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Recent Advances in Supramolecular Affinity Separations: Affinity Chromatography and Related Methods

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Abstract

Affinity chromatography is a technique that uses a stationary phase based on the supramolecular interactions that occur in biological systems or mimics of these systems. This method has long been a popular tool for the isolation, measurement, and characterization of specific targets in complex samples. This review discusses the basic concepts of this method and examines recent developments in affinity chromatography and related supramolecular separation methods. Topics that are examined include advances that have occurred in the types of supports, approaches to immobilization, and binding agents that are employed in this method. New developments in the applications of affinity chromatography are also summarized, including an overview on the use of this method for biochemical purification, sample preparation or analysis, chiral separations, and biointeraction studies.

Keywords: affinity chromatography; affinity supports; immobilization methods; affinity ligands; affinity separations

1 Introduction

Affinity chromatography is a specific type of liquid chromatography in which the separation mechanism is based on an interaction between an immobilized and biologically related binding agent with an applied analyte [1-3]. This method is closely tied to supramolecular interactions in that it makes use of the selective and reversible interactions that occur in many complexes that are formed in biological systems, or mimics of such systems. Examples of biological supramolecular interactions are those that take place between antibodies with their antigens, enzymes with their substrates or inhibitors, and hormones with their receptors [1-8]. Advantages of using these interactions in affinity chromatography include the high selectivity, strong binding, and good resolution that are made possible in this method for specific target compounds [5-8]. The stationary phase in affinity chromatography, which makes up the immobilized component of the supramolecular complex, is often called the "affinity ligand". The support that contains this stationary phase is packed or placed into a column and used to selectively retain the complementary target to the affinity ligand, where this target represents the second part of the supramolecular complex [5-7].

The most common format in which immobilized ligands and columns are used in affinity chromatography is the on/off elution mode, as shown in Figure 1 [5,9]. In this format and in the presence of an application buffer, a sample mixture containing the target analyte is applied to the column. Non-retained components pass through the column and are quickly eluted, while the target interacts with the immobilized affinity ligand. To obtain and release the captured agent, a separate elution buffer is typically used in a situation where the analyte and binding agent have strong interactions, such as occurs for systems with association equilibrium constants of 10^6 M^{-1} or higher [1,7-9]. To weaken these interactions, the elution buffer has a change from the

application buffer in its pH, polarity, or ionic strength; this approach is known as "non-specific elution". Alternatively, the elution buffer may contain a competing agent to promote analyte elution by mass action, giving a method referred to as "biospecific elution" [5,9]. After the analyte has been released, the column may then be regenerated by reapplying the original application buffer or some similar solution [5,9]. The system is then ready for the next sample to be processed. In weak affinity chromatography (WAC), the same mobile phase is used for both sample application and elution under isocratic conditions, as well as for column regeneration. This situation is possible when the supramolecular interactions that are involved in retention have an association equilibrium constant that is less than approximately 10^5 or 10^6 M^{-1} [1,7,9-11].

Affinity chromatography has been popular for decades as a tool for the selective purification of biological molecules [1-7]. In addition, this technique has been used as a method for sample preparation during chemical or biochemical analysis and as a tool for the isolation, measurement, or characterization of targets in biological, clinical, and environmental samples [1-6,12-16]. This review will discuss recent developments in affinity chromatography and related supramolecular separation methods. Topics that will be discussed will include advances with regards to the types of supports, biological binding agents, and immobilization methods that are employed in this method. New developments in the applications of affinity chromatography will also be examined.

2 Supports for Affinity Chromatography

Many materials and matrices have been utilized as supports in affinity chromatography. These supports can be divided into three main categories (see Table 1) [1-3,17,18]. The first category is natural supports, such as agarose, dextran, and cellulose. The second group is inorganic

matrices, such as silica or glass. The third group is made up of synthetic materials that can be used as chromatographic supports, such as polystyrene or polyacrylamide [1,17,18].

2.1 Natural Supports and Related Materials

Agarose is the most popular support material used in affinity chromatography for both the large- and small-scale purification of targets [3,19-23]. This was also the material used in the first modern separations that were described for affinity chromatography in 1968 [3,8,19-21]. Agarose has a large pore size, which makes it suitable for many biomedical separations or the immobilization of large biomolecules. The low cost, low non-specific binding of this material for many biological agents, and good stability of agarose over a broad pH range also make agarose appealing as a support for many applications of affinity chromatography [20,21]. However, the relative low mechanical stability of agarose at high operating pressure does limit its use as a support in analytical-scale separations based on high-performance liquid chromatography [3,5,21]. Recently, a microporous membrane emulsification technique has been used to develop monodisperse microspheres based on agarose [24]. Beaded agarose in the form of microspheres with selected diameters and porosity is commercially available under the tradename Sepharose [3,21,25]. The hydroxyl groups of agarose can be partially or fully derivatized, and several functional groups can be incorporated into this support, such as amine, carboxyl, cyano, or sulfonate groups [21,26-28].

Cellulose is another natural material that has been used as a support in affinity chromatography, specifically for the purification of antibodies or enzymes [3,20,29]. Currently, cellulose is not as popular as agarose as a support for affinity chromatography due to its lower surface area and lower mechanical stability [3,20]. However, cellulose in a membrane format can provide low backpressures and be used in preparative work at high flow rates [3,30,31].

Adsorptive cellulosic fibers have been emerging as a new support matrix for affinity chromatography due to their high swelling capacity and good mechanical strength [32-34].

Besides agarose and cellulose, dextran based matrices such as Sephadex, Superdex, and Sephacryl are also widely used in bioaffinity chromatography [30,35]. Supports for which strong one-point interactions with target proteins are required can be modified with dextran [36,37]. In affinity chromatography this can be an advantage because multipoint interactions with other proteins can lead to non-specific adsorption.

Carbohydrate-based hybrid supports with a dense core (e.g., quartz) have been used to prepare expanded-bed adsorbents for affinity separations [38-41]. Agarose [38-40] and cellulose [41] have often been employed for developing these hybrid supports. The use of expanded-bed adsorbents helps to avoid column clogging by solid contaminants by allowing the creation of a fluidized or expanded bed during sample application [3,20,21,23]. Expanded bed adsorbents have been utilized in such applications as protein purification [42] and isolation of monoclonal antibodies [43,44].

2.2 Inorganic Supports

Inorganic materials such as porous silica particles and glass beads, both modified with hydrophilic groups, are commonly used in high-performance affinity chromatography (HPAC) [3,5,20,21]. These inorganic supports have the advantages of being available in various pore sizes and particle diameters and show good mechanical strength at the pressures and flow rates that are used in HPLC conditions [3,5,20,45]. Non-modified inorganic supports do have some disadvantages when compared to many carbohydrate supports, including their lower pH range stability and possible presence of higher non-specific binding [3,20]. However, these materials can be modified and used with a wide range of affinity ligands, and they are compatible with target

purification or analysis in applications that span from pharmaceutical and biomedical analysis to flow-based immunoassays [3,20,22,45-47]. For instance, HPLC-grade silica has been used as a support to prepare lectin affinity microcolumns to examine binding by drugs with structural variants of alpha$_1$-acid glycoprotein (AGP) [48]. In another report, rapid protein purification was obtained by using a surface imprinted silica support [49].

Monolithic silica supports have also been receiving attention in affinity chromatography [50-52]. There are several advantages of monoliths over traditional particulate supports, including their availability in a range of sizes and shapes [21,50]. Other advantages of monolithic supports consist of their low backpressures, high permeability, and good separation efficiencies [21,50]. In one report, a hybrid organic-silica affinity monolith support was made using 'thiol-ene' click chemistry to immobilize an aptamer that was then applied to recognize a trace protein [53]. Weak affinity chromatography has been increasingly applied to study interactions in biological systems [54,55]. In another recent study, silica-based monolith nanocolumns were made using a small amount of targeted protein and used in weak affinity chromatography coupled with mass spectrometry for rapid fragment screening, as may be used in the future as a tool for drug discovery [56].

Other inorganic-based supports have also been considered for use in affinity columns. An example of one such material is titania, or TiO_2 [57-62]. Supports that contain titania have been employed in a method known as metal oxide affinity chromatography, or MOAC, to bind and capture targets such as phosphopeptides [57-60]. In addition, dendritic polyglycerol-coated chitosan nanomaterials that contained Ti^{4+} and titania nanoparticles have been utilized for the isolation and separation of glycopeptides and phosphopeptides [61,62].

2.3 Synthetic Supports

Synthetic polymers such as polystyrene and polymethacrylate have also been used as supports for both traditional and high-performance applications of affinity chromatography [3,21,30]. Polystyrene supports have been used as a perfusion media and in other separation formats in affinity chromatography [21,63,64]. The long hydrophobic backbone of polystyrene can lead to high levels of non-specific interactions with biological molecules like proteins, but this can be addressed by coating a hydrophilic layer on this material [21,63,64]. Polymethacrylates are more hydrophilic in nature and can be used either in their original or modified forms for affinity separations [46,50]. Other synthetic polymers such as polysulfones and polyamides are also used in affinity chromatography as membrane supports [65]. These organic polymers are often stable over a broad pH range, which is also appealing for their use in affinity separations [3,21,46].

Organic polymers, agarose, and cryogels as monolithic supports are also gaining attention in affinity chromatography [21,50,52,66-77]. Polymeric monolithic supports based on glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) have been used with immobilized agents such as antibodies, enzymes, proteins, and peptides [52,66-69]. For instance, polymethacrylate monoliths known as convective interaction media (CIM), have been used in a wide range of applications ranging from the purification of large biological agents such as viruses, proteins, and DNA to antibody-based separations and immobilized metal-ion affinity chromatography (IMAC) [51,70-74]. The large pore sizes of cryogels have made these materials useful in the separation or capture of large analytes such as eukaryotic cells and bacteria [76,77]. The low surface area seen in cryogels has been noted to lead to a possible decrease in ligand density and binding capacity [75]. However, this limitation can be overcome by using small binding

agents (e.g., metal ion chelates), surface grafting of the ligand, or embedding functionalized particles within a cryogel [78-80].

2.4 Magnetic Beads and Particles

Magnetic particles such as magnetic beads and nanoparticles have been used in some studies as supports for affinity chromatography [81–84]. Magnetic beads are often prepared by depositing magnetic iron oxides (i.e., Fe_3O_4) into porous mono-sized polymer particles, although polymers containing colloidal magnetite or other magnetic metals such as cobalt and manganese have been used as well. Binding agents can be attached to these particles by placing on the polymers reactive functional groups such as isocyanate, vinyl, and epoxy residues [81–84].

The use of magnetic beads or particles with immobilized binding agents was described as early as 1977, when agarose-polyacrylate beads containing magnetic cores were prepared and used in a sandwich immunoassay with an enzyme label [81,83]. Several other types of magnetic beads that could be used with affinity ligands were developed after this, including materials made from starch, carbohydrate, cellulose, or dextran and containing iron oxide; magnetic beads were also prepared from iron oxide and polymers such as acrylates, methacrylates, or styrene [81,83,85]. The use and performance of these initial forms of these beads in a magnetic field suffered from variability in beads' sizes and level of magnetism [81,83]. These limitations were later overcome when beads with the preparation of magnetic beads with more uniform sizes (e.g., spherical monodisperse particles with sizes in the range of 0.5 to 100 μm) [83].

Magnetic particles have been used as supports in several affinity separations [86–95]. These materials have been used to bind targets such as antibodies or other proteins, antigens, and DNA or RNA [86–94]. For instance, magnetic poly(2-hydroxyethylmethacrylate) beads were used to isolate lysozyme from aqueous solutions and egg white and cytochrome c from an aqueous

sample [84,88]. Magnetic attapulgite, containing boronic acid as a binding agent, was used as a support for the selective enrichment of several nucleosides from urine samples [90]. Trace ginsenosides were separated from rat plasma by using magnetic nanoparticles and a boronate as the binding agent [93]. The dye Cibacron Blue 3GA was coupled to magnetic support made of Fe_3O_4 particles entrapped by polyvinyl alcohol and used for the purification of lysozyme and alcohol dehydrogenases from clarified yeast homogenates [94]. Magnetic nanoparticles containing Fe_3O_4 were used for the extraction of luteolin from honey samples using boronic acid as the affinity ligand [96].

