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Affinity monolith chromatography: a review of general principles and recent developments

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Abstract

Affinity monolith chromatography (AMC) is a liquid chromatographic technique that utilizes a monolithic support with a biological ligand or related binding agent to isolate, enrich, or detect a target analyte in a complex matrix. The target-specific interaction exhibited by the binding agents makes AMC attractive for the separation or detection of a wide range of compounds. This article will review the basic principles of AMC and recent developments in this field. The supports used in AMC will be discussed, including organic, inorganic, hybrid, carbohydrate, and cryogel monoliths. Schemes for attaching binding agents to these monoliths will be examined as well, such as covalent immobilization, biospecific adsorption, entrapment, molecular imprinting, and coordination methods. An overview will then be given of binding agents that have recently been used in AMC, along with their applications. These applications will include bioaffinity chromatography, immunoaffinity chromatography, immobilized metal-ion affinity chromatography, and dye-ligand or biomimetic affinity chromatography. The use of AMC in chiral separations and biointeraction studies will also be discussed.

Keywords

Affinity monolith chromatography; Bioaffinity chromatography; Chiral separations; Immobilized metal-ion affinity chromatography; Immunoaffinity chromatography

1 Introduction

Affinity chromatography is a form of liquid chromatography that makes use of a biologically-related agent as a means to capture or purify a target analyte from a sample or complex mixture [1–5]. Retention of the target in this method relies on the specific, reversible interactions that frequently exist in biological systems, such as antibody-antigen or enzyme-substrate interactions [3,6,7]. The immobilized binding agent, or affinity ligand, acts as the stationary phase in this method. The support that is used to contain this binding agent can range from carbohydrate- or polymeric-based beads and gels in low-performance

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affinity separations to silica particles and glass beads in high-performance affinity methods [8–11]. However, an alternative type of support that can be used is a monolith, resulting in a technique known as affinity monolith chromatography (AMC) [8,12–21]. This report will present an overview of AMC, building on a previous review on this topic [20], and with an emphasis on recent developments in this field.

Monoliths, as used in chromatography, are materials that have a single and continuous porous structure with 1) large flow-through pores and 2) smaller side pores that analytes can enter via diffusion (i.e., “diffusion” pores) [22]. The most common commercial monoliths for liquid chromatography have flow-through pores with diameters of 1.5-1.7 μm and diffusion pores with diameters below 100 nm [8]. The external porosity for these monoliths (i.e., the ratio of interstitial volume to the total column volume) ranges from 0.6-0.8 for the flow-through pores [23,24]. The flow-through pores and small diffusion pores can provide low backpressures, good efficiencies, and faster separations for monolith columns compared to columns consisting of packed bed supports [14,23]. Another advantage of monoliths is they can be prepared and used in several formats, including columns, capillaries, disks, microfluidic devices, spin columns, syringes, 96-well plates, and pipette tips [20,21].

The first instance of a monolith, or continuous separation bed, being used for water-soluble compounds can be traced back to 1967 [25]. Using a free-radical polymerization of 2-hydroxyethyl methacrylate and ethylene dimethacrylate, a heteroporous structure was prepared for gel permeation chromatography. The porosity of this monolithic gel was controlled by the concentration of water in the polymerization mixture. In the early 1970s, several chromatographic columns based on open-pore polyurethane were developed [26–28]. These rigid, highly cross-linked gel structures could be made in a variety of configurations and with a variety of functional sites; however, they also suffered from a lack of good permeability and had excessive swelling in some solvents, making them incompatible for use in HPLC and GC [26–28]. The next generation of monolith materials was based on methacrylate disks (e.g., with a diameter of 10-55 mm and a length of 1-7 mm) [29] and membranes (e.g., sheets or related materials with a thickness of 1-3 mm) [30,31], in addition to compressed polyacrylamide gels [32]. However, these membranes and disks tended to have limited sample capacities [33].

A number of alternative monoliths began to appear in the 1990s [12–21]. One such material was prepared by polymerizing methacrylate monomers in the presence of porogenic solvents [33]. These solvents (i.e., porogens) led to the creation of a polymer with interconnected pores that were permeable to both solutes and solvents, with the pore size distribution being adjustable by selecting a suitable combination of porogenic agents [14,33]. These cross-linked and macroporous materials with large through-pores provided supports that could be operated at high flow rates and with lower back pressures compared to the standard particulate columns [24,34,35]. Similar efforts were later extended to the development of silica monoliths as porous inorganic supports for HPLC [36–40].

The first report of affinity separations with monolithic supports appeared in the late 1990s [41,42]. Since that time, there have been many studies that have combined biologically-related binding ligands with monolithic supports for use in chromatography and related

methods [8,14,15,19,43–50]. Figure 1 illustrates this through the number of publications that have appeared in this area over the last 22 years. There were 491 papers in this set that included the concept of “affinity monolith” as part of their discussion, with 66% of these papers appearing from 2011 to 2021.

This review will examine the principles and applications of AMC, with an emphasis on recent developments and reports that have appeared in the last 10 years. The supports that have been used in this field will be discussed, along with the types of immobilization techniques that have been employed in this method. The benefits of these supports and techniques, along with their potential limitations, will also be described in this context. Applications of AMC will then be examined in terms of the types of binding agents that have been utilized in this field. This will include the use of AMC in applications such as bioaffinity chromatography, immunoaffinity chromatography, immobilized metal ion chromatography, dye-ligand, and biomimetic affinity chromatography, chiral separations, and biointeraction studies. Future trends and possible developments in the use of monolith supports in affinity separations will then be considered.

2 General principles of AMC

The format most commonly applied in affinity chromatography and AMC for target capture or isolation involves a step gradient and the use of two mobile phases or buffer solutions. Figure 2 illustrates this format, which is frequently known as the “on/off” elution mode of affinity chromatography [3,51]. Initially, one of a pair of interacting substances is physically adsorbed or chemically immobilized onto a support; this immobilized agent is used as the stationary phase. The binding agent attached to the support is commonly referred to as the affinity ligand [9,52,53]. A sample containing the target is passed through this support by using an application buffer, which acts as the weak mobile phase (i.e., a solution or solvent that allows for strong retention of a target by the column and the stationary phase and is therefore used for sample injection or application). The selection of the pH and ionic strength of this application buffer, along with the temperature, is crucial for getting good retention because these factors contribute to the binding strength of the target with the affinity ligand [3,51,53]. Any non-targeted components of the sample will ideally demonstrate weak binding or no retention with the stationary phase under these conditions and wash away in the presence of the application buffer.

Several approaches can be employed to release the retained target from the immobilized affinity ligand. The approach selected for this purpose will depend primarily on the strength of the target’s interaction with the binding agent on the support. If the target and binding agent have a weak-to-moderate affinity, as seen for interactions with association equilibrium constants of 10^6 M^{-1} or less, isocratic conditions can be used for elution [51,53,54]. In this instance, the elution buffer will have the same pH and composition as the application buffer. This technique, referred to as “weak affinity chromatography” or “dynamic affinity chromatography”, is often employed in solute-protein binding studies and chiral separations [14,51,55–58].

Strong biological interactions, such as typical antibody-antigen binding, often have an association equilibrium constant greater than 10^6 M^{-1} (or a dissociation equilibrium constant below 1 μM) under physiological conditions [51]. For this type of strong interaction, the elution buffer will usually be required to have a pH, ionic strength, or polarity that is quite different from that of the application buffer. This alteration in buffer composition, coupled sometimes with a temperature change, can be used to promote target dissociation from the binding agent. This approach is particularly suitable for the rapid release of a target from the affinity ligand during analytical applications and is known as "non-specific elution" [3,4,51]. If mild and selective elution conditions are instead desired, an elution buffer can be made by placing a competing agent into the application buffer, thus allowing an approach known as "bio-specific elution" [3,51,53]. The buffer additive in this second case is used to compete with the target or immobilized binding agent at the site of their interaction, thus preventing the target from binding with the affinity ligand. As a result, this approach elutes the target from the affinity support through mass action [14,53].

After the target has been released from the affinity ligand, it can be passed through an online detector or collected for further analysis [20]. If the column is to be reused, the stationary phase and support can be regenerated by again applying the initial application buffer. This step allows the immobilized affinity ligand to go back to its initial state and to re-equilibrate itself with the application buffer prior to the next application of the sample and target [3,51].

3 Supports for AMC

As the name implies, AMC utilizes monolithic supports [8,14,35] instead of the more traditional particulate materials that are usually employed in affinity separations [1–5,8,59,60]. Examples of supports that have been used in AMC are shown in Figure 3, as based on a survey of the literature in this area. Many of these monoliths are made using organic polymers or silica; however, cryogels, hybrid materials, and carbohydrate-based monoliths have also been employed. This section will examine each of these materials and look at their recent use in AMC.

3.1 Organic-based monoliths

The most frequently-used organic monoliths in AMC are based on copolymers of glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) [19,44,50,61–65]. GMA/EDMA monoliths can be readily synthesized and are commercially available under the trade name "convective interaction media" (CIM) [15,20,44]. The use of GMA as a functional monomer provides the monolith with epoxy groups on its surface; these groups can be utilized directly or modified for affinity ligand attachment by a number of covalent methods, as described in Section 4.1 [14,47]. A hydrophilic form of these monoliths can be achieved by converting the epoxy groups into diols, which have low non-specific binding for many biological agents [3,4,8,19]. One useful feature of this type of organic monolith is the ability to control its shape, pore size, and surface area, which can allow its use with a large binding agent (e.g., a protein) as the affinity ligand [14,20]. One disadvantage of GMA/EDMA monoliths is these materials tend to have a lower surface area than silica monoliths or silica

particulate supports [14,20], which can limit the amount of an affinity ligand that can be placed within this support [20,44,47].

