobilized. Many methods that use affinity chromatography for only sample pretreatment or target isolation employ a carbohydrate support such as agarose or cellulose. This type of material can be easily modified for ligand attachment, can be used with a wide range of elution conditions, and has low non-specific binding for many biological compounds. However, the limited mechanical stability and relatively low efficiency of many carbohydrate-based materials means that they tend to work best for off-line methods and for columns that will be operated at only low pressures and flow rates [5,9,10].

An alternative to traditional carbohydrate-based supports is to place the affinity ligand onto supports that consist of HPLC media like silica particles or, more recently, monolithic materials [5,9–13]. This alternative approach is known as high performance affinity chromatography (HPAC), or high performance liquid affinity chromatography (HPLAC). The use of affinity ligands with monolithic supports is also referred to as affinity monolith chromatography (see example in Fig. 1) [12–17]. The more rigid and efficient particles or synthetic polymers that are employed in HPAC are often capable of withstanding much higher flow rates and pressures than traditional carbohydrate-based supports and possess better mass transfer properties than these other materials. The result is that HPAC columns can be used on-line with other HPLC columns or analytical methods while providing an increase in speed, precision, and ease of automation versus traditional affinity supports [5,9–13]. This review will discuss recent examples in which both traditional and high performance affinity columns have been used in biomedical and pharmaceutical analysis. However, the emphasis will be placed on methods in which affinity columns are used as part of HPLC systems or in combination with other analytical methods (e.g., mass spectrometry).

3. General formats for affinity chromatography

The most common scheme for performing a separation in traditional affinity chromatography and HPAC is shown in Fig. 2 [5]. First, a sample is injected onto the affinity column under conditions that allow strong binding by the target or analyte of interest with the immobilized ligand. These application conditions typically involve the use of an aqueous buffer that has a pH and ionic strength that mimic the native environment of the ligand and its target. Compounds in the sample that have little or no interaction with the ligand will elute from the column during this step, giving a non-retained peak. The next step utilizes an elution buffer to dissociate the target from the affinity ligand. This elution step often requires changing the mobile phase composition to promote target elution, as can be accomplished by altering the pH or by adding a competing agent to displace the target from the column. During this elution step, the released target can be collected for later analysis or use. If an HPLC support is used in the affinity column, it may also be possible during this step to monitor the eluting target directly by an on-line method. Both the on-line and off-line approaches can be combined with detection methods such as absorbance, fluorescence or mass spectrometry [18]. After the target has been eluted, the column can then be regenerated prior to the next sample application by passing through the original application buffer.

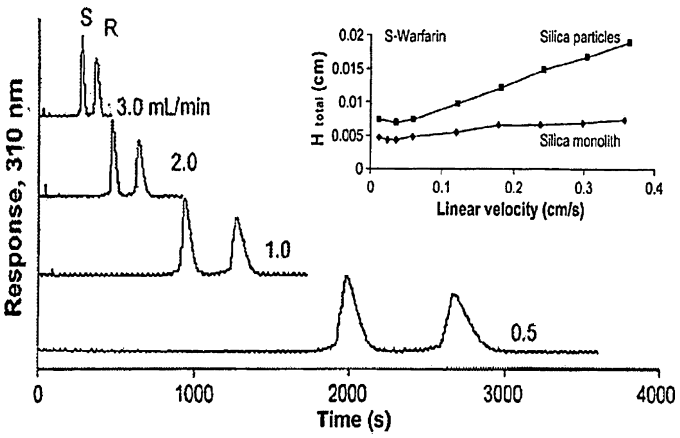

Fig. 1. Separation of *R*- and *S*-warfarin on a 10 cm × 4.6 mm I.D. silica monolith column containing immobilized α_1-acid glycoprotein. These separations were obtained at room temperature using pH 7.0, 0.067 M phosphate buffer as the mobile phase. The inset compares the total plate height (H_{total}) that was measured for *S*-warfarin on the AGP silica monolith column to the total plate height that was determined for the same target and affinity ligand when using 7 μm silica particles as the support. Adapted with permission from Ref. [17].

The format that is shown in Fig. 2 for target capture and elution is sometimes known as the "on/off" or step elution mode of affinity chromatography [5,19]. This approach has been widely employed for compound isolation and sample pretreatment in biomedical and pharmaceutical analysis because of its simplicity, flexibility, selectivity, and ease of use [5–8,18]. This format can also be utilized with many types of affinity ligands, such as lectins, boronates, and antibodies [3]. In addition, it is relatively easy to automate this format when using affinity columns that are appropriate for work in HPAC or as part of HPLC systems; it is even possible to use this mode in some cases for the direct detection of analytes [18]. Direct detection in the step elution mode is commonly performed using on-line absorbance or fluorescence detectors, but detection based on mass spectrometry or a post column reactor is also possible [18–22].

Methods that employ isocratic elution can be developed in affinity chromatography if the retained target has weak or moderate affinity for the immobilized ligand. This situation allows a single mobile phase to be used as both the sample application and elution buffer. The result is an approach known as weak affinity chromatography (WAC) or dynamic affinity chromatography [19,23–27]. WAC has been used in several pharmaceutical and biomedical applications. One common example is the use of isocratic elution for chiral