2.5 Smart Materials

A number of smart materials have been explored for use in affinity chromatography. Smart materials, or stimuli-responsive intelligent materials, are polymers which respond to external chemical, physical, or biochemical factors [97–99]. These polymers change their structure or function (e.g., an alteration in their shape, phase, or ability to take part in molecular recognition) as they respond to changes in factors such as temperature, ionic strength, solvent concentration, UV radiation, magnetic field, pH, or the presence of given binding agent or type of ion. These polymers are then able to return to their initial state once the source of the external stimulus has been removed or reversed [97–99]. Stimuli-responsive polymers can also be either mixed physically or conjugated with a biomolecule to form materials for affinity-related applications [99–101].

The smart materials that are most often used in affinity chromatography are those that respond to a temperature change (i.e., thermoresponsive polymers) [100–103]. These polymers show temperature-dependent hydrophobic/hydrophilic variations and conformational changes in their polymer chains in response to variations in the surrounding temperature [100–104].

Examples of common thermoresponsive polymers that have been used in bioseparations are poly-(*N*-isopropylacrylamide), poly(vinyl) ether, oligo(ethylene glycol) methacrylate, and related derivatives [102,104–107]. Among these, oligo(ethylene glycol) has been of particular interest [100–107]. An application mentioned in Section 2.4 was the use of boronic acid on oligo(ethylene glycol)-based magnetic nanoparticles as thermoresponsive supports for the extraction of luteolin from honey [96]. A support for the capture and release of lysozyme from human urine was described in that made use of oligo(ethylene glycol) and a methacrylate-based molecularly imprinted polymer [108]. Bovine serum albumin (BSA) was purified from protein mixtures and samples by using a thermal- and salt-sensitive MIP as the support [109].

2.6 Nanomaterials

Nanomaterials have yet to be widely examined for use as affinity ligands [110]. However, there have been reports of using nanomaterials to develop stationary phases that can be used in some chiral separations [111-113]. Carbon nanotubes have been shown to have some chiral recognition [114]. Both multi-walled and single-walled carbon nanotubes have been embedded in monoliths and used to separate enantiomers of various organic small molecules [115,116]. For instance, an organic monolith containing multi-walled carbon nanotubes was used to separate the enantiomers of bupivacaine, dansyl-methionine, dansyl-phenylalanine, and 2,4-dichlorophenoxypropionic acid [115]. A monolith containing single-walled carbon nanotubes was used in chiral separations for sulconazole, nomifenzine, etozoline, chlorpheniramine, and celiprolol [116].

2.7 Support Formats

Several forms of supports have been utilized in affinity chromatography [3,20,21]. The majority of reports on this method have used packed columns for either low-to-medium performance affinity chromatography (e.g., using agarose or cellulose) or HPAC (e.g., based on silica or glass beads) have used packed columns [3,21,30]. Small volume columns, including short length or small internal diameter columns, have also been of interest in recent years [3,117-119]. Over the last decade, monoliths and perfusion-based media have both gained popularity in analytical and preparative applications of affinity chromatography [3,21]. Expanded or fluidized beds have also become popular for preparative uses of affinity chromatography [3,20,21].

Several types of supports (e.g., capillaries, packed beds, and monoliths) have been considered for use in microchips and miniaturized devices for affinity-based separations and methods [21,120-126]. The advantages of using these supports for affinity chromatography are that they require a smaller amount of binding agent than more traditional sized columns phase and consume less mobile phase. They also usually require smaller sample injection volumes and can often provide short analysis time [120,126].

Packed capillaries in these methods usually have an internal diameter of 75-500 μm and a length of 5-15 cm [120,127-129]. They are typically used at low flow rates (i.e., nL/min to μL/min) and are often employed with nano-HPLC or microbore HPLC systems [120,127-129]. Capillary columns with IMAC-based stationary phases have been used for in the purification and biointeraction studies of several proteins [127,128]. For example, Ti^{4+} complexed on monodisperse-porous silica microspheres was used in a capillary column for the purification of phosphoproteins from milk and human serum [128]. Monolithic capillary columns containing Cu^{2+} were employed for the isolation of immunoglobulin G (IgG), transferrin, and albumin from

human plasma [127]. A microfluidic affinity capillary microcolumn was made that contained a MIP formed by the interaction of a cis-diol containing template, β-nicotinamide adenine nucleotide, and the functional monomer, 4-vinylphenylboronononic acid; this boronic acid monolith was then employed for the purification of β-nicotinamide adenine nucleotide [129]. Packed capillaries as affinity columns have also been employed for studying the interaction of drugs with serum proteins and identification of ligands specific to the adenosine A_{2A} receptor [120,130].

Short affinity microcolumns having length of 5-50 mm and a typical internal diameter of 2.1 mm have also been employed in several studies [48,131-142]. These microcolumns usually have low backpressures, which allows their use at high flow rates [48,131-137,139-142]. These microcolumns have been used in applications such as studies of drug interactions with $alpha_1$-acid glycoprotein (AGP) or human serum albumin (HSA) by zonal elution, frontal analysis, and ultrafast affinity extraction [48,131-137,139-142]. These microcolumns have been employed in biointeraction studies involving AGP and the lectins concanavalin A (Con A) and *Aleuria aurantia* lectin (AAL) [48] and in work examining binding by drugs such as tapsin derivatives with epidermal growth factor receptors [138].

Two other microscale formats in which affinity chromatography are microchips and microfluidic flow cells [121,126,143,144]. For example, a polymer chip that contained TiO_2-ZrO_2 was employed for the enrichment of phosphopeptides from a tryptic digest of β-casein based on metal oxide affinity chromatography [143]. Con A was immobilized on regenerated bacterial cellulose in a microchip and was used for the separation of lysozyme from transferrin [144]. Microfluidic frontal affinity chromatography based on Ste2-G-protein coupled receptor on a gold substrate held in a flow cell was used to study the interaction of this receptor with α-factor, a natural pheromone peptide [121]. Biotin-avidin interactions in a flow cell have been exploited for

the detection of HIV-1, based on dual labeling of amplification products specific to the HIV-1 gene by using biotin and fluorescent tags and passing these products through streptavidin agarose beads [145].

3 Immobilization Methods

The choice of immobilization technique for preparing affinity columns is an important factor to consider. Figure 2 shows several immobilization techniques that can be used when preparing affinity columns. It is essential that the correct immobilization scheme is selected for a particular application because several effects that can result from immobilization schemes can alter the actual or apparent activity of an immobilized binding agent [1,146-148]. These factors include steric hindrance, multisite attachment, and improper orientation of the affinity ligand [3,147,148].

3.1 Non-covalent Immobilization

Non-covalent immobilization techniques based upon the physical adsorption of affinity ligands to chromatographic supports have been employed initially. In this approach, depending on the properties of supports and affinity ligands, physical adsorption of the binding agent to the support may occur through electrostatic interactions, hydrophobic interactions, or hydrogen bonding [44,148]. Both natural supports (e.g., agarose and cellulose) and inorganic supports (e.g., alumina and silica) have been used for non-covalent immobilization [149,150]. This approach is simple [3,149] but suffers from the possibility of limited stability of the resulting affinity support and the loss of binding agent activity through random orientation [149].

Non-covalent immobilization can also be obtained through biospecific adsorption. In this approach, a secondary ligand is attached to a support that can then bind and immobilize the primary affinity ligand [3,151]. The most common examples of such an approach are to use biotin as a tag

on a binding agent that is then allowed to bind to a support that contains avidin or streptavidin [149,152,153]. Another popular example of biospecific adsorption is the use of protein A or protein G (i.e., immunoglobulin-binding proteins) on supports to bind and immobilize antibodies [154-156].

Entrapment or encapsulation is another form of non-covalent immobilization technique. This approach makes use of the physical encapsulation or trapping of an affinity ligand within the pores of a support or within a cross-linked support or polymer network [157]. Enzymes, proteins, and other affinity ligands have been entrapped for a number of years within silicate-based sol-gels [157-159]. Another pathway for entrapment is the use of a cross-linking agent to immobilize an affinity ligand onto a support [3,131,160-162]. Hydrazide-activated silica support combined with oxidized glycogen as a capping agent has been used to entrap HSA, AGP, IgG, and other agents [131,160,162-164]. This latter method was also used with an online immunoextraction/entrapment system to extract and place a serum protein (i.e., HSA and modified forms of this protein) within small affinity columns to use in drug-binding studies [163].

Another immobilization method that has seen much interest in recent years is one that is based on boronic acids [165-170]. This approach makes use of the interaction of a boronic acid with the vicinal diol groups in many carbohydrate groups to generate a boronate esters. To accomplish this, the surface of the support should first be modified with a with boronic acid derivatives such as aminophenylboronic acid [166-170]. Although this technique avoids the need to carry out prior oxidation of the carbohydrate groups, the reaction of diol groups with boronic acids are usually reversible at a physiological pH [165,171,172]. In addition, there may be competition between different classes of glycoproteins or carbohydrate-containing agents for immobilization onto this type of support [165]. However, such competition can be avoided by the

formation of aldehydes via glycoengineering and/or enzymatic oxidation methods and incorporating unnatural fucose derivatives into the carbohydrate moieties [171,173-176].

3.2 Covalent Immobilization

Covalent immobilization is the most widely used method for coupling an affinity ligand to a support material [1,146,148]. This technique usually requires that the ligand or support, or both, be activated prior to immobilization. Examples of covalent immobilization methods are the cyanogen bromide (CNBr) method; the Schiff base (reductive amination) method; and approaches that use activating agents such as carbodiimide [146,148,177-179]. Many other techniques for this purpose have also been described, as reviewed previously [146,148].

The CNBr method is a two-step process which involves an initial modification of hydroxyl groups on the support to obtain active cyanate esters or imidocarbonate groups; these groups can then undergo a reaction with primary amine groups on an affinity ligands to form a covalent linkage [146,148,180]. Examples of recent applications for this method include its use in the activation of a Sepharose 4B support for the immobilization of sulfanilamide and the activation of a cryogel monolith for the immobilization of HSA [181,182]. Although this method remains a common technique for the immobilization of affinity ligands to materials like agarose, it does suffer from health and safety issues related to the toxicity of its reagents and side-products [18].

The Schiff base method involves the use of a support that has been activated or converted into a form that contains aldehyde groups or amine groups. These groups are then combined with a ligand that has primary amine groups or aldehyde groups, respectively, to form a Schiff base. Because a Schiff base is a reversible bond, this reaction is usually conducted in the presence of a mild reducing agent such as sodium cyanoborohydride to create a more stable secondary amine linkage [148]. This immobilization method has been used in a number of recent reports for the

immobilization of HSA to silica for drug-protein binding studies [132,183]. This method has also been employed in the immobilization of anti-human serum transferrin antibodies, anti-HSA antibodies, and the lectin Con A onto maltose-bonded silica for use in HPAC [184].

One example of a carbodiimide activation method is to activate carboxylic groups on a support by using a carbodiimide such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in the presence of *N*-hydroxysuccinimide (NHS) [185]. The result of this process is stable but reactive NHS ester. The activated NHS ester groups can then be reacted directly with primary amine groups on an affinity ligand such as protein or peptide through amide bond formation [185]. The carbodiimide activation method has been used recently for the immobilization of gelatin onto a poly(hydroxyethyl methacrylate) cryogel and peptides onto agarose supports [186,187]. The carbodiimide activation technique has also been used for the immobilization of Con A onto silica [188]. Other studies have used NHS-activated supports that have been produced without the use of carbodiimides. For example, NHS-activated silica has been created by reacting succinic anhydride with aminopropyl silica and subsequently reacting this product with NHS, as well as by combining disuccinimidyl suberate with aminopropyl silica [146,148,177-179].

Multisite attachment and improper orientation remain as challenges that are often associated with covalent immobilization through primary amine groups and other relatively common functional groups [148]. These challenges can be avoided by employing covalent immobilization methods that use more site selective sites on an affinity ligand, such as free sulfhydryl groups [146,148,189,190]. This latter method has been used for immobilization of protein A, protein G, and HSA onto supports such as agarose beads and silica for affinity-based separations [190-192]. This approach has also been used to immobilize peptides such as glutathione onto agarose for the purification of fusion proteins and peptide-specific antibodies

[193,194]. Although covalent attachment of affinity ligands to supports via sulfhydryl groups is attractive as site-selective immobilization option, the aggressive conditions required to generate the sulfhydryl groups on certain ligands may also result in undesired reactions that can alter the binding characteristics of the ligand [165,195-197].

For sugars and glycoproteins, including antibodies, improper orientation and multisite attachment effects can be significantly minimized by covalent coupling through carbohydrate groups [146,148,198-204]. In this approach, initial oxidation of the carbohydrate group is first carried out by oxidizing agents like periodate or by an enzymatic reaction to form aldehyde groups [148,199,203,205]. Hydrazide or amine groups that have been placed on the support can then be reacted with these aldehydes to covalently attach the ligand [200-203,205]. The main challenge with this approach is that certain important amino acids such as histidine, methionine, and tryptophan may be oxidized along with the carbohydrates by the periodate, thus altering the site selectivity and binding properties of the binding agent [195-197]. Thus, care must be taken to use mild oxidation conditions [148].