The average pore size and surface area of the organic monoliths can be regulated by adjusting the porogenic solvents of the polymerization mixture [47,66–68]. The most common porogens used in the preparation of GMA/EDMA monoliths are cyclohexanol and 1-dodecanol [19,44,67]. The process of preparing a GMA/EDMA monolith generally involves thermally initiated free-radical polymerization [14,15]; however, if faster polymerization is desired photoinitiators can also be used as an alternative to the thermal initiators [67,69]. Photoinitiators are also attractive for placing monoliths within specific regions (e.g., within the channels of a microfluidic device) and when working with thermally labile materials [67,70]. The polymerization mixture containing the monomer, crosslinker, porogens, and thermal initiator or photoinitiator are combined and placed in the desired casing. After the polymerization process has been carried out for a certain period, the formed monolith is washed to remove any remaining polymeric components. At this stage, the support can be activated and used for immobilization by passing coupling reagents and the binding agent through this material [19,44].

Several other organic monoliths that use monomers other than GMA or EDMA have been described. In one report, lectins were immobilized onto an organic monolith prepared with glyceryl monomethacrylate (GMM) and EDMA [71]. Affinity monolith stationary phases containing immobilized metal ions (e.g., Cu^{2+} or Fe^{3+}) and chelating agents were prepared by combining GMA with divinylbenzene (DVB) [72,73]. The trivalent crosslinker (trimethylolpropane trimethacrylate, or TRIM) was used with GMA to create monoliths that were used to immobilize human serum albumin (HSA) for chiral separations and to couple protein A for the analysis of human immunoglobulin G (IgG) [66,74]. GMA has also been used with ethylene glycol diacrylate to create an aptamer monolith for mycotoxin analysis in ultra-high performance liquid chromatography [75]. DVB alone was employed to prepare an organic monolith support that was doped with metal oxide nanoparticles (e.g., TiO_2 or ZrO_2) and used to enrich phosphorylated peptides [76]. A polar organic monolith based on GMM and pentaerythritol triacrylate (PETA) was developed to immobilize antibodies and evaluated for its ability to provide low nonspecific binding [77].

An organic polymer based on *N*-acryloxysuccinimide (NAS), acrylamide, and *N,N'*-methylenebisacrylamide was used to create an immobilized enzyme reactor (IMER) [78]. Multiple proteases were immobilized within this polymer and utilized in proteomic studies involving mouse serum, yeast and human cell lysates [78]. Functional monomers containing boronic acid have recently been used in organic monoliths for AMC. In one study, 3-arylamido phenylboronic acid was combined with EDMA to provide a monolith that could be used to bind and analyze several *cis*-diol compounds, including glycoproteins and nucleosides [79].

3.2 Inorganic-based monoliths

Inorganic monoliths based on silica have also been employed in AMC [44,80–82]. Silica monoliths combine the surface characteristics of particulate silica supports with the mass transfer properties of monoliths. This means a silica monolith tends to show improved

column efficiencies vs silica particles but can be used with similar immobilization methods to those employed with the particulate supports [19,44,80]. Silica monoliths can also have a greater accessible surface area for biomolecules than GMA/EDMA monoliths [83,84], which can increase the immobilization of binding agents within this affinity support [85]. However, silica monoliths do have a more limited pH range over which they can be used when compared with organic monoliths (i.e., about pH 2 to 8) [8,10]. Silica monoliths may also undergo shrinkage during their formation, which makes them difficult to make directly within a column; instead, these monoliths are transferred to their final housing after preparation [37,38,40,86].

Silica monoliths can be challenging to make in a laboratory setting, but these materials are available commercially as both bare silica and as aminopropyl functionalized silica [81,84,85,87,88]. In AMC, a bare silica monolith can be converted to an epoxy or diol form for use in the covalent immobilization of affinity ligands [8,10,14]. The sol-gel method is the most common means to prepare a silica monolith [44,83]. The sol-gel process has also been employed for the entrapment of affinity ligands [14]. The starting reaction mixture contains an aqueous solution of tetramethyl orthosilicate (TMOS) or tetraethyl orthosilicate (TEOS) and poly(ethylene oxide) (PEO) [20,89]. As the PEO is hydrolyzed under acidic conditions, the hydrolysate undergoes polycondensation. The combined effect of these processes results in phase separation and gelation of silica, leading to the creation of flow-through pores and smaller diffusion pores within the support. After aging, the prepared support is dried to remove any excess solvent, and the monolith is encased in a housing to prepare the final column [44,80,83].

Several modifications of this sol-gel process have been reported. For example, alkoxysilanes derived from sugars and sugar alcohols have been used in place of conventional alkoxysilanes for the preparation of silica monoliths [89–92]. Silica prepared in this manner (e.g., from diglycerylsilane, monosorbitylsilane, or maltosyldisilane) has been found to show less susceptibility to shrinkage than when using more traditional alkoxysilanes for monolith preparation [89]. In other work, the use of (3-aminopropyl)triethoxysilane (APTES) has been observed to provide monoliths with cationic sites that counterbalance the anionic charges on the silica and reduce non-specific binding by the monolith [90,93].

In AMC, immobilized serum transport proteins such as HSA and alpha$_1$-acid glycoprotein (AGP) have been placed within silica monoliths for use in chiral separations and drug-protein binding studies [84,85,88,94]. Affinity silica monoliths containing serum proteins or antibodies have also been used to estimate dissociation rate constants for solutes with these binding agents, as well as in biointeraction studies [95–97]. Other binding agents that have been coupled with silica monoliths have been vancomycin, for use in various chiral separations [98]; iminodiacetic acid (IDA) and immobilized metal ions, for phosphoproteome analysis [99]; and trypsin, for protein digestion [100,101]. Silica monoliths have also been used with 3-(2-aminoethylamino)propyl ligands for the separation of phenols, nucleic acid bases, nucleosides, and nucleotides by hydrophilic interaction chromatography (HILIC) [102]. In addition, silica monoliths with entrapped enzymes have been employed as tools for screening enzyme inhibitors or to create IMERs [90,103].

3.3 Hybrid organic-inorganic monoliths

Hybrids of organic and inorganic monoliths were introduced in the early 2000s [104–115]. This type of monolithic column has been made by using two sol-gel precursors, *N*-octadecyl-dimethyl[3-(trimethoxy-silyl)propyl] ammonium chloride and TMOS, with trifluoroacetic acid as the catalyst. This single-step approach provided an *in situ* way to prepare a silica-based monolith functionalized with various organic side chains [104,105]. These side chains have included aminopropyl, phenyl, allyl, mercaptopropyl, propyl, methyl, octyl, vinyl, and chloropropyl groups [106–114].

Hybrid monoliths were first applied in AMC by immobilizing Ti^{4+} onto a monolith prepared by the sol-gel method with TEOS and APTES being used as precursors [116]. This hybrid affinity monolith isolated 15 phosphopeptides from a digest of α-casein and bovine serum albumin (BSA) [116]. A “one-pot” approach was utilized to develop a hybrid affinity monolith based on TMOS and 3-methacryloxypropyltrimethoxysilane as co-precursors and 4-vinylphenylboronic acid as a functional monomer; this monolith was then used to bind glycoproteins such as ovalbumin, horseradish peroxidase, and transferrin [117]. Boronic acid and its derivatives have also been incorporated with these hybrid monoliths to specifically capture various biomolecules that contain *cis*-diol groups (e.g., catechols, nucleosides, and ribonucleotides) [118–122] and glycoproteins [123,124]. Aptamers, sometimes coupled with gold nanoparticles, have been used in hybrid organic-inorganic monoliths for the selective binding of thrombin [125] and ochratoxin A [126,127].

A hybrid monolith based on hierarchical periodic mesoporous organosilica was developed for protein and enzyme immobilization [128]. This material was synthesized from a bridged silane precursor ($[(R'O)_3SiRSi(OR')_3]$) that provided a regular arrangement of active organic species in the monolith’s structure. This approach was used for horseradish peroxidase, cytochrome C, and hemoglobin. The inorganic/organic composition in this framework provided a high organic content along with an enhanced adsorption capacity and improved immobilization stability [128].

3.4 Carbohydrate-based monoliths

Carbohydrates such as agarose have long been popular supports in affinity chromatography [4,59]. Agarose is a polysaccharide containing repeating units of agarobiose in a linear chain, in which agarobiose is a disaccharide composed of D-galactose and 3,6-anhydro-L-galactopyranose [8]. Agarose has good chemical stability over a wide pH range, and most biological ligands show low non-specific interactions towards this hydrophilic material [3,4]. Furthermore, the large pore size of agarose is useful for the immobilization of large binding agents [8]. Agarose can also be readily modified for ligand immobilization and can be prepared in a range of shapes, including rods, membranes/disks, and fibers; however, the low mechanical stability of this material has often limited its use to low- or medium-performance separations in liquid chromatography [3,8,43].

A monolith can also be prepared by modifying agarose with other agents [8]. In one such method, an agarose suspension in water was first heated to 90-100 °C [8,43]. A water-immiscible organic solvent (e.g., cyclohexane, for controlling pore size) and a surfactant

(e.g., Tween 80) were then blended into the agarose mixture and shaken to form an emulsion [14]. This agarose emulsion was then poured into a suitable mold and placed in a water bath at 60 °C. After slow cooling and thorough washing, the product was an agarose gel in the form of a monolith with flow-through pores [8,43].

Agarose monoliths have been used in AMC for various types of biomolecule purifications. For instance, agarose monoliths containing an immobilized NAD^+ derivative or Cibacron Blue, a synthetic dye, have been utilized for enzyme purification [129,130]. Purification of IgG and other glycoproteins has been achieved by employing monoliths made from an agarose-chitosan composite [131,132]. Hybrid monoliths based on agarose have been used with a biomimetic ligand based on protein A for the selective purification of antibodies from cell culture extracts [133]. An agarose monolith has also been used to bind BSA by employing immobilized Cu^{2+} as a binding agent [134].

Other types of carbohydrates have also been used as part of monoliths for AMC. As an example, β-galactose, β-glucose, and α-mannose have been used with an alkene-terminated tetraethylene glycol spacer during the formation of monoliths [135]. These modified carbohydrates were used with the co-monomer 2-hydroxyethyl methacrylate (HEMA) to form monolith capillary columns. These carbohydrate monoliths were then utilized to analyze the interactions of these carbohydrates with lectins such as concanavalin A (Con A), *Lens culinaris* agglutinin, and peanut agglutinin [135].