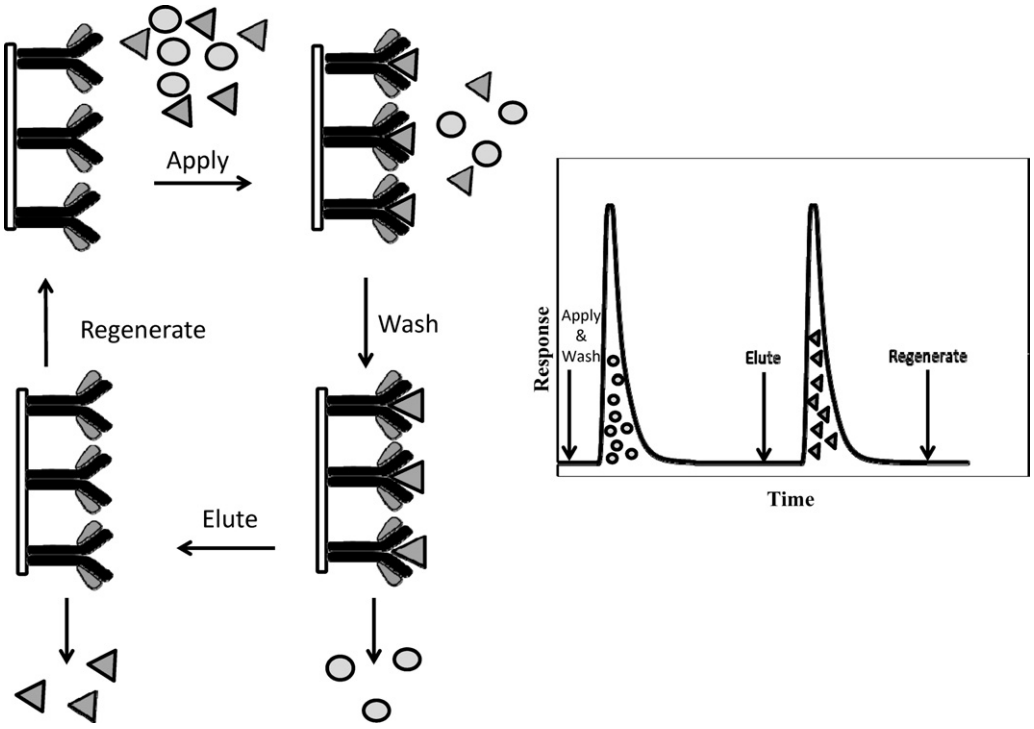

Fig. 2. The on/off elution format for affinity chromatography and a general chromatogram for this format.

separations based on affinity columns that contain immobilized enzymes such as trypsin, α-chymotrypsin and cellobiohydrolase I or serum proteins such as human serum albumin (HSA), bovine serum albumin (BSA) and α_1-acid glycoprotein (AGP) [19,28,29]. An example of such a separation is provided in Fig. 1. In addition, WAC has been explored as a tool for drug screening assays. For instance, this method has been used with trypsin and thrombin columns plus HPLC and mass spectrometry for the fragment-based drug discovery/design screening of amidines, or arginine mimetic ligands [30]. This screening approach has also been utilized in the evaluation of ligands or inhibitors of cholera toxin [31,32]. The combined use of affinity microcolumns with HSA is another option that has been recently explored as a means for the high-throughput screening of drug–protein interactions [33,34].

Another format that is often utilized for affinity chromatography in biomedical analysis is affinity extraction. In this approach, a specific analyte or group of analytes is removed or extracted from the sample by a column that contains an immobilized affinity ligand. The retained targets are then eluted and passed to a second method for analysis. Antibodies are often placed in columns for use in affinity extraction, giving a method known as an immunoextraction [18,35]. However, other ligands (e.g., boronates or lectins) can also be used in this format [36,37]. When it is part of an HPAC system, affinity extraction can be utilized with some ligands to remove targets from samples in less than a few seconds (see Fig. 3). This feature has been used to measure the free fractions of drugs and hormones in serum or drug/protein mixtures with columns that contain immobilized antibodies or HSA as the affinity ligand [38–41].

A closely related method to affinity extraction is affinity depletion. In this technique an affinity column is used to remove abundant compounds from a complex sample prior to analysis of the non-retained sample components by a second method. This format also frequently uses an antibody as the affinity ligand [35]. A typical application of this method is in proteomics, in which antibody columns have been used to remove highly abundant proteins such as HSA and immunoglobulin G (IgG) from serum prior to the analysis of the lower abundance proteins in such a sample [37,42].

4. Lectin affinity chromatography

One particular technique that has seen growing interest over the last few years is lectin affinity chromatography. This is a type of affinity chromatography in which an immobilized lectin is used as the stationary phase. Lectins are non-immune system proteins that have the ability to recognize and bind certain types of carbohydrate residues [43,44]. These carbohydrate-binding proteins are found in plants and animals, as well as in microorganisms [44–48]. Due to their ability to bind to carbohydrate residues, lectins have been widely used to isolate and identify glycoproteins, glycopeptides, glycolipids and oligosaccharides [43–45]. The most common types of lectins that are used in affinity chromatography are concanavalin A (Con A), wheat germ agglutinin (WGA), and jacalin [8,43,49]. Con A specifically binds to targets that contain α-D-mannose or α-D-glucose residues. WGA binds to D-*N*-acetylglucosamine residues, and jacalin binds to galactose or mannose residues [43–45,49].

Lectins have often been used for sample pretreatment and target purification in applications where agarose is employed as the support [8,43]. For example, in one recent study galactrox (i.e., a lectin that binds to galactose residues and that is found in snake venom from *Bothrops atrox*) was purified using a lactosyl affinity column that made use of an agarose support [50]. Small columns containing *Sambucus nigra* agglutinin (SNA) on agarose have been used for glycoprotein enrichment and to capture sialylated glycopeptides prior to glycosylation site mapping by mass spectrometry [51].

Other reports have examined the use of lectin supports along with other HPLC methods and/or mass spectrometry. In one study, glycoproteins were isolated from human serum using lectin affinity columns, followed by tryptic digestion of these glycoproteins and sequential capture of their acidic peptides by using a strong anion-exchange column and a copper-immobilized metal ion affinity column [52]. Other reports have placed several lectins (i.e.,

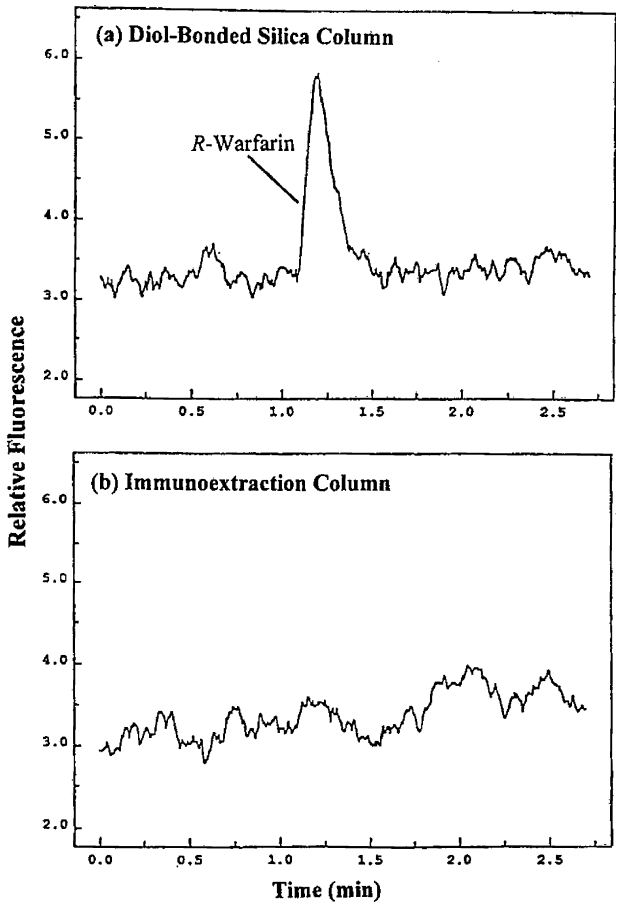

Fig. 3. (a) Injection of *R*-warfarin onto an inert control column and (b) affinity extraction of *R*-warfarin by an immunoaffinity microcolumn of the same size but that contained anti-warfarin antibodies. The contact time between the injected sample and the immunoaffinity layer in (b) was only 60 ms.
Adapted with permission from Ref. [38].