4 Binding Agents used in Affinity Chromatography

4.1 Biological Agents as Affinity Ligands

The first use of biological binding agent as a stationary phase in affinity chromatography can be traced back to 1910 when Starkenstein immobilized starch on a support to isolate α-amylase [206]. This type of affinity chromatography is now commonly called bioaffinity chromatography [1,3]. This general method gained popularity for the isolation of enzymes and other biological agents following the work in 1968 by Cuatrecasas et al., who used columns containing nuclease inhibitors to isolate α-chymotrypsin, carboxypeptidase A and staphylococcal nuclease [3,19,151]. Over the past fifty years, bioaffinity chromatography remains one of the most common types of

affinity chromatography [3,18,22,30,151]. Examples of biological agents that can be used as affinity ligands are shown in Table 2. This section will examine the major types of affinity ligands that consist of biological agents and new developments that have taken place with such stationary phases.

4.1.1 Immunoaffinity Chromatography

Immunoaffinity chromatography (IAC) is the most common form of bioaffinity chromatography [207-210]. This method is based on the highly specific and strong interactions that often take place between antibodies and their target antigens, which are used in IAC to capture, isolate, and purify various biochemicals and chemical agents [208-211]. The versatility of antibodies with regards to the many types of targets against which they can be prepared has made IAC a powerful purification method for use in many applications. These applications have included sample preparation, sample cleanup, and clinical or diagnostic assays for drugs, hormones, toxins, and biomarkers [207-214].

Applications of IAC may involve the use of various elution and detection methods. For example, this approach has been used with pH step elution for the isolation of plasma and serum proteins in biological matrices, as followed by using detection based on absorbance measurement or tandem mass spectrometry [207-209]. IAC has also been used to isolate other agents, such as glycoproteins and carbohydrates [207,208,212,214]. When used for sample pretreatment, IAC has aided in the isolation of compounds from water or food samples for further analysis by other LC methods, as employed for the determination of antibiotics and toxins in such matrices [213,215]. In addition, IAC has also been used as the basis for chromatographic immunoassays, as have been developed using both competitive and non-competitive binding formats [213,216-219]. More details on recent developments in these analytical applications are provided in Section 5.

4.1.2 Immunoglobulin-binding Proteins

Immunoglobulin-binding proteins are another important set of biological binding agents that are used in bioaffinity chromatography [207,220-229]. Two common examples of these agents are proteins A and G [221-224,228,229]. Protein A is a surface protein found in the cell wall of the bacterium *Staphylococcus aureus* [220]. Protein G is an immunoglobulin-binding protein from Streptococci bacteria. Both these binding agents have a high affinity for the constant, or Fc, region of many types of polyclonal or monoclonal antibodies, which makes these useful in the detection and purification of many subclasses of immunoglobulins [207,220-223]. Although protein A and G both bind to antibodies from various species, they differ in their affinity and dependence between species and immunoglobulin types [207,220,224]. For example, proteins A and G both have strong binding to most forms of human IgG-class antibodies [207,230]. However, protein A and G differ in their binding to human IgM- and IgA-class antibodies [207].

Other types of immunoglobulin-binding proteins have been reported as well. An example is protein L, which is isolated from the bacterial strain *Peptostreptococcus magnus* [226,227]. Unlike protein A and protein G, protein L binds through the light chains instead of the Fc region of immunoglobulins. This feature gives protein L an ability to bind to a wider range of immunoglobulin classes than proteins A and G (e.g., human IgA, IgD, IgE, IgG, and IgM) [226,227]. Recombinant forms and mixtures of these immunoglobulin proteins have also been employed in affinity methods used to capture, purify, and measure immunoglobulins in samples [207,220-224,228,229,231]. For example, the adsorption kinetics of recombinant human IgG antibodies with protein A was studied by affinity chromatography [222]. The affinity of protein A for IgG from various species was also examined [223]. Protein G affinity chromatography was also used to determine the amount of soluble IgG in bovine colostrum products [224].

4.1.3 Lectins

The use of lectins as binding agents in chromatography results in a method known as lectin affinity chromatography (LAC) [3,48,220,232,233]. Lectins are carbohydrate binding proteins that are highly specific for targets that contain complementary sugar groups [220,232,233]. LAC has frequently been used for the isolation and separation of glycoproteins and their glycoforms, as well as glycolipids and polysaccharides, thus making this technique a valuable approach to glycan analysis [48,232,233]. For example, this method has been employed for the separation and analysis of glycoforms for AGP based on lectin microcolumns [48].

Many types of lectins have been used in LAC. Two common examples are Con A and wheat germ agglutin (WGA), which can be utilized to bind agents containing high-mannose type glycans or *N*-acetyl-D-glucosamine and sialic acids, respectively [220]. Additional lectins have been employed in LAC are jacalin, AAL, various types of *Phaseolus vulgaris* agglutinin (PHA), *Datura stramonium* agglutinin (DSA), *Lens culinaris* agglutinin (LCA), *Maackia amurensis* lectin (MAM), *Ricinus communis* agglutinin (RCA), and *Sambucus sieboldiana* agglutinin (SSA) [3,48,220]. The coupling of lectin columns for the characterization of glycans on the same glycoconjugate is referred to as serial lectin affinity chromatography (SLAC) [234]. For example, N-glycosylated and O-glycosylated proteins have been examined by using a set of lectin columns for the determination of glycan structures [233].

4.1.4 Enzymes

Enzymes are another group of biological agents that are often employed as ligands or targets in affinity chromatography [3,11,220]. Enzymes can be coupled to various chromatographic supports, including silica or monoliths, and through the use of various immobilization methods [3,11,220]. Enzymes can undergo selective and stable interactions with

their substrates and inhibitors, which can make enzymes valuable for the purification of enzyme inhibitors as well as for scavenging impurities [3,11,220,235-237]. For example, catalase has been used for scavenging perborate and hydrogen peroxide, horseradish peroxidase has been employed for the removal of chlorophenols from wastewater, and rhodanese has been utilized employed for the removal of cyanides in effluents and air [220,236,237]. Enzyme-inhibitor interactions have further been exploited for the purification of enzyme inhibitors, such as the use of trypsin to isolate trypsin inhibitor, and DD-peptidase to bind penicillin [220]. Trypsin has recently been entrapped in a sol-gel monolithic column as a means to screen for *N*-alpha-benzoyl-DL-arginine-4-nitroanilide hydrochloride [235]. Enzyme inhibitors have also been extensively used for the purification of enzymes, as will be discussed later in Section 5 [3,11,220].

Other applications of enzymes have involved their use in solid-phase extraction (SPE) and immobilized enzyme reactors (IMER) for the screening of bioactive compounds from natural products [22,238-242]. In such work based on IMER, a substrate that is specific for the immobilized enzyme is introduced along with an extract of the natural product is to be examined [22,239-242]. The immobilized enzyme is then used to catalyze the formation of a product from the substrate and to see how this process is affected by bioactive compounds in the natural product extract (e.g., through competitive or non-competitive binding with the enzyme to prevent the formation of the product) [22,239-242]. Enzymes such as acetylcholinesterase, gelatinase, xanthine oxidase, and adenosine deaminase have been employed in this type of scheme with as supports such as silica or sol-gel capillary monoliths and in combination with two-dimensional liquid chromatography or LC coupled with mass spectrometry [22,237-239]. Enzymes have also been immobilized on silica and used in a microextraction column to fish for xanthine oxidase

inhibitors in natural products by using LC coupled with a diode array detector and tandem mass spectrometry [22,238].

Some enzymes have been employed as chiral selectors [243-247]. Examples of enzyme-based CSPs are those based on penicillin G acylase [243-245], glucoamylase [246], and cellulase [247]. For instance, penicillin G acylase has been covalently immobilized with a silica monolith for the chiral separation of 2-aryloxyalkonic acid methyl esters and isosteric analogs [245]. Cellulase was recently employed as a CSP by covalently immobilizing it onto aminopropyl silica and using this stationary phase for the chiral separation of various β-blockers [247]. Other examples are described in Section 5.3.

4.1.5 Serum Proteins

Serum proteins are one set of biological binding ligands that have been used for sample preparation and binding studies in affinity chromatography [11,248,249]. HSA, BSA, AGP, and lipoproteins are the examples of these binding agents. HSA and AGP act as the main carrier proteins for numerous drugs, low mass hormones, and fatty acids in the human circulatory system [11,250,251]. HSA is primarily involved in delivering acidic and neutral drugs, while AGP is the main carrier for basic drugs and steroids [250,251].

Applications of these serum proteins in affinity separations has been the subject of several recent reviews and papers [252-263]. One example is their use as stationary phases in chiral separations [11,252,253], as is described in more detail in Section 5.3. Another use of these serum proteins has been in biointeraction studies (see Section 5.4) [254-262]. For example, several studies in recent years have used affinity columns to investigate the overall affinity and site-specific binding of various solutes toward these carrier proteins [135,141,254-262]. The use of affinity chromatography to develop structure-affinity relationships for solutes with serum proteins

has also been described [11,135,253,256,257,261]. In these studies, a change in structure for either a group of solutes or in the immobilized binding agent is made to see how these affects the solute-binding agent interaction. Specific examples include reports that have looked at how changes in the glycation of HSA or glycosylation of AGP affect the ability of these serum proteins to bind to various drugs and drug classes [11,135,141,262].

4.1.6 Biotin, Avidin, and Streptavidin

The interactions of biotin with the proteins streptavidin and avidin have often been employed in affinity chromatography [220,264-266]. Biotin, which is also known as vitamin H and vitamin B_7, is a cofactor found in all living cells [220,264-266]. Avidin is a glycoprotein that is found in egg whites, while streptavidin is a protein produced by *Streptomyces avidinii* bacteria [220,264-267]. Biotin affinity columns are usually prepared by immobilizing 2-iminobiotin or native biotin to an LC support by employing the valeric acid side chain of these compounds [220]. Avidin or streptavidin can also be immobilized by using an approach such as the cyanogen bromide method [220].

It has been known for many years that biotin can undergo strong non-covalent interactions (i.e., with dissociation equilibrium constants as low as the picomolar-to-femtomolar range) with avidin and streptavidin [265-267]. Although there are similar biotin interacting sites in both these proteins, streptavidin possesses stronger binding to biotin than avidin as it can form a greater number of hydrogen bonds with the valeryl carboxylate group of biotin [220,264].

Biotin-avidin interactions have been employed for carrying out the purification of biotinylated proteins using native elution conditions as well as for the determination of biotin in food and beverages [268,269]. Biotin-avidin interactions have also been recently used to study the interactions of avidin on quantum dots with biotinylated immunoglobulin G [270]. Interactions

based on biotin with streptavidin have been used for the identification and characterization of ligands specific to nanodisc-embedded G-protein coupled receptors, the detection of human immunodeficiency virus-1 (HIV-1) in sub-attomole levels, and the extraction of streptavidin in presence of green fluorescent protein (EGFP) and a cell lysate of *E. coli* [130,145,152]. A biotinylated ligand complex prepared by coupling an azo-based linker through click chemistry was immobilized on streptavidin support for the affinity independent elution of ligands [271].

Monolithic columns have been combined with biotin-streptavidin/avidin systems for various applications [259,272]. For instance, monomeric avidin that was attached to monoliths based on co-polymers of glycidyl methacrylate with ethylene dimethacrylate and acrylamide have been employed for the enrichment of biotinylated peptides and proteins [272]. Streptavidin-biotin interactions were also exploited by applying biotinylated HSA to streptavidin immobilized on silica- and organic-based monoliths that were used in nano-weak affinity chromatography [259].

The fact that avidin and streptavidin have essentially irreversible binding to biotin means avidin/streptavidin-biotin complexes can only dissociate under harsh conditions, which can lead to the inactivation of some biotinylated biomolecules [267,273,274]. This has made genetically or chemically modified forms of avidin or streptavidin of interest for use in affinity applications [275]. Tamavidin 2-REV, a mutein engineered from tamavidin, was immobilized on agarose for the purification of biotinylated BSA and exhibited reversible binding for this target (dissociation equilibrium constant, 10^{-7} M) [275]. Core streptavidin, a mutant of streptavidin, can undergo reversible interactions with a strong affinity with biotin without being affected by harsh experimental conditions [276-279]; this form of streptavidin was fused with the transmembrane glycoprotein CD47 and isolated by using biotinylated agarose [279].