3.5 Cryogel monoliths

Another type of support that has been employed in AMC is cryogels [14,19,44]. This type of material can be developed initially by freezing an aqueous solution of monomeric or polymeric precursors to a temperature below −10 °C. A mixture of acrylamide, allyl glycidyl ether, and *N,N'*-methylenebisacrylamide is typically used for polymerization, along with an appropriate initiator [14]. Ice crystals that form during cooling as porogens when polymerization is initiated. Subsequent thawing and removing of these ice crystals leads to the formation of interconnected macropores of 10-100 μm in diameter [14]. The large pores of cryogels can allow the immobilization of high-mass biological agents that range from proteins to microbes and even animal cells [136,137]. The larger pores also provide low backpressures for these materials; however, the same feature contributes to a much lower surface area for this material (e.g., for use in immobilization) compared to other chromatographic supports [136,137].

Some of the monomers that have been used for cryogel preparation in AMC are agarose, HEMA, acrylamide, and 2-(dimethylamino)ethyl methacrylate [14,44,136,138–146]. Affinity ligands that have been employed with cryogels include metal ions (e.g., Cu^{2+}, Zn^{2+}, and Ni^{2+} bound via IDA), lectins (Con A), protein A, synthetic dyes (Cibacron Blue), and amino acids [136,138,139,141–153]. These binding agents have been applied to the separation and purification of yeast and bacteria [136,139,141,150], blood cells [153], general proteins (e.g., HSA, BSA, interferon, cytochrome c, and lactoferrin) [138,142,145,146,149,154–157], enzymes (e.g., chicken egg lysozyme, urokinase, and β-glucosidase) [138,143,147,148,158], antibodies (immunoglobulin M and IgG) [144,152,159], DNA [151], and glycoproteins (e.g., horseradish peroxidase) [160].

A cryogel monolith based on poly(vinyl alcohol)/polyethyleneimine and containing Cu^{2+} has been used for the removal of hemoglobin from blood [161] and the carbohydrate *N*-acetyl-D-glucosamine was immobilized onto a cryogel monolith for the purification of Con A [162].

4 Immobilization methods for affinity monoliths

Another important factor to consider in AMC is the way the affinity ligand is attached to or incorporated within the monolith. The immobilization method should be selected carefully because ligand activity can be altered or lost if the wrong technique is employed. Ideally, an immobilization method should not alter or change the activity of the ligand through improper orientation, multisite attachment, or steric hindrance [3,60]. Various immobilization approaches have been considered for use in AMC, as illustrated in Figure 4 [11]. These approaches generally involve covalent methods, biospecific adsorption, and other methods based on entrapment, molecular imprinting, or coordination-based immobilization [14,20,44,60].

4.1 Covalent immobilization

Covalent immobilization is the most common means for attaching affinity ligands within monolithic supports [14,20,44]. In this method, the monolith is activated and placed in contact with the desired affinity ligand [14]. The activation and immobilization steps can be carried out by either circulating the reagents and ligands through the monoliths or by dipping the monoliths into solutions of reagents and ligands [14,47,50]. Many types of covalent methods have been employed to immobilize affinity ligands to monoliths. The most common examples, and recent developments in these methods, are discussed in this section.

One common covalent immobilization technique is the epoxy method. In this method, an epoxy group on a monolith is usually coupled with amine groups on an affinity ligand to generate a secondary amine bond [14,60]. Depending on the immobilization conditions, this method can also be used with ligands that have sulfhydryl or hydroxyl groups [60]. An advantage of this method is the ease with which it can be used with monoliths that already possess epoxy groups as part of their structure, such as GMA/EDMA monoliths (see Section 3.1) [14,19,44]. Although this method is simple and relatively fast, it does tend to produce a lower amount of immobilized binding agent than other covalent methods, as the epoxy groups are susceptible to loss by hydrolysis [50,64]. This technique has been used to immobilize a variety of ligands for AMC. Examples of binding agents that have been coupled by this approach are protein A, protein G, protein L, BSA, HSA, antibodies, amino acids, enzymes, synthetic dyes, boronates, and antibodies [47,50,64,74,132,152,163–172].

The Schiff base method, or reductive amination, is another common immobilization technique used in AMC [14,20,44]. This method often starts with a monolith that contains diol groups. These diol groups are then oxidized to form aldehyde groups [14,20,44,60]. Affinity ligands that contain primary amines are then reacted with the aldehyde groups to form a reversible Schiff base, which can be reduced upon its formation by sodium cyanoborohydride to create a stable secondary amine. Sodium borohydride or a small amine-containing capping agent (e.g., ethanolamine) can later be used to reduce or remove any

remaining aldehyde groups on the support [60,173]. This method tends to provide a higher activity for affinity ligands than the epoxy method; however, some loss of activity can occur if this technique is used with a harsh reducing agent [50,60]. Affinity ligands that have been immobilized within the monoliths by this method are protein A, HSA, aptamers, enzymes, heparin, chondroitin sulfate A, boronates, and antibodies [47,50,60,64,88,163,174–180].

The glutaraldehyde method is related to the Schiff base method but instead uses a support that contains amine groups. These amine groups are reacted with glutaraldehyde to form aldehyde groups, which are then reacted with amines on affinity ligands in the same manner as described for the Schiff base method. This method requires more steps than the Schiff base method but places a longer spacer between the affinity ligand and support, which is useful in avoiding steric hindrance when dealing with small binding agents. This method has been employed in AMC for the immobilization of protein A, L-histidine, HSA, enzymes, modified carbohydrates, and aptamers [165,171,181–187].

The carbonyldiimidazole (CDI) method is another approach for covalent immobilization in AMC [14]. This method makes use of alcohol groups that are present on a monolith; for instance, GMA/EDMA monoliths can be used for this purpose by first converting epoxy groups to diols. These alcohol groups are then activated by reacting them with 1,1 ´-carbonyldiimidazole. An affinity ligand is then immobilized by nucleophilic substitution, which occurs between the activated groups of the support and primary amine groups on the ligand [173]. This method is simpler than the Schiff base and glutaraldehyde methods, but also tends to provide lower ligand activities [60]. This method has been used in AMC to immobilize protein A, HSA, L-histidine, glycosaminoglycan, and antibodies [47,50,163,167,187–189].

The disuccinimidyl carbonate (DSC) method is another approach for covalent immobilization in AMC [14]. This method can make use of a monolith that contains either alcohol or amine groups, which are then reacted with DSC to create a succinimidyl carbonate-activated form [47,50,60,190,191]. These groups can then be reacted with primary amine groups of affinity ligands to form a stable carbamate linkage [173]. Although this method is relatively fast, it does tend to show susceptibility towards hydrolysis and a consequent decrease in the amount of affinity ligand that can be immobilized [60,173]. This approach has been used to immobilize HSA on GMA/EDMA monoliths and to place protein A on an amine-containing silica monolith [50,191].

The hydrazide method is a covalent method that can be used to immobilize glycoproteins and carbohydrate-containing ligands [19,60,173,187,192]. This method requires that the support first be activated to contain hydrazide groups. An affinity ligand that contains aldehyde groups (e.g., as occurs in carbohydrate chains that have been mildly oxidized) is then reacted with this support to form a hydrazone bond [192]. The remaining aldehyde groups can be reduced (e.g., using sodium borohydride) or capped with a small hydrazide agent [60,171]. This method involves more steps than several of the other coupling methods that have already been discussed but does provide a means for the site-selective immobilization of glycoproteins through their carbohydrate chains. This method has been

utilized to immobilize antibodies within organic monoliths and to couple AGP in a silica monolith [47,85,188].

The cyanogen bromide (CNBr) method has also been applied in AMC, especially for carbohydrate-based materials such as agarose [20]. In this technique, alcohol groups on the support are reacted with CNBr. The activated support can be used to immobilize affinity ligands with amine groups through the formation of an isourea linkage [3,19,173]. This method is simple and requires relatively mild conditions for ligand attachment [60]. However, the use of CNBr requires special precautions, as this chemical is a toxic material [187]. In addition, the isourea linkages are positively charged at a neutral pH and can act as anion-exchange sites, leading to a potential source of non-specific binding [60,173]. Also, this linkage can be unstable, resulting in loss of the affinity ligand over time [60,187]. This method has been applied in AMC for the immobilization of NAD^+ and L-chlorosuccinamic acid [129,173].

A method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) has also been used for covalent immobilization in AMC [193–195]. Affinity ligands with carboxyl groups can be immobilized by this method. This process starts with a monolith that contains amine groups. Carboxyl groups on the ligands are then activated with EDC/NHS to give a succinimidyl ester, which can react with the amine groups on the support to form an amide bond. Advantages of this method are that the reagents are water-soluble and this approach allows for easy purification of the conjugated products [195]. This approach has been used in AMC to immobilize heparin onto monolithic supports [193,194].

Another covalent immobilization approach that has been employed in AMC is “click chemistry”, or copper-catalyzed azide-alkyne cycloaddition [196]. This approach is relatively simple and is compatible with a wide range of functional groups [123]. This method has been used to place boronate affinity ligands within monoliths [123,197]. This approach starts with activating the monolith with sodium azide (NaN_3). A boronate ligand containing an alkyne group can then be immobilized onto the azide-activated monolith in the presence of CuI and a methanol/water mixture [123].

4.2 Biospecific adsorption

Biospecific adsorption is an indirect immobilization technique where an affinity ligand is held in place by a secondary binding agent that is immobilized to the support [14,60]. This method starts with the covalent immobilization of the secondary binding agent. The affinity ligand is then allowed to adsorb to this agent. In some cases, the adsorbed affinity ligand can be held in a more stable manner by crosslinking it with the secondary binding agent [60].

Protein A and protein G are two examples of secondary agents that are frequently used in biospecific adsorption for antibodies, due to the ability of these bacterial cell wall proteins to bind the lower stem region of antibodies from various species [60,64]. Other examples are avidin and streptavidin, which can be used to capture and bind biotinylated affinity ligands [64]. Biospecific adsorption can allow for easy regeneration of the support through the use of an elution step to release the affinity ligand and an application step to reapply a fresh

batch of the affinity ligand. However, biospecific adsorption can be more expensive than covalent immobilization due to its requirement for multiple binding agents. The presence of the secondary binding agent may also increase the possibility for non-specific binding and result in a lower binding capacity for the affinity ligand due to steric hindrance. Biospecific adsorption in AMC has been used to prepare monoliths for the capture of antibodies by protein A or protein G; in addition, streptavidin monoliths have been used to adsorb biotinylated HSA, aptamers, and membrane proteins [60,64,172,187,198–200].