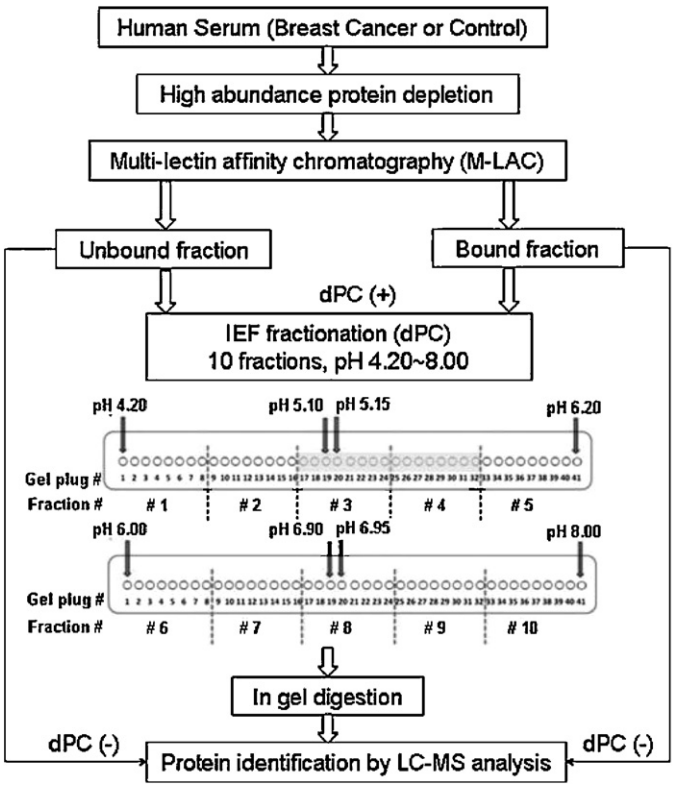

Fig. 4. Protocol used with multiple lectin affinity chromatography (M-LAC) for the study of glycoproteins in serum. Four techniques were used in this separation: immunodepletion, glycoprotein fractionation, isoelectric focusing (IEF) fractionation using a digital proteome chip (dPC), and reversed-phase liquid chromatography in conjunction with mass spectrometry (LC–MS).
Reproduced with permission from Ref. [55].

Con A, WGA and jacalin) in a single agarose column for use in a technique called multilectin affinity chromatography (M-LAC). For instance, one study used immobilized antibodies and an affinity depletion method to remove highly abundant proteins such as HSA and IgG from serum samples, followed by passage of the non-retained fraction of this support through an M-LAC column to separate glycoproteins from the other remaining proteins in the sample. These protein fractions were later digested and analyzed by liquid chromatography/mass spectrometry (LC–MS) to locate potential biomarkers for a particular disease [53].

Additional supports have been used with lectin columns for biomedical analysis. As an example, M-LAC columns have been prepared using a coated polystyrene–divinylbenzene support. This lectin column was then used along with an antibody column as part of an automated HPLC platform [37]. The same affinity methodology has been combined with isoelectric focusing and a digital proteome chip for proteomic studies. In each case, LC–MS was used to detect changes in the various protein fractions that were obtained from healthy individuals versus those with a given disease state (see scheme in Fig. 4) [54,55].

Sets of lectin columns have been employed for the isolation and purification of glycoproteins and glycoconjugates in an approach known as serial lectin affinity chromatography (S-LAC). This method has been used with small agarose columns containing Con A, WGA or SNA and a hydrophilic interaction column to extract and enrich glycoproteins and glycopeptides prior to their analysis by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) and liquid chromatography/tandem mass spectrometry (LC–MS/MS) [56].

5. Boronate affinity chromatography

Boronate affinity chromatography is a separation method that uses a boronate as the affinity ligand [57]. At a basic pH, most boronate derivatives will bind to targets that contain *cis*-diol groups, such as are present in many carbohydrate-containing compounds. Boronate columns have been used for several decades in clinical laboratories for the quantitation of glycated hemoglobin as an assessment of long-term diabetes management [58–61]. Methods employing this technique have used both agarose and supports that can be employed in HPAC methods [59–66]. Similar methods have been utilized to look at other types of glycoproteins, such as glycated albumin and some apolipoproteins [67,68].

Concerning the analysis of glycated hemoglobin, hemoglobin A1c (HbA1c) is the major component of glycated hemoglobin found in human blood [69,70]. A recent study evaluated the influence of different hemoglobin variants on the HbA1c values that are measured when using ion-exchange HPLC or boronate affinity-based HPLC. Of particular interest was the influence of hemoglobin variants Hb G-Taichung, Hb E, and Hb H on the measurement of HbA1c [71]. It was found that HbA1c measurements may be affected by the presence of Hb E and Hb H but not Hb G-Taichung. Boronate chromatography has also been combined with other analytical methods for HbA1c measurements. One report utilized a boronate affinity column and a cation-exchange column that were coupled with isotope dilution analysis and inductively coupled plasma mass