4.1.7 Carbohydrates

Carbohydrates have been used as immobilized ligands in chromatographic methods to carry out chiral separations and other forms of selective separations (see Section 5 for examples of applications) [3,11,220,280-284]. Polysaccharide based stationary phases have been extensively used for the separation of chiral drugs and solutes as a result of their excellent enantioselectivity an ability to be used in a variety of separation modes [3,11,112,282,283,285,286]. Most chiral stationary phases (CSPs) that employ polysaccharides are based on 3,5-dimethyl phenyl carbamate or 3,5-dichloro phenyl carbamate derivatives of cellulose and amylose, which can be prepared by coating or immobilizing these chiral agents onto supports such as porous silica particles, silica monoliths, or superficially porous silica [11,280,283,285-287].

Other polysaccharides such as derivatives of chitin and chitosan have also been recently employed for chiral separations. Some attractive features of these polysaccharides are their insolubility in many organic solvents (e.g., as may be used as a mobile phase) and their comparable enantioselectivity to cellulose or amylose derivatives for some applications [281,282]. In the case of chitin, *N*-acetyl-D-amine glucose units are bonded together by β-(1→4) glycoside bonds, with deacetylation of chitin resulting in the formation of chitosan [281,282,284]. Most chitosan and chitin based chiral selectors have made use of arylcarbamate or 3,5-dimethyl carbamate derivatives of chitosan and chitin [284,288-295].

Cyclic oligosaccharides composed of α-1,4-linked D-glucopyranose units, such as β-cyclodextrin, have also been frequently employed as CSPs [11,112,282,283,296,297]. The glucopyranose units in these cyclodextrins are arranged in a circular polymer which has a hydrophobic cavity and an exterior that is hydrophilic [112,282,283,296,297]. Some chiral solutes

can form inclusion complexes with the hydrophobic cavity and undergo differential interactions to the hydroxyl groups that are at the mouth of the cyclodextrin cavity [112,282,296,297]. Host-guest interactions between chiral solutes and cyclodextrins mainly occur through hydrogen bonds, hydrophobic forces, π-π bonding, and/or ionic interactions [112,282,296,297]. These CSPs can be prepared by coating or covalently immobilizing a cyclodextrin to the desired chromatographic support [11,112]. Recently, immobilization strategies for β-cyclodextrin using click chemistry [298-302], monolithic supports [303], hybrid chromatographic supports [304,305], and light-assisted preparation of the CSP [306] have also been reported.

CSPs that are based on derivatized β-cyclodextrins may suffer from a loss of enantioselectivity due to exhaustion of hydroxyl groups that were originally present on the rim of the cyclodextrin or due to the steric hindrance caused by a large number of substituents at or near the rim of the cyclodextrin [307,308]. Several new forms of cyclodextrin supports, or studies of current cyclodextrin materials, have been recently reported to overcome these disadvantages. For example, a bridged bis(β-cyclodextrin) has been utilized as a new ligand for chiral separations and has been noted to possess strong hydrogen bonds, synergistic inclusion effects, and good molecular assembly, which can lead to improved chiral separations [307,308]. This type of stationary phase consists of two β-cyclodextrin units that are connected together by a linker such as ethylene diamine dicarboxyethyl diamido or stilbene diamido, which have been proposed to provide additional binding regions by creating pseudo-cavities [307,308]. A chiral mixed-mode stationary phase has also been prepared by grafting ionic liquids with β-cyclodextrin and C18 on silica; these materials have been found to exhibit retention based on reversed-phase, hydrophilic, and ion-exchange mechanisms [309].

The methods of peak profiling and peak fitting, respectively, have been used to study the kinetics and thermodynamics of interactions by immobilized β-cyclodextrin with various solutes [310-313]. For example, peak profiling based on both single- and multi-flow rate methods was used to measure the apparent dissociation rate constants for various drugs with immobilized β-cyclodextrin [310,311]. Peak fitting was used under non-linear conditions to determine the binding and rate constants for acetaminophen, trimethoprim, ketoprofen, indapamide, and uracil with immobilized β-cyclodextrin [310].

Macrocyclic oligosaccharides such as aliphatic and aromatic cyclofructans (CFs) have also been utilized in chiral separations [281,314]. These compounds are classified based on the number of fructose units they contain in their structure, which examples being cyclofructan 6, cyclofructan 7, and cyclofructan 8 [281,314]. These cyclofructans are either partially derivatized into an aromatic or aliphatic form and coupled to silica or they are first combined with silica support and then derivatized into one of these forms [281,314-318]. Click chemistry has also been used to prepare cyclofructan CSPs [319].

Another type of carbohydrate-related agent that has been used in affinity separations is heparin. Heparin is a glycosaminoglycan that is structurally related to heparan sulfate and is an important receptor for virus attachment in mammalian cells [320]. This property also means heparin can be used to bind to viral particles [320]. Based on this ability, heparin has been employed in affinity chromatography for the purification of porcine reproductive and respiratory syndrome viral particles, and human papillomavirus tyle 16 L1 protein from *Saccharomyces cerevisiae*, as well as the separation of HIV-1 gag viral like particles and extracellular vesicles [320-322].

4.1.8 Lipids

Lipids are another class of biological ligands that have been employed in affinity chromatography and related separation methods. Phosphatidylcholine is a phospholipid that is extensively employed in applications that involve lipid supports, such as immobilized artificial membrane chromatography and immobilized liposome chromatography [3,11]. Phosphatidylcholines are glycerophospholipids that contain a glycerol backbone, two fatty acids coupled together by ester linkages, and a choline head group [323,324]. Other lipids such as sphingomyelin and cholesterol have also been employed in immobilized artificial membranes and other supports that have been used in separations [3,11,325]. Sphingomyelins are based on ceramide and consists of a long-chain sphingoid base with an amide-linked acyl chain and a phosphorylcholine head group [323,324]. Cholesterols are sterols that are contain a four-ring hydrocarbon structure [323,324].

Immobilized artificial membrane chromatography, or IAM, is one format in which lipids have been employed as affinity ligands to study membrane protein-substrate interaction and membrane-drug interactions [311,326]. The stationary phase in IAM is prepared by covalently immobilizing a monolayer of phospholipid analogs with functional head groups, which are attached to silica or a polymeric monolithic support. This type of support creates a hydrophobic environment for the adsorption and immobilization of transmembrane proteins such as receptors, transporters, ion channels, and enzymes [326-336]. Solubilized cellular membrane fragments can be immobilized on IAM supports through entrapment or adsorption after dialysis [326-336]. For example, voltage-dependent anion channel isoform 1 (VDAC-1) has been immobilized on lecithin functionalized silica gel for the screening of anti-cancer compounds found in traditional Chinese medicine [330]. This receptor support was also used to study the interactions of VDAC-1 with

ligands such as NADH, ATP, and NADPH [330]. IAM has further been used to study the partitioning of drugs into lipid monolayers to predict drug absorption and cell permeability [337-339].

Immobilized liposome chromatography, or ILC, is a type of affinity chromatography in which lipid bilayers or proteoliposomes are immobilized on a support through non-covalent steric, electrostatic, hydrophobic, or covalent interactions [340-347]. The liposome membrane is composed of self-aggregates of a structured phospholipid with charged choline or phosphate groups, a carbonyl group, and a hydrophobic environment; the result is considered structurally similar to a cell membrane [121,348-350]. Proteoliposomes can act as biomimetic membranes to reconstitute membrane proteins [121,348-350]. For instance, a detergent-solubilized membrane protein, photosynthetic reaction center (RC) from *Rhodobacter spaheroides*, was functionalized with a biotinylated lipid and immobilized on streptavidin beads to study the interactions by RC to c-type cytochromes [350]. ILC has also been applied to study the interactions of drugs with membranes and to screen for membrane penetrable compounds [340-347]. As an example, liposomes immobilized in capillary columns containing silica monoliths have been utilized to separate acidic, basic, and neutral drugs [346]. Liposomes immobilized on silica microspheres have been employed for screening of bioactive compounds in some types of traditional Chinese medicine [340]. A support containing *N*-hexadecyl iminodiacetic acid was used to adsorb Cu^{2+} on liposomes for the purification of his-tagged proteins [351]. Lipid-metal ion interactions have also been studied using ILC [352,353], as has been demonstrated in the use of a zwitterionic lipid immobilized on non-porous Stöber silica particles to examine interactions of the lipid with group I and II metal ions [352].

Lipids can also be employed as biomimetic membranes in the form of lipodiscs, nanodiscs, and lipid rafts [130,321,322,351]. Lipodiscs are made up of a circular planar bilayer that is usually stabilized by lipids modified with polyethylene glycol head groups; these lipodiscs can then be covalently immobilized on diol silica [354]. The intra- and extra-cellular sides of the lipodiscs are exposed to the bulk solution; this means that when the lipodiscs are functionalized with membrane proteins, the active site of the membrane protein will be exposed to the surrounding environment [354]. In one recent report the integral membrane protein human aquaporin-1 was immobilized on lipodiscs and used in weak affinity chromatography for fragment screening of integral membrane proteins [354]. Nanodiscs are composed of self-assembled bilayers of phospholipids and a membrane scaffold protein (e.g., from human serum apolipoprotein A1) [130]. The nanodiscs have been immobilized on monoliths modified with streptavidin and by using a biotinylated membrane scaffold protein (adenosine A_{2A} receptor) for the detection of G protein-coupled receptor-bound weak affinity fragments [130]. Lipid rafts consist of microdomains that are enriched in dynamic assemblies of sphingolipids or cholesterol, as have been used on silica for the screening of anti-cancer agents in traditional Chinese medicine [324,325].

4.1.9 Nucleic Acids

Another set of biological binding agents that have been used in affinity chromatography are nucleic acids [355-364]. For instance, DNA is used as the affinity ligand in the method of DNA affinity chromatography to bind and isolate DNA-binding proteins [355-362]. Examples of proteins that bind DNA and that have been purified or isolated by this approach include helicases, restriction enzymes, polymerases, primases, topoisomerases, teleomerases, DNA repair proteins, histones, and transcription factors [355-359,363,364]. The binding agent that is used in this approach may be either a general preparation of fragmented nuclear DNA or a specific section of

DNA. A general preparation would be used to separate bind DNA-binding proteins from sample components that are not able to bind DNA [355]. A specific section of DNA would be employed as the ligand in a situation where the goal is to bind a target that has a specific interaction with the selected DNA section or sequence [355,364].

Another group of ligands that are typically based on nucleic acids are aptamers [365]. Aptamers are synthetic oligonucleotide sequences (i.e., DNA and RNA) that are tailored to bind to specific target molecules [365-367]. These targets may include proteins, nucleotides, viruses, or small molecules. Aptamers are often generated through a selection process called as the systematic evolution of ligands by exponential enrichment (SELEX) [365-370]. SELEX is a technique that can be used to screen for and amplify (e.g., the polymerase chain reaction) a specific oligonucleotide sequence that has high affinity toward a desired target, beginning with an initial large library of potential binding agents [365].

In affinity chromatography, an aptamer can serve as a stationary phase by immobilizing this agent onto a column and using this to interact with a particular target analyte [366,370]. Target recognition with this type of binding agent is based on the unique three-dimensional structure of the aptamer, which can form pockets and interaction regions with relatively high affinities and good specificities [368,369,371]. These advantages, along with the ability to generate aptamers by synthetic means and through combinatorial methods, have made this group of affinity ligands attractive in recent studies as alternatives to antibodies [372-375].

Aptamers has been used as binding agents in columns to bind both small and large targets [376-386]. Small molecules like mycotoxins and aflatoxin have been purified and enriched by aptamer affinity chromatography [379,381-383,386]. Columns containing aptamers have also been used to isolate recombinant proteins and therapeutic monoclonal antibodies [377,379,384].

Monolith columns containing aptamers have been described [376,378,383,386], and aptamers have been combined with multi-walled carbon nanotubes and gold nanoparticles for protein purification [376,380].

4.2 Non-biological Agents as Affinity Ligands

A variety of non-biological binding agents are also often considered to be stationary phases that fall within the field of affinity chromatography (see Table 3). Examples of these binding agents are boronates, dye-ligands, immobilized metal ion chelates, and molecularly imprinted polymers [1,3]. This section will examine the basis behind the use of each of these binding agents as affinity ligands and recent developments that have occurred with these stationary phases.