4.3 Other immobilization methods

Several other formats for immobilization and ligand preparation have been employed in AMC. One example is entrapment, which is a noncovalent approach in which ligands are encapsulated within a support [60]. In this method, the support usually has pores or openings that are smaller than the entrapped affinity ligands but allow access by smaller targets to these ligands. In AMC this method has typically been done by using sol-gel chemistry and adding the affinity ligand (e.g., an enzyme or protein) to the reagent mixture that is used to make the monolith [60,90,178]. If done correctly, entrapped affinity ligands will remain in a soluble form without significant denaturation [60,90,178]. However, this approach can lead to materials with slow mass transfer, problems with controlling the pore size of the support, and issues with shrinkage (e.g., sol-gels) [60,90].

Molecular imprinting is another method that has been used in AMC to prepare supports with binding regions to a given target [60,201–204]. This is often done by allowing a mixture of functional monomers and a crosslinking agent to polymerize in the presence of a template molecule that is related to the desired target [201–203]. The template is removed after the polymer has formed, leaving cavities that are complementary to the final target in their shape and arrangement of functional groups [202,203]. This type of material that is created by this process is known as a molecularly imprinted polymer (MIP) [201–204]. This method has been used in AMC with an imprint of protein G to capture a recombinant form of this protein from cell lysates, with an imprint of cytochrome c to recognize cytochrome c vs lysozyme, and in a hybrid aptamer/MIP monolith for the analysis of ochratoxin A in beer [201,205,206].

Coordination chemistry has also been used to immobilize affinity ligands for AMC. This approach is similar to covalent immobilization and is typically used in combination with immobilized metal-ion affinity chromatography (IMAC) [20]. This method is based on the specific interactions that can take place between immobilized metal ions and some amino acids in proteins and peptides, such as histidine, tryptophan, or cysteine residues [207–209]. This method starts with the coupling of metal ions such as Cu^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+}, or Co^{2+} with an immobilized chelating agent (e.g., IDA or nitrilotriacetic acid) [209]. Organic monoliths based on GMA/EDMA or GMA/DBV polymers and cryogels have been used in IMAC and with immobilized metal ions for the separation of histidine-tagged proteins, the enrichment of N-glycans, and the isolation of antibodies or IgG from plasma and biological samples [14,210–215].

5 Applications of AMC

Many types of binding agents have been employed with AMC, leading to several sub-categories of AMC based on the immobilized binding agent. This section will discuss many of these sub-categories, including bioaffinity chromatography, immunoaffinity chromatography, IMAC, and dye-ligand or biomimetic affinity chromatography. The use of AMC in chiral separations and biointeraction studies will also be examined.

5.1 Bioaffinity chromatography

The term “bioaffinity chromatography” refers to the use of a biological binding agent as the stationary phase in affinity chromatography [216]. The ligands most commonly included in this type of affinity chromatography are immunoglobulin-binding proteins, enzymes, lectins, carbohydrates, and agents related to avidin/streptavidin-biotin interactions [14,15,19–21,216]. Each of these groups of binding agents will be discussed in this section. A special set of bioaffinity chromatography, immunoaffinity chromatography, will be discussed in Section 5.2.

AMC has been used with two types of immunoglobulin-binding proteins: protein A and protein G [14,15,44,216]. These binding agents, which are both bacterial cell surface proteins, can specifically interact with the lower stem (F_c) region of many types of immunoglobulins and antibodies [217–219]. Protein A is produced by *Staphylococcus aureus*; the native protein has a molar mass of 42 kDa and is composed of five domains, four of which are F_c-binding domains [217,218,220]. Protein G is produced by groups G and C *Streptococci* and is often used in affinity separations as a recombinant form that lacks the albumin-binding region present in the native form [219,221]. The recombinant form of protein G has a molar mass of 23 kDa and contains two F_c-binding regions [221].

Several applications have been reported for protein A and protein G in AMC. The fact that protein A has different affinities towards various subclasses of human IgG [170,216] has been exploited by using protein A in a GMA/EDMA monolith for the separation of these subclasses [222]. The same type of affinity column has also been used to evaluate a method for the quantitation of IgG in both purified samples and crude supernatants from Chinese hamster ovary cells [223]. Protein A, either in its native or recombinant forms has been used in GMA/EDMA, GMA/TRIM, and oligoepoxide-based monoliths for the separation and purification of IgG in human serum and rabbit serum [74,171,188,224]. The simultaneous isolation of IgG subclasses and immunoglobulins A plus M in human serum has been done by using protein G and a related binding agent (protein L) in CIM monoliths [225]. A protein A cryogel monolith was employed to remove immunoglobulin M-based autoantibodies from human plasma [144]. A chromatographic method that combined anion-exchange with a protein G monolith disk was used for the simultaneous detection of IgG, transferrin, and insulin in cell culture media (see Figure 5) [226]. A similar approach was employed to isolate ruthenium complexes from IgG, transferrin, and HSA in human serum [227].

AMC can be utilized to capture solutes and screen for targets that bind to enzymes that have been placed in monoliths. For example, dihydrofolate reductase in a silica monolith

has been used to detect inhibitors for this enzyme [90], and screening of trypsin inhibitors has been achieved by integrating a GMA/EDMA monolithic trypsin reactor offline with LC-MS [65]. However, it is more common for an enzyme to be used in a monolith to create an IMER for biocatalysis [100,228–231]. Trypsin is the most common enzyme employed for this purpose [100,101,228,229,232]. A trypsin silica monolith has been coupled with HPLC and ESI-MS/MS for the analysis of peptides and proteins of interest in biotechnology [100,229]. Trypsin has also been immobilized in GMA/DVB monoliths and used in pipet tips for protein digestion [233]. Peptide-*N*-glycosidase F, an endoglycosidase frequently used to release *N*-linked glycans from glycoproteins, has been immobilized into a GMA/EDMA monolith and used for *N*-glycan release from glycoproteins such as ribonuclease B, fetuin, and IgG [234]. An NAS organic monolith with several proteases (e.g., trypsin, Lys-C, and Lys-N) has recently been studied for rapid on-column protein digestion [78]. Simultaneous online protein digestion and peptide mapping has been achieved by using trypsin in a poly(NAS-co-EDMA) monolith column [235]. The kinetic properties of γ-glutamyl transpeptidase, as well as naproxen binding by BSA, were examined using silica monoliths [103,236]. Lactase, acetylcholinesterase, and glucose oxidase have been coupled to agarose monoliths for use as bioreactors [43].

Lectins are non-immune system proteins that can bind specific carbohydrate moieties [70,216,237,238]. This feature has made lectins an attractive binding agent for glycoproteins and other carbohydrate-containing agents in AMC [19,70]. Con A and wheat germ agglutinin (WGA) are two lectins that have frequently been used as affinity ligands with monoliths [15,44]. These two lectins have been immobilized onto a neutral GMA/EDMA monolith and a related cationic monolith (prepared using GMA/EDMA plus [2-(methacryloyloxy)ethyl]trimethylammonium chloride) for the pre-concentration of glycoproteins and their corresponding glycans [239]. Con A on a GMA/EDMA monolith has been used to isolate high mannose *N*-glycans from a tryptic digest of ribonuclease B prior to analysis by MS/MS [240]. A sandwich capillary monolith prepared by coupling Con A to a GMA/EDMA support through Cu^{2+} bound to IDA was considered for use in capturing glycoproteins from mouse urine [241]. The lectins WGA and *Lens culinaris* agglutinin were placed in GMA/EDMA monolith capillaries to isolate glycoproteins by capillary electrochromatography [62]. The same type of organic monolith has been utilized with the lectin *Pisum sativum* agglutinin for separating chicken ovalbumin, ovomucoid, and turkey ovalbumin into glycoform fractions [70]. A tandem column platform based on WGA, Con A, and *Ricinus communis* agglutinin-I in GMM/EDMA monoliths was developed to capture glycoproteins from breast cancer and disease-free human sera [71]. A Con A cryogel was also examined for use in the separation of yeast cells from a culture medium [150].

Carbohydrates can also be coupled to monolithic supports [216,242–245]. β-Cyclodextrin (β-CD) is a cyclic oligosaccharide that has been used with silica monoliths [242,243]. These monoliths were employed for the separation of positional isomers (e.g., for naphthalenedisulfonic acids and cresols) and chiral analytes (e.g., dansyl-amino acids, benzodiazepine, methadone, methylphenobarbital, and carprofen) [242,243]. Recently, a glycocluster (i.e., thiolsaccharide pendant) was grafted to β-CD and was used with a HEMA/PETA monolith for glycoprotein enrichment from a horseradish peroxidase digest [246]. Several other carbohydrates, such as *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine,

and *N*-acetyl-D-mannosamine, have been immobilized on cryogel monoliths to purify lectin Con A [162,244]. The acidic oligosaccharide sialyllactose has been immobilized on a GMA/EDMA monolith column to purify the influenza virus from cell cultures [245].

The strong non-covalent binding that occurs between avidin or streptavidin with biotin has been used in several applications of AMC [21,216]. Avidin is a glycoprotein found in egg whites, and its bacterial counterpart, streptavidin, is produced by *Streptomyces avidinii* [247]. The association equilibrium constants for binding by these proteins with biotin range from 10^{13} to 10^{15} M^{-1} [247,248]. Biotinylated aptamers have been coupled to monolithic supports containing avidin or streptavidin [249–252]. Monomeric avidin has also been placed in organic monoliths to enrich biotinylated cytochrome C and its corresponding peptides [253]. A streptavidin GMA/EDMA capillary was developed to adsorb biotinylated HSA and to examine binding by drugs known to bind HSA (e.g., warfarin, furosemide, and ibuprofen) [64].