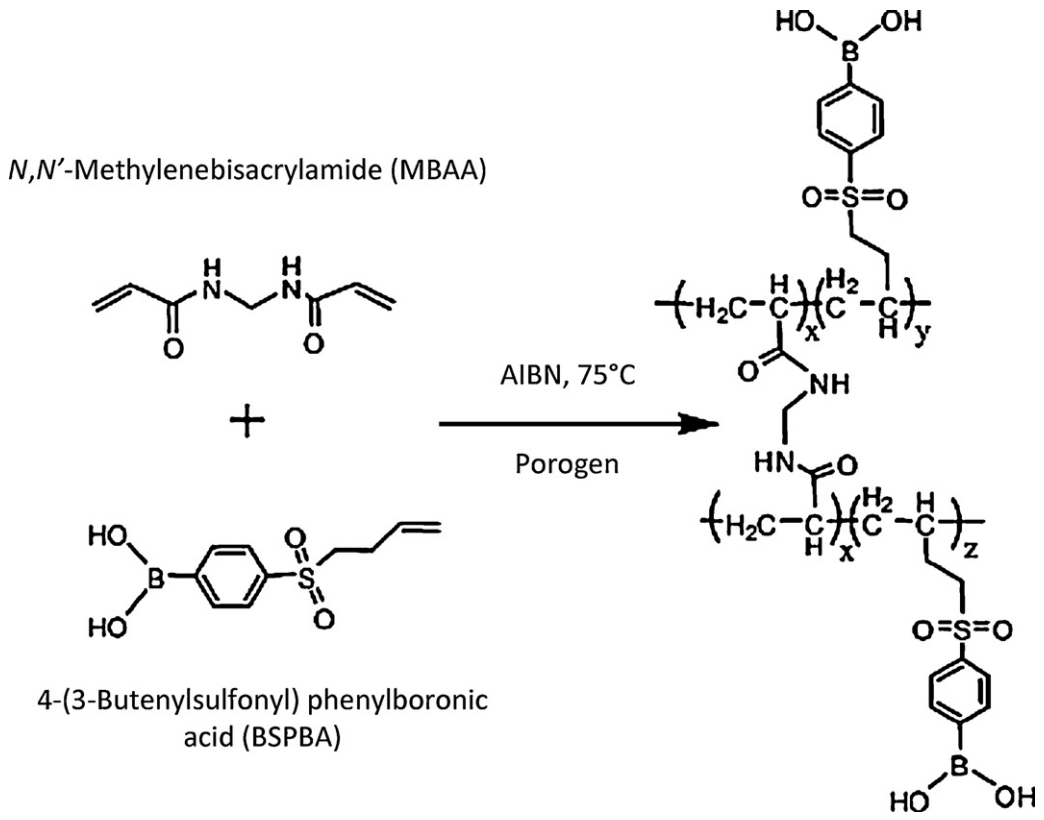

Fig. 5. Synthesis of a monolith containing a boronate, as based on in situ free radical polymerization using 4-(3-butenylsulfonyl) phenylboronic acid and *N*,*N*′-methylenebisacrylamide. The azobisisobutyronitrile (AIBN) was used to initiate polymerization. The porogen was a mixture of two solvents that was used to promote the formation of pores in the monolith during its formation.
Adapted with permission from Ref. [73].

spectrometry for the quantitation of HbA1c in diabetic patients [36].

Several recent studies have considered new ways of preparing boronate affinity columns for work with biological samples. A capillary boronate affinity monolith was prepared at a neutral pH to capture glycoproteins like horseradish peroxidase and lactoferrin [72]. The synthesis of this monolith was achieved by the in situ free radical polymerization of 4-vinylphenylboronic acid (VPBA) in the presence of *N*,*N*′-methylenebisacrylamide (MBAA) as a hydrophilic cross-linking agent. By substituting VPBA with 4-(3-butenylsulfonyl) phenylboronic acid (BSPBA), as shown in Fig. 5, it was possible to obtain strong retention of the glycoconjugates at a neutral pH [73]. Another study used "click chemistry" to create a new boronate ligand. In this work, 3-(prop-2-ynyloxycarbonylamino) phenylboronic acid was coupled to azide-activated agarose using a Cu(I)-catalyzed 1,3-dipolar cycloaddition reaction to create a material that could be used for the separation of glycoproteins such as ovalbumin and RNase B [74].

A number of hybrid affinity methods using boronates have been described for the analysis of biomedical samples. One report combined the use of boronates and lectins in a single column to separate glycoproteins. This technique, referred to as boronic acid-lectin affinity chromatography, was created by combining an agarose support that contained immobilized Con A and WGA with an agarose support that contained immobilized *m*-aminophenyl boronic acid. This mixed-bed column was then used to separate a set of glycoproteins that included RNase B, glutathione peroxidase, and myoglobin [75]. Another report used a silica support and immobilized *m*-aminophenyl boronic acid along with ion-pair chromatography and flow injection analysis to simultaneously determine several catecholamines in urine samples [76].

6. Immunoaffinity chromatography

The use of affinity columns that contain immobilized antibodies or related agents is a technique known as immunoaffinity chromatography [18,77]. Many immunoaffinity methods have been developed in the past for the isolation and purification of hormones, enzymes, peptides, viruses, and other biologically relevant substances [77,78]. Like other affinity ligands, antibodies can be used on traditional affinity supports like agarose or attached to supports that can be used in HPAC, such as silica or monolithic materials [11–13,18,77]. The combination of immunoaffinity chromatography and these latter supports produces a method known as high performance immunoaffinity chromatography (HPIAC) [18,77].

There are several elution formats that can be utilized in immunoaffinity chromatography and HPIAC. The most common approach involves the step elution mode that is illustrated in Fig. 2 [18,77]. Elution of the retained target in this case is often accomplished by using a change in the pH of the mobile phase, although the addition of a chaotropic agent or organic modifier can also be employed [77]. Step elution is frequently used during the isolation of target compounds by immunoaffinity columns prior to the analysis of these targets by another method [18,35,37,42,77]. In addition, this approach can be used for the direct detection of targets if they are present at sufficient levels in the sample. Analytes that have been measured by this approach have included acetylcholinesterase, benzidine, HSA, IgG, insulin, and transferrin [35,77]. Detection in this situation may be carried out by using many of the methods that were discussed earlier (e.g., absorbance, fluorescence, and mass spectrometry). Other detection schemes that have been employed are pulsed amperometry and radiometric detection [18,35,77].