4.2.1 Boronates and Related Mixed Ligands

Boronates have emerged as a useful and reliable set of affinity ligands for the selective recognition, separation, and enrichment of glycoproteins, nucleosides, catecholamines, and other cis-diol containing compounds [387-390]. Targets that contain cis-diol groups are often important in fields such as glycomics, cancer-cell targeting, and disease diagnostics [391-398]. The principle of boronate affinity chromatography (BAC) is based on the covalent formation of a cyclic ester when boronic acid or one of its derivatives reacts with a cis-diol-containing molecule. This reaction usually takes place when the pH of the reaction environment is greater or equal to the pK_a of the boronate that is used as the binding agent. This cyclic ester dissociates when the pH of the environment is below the pK_a of the binding agent, which allows for elution and release of the captured target [387-390].

Some common boronates that are used as ligands in BAC are 3-aminophenylboronic acid and 4-vinylphenylboronic acid, which have pK_a values of 8.8 and 8.2, respectively [389,390].

However, use of these boronate ligands is hampered in terms of weak binding properties they have at the typically more neutral pH of biological samples [389,390,399-402]. This has given rise to the synthesis and use of new boronates with lower pK_a values, such as Wulff-type boronic acids, benzoboroxoles, phenylboronic acids with electron-withdrawing groups (e.g., sulfonyl, fluoro, or carbonyl groups) on the phenyl ring, and heterocyclic boronic acids [90,390,403-417]. These boronic acid ligands have been shown to have enhanced binding at neutral or acidic pH values than conventional boronates, leading to improved binding strength and extraction efficiency [390,403,418-420].

Boronate ligands and BAC have been used in many recent applications [402]. Examples are the use of boronates and BAC for the enrichment, selective separation, and analysis of nucleosides, flavone, glycoproteins, glycopeptides, carbohydrates, and other cis-diol containing compounds. These applications have been carried out with such samples as urine, serum, and other biological samples that could be processed under acidic or neutral pH conditions [402,404-407,410,415-418,421-431].

Mixed affinity ligands based on boronates plus other binding agents have also been used for the separation of biomolecules in affinity chromatography. One example is the combination of a boronic acid and lectin to give a method known as boronic acid-lectin affinity chromatography, which is also referred to as BLAC or BAD-lectin affinity chromatography [432–435]. This combination overcomes the difficulty of using boronic acids to differentiate different glycoforms, as can be achieved by adding a lectin to the same stationary phase [432,435]. Although the number of lectins that have been used in this approach is still limited, these binding agents have been used with boronates to purify, with high specificity, some classes of glycans with cis-diol groups with high specificity [435,436]. The stationary phase in this method is often prepared by mixing a

support that contains an immobilized lectin with another support that contains a boronate [433,435]. BLAC is usually used for comparative glycosylation profiling. For example, a BLAC column was used to isolate and enrich *N*-linked glycans labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) from prostate cancer patients [433] and *N*-linked glycopeptides from HeLa cells [435].

4.2.2 Dye-ligands

A dye-ligand is another type of synthetic binding agent that has been used in affinity chromatography [365,437,438]. This combination is sometimes called dye-ligand affinity chromatography, which is a type of biomimetic affinity chromatography [365]. A common example of a dye-ligand is Cibacron Blue 3GA. Other examples are Procion Red HE-3B and Procion Yellow H-A [365,437]. Dye ligands typically consist of two parts: a portion that binds to the given target and a reactive group for covalent attachment of the dye to a support [365,437,438].

The earliest application of a dye-ligand for affinity chromatography was the use of Blue Dextran for enzyme purification [439]. Dye-ligands are now commonly used in columns for protein and enzyme purifications due to the binding ability of such ligands to interaction with a variety of proteins and the ability to modify the structure of the dye-ligand to alter its specificity [437,438,440,441]. The modification of a dye-ligand and the design of a derivatives for a given target can be aided by using computational chemistry [440]. Recent reported applications of dye-ligands as binding agents in affinity chromatography have involved the use of these agents for purification of fucosidan, lactoferrin, and lactoperoxidase from food by-products and biological matrices [442-445].

4.2.3 Immobilized Metal-ion Chelates

Metal ions held in the form of immobilized chelates are another type of non-biological binding agent that has been employed in affinity chromatography [3,446]. This method is known as immobilized metal-ion affinity chromatography (IMAC) [3,446]. IMAC is based on the interaction between chelated metal ions and residues on some amino acids (e.g., cysteine, histidine, and tryptophan) that can act as electron donators [446-448]. In IMAC, a chelating agent is immobilized onto a material such as silica, cellulose, agarose, or a cryogel and used to hold metal ions through coordination bonds [446-451].

Selection of the proper chelating ligand and metal ions can affect the stability of this metal-chelating agent complex and the resulting retention of targets that bind to this support. Iminodiacetic acid (IDA), nitrilotriacetic acid (NTA), carboxymethylated aspartic acid (CM-Asp), diethylene triamine pentaacetate (DTPA), and tris(carboxymethyl) ethylene-diamine (TED) are some examples of chelating ligands that have been used in IMAC [446-448,450]. Metal ions like Ni^{2+}, Cu^{2+}, and Zn^{2+} are typically used to bind targets that contain nitrogen, oxygen or sulfur atoms that are accessible. On the other hand, metal ions such as Cu^{+}, Ag^{+}, Pd^{2+}, Cd^{2+}, and Hg^{2+} can be used to bind targets that contain sulfur [447,449,450,452].

IMAC has been employed in the selection of DNA aptamers and in protein purification for targets such as Fab fragments, monoclonal antibodies, and phosphopeptides [451,453-465]. In proteomics, IMAC has taken on an important role in the enrichment and extraction of phosphopeptides from samples [454-458]. Some recent platforms that have been reported include the use of IMAC with magnetic microspheres, *O*-carboxymethyl chitosan Schiff base complexes, and capillary-channeled polymer fibers [458,463-465]. Hybrid methods that combine IMAC and

capillary electrophoresis (CE) or that use multi-dimensional IMAC to purify analytes from a complex sample have also been described [459,466].

4.2.4 Molecularly Imprinted Polymers

Molecular imprinting is an alternative strategy for preparing supports and stationary phases for affinity chromatography [467-469]. This technique makes use of a support that has binding pockets prepared within it for binding recognition with the desired target [469]. A support that is prepared in this way is often known as a molecularly imprinted polymer (MIP) [467]. An MIP is often made by either a covalent or noncovalent technique. In the noncovalent imprinting approach, the target analyte or template is mixed with a functional monomer and cross-linking agent. After this mixture is allowed to polymerize, the template or target is washed away, leaving behind binding pockets that have the correct shape and arrangement of functional units to bind to the desired target [467-469]. In covalent imprinting, the target molecule is covalently coupled to a functional monomer that is combined with the rest of the polymerization mixture. After polymerization, the target is cleaved away by a chemical means, leaving being a cavity that can bind the target molecule [467,469,470]. Ionic imprinting can also be utilized, in which metal ions act to enhance binding by the target analyte and functional monomer in an aqueous solution [467,469,470].

Molecular imprinting has been used in many reports to make supports for chiral separations; however, it has also been widely used in solid-phase extraction and some chromatographic techniques [467-479]. For example, a MIP support has been used for solid-phase extraction and coupled with ligand exchange chromatography to screen for chiral drugs in urine [475]. MIPs have also been employed in many studies for the binding and isolation of low-mass

targets and have developed for the use with larger molecules such as proteins in applications such as diagnostics, drug delivery, environmental analysis, and proteomics [467-482].

5 Applications of Affinity Chromatography

The applications of affinity chromatography have grown over the course of the last decade [3]. These applications have included the utilization of this method for the isolation of various chemicals and biochemicals, as well as the use of affinity chromatography for sample pretreatment or analysis [1-8]. There has also been continued work in using affinity-based separations as tools to study biochemical interactions [1,3]. As shown in Figure 3, fields in which one or more of these formats have been employed include biochemistry and biochemical research, molecular biology, biotechnology, microbiology, cell biology, immunology, analytical chemistry, pharmacology or pharmaceutical sciences, and biophysics [1-8]. Other areas of chemistry and related fields in which affinity chromatography has been used are clinical chemistry, environmental chemistry, and food science [1,3]. This section will examine the recent developments that have taken place in applications of affinity chromatography and the use of this method in these various areas.

5.1 Preparative Applications

One of the most popular uses of this technique has continued to be in the purification of biological ligands. A variety of binding agents can be used for this purpose. Examples are immunoglobulin-binding proteins, antibodies, and antigens, as discussed earlier in Section 4.1 [221-229].

5.1.1 Enzyme and Protein Purification

The separation and purification of enzymes have long been an important application of affinity chromatography [3,438,439]. Examples based on triazine dyes as affinity ligands are the

use of Cibacron Blue F3GA to isolate microbial cellulase from rumen liquor [483], microbial xylanase from rumen liquor [484], and proteases from pancreatic and stomach extracts of fish by-products [485]. Hemicyanine dye has been used in a similar manner to isolate peroxidase from a chewing stick [486], and aminosquarylium cyanine dye has been employed for the purification of lysozyme, α-chymotrypsin, and trypsin [487]. Azo dyes such as Reactive Orange 4 were utilized for the purification of malate dehydrogenase from a yeast cell homogenate [42].

IMAC can also be employed for the separation of enzymes. This method is based on the interactions between a metal ion conjugate such as Cu^{2+}- or Zn^{2+}-iminodiacetic acid that is placed onto a chromatographic support and used to bind to histidine or cysteine residues [488-490]. In recent work, a support containing Cu^{2+} complexed with iminodiacetic acid was employed for the isolation of metalloprotease from a marine bacterium and for the isolation of his-tagged chitinase [488,489]. In a similar manner, Zn^{2+}-iminodiacetic acid has been utilized in an affinity method for the capture of camel liver catalase [490].

Coenzymes and enzyme inhibitors are additional affinity ligands that have utilized for the purification of enzymes [25,181,491-494]. 4-Aminobenzohydrazide and its derivatives have been used as enzyme inhibitors for the purification of plant peroxidase enzymes from red cabbage and radishes [492,493]. In addition, the enzyme inhibitor β-lactosylamidine has been utilized for the purification of a cellulolytic β-glucosidase mixture of endo-acting endo-β-(1→4)-glucanase I and exo-acting cellobiohydrolase I from *Hypocrea jecorina* [491]. Sulfanilamide has been recently used as an inhibitor-based binding agent for lactoperoxidase in milk [181]. Riboflavin, a coenzyme which is flavin adenine dinucleotide (FAD)-dependent, was immobilized on Sepharose beads and used to isolate cholesterol oxidase, a FAD-dependent enzyme expressed in *E. coli* [494].

Affinity chromatography is also an important tool for the purification of other types of biomolecules [1,2,4-8]. This includes the utilization of affinity chromatography for the small- and large-scale purification of native proteins and recombinant proteins. Dye-ligand affinity chromatography are related methods are often used for this purpose [1,2,4-8,365,438,440,495,496]. Smaller scale applications of affinity methods for purification include the use of IMAC and Ni^{2+} chelate columns for the isolation of his-tagged proteins and the capture of antibodies or specific antigens by immunoaffinity columns [207,208,210,220,446,448,497,498].

5.1.2 Purification of Viral Particles, Cells, and Related Targets

Affinity ligands attached to polysaccharide supports such as agarose or cellulose have been employed for the purification of viral particles and related agents [320]. Heparin is one ligand that has been covalently immobilized on agarose beads for the purification of whole viruses and virus-like particles [320-322]. Con A and camelid antibody fragments have both been used as binding agents for the purification of recombinant virus vectors in gene and cellular therapy [499,500]. Affinity chromatography utilizing Cellufine sulfate as a binding agent has been used for the isolation of Japanese encephalitis virus derived from Vero cells, as well as for purification of the whole virus produced in Vero cell cultures for development of a yellow fever vaccine [501-505].

The interactions between metal ion chelates and amino acid residues in IMAC has been used in several reports for applications related to viruses and viral proteins. This approach has been employed for the purification of his-tagged nucleocapsid and matrix proteins of the Nipah virus [506,507]. This method has also been used to isolate his-tagged recombinant viral vectors that are related to gene therapy [508] and the virus responsible for foot-and-mouth disease virus for use in vaccine formulation [509]. IMAC has further been used to purify the Hepatitis B viral

protein HBcAg from *E. coli* cell lysates [510,511]. Cu^{2+}-based IMAC has been employed for the inactivation of viruses during the manufacturing process of plasma coagulation factors such as plasma derived coagulation factor VIII and coagulation factor IX (Replenine-VF) [512,513].