5.2 Immunoaffinity chromatography

Immunoaffinity chromatography (IAC) is a type of bioaffinity chromatography that utilizes antibodies or related biological agents as affinity ligands [254–257]. This set of methods includes immunoextraction and immunodepletion [256–258]. In immunoextraction, isolation of a specific target from a sample is accomplished by using IAC prior to analysis of the target by a second method [255–257]. In immunodepletion, IAC is used to remove a given component from the sample, such as the removal of high-concentration proteins so that other minor proteins can be more easily examined [256,258].

A variety of monolith types have been used in AMC with immobilized antibodies [47,77,259–264]. For example, GMA/EDMA monoliths were used with rabbit IgG and anti-fluorescein antibodies to maximize the content of such affinity ligands for rapid target capture [47]. A GMA/EDMA monolith was used with antibodies to purify tagged blood group antigens [263]. An online immunoextraction based on a GMA/EDMA monolith was used with HPLC to quantify pyrethroid insecticides [262]. The same approach was used to extract and analyze the mycotoxin aflatoxin B1 by combining an anti-aflatoxin B1 monolith with a reversed-phase HPLC column [259]. A similar method was used for the online extraction of trace bisphenol A from environmental water [260]. A polymeric microchip based on GMA/EDMA and containing anti-FITC antibodies was developed for the capture and analysis of FITC-labeled amino acids by CE [261]. Several organic monoliths were evaluated for use with immobilized antibodies to haptoglobin, apolipoprotein B, and apolipoprotein A1 [77].

A number of recent applications have been reported for monoliths in IAC. In one study, antibody monoliths based on EDMA and vinyl azlactone were used to extract proteotypic epitope peptides from protein ProGRP, a cancer biomarker, for detection in human serum by LC-MS/MS (see Figure 6) [265]. Several commercial organic monoliths were employed with IAC and anti-human fibrinogen antibodies for the isolation of fibrinogen from human blood samples [266]. CIM disks were used to immobilize protein G, which was then used to adsorb antibodies for the capture of ammodytoxins (i.e., the most toxic components of the snake venom) and their interacting protein partners from the snake venom [267]. Protein

A and protein A/G monolithic microcolumns were developed to bind recombinant bovine somatotropin [268]. A 96-spot organic monolithic array using monoclonal antibodies to the mycotoxins deoxynivalenol, zearalenone, T-2 toxin, and fumonisin B_1 was used to detect these compounds in food samples [269]; a paper version of these mycotoxin IAC arrays was also developed [270]. An immunodepletion method using anti-HSA antibodies on a commercial CIM support was used to remove HSA from culture media [271].

IAC with monolith supports has also proven to be a useful technique for large-scale purification. This has been demonstrated for cells, cell organelles, viruses, and plasmid DNA [272]. Recently, extracellular vesicles, including exosomes and microvesicles, have been isolated by using a CIM disk with immobilized antibodies to human $CD6_1$, a glycoprotein with specific binding towards platelet-derived extracellular vesicles [273]. Low-density lipoprotein has been selectively captured by a combination of a chondroitin-6-sulfate monolith disk followed by a monolith containing antibodies to apolipoprotein B-100 [189]. Monolith supports based on GMA/EDMA were polymerized inside 3D printed microfluidic devices and used with antibodies to bind ferritin (a preterm birth biomarker) from human serum [274].

5.3 Immobilized metal-ion affinity chromatography

IMAC, in which a metal ion is held by a chelating group on a support, is another format that has been explored in AMC [19,20,208,209]. This combination has been used mainly for the purification and capture of specific amino acids, peptides, DNA, or proteins (e.g., histidine-tagged agents or phosphoproteins) [19,20]. Monoliths based on agarose, silica, organic polymers, and hybrid materials have all been used with IMAC [67,68,71–86].

The IMAC format in AMC has been utilized in several studies for the purification and analysis of proteins, enzymes, N-glycans, peptides, and DNA [134,159,210,275–284]. For instance, a hybrid cryogel containing Cu^{2+} on bentonite particles has been used to bind hemoglobin from aqueous solutions (see Figure 7) [280]. Another hybrid cryogel containing Ni^{2+} attached to O-carboxymethyl chitosan was used to retain lysozyme [278]. Recombinant human histidine-tagged ubiquitin was captured from an *E. coli* lysate by using GMA grafted with nylon-6 capillary channeled polymer fibers, which were then coupled with IDA and Cu^{2+} [281]. Plasmid DNA has been purified by using a GMA/EDMA monolith that contained Cu^{2+} [210]. A recent report used commercial affinity monoliths containing various charged metal ions to separate empty vs full capsids during the preparation of adeno-associated virus for use in gene therapy [277].

IMAC has often been used in AMC to isolate peptides and phosphopeptides for proteomic studies [99,116,276,285–289]. This work has made use of monoliths that contained chelates of Fe^{3+}, Ti^{4+}, Zr^{4+}, as well as metal oxide nanoparticles [99,116,285–289]. A specific use of IMAC in AMC has been the enrichment of low abundance phosphopeptides from human serum and HeLa cell digests by employing a phosphate-functionalized hybrid monolith that also contained Ti^{4+} [287]. IMAC has also been used with monoliths to analyze the mitochondrial phosphoproteome [116] and the phosphopeptides obtained from α-casein or β-casein digests [99,285,289,290].

5.4 Dye-ligand and biomimetic chromatography

Dye-ligand affinity chromatography is a method that uses a synthetic dye as the affinity ligand [19,146,209,291–293]. This method uses the fact that some synthetic dyes mimic natural ligands (e.g., an enzyme co-factor) and can bind specifically to some biomolecules [209]. Triazine dyes are often used for this purpose as dye ligands, with one common example being Cibacron Blue [19,209]. Dye-based ligands have been found to have several potential advantages over biologically-based binding agents, such as their low cost, good stabilities, ease of preparation, and high binding capacities [292]. Dye-ligand affinity chromatography has been used in AMC to purify proteins such as BSA, HSA, and IgG, as well as DNA [168,291–293]. Dye ligands have also been employed in AMC for the removal of proteins from samples, such as the use of Cibacron Blue on a cryogel to deplete HSA from human serum [146].

Other synthetic compounds can also be used as mimics or replacements for biological ligands [209]. Examples include affinity ligands based on dye-scaffolds, boronates, and peptides, amino acids, RNA, or DNA that have been selected from large libraries and that can bind a given target [294–297]. In AMC, a triazine-based biomimetic ligand was used with a chitosan and PVA monolith to purify IgG [298]. Boronates have been used in AMC for the separation of glycoproteins, saccharides, nucleosides, and other *cis*-diol compounds [197,297,299–302]. Types of monoliths that have been used with boronate affinity ligands have included cryogels, MIPs, inorganic monoliths, organic monoliths, and hybrid monoliths [123,197,297,300,301]. An example is a restricted access boronate monolith that was developed to bind IgG in the carbohydrate groups of its F_c region [297].

Peptides are another set of affinity ligands that have been used in AMC [15,166,303,304]. Arginine homopeptides have been immobilized on epoxy-activated CIM to purify plasmid DNA [166]. Phage display libraries and peptides from combinatorial libraries have also been explored for use as biomimetic ligands in AMC [303,304]. As an example, a peptide expressed by phage display was used in a cryogel for the purification of lactoferrin from milk [303]. A synthetic hexapeptide from a combinatorial library was immobilized on a GMA/EDMA monolith to bind IgG [304].

Aptamers are another set of biomimetic ligands that have been employed in AMC [305]. These ligands are typically obtained based on sequences of single-stranded DNA or RNA with 3D structures that can specifically interact with a given target, which can range from a small molecule to a protein, cell, or virus [306]. Advantages of using aptamers are their good selectivity, ability to be quickly regenerated, relatively low production cost, ease of modification, and good chemical or thermal stability [306]. Aptamers have been immobilized on both organic and hybrid monoliths [180,186,206,307–313]. Several studies have demonstrated the capture of thrombin by anti-thrombin aptamers attached to GMA/EDMA or hybrid monoliths [186,307,311]. An RNA aptamer was immobilized on an organic monolith to purify lysozyme from egg white [180]. Aptamer monoliths have been employed to bind ochratoxin A from food samples [309,310,314]. Similar studies have been done with aptamers on miniaturized silica monoliths for the preconcentration and analysis of ochratoxin A in beer and wine [315]. The removal of trace bisphenol A and zearalenone

from food samples using aptamers on gold nanoparticles and a hybrid monolithic column has also been reported [308,313].

5.5 Chiral separations

AMC can also be used with biologically-related binding agents to separate analytes that have more than one chiral form [14,19,20,44,316]. Several serum transport proteins (e.g., HSA, BSA, and AGP) have been used to develop chiral stationary phases with monoliths [50,66,84,85,94,317,318]. For example, HSA has been coupled to organic monoliths based on GMA/EDMA or GMA/TRIM by several covalent immobilization methods; these monoliths were then evaluated as chiral stationary phases for *R*/*S*-warfarin and D/L-tryptophan [50,66]. The same types of materials based on HSA as a chiral stationary phase were used for the enantioseparation of D/L-amino acids [317]. AGP and HSA in silica monoliths were examined for their use in resolving chiral solutes such as *R*/*S*-warfarin, *R*/*S*-propranolol, D/L-tryptophan, and *R*/*S*-ibuprofen [84,85]. Supports have been prepared for capillary electrochromatography by placing BSA in silica monoliths, such as used for the chiral separation of pantoprazole and atenolol (see Figure 8) [94,318]. The chiral selectivity of BSA and ovomucoid have been examined when these proteins were placed into silica monolith capillary columns; these stationary phases were then tested for use in separating D/L-tryptophan and benzoin enantiomers (by BSA) or the enantiomers of benzoin, eperisone, and chlorpheniramine (by ovomucoid) [94].

Carbohydrates have also been used with monoliths for chiral separations. One report used β-CD with silica monoliths to develop chiral stationary phases for CEC, which were then used to separate the chiral forms of several negatively-charged analytes (e.g., naphthalenedisulfonic acids, dansyl-amino acids, and carprofen) and neutral aromatic agents (e.g., cresols) [243]. In another study, a β-CD silica rod was compared with a β-CD particulate support for the resolution of chiral compounds such as chromakalin, prominal, oxazepam, and methadone [242].