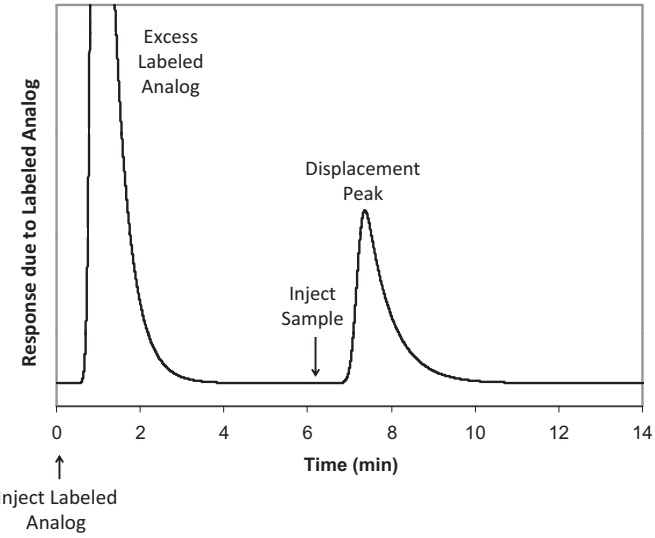

Fig. 6. An example of a displacement immunoassay. Adapted with permission from Ref. [40].

Another format that can be used with immunoaffinity chromatography and HPIAC is a flow-based immunoassay, or "chromatographic immunoassay" [79,80]. These methods can be classified as either competitive or non-competitive immunoassays. Competitive binding immunoassays typically involve competition between the target analyte and a labeled analyte analog for a limited number of binding sites in an antibody column. Either the retained or non-retained fraction of the labeled analog is then detected, such as through the use of fluorescence, absorbance, chemiluminescence, or electrochemical formats [18,80]. The resulting signal provides an indirect measure of the amount of target that was in the original sample [79,80].

There are several ways to perform a competitive binding immunoassay by immunoaffinity chromatography. Three possible approaches are the simultaneous injection mode, the sequential injection mode, and a displacement immunoassay [35,79–82]. The simultaneous injection method involves application of the labeled analog and the target at the same time onto a column that contains a limited amount of immobilized antibodies for these agents [82]. The sequential injection format involves application of the target followed by injection of the labeled analog [81,82]. The displacement format (see Fig. 6) uses the adsorption of an excess of the labeled analog onto a column that contains the immobilized antibody; a sample of the target is later applied to the column and displaces some of the labeled analog [79,80]. Simultaneous injection immunoassays have been utilized in the analysis of adrenocorticotropic hormone, caffeine, cortisol, digoxin, gentamicin, HSA, IgG, methotrexate, testosterone, and theophylline [35,80]. Sequential injection immunoassays have been used to measure α-amylase, digoxin, HSA, and IgG [35]. Displacement immunoassays have been employed for the analysis of benzoylecgonine, cocaine, cortisol, phenytoin, and thyroxine [18,35,39,40,79,80].

There are two common types of non-competitive immunoassays: the one-site immunometric assay and the two-site immunometric assay [79,80]. A one-site immunometric assay is performed by incubating the sample with a known excess of labeled antibodies that bind to the target. This mixture is then injected onto a column that contains an immobilized analyte analog. The immobilized analog binds to any antibodies that are not already bound to the target, while antibodies that are bound to the target elute as a non-retained fraction. Either the amount of non-retained or retained antibodies can then be measured to determine the amount of the target that was in the original sample [83]. This type of immunoassay has been used alone or as part of post column detection schemes for analytes such as digoxin, α-fetoprotein, granulocyte colony-stimulating factor, interleukin-10, and thyroxine [35,83].

A two-site immunometric assay, or sandwich immunoassay, utilizes two different antibodies that can bind simultaneously to the same target. In this assay, one of the antibodies is immobilized to a support and used to extract the target, while the other antibody contains a label that is used for detection. After non-retained components and excess labeled antibodies have been washed from the column, the retained target and labeled antibodies are also eluted. The signal that is generated by the labeled antibodies is used as a direct measure of the amount of target in the sample [80]. Examples of analytes that have been measured by this approach are human chorionic gonadotropin, HSA, IgG, interleukin-5, parathyroid hormone, and thyroid-stimulating hormone [35,80].

Many reports have used immunoaffinity chromatography in combination with other techniques for the analysis of complex biological samples. Examples of these methods that have already been discussed are immunoextraction [18,35,38–41] and immunodepletion [37,42]. Off-line immunoextraction has been coupled with HPLC, gas chromatography (GC) and other methods for the determination of such analytes as α_1-anti-trypsin, BSA, chloramphenicol, cortisol, clenbuterol and phenytoin, among others [18,35,77]. On-line immunoextraction methods have also been reported for a wide range of drugs, proteins and other compounds of biomedical interest [18,35]. These on-line methods have been created by combining immunoaffinity columns with GC, mass spectrometry, size-exclusion chromatography, ion-exchange chromatography, and capillary electrophoresis. However, most of these on-line methods have involved immunoextraction and reversed-phase HPLC. This combination reflects the popularity of reversed-phase HPLC in biological separations and the relative ease with which an immunoaffinity column can be coupled with a reversed-phase column [18,35,77].

7. Immobilized metal ion affinity chromatography

Immobilized metal ion affinity chromatography (IMAC) is another type of affinity chromatography that has seen growing use in biomedical analysis. IMAC makes use of the specific interactions that can occur between immobilized metal ions and targets such as amino acids, peptides, proteins, and nucleic acids [84–87]. The metal ions are immobilized within a column through the use of chelating agents like iminodiacetic acid, nitrilotriacetic acid, carboxymethylated-aspartic acid, and L-glutamic acid. Metal ions that are often chelated to these groups include Ni^{2+}, Zn^{2+}, Cu^{2+} and Fe^{3+} [88–90]. IMAC was originally developed for the isolation and separation of metal- and histidine-containing proteins [87]. During this type of analysis, the sample is passed through an IMAC column, and targets that can bind to the immobilized metal ions will be retained. These targets are later eluted through the addition of a competing agent (e.g., imidazole) and/or by changing the pH [87–90].

IMAC has become a powerful tool for analyzing membrane proteins, histidine-tagged proteins, and phosphorylated proteins [89,91–93]. In recent work, IMAC has also played a role in sample pretreatment for the detection of drugs. Examples of drugs that have been examined through the use of IMAC are tetracyclines, quinolones, macrolides, β-lactams, and aminoglycosides [94,95].