Antibody binding proteins and antibodies attached to agarose-based supports have been both used to examine viral clearance [320,514-517]. For example, native protein A [516] and alkali-stabilized recombinant protein A [515] have been employed for clearance studies involving endogenous retroviral particles that are present in antibody feedstock. Monoclonal antibodies to recombinant coagulation factor VIII have been used for the purification of antihemophilic factor VIII compounds [513,518-520]. The latter approach has also been used in a mixed-mode resin to extract and purify a recombinant factor VIII with a truncated B-domain [521].

Cell affinity chromatography is a recent form of bioaffinity chromatogrpahy that has been used to isolate and separate certain cells with high purity [22,522,523]. Cell separations in this technique are carried out based on the interaction between binding agents such as lectins or monoclonal antibodies with cell surface markers such as glycoproteins and receptors [522-524]. Some supports that have been used in cell affinity chromatography are magnetite and several forms of monoliths [524-527]. The separations of cell subsets with a high purity and on a small scale has been accomplished by combining with a microfluidic device with the use of aptamers as binding agents [528-532]. Cell affinity chromatography has also been recently employed for counting cancer cells in samples, examining microbial interactions with specific glycans, and following the viral infection of cells [525,530-533].

5.2 Analytical Applications

Many of the same binding agents have made affinity chromatography a useful tool in analytical applications employed for sample pretreatment and preparation [3,11,219,534-536].

Most of these applications employ the on/off elution mode of affinity chromatography, as shown earlier in Figure 1, due to the relatively simplicity and speed of this approach [3,5,219]. However, isocratic elution is also used when the interactions involved have weak or moderate binding strengths, as occurs in weak affinity chromatography. This second situation is commonly used in the use of affinity chromatography in chiral separations, as discussed in the next section.

One way in which affinity chromatography is often used for chemical analysis is as a tool for sample pretreatment [1,3,219,534-536]. For example, MIPs have been employed in solid-phase extraction as a means of sample preparation for the analysis of various target compounds [211,473,475]. Columns and support that contain antibodies have been used in many reports for the immunoextraction of a specific target from a mixture prior to the use of a second analytical method such as HPLC, gas chromatography (GC), mass spectrometry, or CE [208,210,219]. These techniques, which are a type of immunoaffinity chromatography, have been used for examining targets in a variety of clinical, pharmaceutical, and environmental samples [210,219,537]. A related application of affinity chromatography is immunodepletion, in which a set of specific agents are removed from a sample prior to the analysis of other sample components [46]. Immunodepletion has been used in a recent studies as a means for removing proteins that occur at a high abundance prior to analysis of lower abundance proteins [46,537-539].

It is also possible to use affinity chromatography to directly measure a particular target in a sample [3,219]. This is often done by using affinity chromatography as part of an HPLC systems, and often in the form of HPAC. Binding agents have been used for this purpose are antibodies/antigens, immunoglobulin binding proteins (e.g., protein A or protein G), immobilized metal-ion chelates (i.e., IMAC), boronates, and lectins [534-536]. Direct detection of the captured and eluted target can be carried out in these methods if this target is present at a sufficient

concentration to allow it to be monitored by its absorbance or fluorescence, by using mass spectrometry, or by employing a post column reactor for detection [208,219]. Affinity columns have further been employed in multi-dimensional methods by combining them with reversed-phase liquid chromatography, CE, or GC [219].

Targets that are present at low concentrations can be detected by using affinity chromatography with indirect detection. This strategy approach is often employed when affinity columns are used with antibodies or related binding agents to create a chromatographic immunoassay [208,210,219]. For example, a target analyte may be required to compete with a labeled analog for binding sites on an immunoaffinity column that contains a limited amount of immobilized antibodies for the target and analog. This creates a situation in which the amount of labeled analog that is captured will be affected by the amount of target that was present in the sample, thus providing a means for indirectly determining the sample concentration for the target [208,210,219]. Many formats have been described for chromatographic immunoassays. These formats include traditional competitive based methods, as well as displacement assays, sandwich assays and one-site immunometric assays [210,217,219,540-544].

5.3 Chiral Separations

Chiral separations are another important application of affinity chromatography [3,11,220,280-284]. Polysaccharides such as amylose and cellulose have been used as CSPs to carry out enantioseparation of various chiral drugs and solutes [3,112,280,282,283,285,286]. Examples that have used amylose-based CSPs have included the chiral separation of chemopreventive chiral isothiocyanates, pesticides, β-amino acid derivatives, lysine derivatives, coumarin derivatives, butyrolactones, and fungicides [545-551]. Other applications have included sulfoxide-containing drugs, anti-histamines, simendan, piperine-2,6-diones, local anesthetics,

calcium channel blockers, clausenamidone/neuclausenamidone, anti-cholinergic drugs, and various profens [547,552-558]. Additional examples have used cellulose-based CSPs in HPLC for the separation of sulfoxides, alkaloid analogs, β-blockers, local anesthetics, β-amino acid derivatives, anti-fungal drugs, and psychoactive drugs [548,555,559-564].

Various chitin and chitosan derivatives have been used as CSPs [281,282,288-295]. These stationary phases have been used for the chiral recognition of solutes such as benzoin, triazole fungicides, Troger's base, and methylphenyl sulfoxide [288,289,291-294]. This class of CSPs has further been employed in enantioselective separations of voriconazole, glutethimide, citalopram hydrobromide, 1-phenyl ethane-1,2-diol, and flavanone [288,289,291-293].

As discussed in Section 4.1.7, derivatives of β-cyclodextrin have often been exploited in affinity chromatography and HPLC systems to carry out chiral separations [11,112,282,283,296,297]. Cationic β-cyclodextrin CSPs prepared by click chemistry have recently gained popularity for the chiral selection of analytes such as β-blockers, racemic β-nitro ethanol derivatives, profens, amino acid derivatives, flavonoids, aromatic alcohols, acidic drugs, benzene homologues, and isoxazolines [298-302]. A hybrid support prepared by coupling β-cyclodextrin with piperidine, L-proline, and an ionic liquid with 3,5-diamino-1,2,4-triazole has been used to separate pyrrolidine and several chiral drugs [304]. Another hybrid support made by hybridizing β-cyclodextrin with ethane, triazinyl, and 3,5-dimethyl phenyl functional groups has been applied for enantioselective separation of anilines, phenols, aromatic hydrocarbons, phenols, and acidic compounds [305].

Several variations on cyclodextrin-based CSPs and related stationary phases have been described in recent reports. β-Cyclodextrin immobilized on monolithic supports has been employed for separation of β-blockers, α-blockers, anti-fungal drugs, catecholamine, sedative

hypnotics, anti-arrhythmic drugs, and antihistamines [303]. β-Cyclodextrin CSPs prepared by light assistance have been utilized for separating promethazine, benzoin, and chlortrimeton [306]. Bridged bis (β-cyclodextrin)-based CSPs have been used for the separation of chiral drugs, pesticides, and triazole fungicides [307,308]. A mixed-mode stationary phase based on β-cyclodextrin has been utilized for the enantioseparation of warfarin, styrene oxide, and 1-phenyl-1-propanol using reversed-phase elution conditions, while profens such as ibuprofen and ketoprofen were separated by this material by employing a polar organic elution mode [309]. Aromatic derivatives of the macrocyclic oligosaccharide cyclofructan has been utilized for the chiral recognition of analytes such as warfarin, furoin, Troger's base, 2-napthol atropisomers, and novel spirobassinin analogs [315-318]. A cyclofructan 6 CSP prepared by click chemistry has been employed for the chiral selection of amine and alcohol derivatives [319].

Proteins such as serum proteins and enzymes have also been employed as CSPs [3,11,112,283]. Serum proteins such as HSA [68,565,566] and AGP [567-570] have been immobilized on silica particles or monoliths to study the interactions of these serum proteins with chiral solutes. For instance, AGP from chicken egg whites was covalently immobilized on aminopropyl silica for the chiral recognition of benzoin, ibuprofen, chlorpheniramine, propranolol and oxprenolol [570]. An affinity support that contained HSA covalently immobilized to silica and modified with the cross-linking agent bis-maleimidohexane was prepared and examined for its chiral selection of drugs such as warfarin, verapamil, and carbamazepine [566]. A related serum protein, BSA, has been used as in a mixed-binary CSP that was prepared by grafting BSA and the antibiotic eremomycin for chiral separation of various profens [571].

The ability of enzymes such as penicillin G acylase, glucoamylase, and cellulase to act as CSPs was discussed in Section 4.1.4 [243-247]. Recently, lysozyme was covalently immobilized

to mesoporous polyimide achiral covalent organic frameworks for the chiral separation of various amino acids and drugs, such as threonine, leucine, tryptophan, ofloxacin, metoprolol, and chlorpheniramine [572]. Pepsin has been covalently immobilized within silica monoliths and used to carry out a chiral separation for the analgesic nefopam [573].

As mentioned in Section 4.2.4, MIPs are another type of material that have frequently been explored for use as CSPs [283,574]. Various polymerization techniques have been applied for the preparation of MIP-based CSPs. Examples of these techniques are imprinting in performed beads, precipitation polymerization, suspension polymerization, multi-step swelling and polymerization, and surface imprinting [575-582]. Supports with which MIPs have been combined for chiral separations include from organic polymer-based particles, monoliths, membranes, and alignate monospheres [583-590]. For example, a MIP-based CSP using monodisperse hybrid silica microspheres was prepared with L-phenylamine as the template, methacrylic acid and acryloyl-β-cyclodextrin as the functional monomers, and ethylene glycol dimethacrylate as the cross-linker; this support was then used to separate a racemic mixture of phenylalanine [583]. Another MIP based on a monolithic column and using 4-vinylpyridine as the functional monomer, ketoprofen as the template, and ethylene glycol dimethacrylate as the cross-linking agent, was used for the chiral separation of ketoprofen [587].

5.4 Biointeraction Studies

Affinity chromatography can also be employed as a tool for biophysical applications such as in solute-ligand interactions. In this technique, useful information on equilibrium and rate constants, and number and types of affinity sites can be obtained [11,13,14,16]. Advantages of using this approach in such a way, especially when carried out on an HPLC system, are the speed, precision, and ease of automation that can be obtained [14]. Methods that have been used for this

purpose, as discussed in this section, are zonal elution, frontal analysis, and various techniques for kinetic studies [13,14,16].

5.4.1 Zonal Elution Methods

Zonal elution is an approach that has frequently been used in affinity chromatography to characterize the strength of solute-ligand interactions [3,11,46]. In this approach, a small volume of an analyte or probe compound is introduced into an affinity column that contains an immobilized ligand [11,46]. The mobile phase used for the elution of the analyte or probe often contains a possible competing agent that is present at known concentration; the pH, ionic strength, polarity or temperature of the mobile phase may also be altered, as desired [11,46,591,592]. Based on the retention time that is observed for the injected compound and the void volume of the column, the binding strength and type of interactions that are present on the affinity ligand for the injected analyte/probe and competing agent can be determined [3,11,46,591,592].

Besides being used to examine interactions of solutes with native serum proteins, zonal elution has been utilized in recent reports to study the interactions of drugs with modified forms of proteins such as HSA and AGP [13,162,163,592-597]. Zonal elution has also been employed with affinity columns for characterizing the interactions of Con A and AAL with AGP [48], the binding of protein G with rabbit IgG [137], and the binding of drugs such as acetaminophen and ketoprofen with β-cyclodextrin [310-313]. This method has also been used to investigate the interactions of drugs or bioactive compounds with epidermal growth factor receptor, β2-adrenoceptor, and α1A-adrenoceptor in cell membrane chromatography [138,598-601].

Various types of information that can be obtained from zonal elution experiments [11,46,591,592]. This information can include the equilibrium constant(s) for an interaction, the location of a given binding site (i.e., through the of site-selective probes), and the type of

competition that is occurring between a given set of solutes for the binding agent. The effect of pH, solvent composition, and temperature on the binding strength and type of interaction that is present can also be examined by this approach [11,46,591,592]. For example, zonal elution has been employed with affinity microcolumns to study the effects of glycation on the binding interactions of the sulfonylurea drug tolazamide with HSA [134]. In another study, a zonal elution format was used with an anti-AGP column and adsorbed samples of AGP to look at the changes in binding by various drugs with purified or normal AGP and AGP from patients with lupus [593]. Zonal elution was also used in a recent report to examine the competition between folic acid and 5-aminosalicylate for HSA [602].