Other types of binding agents have been employed as chiral stationary phases with monolithic supports. Vancomycin, a glycopeptide antibiotic, was immobilized to a silica monolith and employed in the enantioseparation of racemic benzoin, terbutaline, atenolol, metoprolol, alprenolol, pindolol, and propranolol [98]. A pepsin silica monolith was examined for its chiral selectivity with regards to the enantiomers of nefopam, chlorphenamine, and citalopram when used in CEC [319]. A hybrid organic-inorganic monolith was used with an aptamer for the chiral separation of chemotherapeutic anthracyclines such as doxorubicin and epirubicin, as found in serum and urine samples [320].

5.6 Biointeraction studies

Another type of affinity chromatography is biointeraction chromatography or quantitative affinity chromatography, which refers to the use of an affinity column to study biological interactions [321]. AMC has also been used for this purpose [14,19,44,85]. Frontal analysis is one format for biointeraction studies that has often been applied in AMC [14,19]. In frontal analysis (also known as frontal affinity chromatography), a known concentration of

an analyte (or a mixture of analytes) is applied continuously to a column that contains the affinity ligand of interest [14,321]. As the analyte binds to sites on the affinity ligand, this binding leads to the eventual saturation of the column and an increase in the amount of solute eluting from the column, resulting in a breakthrough curve. From the mean position of the breakthrough curve and the known concentration of the applied analyte, it is possible to obtain information on the amount of ligand that is present, the number of analyte/ligand binding sites, and the binding constants for the analyte at these sites. The main advantage of this method is its ability to simultaneously measure both the equilibrium constant(s) and number of binding sites for the analyte within the column [321].

Frontal analysis has been mostly applied to determine the binding strengths and adsorption capacities for affinity monoliths [47,64,84,85,178,322–324]. The interactions between various drugs and immobilized ligands have also been studied by this approach. One report used this method to determine the binding constants of carbamazepine with HSA and AGP on silica monoliths [84,85]. A similar approach was used to examine binding by naproxen to BSA on a GMA/EDMA monolith [236]. Frontal analysis using affinity monoliths coupled with MS has been applied to examine the binding of small molecules to dihydrofolate reductase [90] and to examine small ligands that may bind to adenosine A_{2A} receptor and compete with theophylline for this receptor (see Figure 9) [200]. AMC has also been used in a frontal analysis format to examine the binding of IgG to protein A, protein G, and protein L [170,178,325]. The binding of adenosine triphosphate and *N,N*-diethylvanillamide with the immobilized N-terminal domain of heat shock protein 90 on organic monoliths has been evaluated by frontal analysis [64].

Another popular format for biointeraction studies in AMC is zonal elution [14,44,50,66,84,85,321,325,326]. In this technique, a narrow plug of a target analyte is injected onto an affinity column while the analyte’s elution time or elution volume is monitored. The retention factor obtained for the target can then be used as a measure of the binding strength of the target with the affinity ligand and of the amount of active sites that are available for this binding [321,326]. Zonal elution has been used to optimize the immobilization and binding of serum proteins such as HSA and AGP in monoliths and to study the interaction of various drugs with these proteins [50,66,84,85]. Monolithic microcolumns containing HSA or AGP have further been used in a special type of zonal elution known as peak decay analysis to determine the dissociation rates of various drugs from these proteins [96,97].

6 Concluding remarks

This review has explored the general principles and recent applications of AMC. The types of application and elution formats, supports, and immobilization methods that have been employed in this method have been described. Supports that have been used in AMC have included organic and inorganic monoliths, hybrid monoliths, carbohydrate-based materials, and cryogels. Immobilization and ligand preparation schemes have ranged from covalent immobilization to biospecific adsorption, entrapment, molecular imprinting, and coordination-based coupling.

Many types of AMC have been described using these various classes of binding agents. Bioaffinity AMC has made use of affinity ligands such as protein A or G, enzymes, lectins, carbohydrates, and the biotin-avidin/streptavidin interactions. Immunoaffinity AMC, including both immunoextraction and immunodepletion formats, has also been used in many reports. Non-biological agents and alternative affinity ligands have also been used in AMC, such as immobilized metal ions, synthetic dyes, boronates, peptides, and aptamers. These methods have been utilized in applications spanning from biochemical purification to sample pretreatment and the analysis of targets such as drugs, environmental contaminants, proteins, antibodies, carbohydrate-containing agents, and phosphorylated peptides or proteins. In addition, AMC has seen growing use in chiral separations and biointeraction studies. It is expected that applications in these areas will continue to appear in the future as further developments occur in the types of supports, immobilization methods, and affinity ligands that can be used in AMC. This, in turn, should result in an even greater set of applications for AMC in the purification, isolation, and analysis of chemicals or biochemicals.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations:

AGP	alpha1-acid glycoprotein
AMC	affinity monolith chromatography
APTES	(3-aminopropyl)triethoxysilane
CIM	convective interaction media
CNBr	cyanogen bromide
Con A	concanavalin A
DSC	disuccinimidyl carbonate
DVB	divinylbenzene
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDMA	ethylene glycol dimethacrylate
GMA	glycidyl methacrylate
GMM	glyceryl monomethacrylate
HEMA	2-hydroxyethyl methacrylate

IAC	immunoaffinity chromatography
IDA	iminodiacetic acid
IgG	immunoglobulin G
IMAC	immobilized metal-ion affinity chromatography
IMER	immobilized enzyme reactor
MIP	molecularly imprinted polymer
NAS	N-acryloxysuccinimide
NHS	N-hydroxysuccinimide
PEO	poly(ethylene oxide)
PETA	pentaerythritol triacrylate
TEOS	tetraethyl orthosilicate
TMOS	tetramethyl orthosilicate
TRIM	trimethylolpropane trimethacrylate
WGA	wheat germ agglutinin

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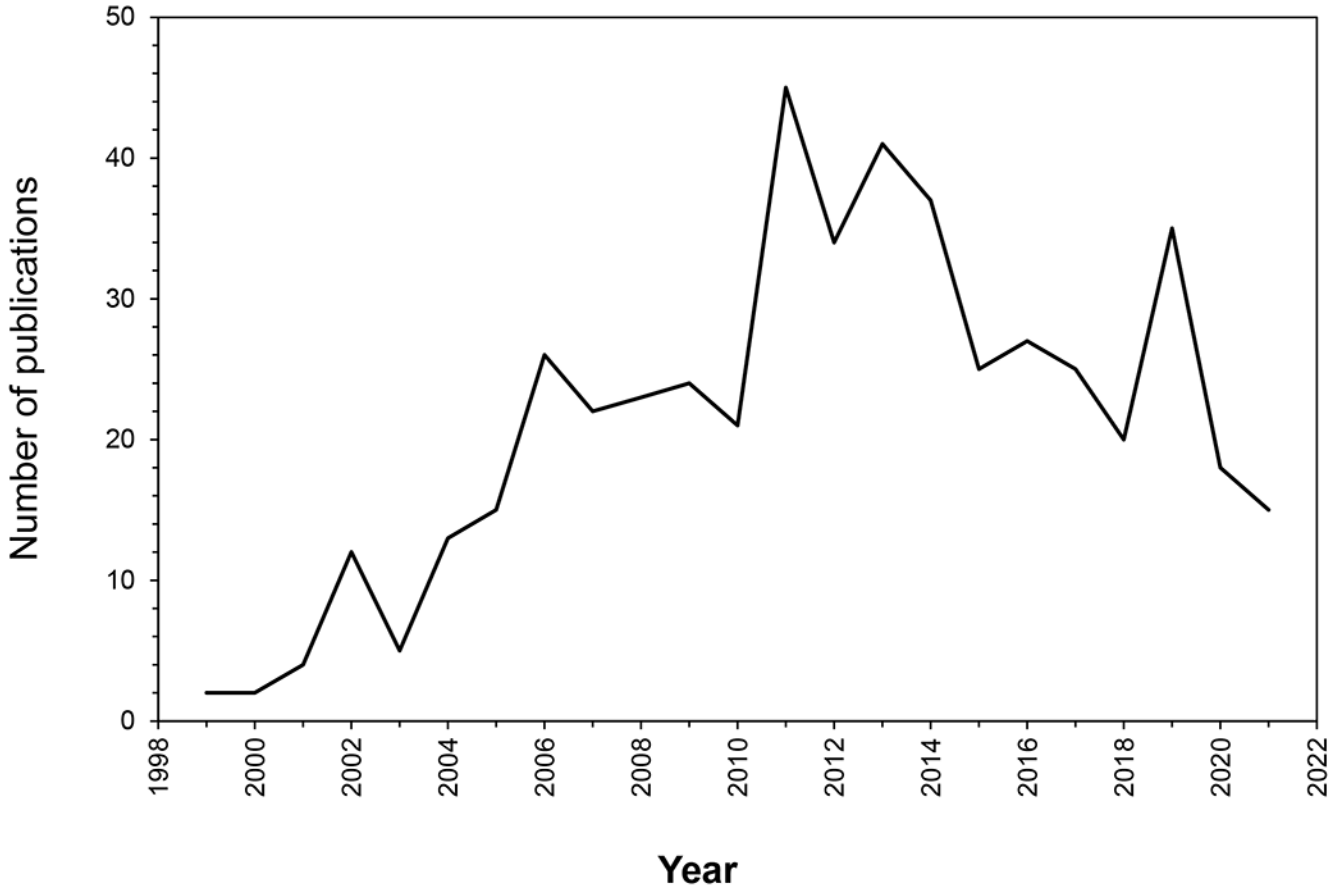

Figure 1.
Number of publications that appeared between 1999 and 2021 containing the concept "affinity monolith" (491 total). These data were obtained through a search that was conducted in May 2021 using SciFinder.