Another growing application for IMAC has been in the detection of biomarkers for disease diagnosis. This type of work has often involved the use of IMAC with mass spectrometry in surface-enhanced laser desorption/ionization (SELDI) [96–98]. An example of this approach is shown in Fig. 7. The first step in this method is the immobilization of the desired metal ions onto a surface such as

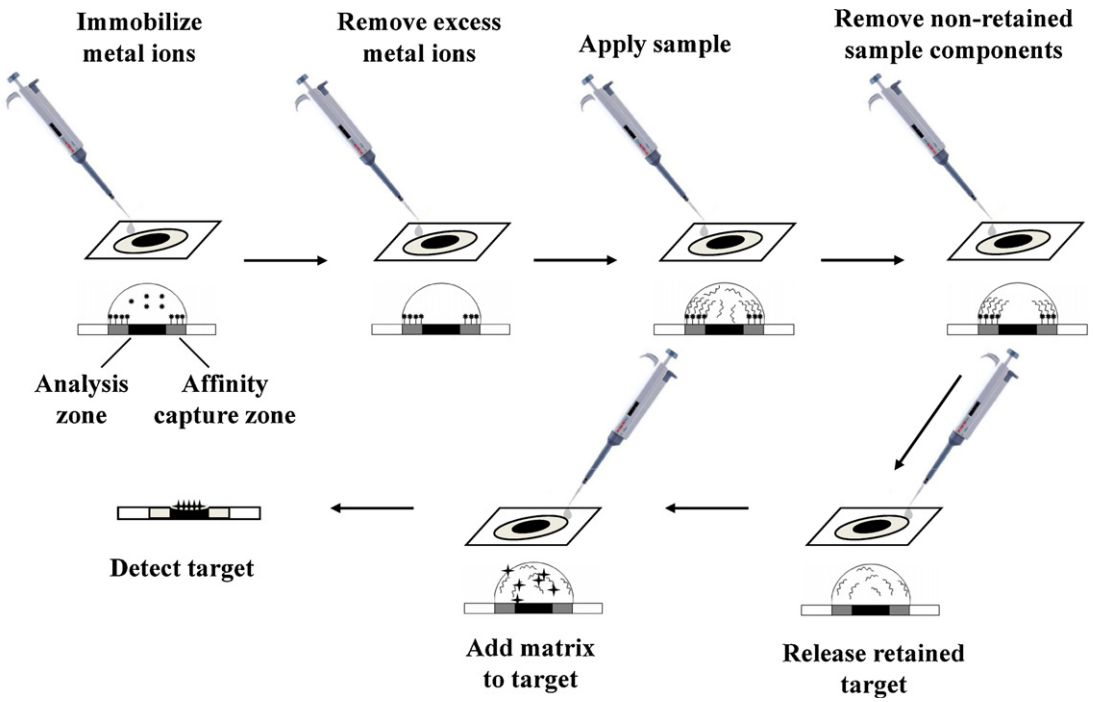

Fig. 7. Scheme for the purification and mass spectrometric analysis of a target by using surface-enhanced laser desorption/ionization (SELDI) with a surface that contains immobilized metal ions.
Based on information provided in Ref. [99].

a chip. After excess metal ions have been washed away, the sample is applied and the target is allowed to bind to the immobilized metal ions while non-retained substances are washed away. An elution buffer is next added to release the retained target from the immobilized metal ions so the target can enter an analysis zone. An appropriate matrix is then placed on this surface to aid in the ionization and detection of the target by mass spectrometry [99]. This technique has been used for detecting biomarkers in various samples, including serum, urine, and tissues [96–98].

The refinement of IMAC for protein purification is another active area of research. Although there have been many studies aimed at optimizing supports and ligands for IMAC, there are still relatively few reports in which IMAC has been used for large-scale protein purification [89,100–102]. One problem that still needs to be addressed for such applications is the potential for leakage of metal ions from IMAC columns. This issue is of concern because the presence of metal ions in the collected fractions may interfere with the purity, shelf-life and apparent activity of a protein that is isolated by IMAC [103–105]. When IMAC is used to isolate recombinant histidine-tagged proteins, other issues that must be considered include the need for eventual removal of the histidine tag from the recombinant protein and the possible co-elution of this target with other proteins from the sample [89,105,106]. Many of the same issues will need to be addressed as applications for IMAC expand in the areas of biomedical and pharmaceutical analysis. This will probably require the development of new chelating ligands for IMAC, as well as the use of focusing methods to improve the selectivity of IMAC during the isolation of proteins from biological samples.

8. Analytical affinity chromatography

Besides its application for isolating and measuring specific targets, affinity chromatography can also be used to study interactions in biological systems. When affinity chromatography is utilized for this purpose, the method is referred to as analytical affinity chromatography, quantitative affinity chromatography or biointeraction affinity chromatography [107–110]. This approach has been used in many recent studies to examine drug binding with agents that include serum proteins, enzymes, and receptors, as well as the interactions of biomolecules with lectins, aptamers, and antibodies [107–114]. Information that can be obtained by this approach includes the overall extent of binding, the equilibrium constants for an interaction, the number and types of sites involved in the binding process, and the rate constants for the interaction [107–110,113].

One method that is often used to conduct these measurements is zonal elution. In this type of experiment, a small plug of a drug or analyte is injected onto an affinity column that contains the immobilized binding agent of interest. The retention factor or peak profile for the analyte is then analyzed under various conditions to obtain quantitative or qualitative information for a biological interaction [108,110]. There are many different applications for zonal elution. For instance, retention factors can be used directly as a measure of the overall affinity of solute–ligand binding [108]. This application has been used in HPAC for the high throughput analysis of drug interactions with HSA [33,34,115]. The measurement of retention factors as the mobile phase or column conditions are changed (e.g., a variation in pH, ionic strength, solvent polarity or temperature) can also provide clues as to the forces and mechanisms that underlie a drug–protein interaction [108,110]. Such an approach has been used in numerous studies to examine the chiral selectivity of proteins such as HSA, AGP, trypsin, α-chymotrypsin and cellobiohydrolase I [19,28,29,108,110,114,116]. A comparison of retention factors that are measured by zonal elution for a set of structurally similar compounds can further be used to create quantitative structure–retention relationships that describe the interaction of these compounds with a given protein or binding agent, as has been used to examine drug binding sites on HSA and AGP [108,114,117–120].