5.4.2 Frontal Analysis Methods

Frontal analysis is another affinity chromatography technique that is employed to study solute-ligand interactions [3,11,46,260,592]. The most common way of carrying out frontal analysis is by using a series of analyte solutions that are applied separately to a column, and with washing steps with buffer being applied each solution [3,11,46,592]. In this format, the column is first equilibrated with the application buffer, with a known concentration of the target then being passed through the affinity column until a breakthrough curve is formed. The retained target is next washed from the column and this process is repeated using another application of the target at the same or different concentration [3,11,46,592]. If relatively fast association and dissociation is occurring between the target and affinity ligand during the time scale of this experiment, the central position of the breakthrough curves can be related to the corresponding concentrations of the applied target to produce a binding isotherm. This isotherm is then fit to one or more binding models to obtain the equilibrium constants and moles of binding sites that the immobilized binding agent and column have for the target [3,11,46,592].

A relatively wide range of targets and binding agents have been examined in recent reports by affinity chromatography and the traditional form of frontal analysis [603-624]. Many of these studies have involved the analysis of binding by drugs or solutes with serum proteins such as HSA and AGP [3,15,162,163,592,593,596,602,603]. This approach has been for studying the interactions of drugs with membrane protein and nuclear receptors, including voltage dependent anion channel 1, G-protein coupled receptors such as GPR17, nicotinic acetylcholine receptors, P-glycoproteins, β2-adrenergic receptors, dopamine receptors, and angiotensin converting enzyme 2 receptors [330-332,334-336,604-610]. Antibody-antigen binding [616,617] and fucose specific lectin-fucosylated glycan interactions [618] have been examined by frontal analysis using affinity columns. Additional systems that have been characterized by affinity chromatography and frontal analysis span from the binding of boronates with cis-diol compounds [611,612] to the binding of thrombin with phenolic acids [613] and the interactions of β_2-agonists with MIPs [614]. Frontal analysis has also been used to determine the complexation stability constant and total moles of binding sites for Cu^{2+} ions with regards to L-glutamic acid [615].

Another format that has been explored for frontal analysis is the stepwise application of target solutions to an affinity column [260,619-621]. In this technique, the column is again first equilibrated with the application buffer with no target present. This is followed by the continuous application of the target in solutions going from a from low to high concentration, producing a continuous breakthrough curve where a plateau is for each concentration and step in this process [260,619-621]. Because this process is continuous and does not require washing steps in between the applied solutions of the target, it can require less of the target than traditional frontal analysis. This last feature has made this method attractive for use in cell membrane chromatography [619,620]. For example, stepwise frontal analysis has been employed to obtain a dissociation

equilibrium constant for the interaction of α_{1A} adrenergic receptor with natural products and drugs such as dehydroevodiamine, tamsulosin, silodosin, oxymetazoline, and schizandrin A [601,619,620].

Frontal affinity chromatography coupled to mass spectrometry (FAC-MS) has become an important tool for the high throughput screening of drug candidates against immobilized membrane receptor protein targets [331,607,622-624]. In this technique, the breakthrough curve for an applied target is followed by MS as the target interacts with an immobilized binding agent [331,607,622-624]. There are two modes in which FAC-MS may be employed: the indicator mode and Q1 scan mode [331,607,622-624]. The indicator mode is based on how the presence of a competitor in the mobile phase causes a shift in the binding of an applied target with the immobilized binding agent [607]. For instance, FAC-MS has been used with adsorption energy distribution calculations to study the adsorption of salbutamol on β_2-adrenogenric receptor that was immobilized on polystyrene amino microspheres [607]. The shift seen in this breakthrough curve for salbutamol was then examined in the presence of mobile phases that contained competing agents such as paeoniflorin, liquiritin, and a mixture of six compounds to see if FAC-MS could be employed for screening of site-specific bioactive chemicals in a complex matrix [607]. The second mode in which FAC-MS can be used is the Q1 mode. In this mode, multiple targets are screened simultaneously against a given binding agent and are ranked based on their affinity for this binding agent when it is used as a stationary phase [331,624]. This mode has been employed for the screening of ligands against human estrogen receptor β and a G-protein coupled receptor [331,624].

5.4.3 Methods for Kinetic Studies

Affinity chromatography has been used to study the kinetics of various solute-ligand interactions [16,625-627]. The most common format for this work is to examine the retention or peak shape that is obtained for a target that applied or injected onto an affinity column that contains an immobilized form of the desired binding agent [16,625-627]. Examples of techniques for kinetic studies that make use of this general format include those that use band broadening measurements (e.g., the plate height and peak profiling methods) [202,310,311,313,628-632], peak decay analysis [137,633-635], the split-peak effect [636-638], and peak fitting [598,632,639-641].

Plate height method and related technique of peak profiling for kinetic analysis both make use of band broadening measurements [202,310,311,313,628-632]. These methods are based on application of a small volume of a target under linear elution conditions onto an affinity column and control column at one or several flow rates; the degree of band-broadening is then compared on these two types of column to obtain the contribution due to stationary phase mass transfer. This value, in turn, can be used with the known flow rate and measured retention factor for the target on the affinity column to determine the dissociation rate constant for this interaction process [628,629]. Peak profiling has been utilized to examine the interactions of various drugs with the serum proteins AGP and HSA [262,630,631] and with β-cyclodextrin [310,311,313]. This method has further been used to examine the interactions of membrane receptors with drugs and bioactive compounds [242,632].

Peak decay is another method that can be employed for kinetic analysis in affinity chromatography [137,633-635,641]. This method involves the application of a target solute onto both an affinity column and control column in presence of high flow rates and/or mobile phase conditions that prevent rebinding of the target as it dissociated for an immobilized binding agent.

This method can be performed in both competitive and non-competitive formats, depending on whether a competing agent is added to the mobile phase to promote target release and to avoid rebinding to the affinity column [137,633-635,641]. Peak decay has been employed with affinity chromatography for the analysis of various systems. Examples include studies of drug dissociation from serum proteins such as HSA and AGP [633-635], work examining antibody-antigen interactions [137], and studies of the interactions of β_2-adrenergic receptor with a number of drugs [641].

Another kinetic technique that employs an immobilized binding agent is the split-peak method. The split-peak method makes use of conditions in which a target binds irreversibly to the affinity ligand but, due to the short time allowed for this binding, a small fraction of the target also passes through the column as a non-retained peak [636-638]. The relative amount of target that is in the free vs retained fractions at various flow rates can be used to obtain information on the association rate constant for the target with the immobilized binding agent, or on the rate of stagnant mobile phase mass transfer in the column [633-635,641]. This technique has been used to study antibody-antigen interactions and the binding of protein A and protein G with immunoglobulins [636-638].

The peak fitting method is performed by injecting various concentrations of a target onto an affinity column and looking at the retention and shape of the resulting peak as the sample concentration is varied [598,632,639-641]. By fitting these peaks to various models, it is possible to obtain the rate constants for interactions by the target with the affinity ligands. Peak fitting has been used in both zonal elution and frontal analysis formats for studying the interactions of drugs with immobilized receptors such as β_2-adrenergic receptor and nicotinic acetylcholine receptor [598,632,639-642].

An alternative format for kinetic studies is to apply a small target in the presence of a soluble binding agent onto an affinity column which contains an immobilized binding agent for the target. This immobilized binding agent may be the same as the soluble binding agent, or a different ligand, and acts as a secondary probe to extract the unbound fraction of the solute from the sample [16,139-142,625-627,643]. This technique is known as ultrafast affinity extraction. This method makes use of a short time for extraction of the target by employing medium-to-high flow rates and small affinity columns. This approach has been used to provide information on both the thermodynamics and kinetics of the interactions between a number of drugs or hormones and serum transport proteins such as HSA, AGP, and sex-hormone binding globulin [139-142,643]. Both single-column systems and two-column systems have recently been described for ultrafast affinity extraction, with the single-column system being used for kinetic studies [139-141].

6 Conclusions

Affinity chromatography and related techniques based on supramolecular interactions have continued to expand and adapt as a tool for achieving selective separations and in examining the components of complex samples. A few examples that were discussed in this review included improvements in natural, inorganic, and synthetic supports for affinity chromatography; the use of magnetic particles, smart materials, and nanomaterials as new supports for affinity methods; and the continued development of support formats based on capillaries, packed beds, monoliths, and microscale separation formats. In addition, advances and new approaches have appeared in the development of new synthetic methods and tools, for covalent immobilization, biospecific adsorption, and non-covalent immobilization in affinity chromatography.

The variety of biological and non-biological binding agents that may be employed as stationary phases in affinity separations has also shown continued growth and development. Biological agents that are now often employed in affinity separations range from enzymes, antibodies, immunoglobulin-binding proteins, serum proteins, and lectins to biotin combined with avidin or streptavidin, carbohydrates, lipids, and nucleic acid-based ligands, including aptamers. Advances and on-going work have appeared as well in the use of non-biological ligands, such as methods that employ boronates, mixed ligands, dye-ligands, immobilized metal-ion chelates, or MIPs.

These advances in the components of affinity chromatography have been associated with improved or expanded capabilities that allow this method to be used for a broad set of applications. Affinity chromatography has remained an important separation method for the purification of enzymes, native proteins, and recombinant proteins. However, this method has also grown as an approach that can be used to purify and characterize viral particles, cells, and related targets. Many analytical applications have continued to appear as well, such as the use of affinity separations for sample pretreatment, the removal of specific solutes from a sample (i.e., as part of immunoextraction or immunodepletion), and for the direct or indirect measurement of a particular target in a complex matrix (e.g., by means of chromatographic immunoassays). A related use of affinity chromatography for chemical purification or analysis has been in the field of chiral separations. Finally, affinity-related separations have seen further advances in approaches by which they can be used to study biological interactions by means such as zonal elution, frontal analysis, or various formats that can be employed for kinetic studies. Overall, the variety of system components and formats that may be used in affinity chromatography and related methods should continue in the future to make these techniques popular and powerful separation and analysis tools

in fields such as analytical chemistry, biochemistry, clinical testing, environmental analysis, molecular biology, and pharmaceutical science.

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Figure Legends

Figure 1. A typical sample application and elution scheme used in affinity chromatography, the on-off elution mode.

Figure 2. General types of immobilization schemes used in affinity chromatography.

Figure 3. Common applications of affinity chromatography. This figure is, based on a search of the literature that was made with SciFinder in January 2019 for papers (46,335 total obtained) in the indicated areas and that were linked to the term "affinity chromatography". The figure is reproduced from Ref. [3] with permission from Elsevier.

Table 1. Types of supports used in affinity chromatography

General type of support	Examples of supports
Natural	Agarose, cellulose, dextran, agarose-chitosan composites
Inorganic	Silica, aluminum oxide, titania
Synthetic	Polystyrenes, polyacrylamides, polysulfones, polyamides, cryogels
Miscellaneous	Magnetic beads and particles (e.g., based on iron oxides)
	Smart materials (e.g., thermoresponsive polymers
	Nanomaterials (e.g., multi-walled and single-walled carbon nanotubes)

Table 2. Types of biological binding agents used in affinity chromatography

Type of binding agent[a]	Targets	Examples of applications
Antibodies & antigens	Complementary antigens or antibodies	Immunoaffinity chromatography; immunoextraction; immunodepletion; chromatographic immunoassays
Immunoglobulin-binding proteins (e.g., protein A, protein G, protein L)	Immunoglobulins, antibodies, and antibody fragments	Antibody purification and analysis; biospecific adsorption of antibodies
Lectins (e.g., Con A, WGA, AAL)	Glycoproteins, glycolipids and polysaccharides	Lectin affinity chromatography; glycan analysis; isolation of carbohydrate-containing targets
Enzymes	Enzyme substrates and inhibitors	Purification of enzyme inhibitors; chiral separations; immobilized enzyme reactors
Serum proteins (e.g., HSA, BSA, AGP	Various drugs and low mass hormones	Biointeraction studies; chiral separations

Biotin	Avidin or streptavidin	Capture or labeling of agents that contain avidin or streptavidin; biospecific adsorption
Carbohydrates	Targets that bind polysaccharides or cyclic oligosaccharides	Chiral separations; purification of agents that bind carbohydrates
Lipids	Targets that interact with lipids or binding agents contained within lipid structures	Immobilized artificial membrane chromatography; immobilized liposome chromatography; biointeraction studies; screening of compounds that bind to membrane receptors
Nucleic acids & aptamers	Targets that bind to general or specific structures or sequences within a nucleic acid or aptamer	DNA affinity chromatography; aptamer affinity chromatography; purification and isolation of DNA binding proteins; purification, enrichment or analysis so small targets that bind to aptamers

Table 3. Types of non-biological binding agents used in affinity chromatography

Type of binding agent[a]	Targets	Examples of applications
Boronates	cis-Diol containing compounds: glycoproteins, nucleosides, catecholamines	Boronate affinity chromatography; boronic acid-lectin affinity chromatography; biospecific adsorption
Dye-ligands	Various enzymes and proteins	Dye-ligand affinity chromatography; purification of enzymes and proteins
Metal-ion chelates	Various biological agents that bind to specific metal ions	Immobilized metal-ion affinity chromatography; purification of his-tagged proteins; enrichment and extraction of phosphopeptides
Molecularly imprinted polymers	Targets that are complementary to the binding pockets formed within the imprinted polymer	Solid-phase extraction; chiral separations