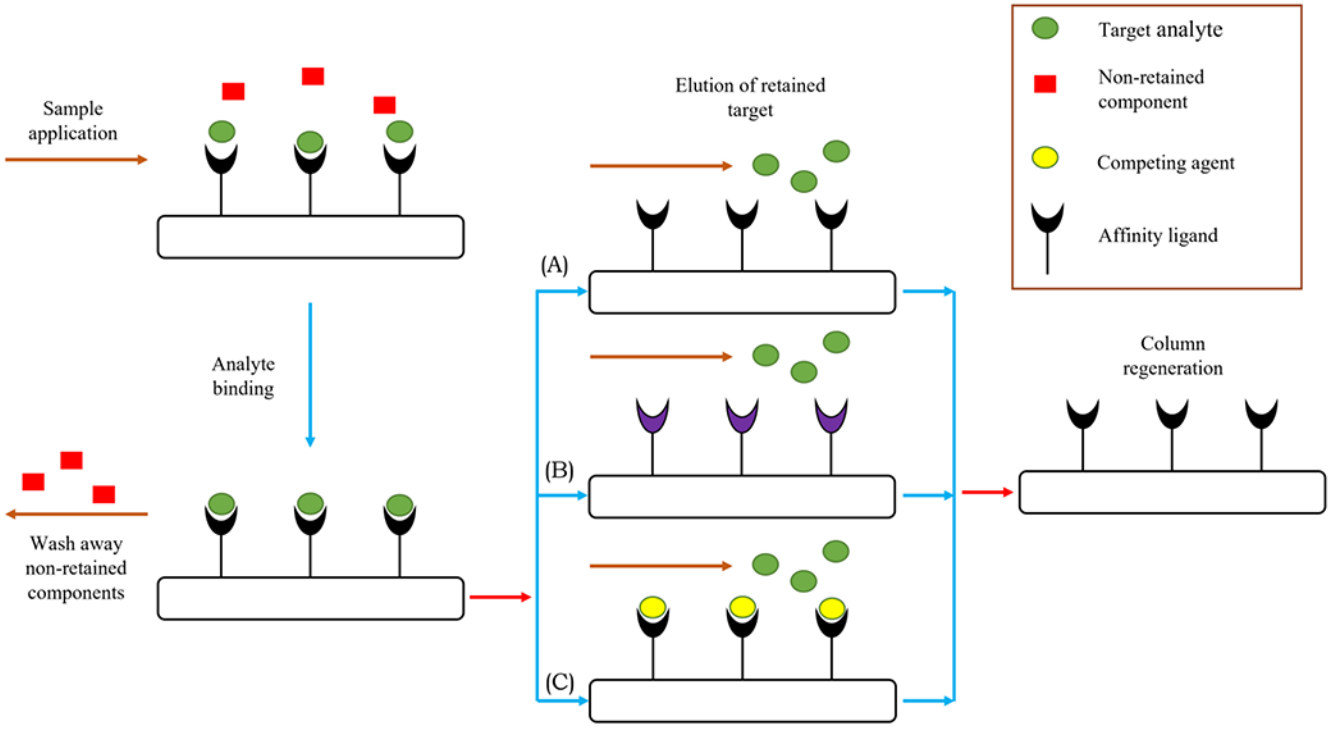

Figure 2.

The “on/off” mode used for elution in affinity chromatography and AMC. The three types of elution schemes shown are (A) isocratic elution, where the elution buffer is the same as the application buffer; (B) non-specific elution, where the elution buffer has a different pH, polarity, or ionic strength from the application buffer; and (C) bio-specific elution, where the elution buffer has the same basic composition of the application buffer with the addition of a competing agent. The bio-specific elution method shown is reversed-role elution, in which the competing agent binds to the immobilized affinity ligand; in normal-role elution, the competing agent instead binds to the target.

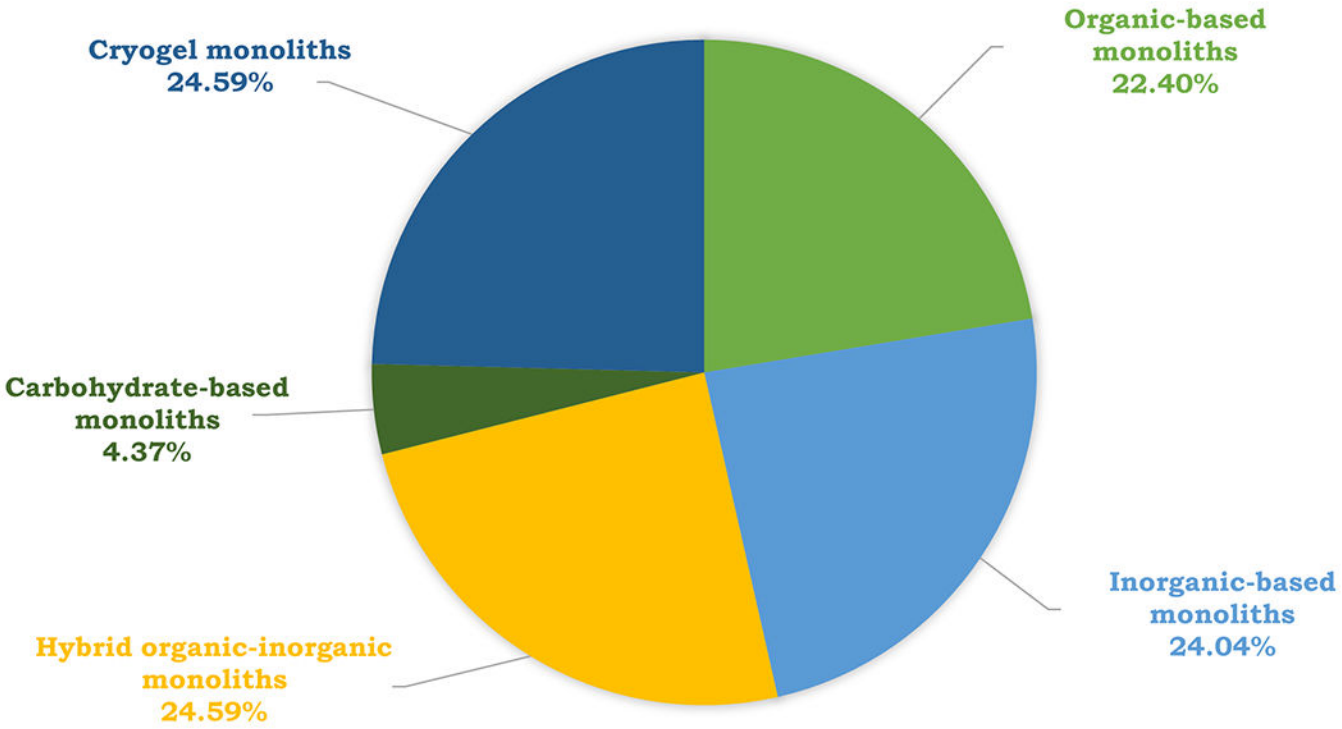

Figure 3.

Use of various support materials in AMC, based on a search of papers that have employed monolith supports in affinity chromatography. This search was conducted in May 2021 using SciFinder and examined papers that appeared between 1999 and 2021 with the phrase or concept “affinity chromatography” and the various types of monoliths that are shown in this chart (183 total).

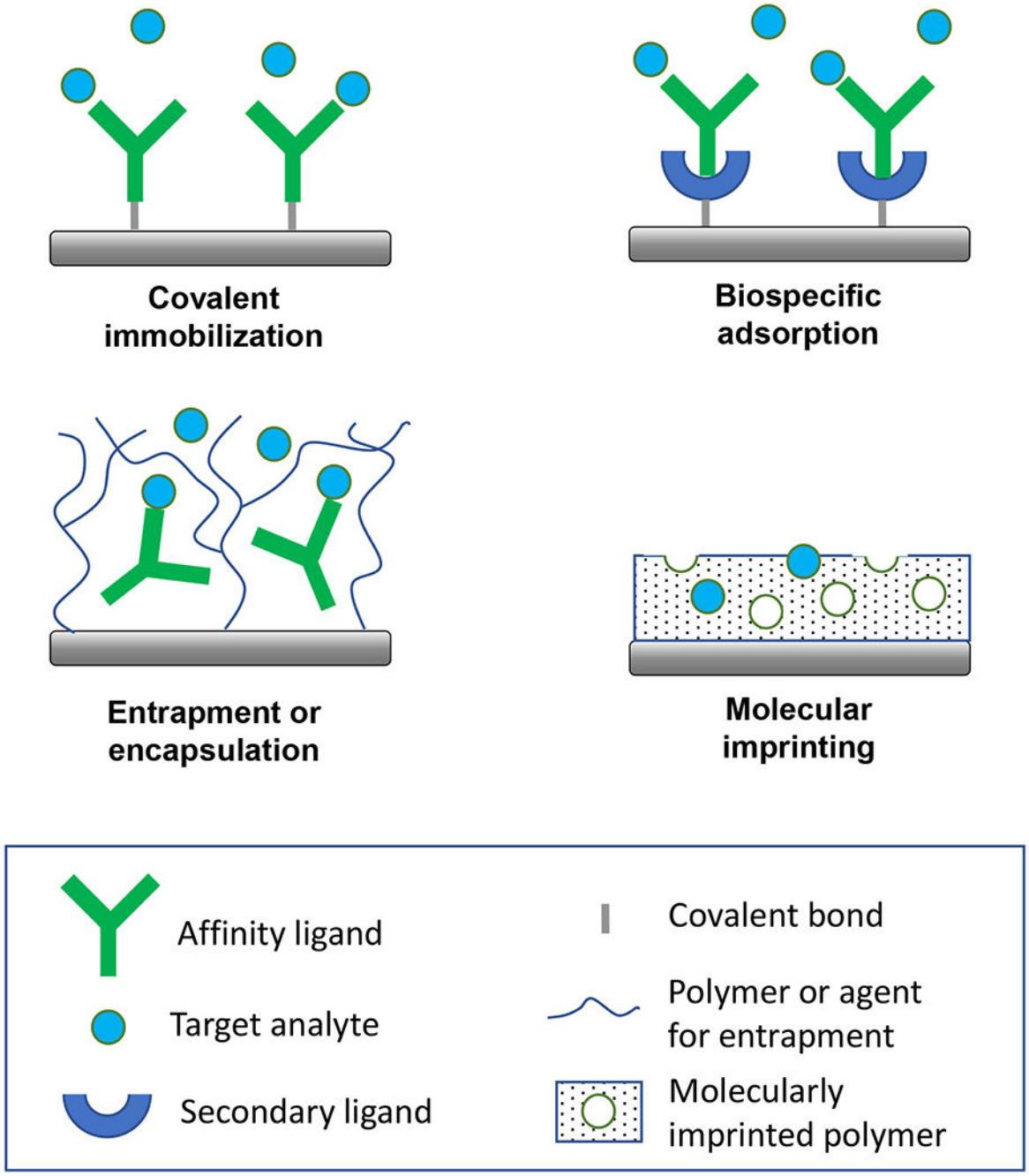

Figure 4.
Strategies that have been used to place or immobilize a binding agent within a support for use in AMC. Reproduced with permission from Ref. [11]. Copyright 2020, with permission from Elsevier.