Another common use of zonal elution experiments is in competition and displacement studies. In this type of experiment, injections of a target or probe are carried out in the presence of a

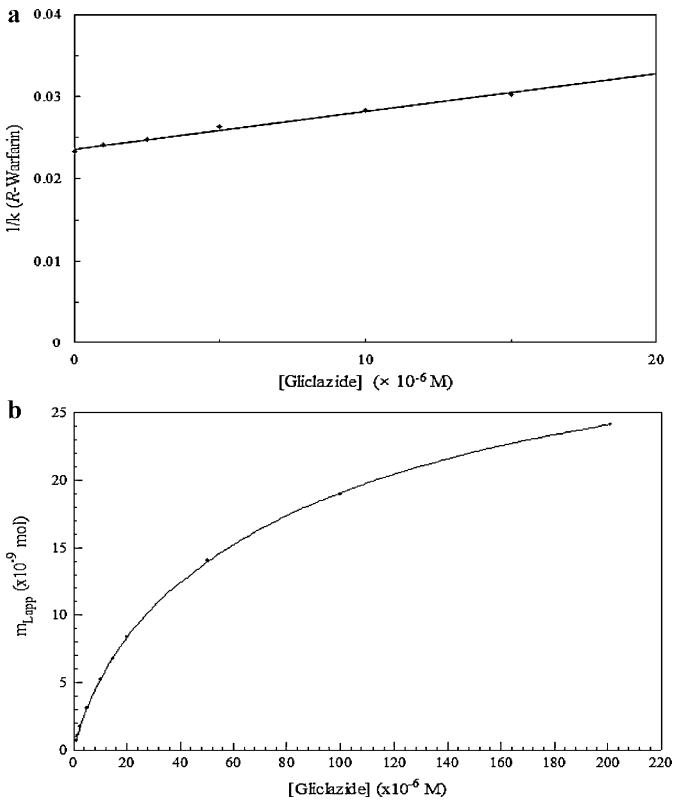

Fig. 8. Examples of (a) a zonal elution competition study with *R*-warfarin and gliclazide as a competing agent on an HSA column and (b) a frontal analysis experiment for gliclazide applied to an HSA column, with data being fitted to a two-site binding model. Terms: *k*, retention factor; $m_{L,app}$, moles of applied target that are needed to reach the mean position of the breakthrough curve at a given concentration of the target.

Adapted with permission from Ref. [121].

fixed concentration of a competing agent in the mobile phase [108]. Analysis of how the retention factor of the target changes with respect to the concentration of the competing agent can then be used to identify the type of competition that is occurring between these two solutes. In some cases, the same data can be further used to determine the association equilibrium constants and number of sites that are involved in the interaction [108,110].

Fig. 8(a) provides an example of a competition study for an HSA column in which *R*-warfarin was an injected probe for Sudlow site I of HSA and gliclazide was employed as a competing agent [121]. As the concentration of gliclazide in the mobile phase was increased during this experiment, the retention factor (*k*) for *R*-warfarin shifted to smaller values. When a plot of 1/*k* versus gliclazide concentration was made, the result was a linear relationship, which is representative of direct competition by *R*-warfarin and gliclazide at a single common region on HSA. It was then possible to use the slope and intercept of this plot to determine the equilibrium constant for gliclazide at the common binding region on HSA [121]. Comparable experiments and plots have been used to examine systems with other types of interactions [108,110].

Similar experiments to those in Fig. 8(a) have been employed in numerous studies. Examples include work that has examined the displacement of D/L-thyronine and D/L-tryptophan from HSA by bilirubin or caprylate [122]; the competition of *R*/*S*-warfarin with oxazepam and lorazepam on HSA [123]; and the effects of fatty acids on the binding of HSA with various drugs (e.g., warfarin, phenylbutazone, tolbutamide, acetohexamide, and gliclazide) [124,125]. This method has also been utilized to compare various coumarin and indole derivatives as possible probes for Sudlow sites I or II of HSA [126,127]. Recent work has used a modified version of this method to provide quantitative information on the allosteric interactions that take place on HSA between ibuprofen and benzodiazepines, L-tryptophan and phenytoin, or warfarin and tamoxifen [128–130]. Competition studies using site-specific probes have further been employed to investigate the changes that occur in solute interactions with HSA that has been modified at specific binding regions through synthetic methods [131,132] or natural processes (e.g., glycation) [121,133–135].

A second approach that can be used for binding studies in affinity chromatography is frontal analysis, or frontal affinity chromatography (FAC) [107–111,136]. In this technique, a known concentration of a target is continuously applied to an affinity column that contains an immobilized binding agent. As the immobilized binding agent becomes saturated with the target, a breakthrough curve will form. The mean position or shape of the breakthrough curve is then analyzed. For instance, the mean position of this curve can be fit to various binding models to determine the type of interaction that is taking place in the column, as is illustrated in Fig. 8(b) [121]. From the best-fit parameters, it is possible to determine the equilibrium constants and number of binding sites that are present between the target and immobilized binding agent [108]. Information on the interaction kinetics can also be obtained from the shape of the breakthrough curve [112].

Frontal analysis has been used to investigate the binding of HSA to *R*/*S*-warfarin [137] or D/L-tryptophan [138,139], to compare the binding of monomeric versus dimeric HSA for various drugs [140], to examine the effects of chemical modification on the binding of site-specific probes with HSA [132], and to examine the multi-site binding of thyroxine with HSA [119,141]. This method has been employed more recently to examine the overall binding of drugs such as acetohexamide, carbamazepine, imipramine, lidocaine, phenytoin, tolbutamide and verapamil with HSA [142–147], as well as the interactions of various coumarin and indole derivatives with HSA [126,127]. Similar experiments have been used to investigate the binding of propranolol, carbamazepine, and lidocaine with AGP [116,145,148] and the interactions of propranolol and verapamil with high-density lipoprotein [149]. Additional work has been conducted in the development and evaluation of methods for the detection of multi-site binding by frontal analysis [141,150].

Frontal analysis can also be utilized to look at how changing the mobile phase or temperature will affect the affinity and number of binding sites that are involved in a solute–ligand interaction. As an example, this approach has been used to examine the changes in binding by D/L-tryptophan to HSA as a function of pH [138]. The effect of temperature on biological interactions has been frequently studied by this method, as illustrated by reports that have investigated the interactions of *R*/*S*-warfarin, D/L-tryptophan with HSA [137,138], the binding of propranolol and carbamazepine with AGP [116,148], and the interactions of propranolol with high-density lipoprotein [149]. This approach has further been utilized to determine the overall changes in drug or solute interactions that can occur during the modification of HSA at its binding regions [121,132–135].