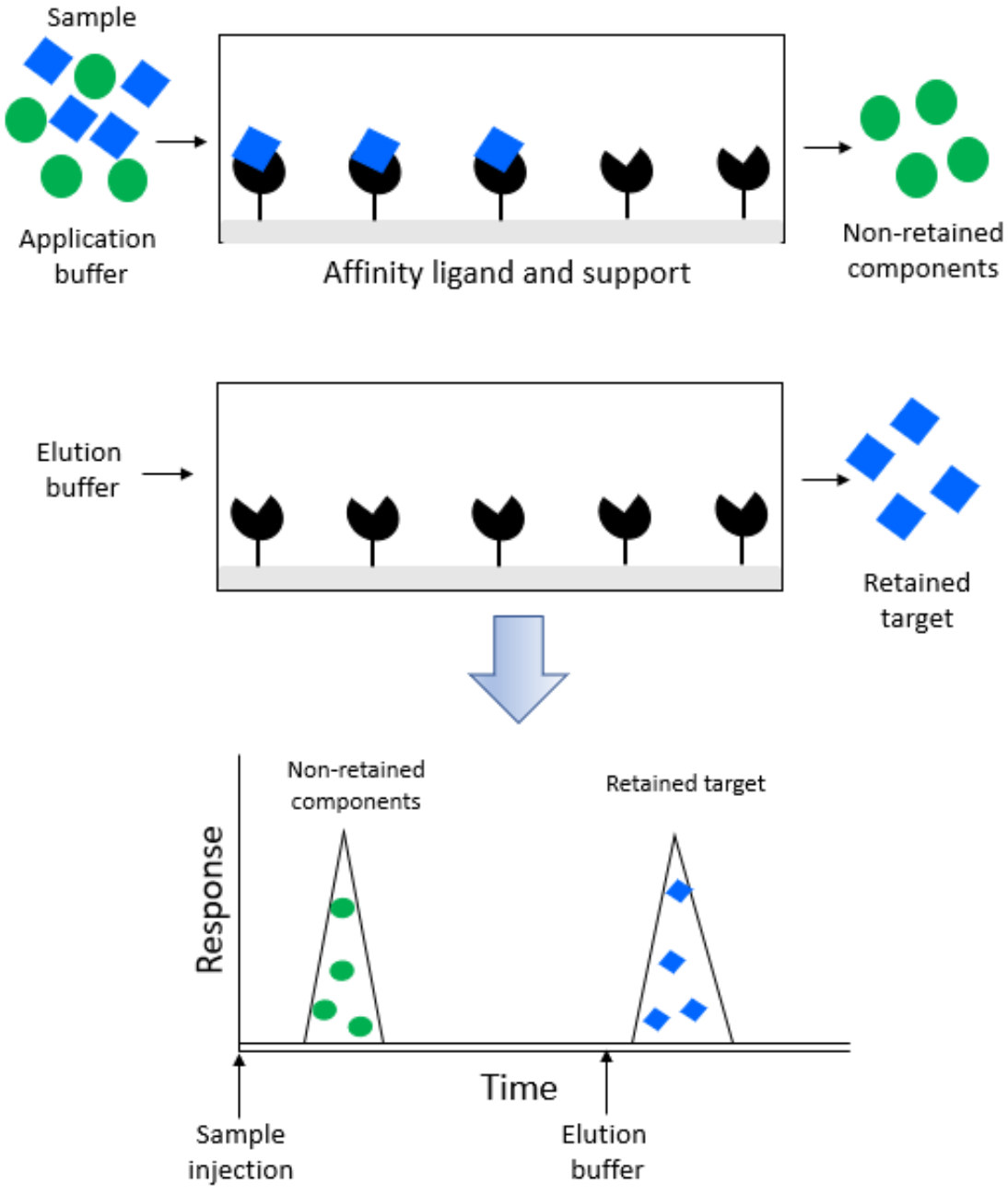
Sample
Application buffer
Affinity ligand and support
Non-retained components
Elution buffer
Retained target
Non-retained components
Retained target
Response
Time
Sample injection
Elution buffer

Figure 1

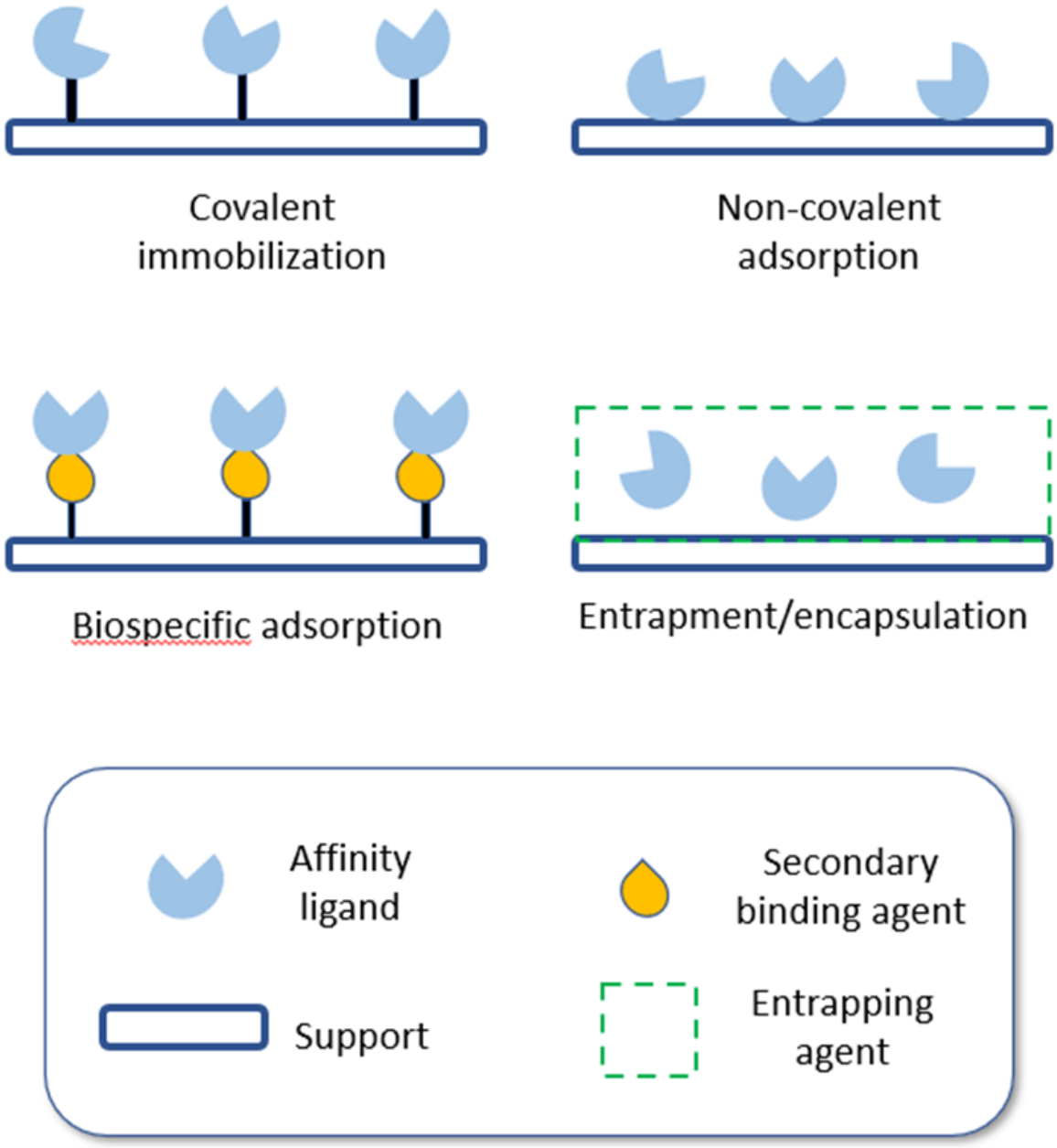
Covalent immobilization
Non-covalent adsorption
Biospecific adsorption
Entrapment/encapsulation
Affinity ligand
Secondary binding agent
Support
Entrapping agent

Figure 2

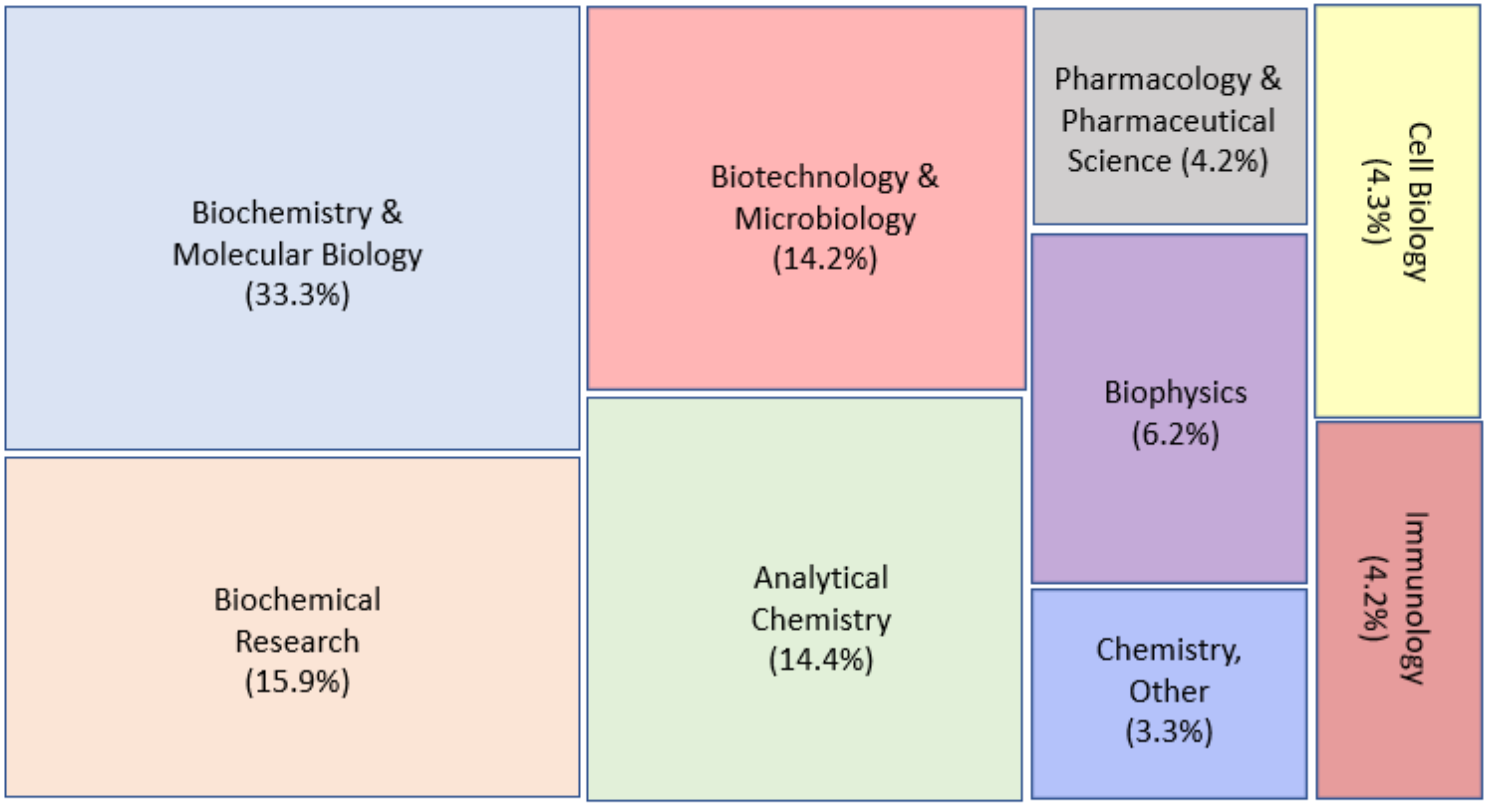
Biochemistry & Molecular Biology (33.3%)
Biotechnology & Microbiology (14.2%)
Pharmacology & Pharmaceutical Science (4.2%)
Cell Biology (4.3%)
Biophysics (6.2%)
Biochemical Research (15.9%)
Analytical Chemistry (14.4%)
Chemistry, Other (3.3%)
Immunology (4.2%)

Figure 3