Another format in which frontal analysis can be employed is as a tool for competitive binding experiments, particularly when used with mass spectrometry. This combination of methods is known as frontal affinity chromatography-mass spectrometry (FAC-MS) [110,136,151,152]. FAC-MS has been explored in numerous reports as a tool for screening mixtures of compounds for binding to a given immobilized ligand [136,151–159]. Binding agents that have been investigated by this technique include lectins [155,159], enzymes [136,152,157] and galactosaminoglycans [154].

Another application of analytical affinity chromatography is as a tool for studying the kinetics of a biological interaction [108,113]. There are several approaches for this type of work. One such

approach is the plate height method, in which band-broadening is measured for a target on a column that contains the desired affinity ligand and on a control column that contains no ligand. The measured plate heights for the analyte are then plotted as a function of flow rate or linear velocity to find the plate height contribution due to stationary phase mass transfer, which is related to the dissociation rate constant between the target and affinity ligand [113]. Two examples of this approach have been its use to examine the interaction kinetics of *R*/*S*-warfarin and D/L-tryptophan with HSA [160,161].

Peak profiling is a variation on the plate height method in which retention times and peak widths are measured for both a target and a non-retained solute on an affinity column [113]. These values are then compared and used to determine the rate constant for target dissociation from the affinity ligand. During this process, the results for the non-retained solute are used to correct for band-broadening processes other than the target–ligand interaction. This method has been used to estimate the dissociation rate constants for L-tryptophan, carbamazepine, imipramine and phenytoin metabolites from HSA [162–165]. Peak profiling methods based on the use of either single flow rates or multiple flow rates have been described [162,163], and techniques have been developed for the use of this technique with solutes that have multiple interactions with a column (e.g., binding to both the affinity ligand and to the support) [164]. In addition, this method has been combined with chiral separations to simultaneously examine the interaction kinetics of HSA with the enantiomers of drug metabolites [165].

Another approach that has been used to examine the kinetics of biological interactions by analytical affinity chromatography is the peak decay method. This technique is carried out by first injecting a pulse of a target onto a small affinity column. The mobile phase is then quickly passed through to prevent re-association of the target as it is released from the affinity ligand. Under the appropriate conditions, the result is a decay curve that can be used to measure the dissociation rate constant for the target from the ligand [113]. This method has been used in recent studies to estimate the dissociation rate constants for various drugs from HSA [166,167] and has been explored as a high-throughput method for such measurements using affinity microcolumns and/or affinity monoliths [167].

Curve fitting has been used in both zonal elution and frontal analysis methods to obtain information on the kinetics of biological interactions [113]. For instance, frontal analysis profiles were recently used to examine the association kinetics of target interactions with antibodies in immunoaffinity columns [112,168]. The same approach has been used to examine binding of some aptamers with their targets [168]. Zonal elution studies carried out under non-linear elution conditions have also been employed in kinetic studies with affinity columns. An example is the use of this approach in several reports to examine the interactions of various inhibitors with immobilized nicotinic acetylcholine receptor membrane columns [169–174].

9. Other biomedical applications of affinity chromatography

There are a number of areas related to affinity chromatography that have also been of great interest in pharmaceutical and biomedical analysis. One such area is in the use of affinity columns for enzyme isolation [43,175]. This approach may use affinity ligands that are substrates, inhibitors, cofactors, or proteins that are associated with the biochemical pathways of the target enzyme [175]. Some examples are the use of immobilized flavin mononucleotides for the purification of flavin adenine dinucleotide synthetase [176] and the use of inhibitors to purify proprotein convertase furin [177]. Other reports have sought to develop and use new types of immobilized enzyme reactors (IMERs) for screening potential enzyme inhibitors, for research in drug metabolism, or for the digestion of proteins for peptide mapping [178–181]. Part of this work has involved the use of improved chromatographic supports for IMERs, such as monolithic media [12,13].

Synthetic dyes are another group of ligands that can be employed in affinity chromatography, creating a method known as dye–ligand affinity chromatography [182]. For instance, columns containing triazine dyes are commonly used to purify albumin and other blood proteins, along with various enzymes and proteins of interest as pharmaceutical agents [182–184]. Dye–ligand columns have also been employed to remove prion proteins, human immunodeficiency virus-1, and hepatitis B viral particles from biological fluids [185–187]. The use of computational methods or combinatorial chemistry to screen dyes and other ligands for a particular target is known as biomimetic affinity chromatography; this approach has been of great recent interest as a tool for the purification of protein-based pharmaceuticals [182,188].

Molecularly imprinted polymers (MIPs) make up another group of alternative ligands and supports that have been undergoing development for use in affinity methods [189,190]. Binding sites in MIPs are created by using functional monomers that can form a complex with a template such as the target analyte. After the monomers have been cross-linked, the template is removed and leaves behind a pocket that will recognize and retain the target. Most applications for MIPs up to the present have been in the area of affinity extractions [189,190], including their recent use for on-line methods [191,192].

Another relatively new class of ligand is the aptamer. Aptamers are made up of nucleic acid sequences that can take on three-dimensional structures that will bind a given target [182]. Aptamers are generated by screening the binding of the target with members of a large, random library of single-stranded DNA or RNA sequences. This method is carried out through a process known as SELEX, or the "systematic evolution of ligands by exponential enrichment" [182]. Aptamer columns have been utilized to purify recombinant proteins [193], as well as for chemical analysis [194–199]. Some aptamers have been used as part of microfluidic systems [196], while others have been immobilized within monolithic supports [197,198].

10. Conclusion

As has been shown in this review, affinity chromatography is a versatile technique that has become a valuable separation tool for biomedical and pharmaceutical analysis. This method can be carried out with traditional carbohydrate supports or with HPLC supports, such as silica and monolithic materials. This method can be used with step elution or isocratic elution and as part of affinity extraction or affinity depletion schemes. It can also be carried out with a wide range of ligands, such as lectins, boronates, antibodies, immobilized metal ions, molecularly imprinted polymers, dye ligands, and aptamers. These affinity methods can either be used alone or in combination with other techniques, such as reversed-phase HPLC or mass spectrometry. In addition, affinity chromatography can be used to study biological interactions and the competition or displacement of solutes on proteins or other binding agents. Based on the many recent applications of this method, it is expected that affinity chromatography will continue to grow in importance and popularity as a tool for the separation and analysis of biological and pharmaceutical agents in complex samples.

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