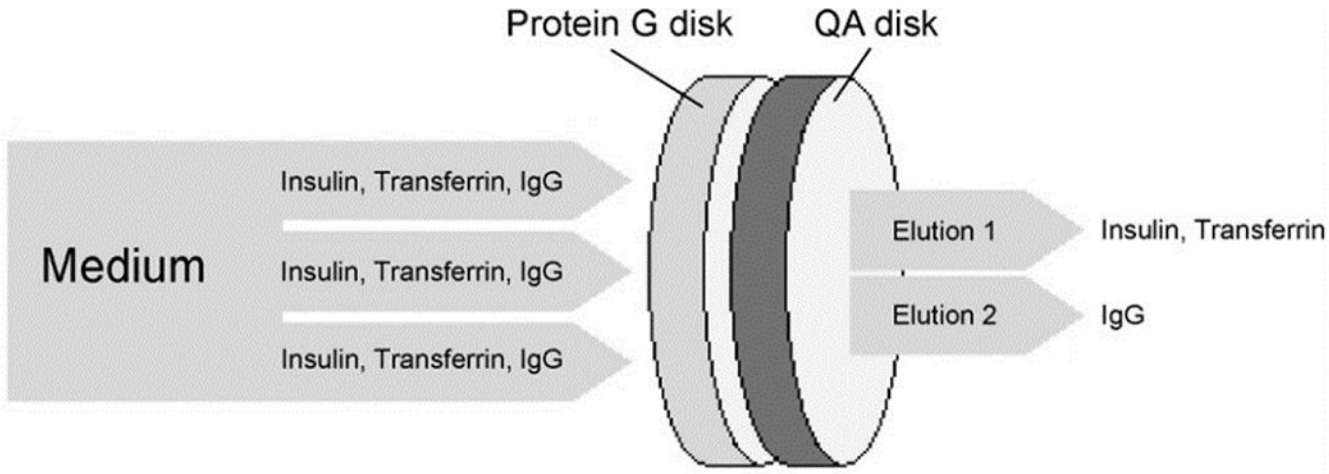

Figure 5.

Combination of a protein G monolithic disk with a quaternary ammonium (QA) anion exchange monolithic disk for use in the sequential analysis of insulin, transferrin, and IgG in cell culture media. Reproduced with permission from Ref. [226]. Copyright 2018, with permission from Elsevier.

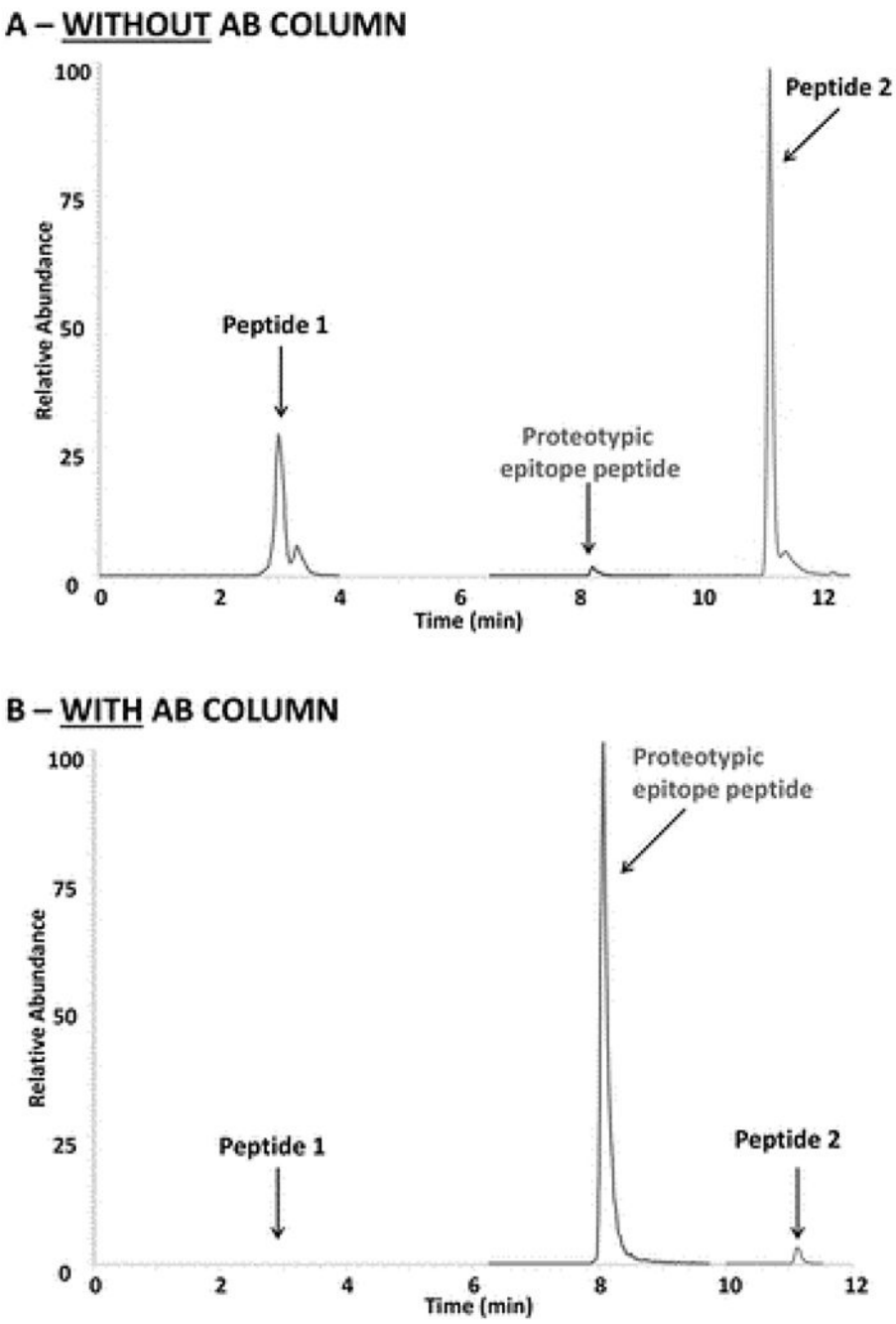

Figure 6.

Extracted ion chromatograms for peptide 1, peptide 2, and a proteotypic epitope peptide from a digest of ProGRP, a protein biomarker for lung cancer, after sample pretreatment using (A) a solid-phase extraction column or (B) immunoextraction for the epitope peptide with an antibody monolith column. Reprinted with permission from Ref. [265]. Copyright 2018 American Chemical Society.

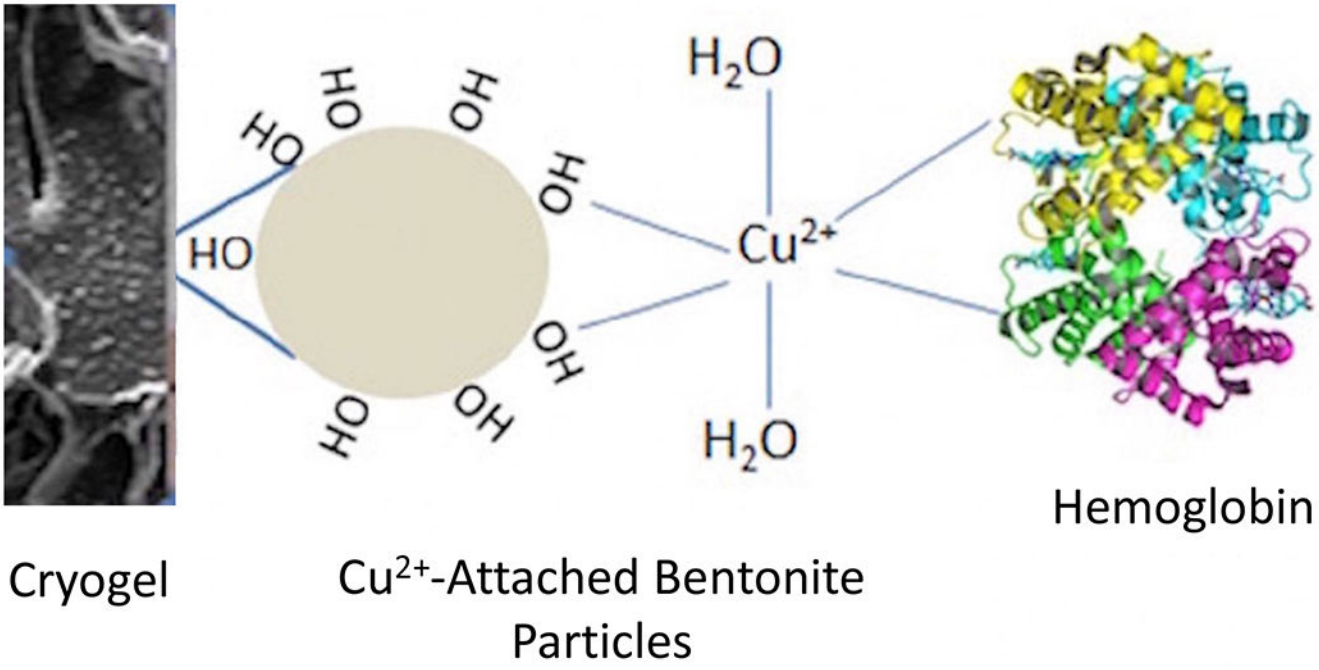

Figure 7.

A hybrid monolithic support for IMAC based on Cu^{2+}-attached bentonite particles embedded in a cryogel and used to bind hemoglobin. Adapted with permission from Ref. [280]. Copyright 2017, with permission from Elsevier.

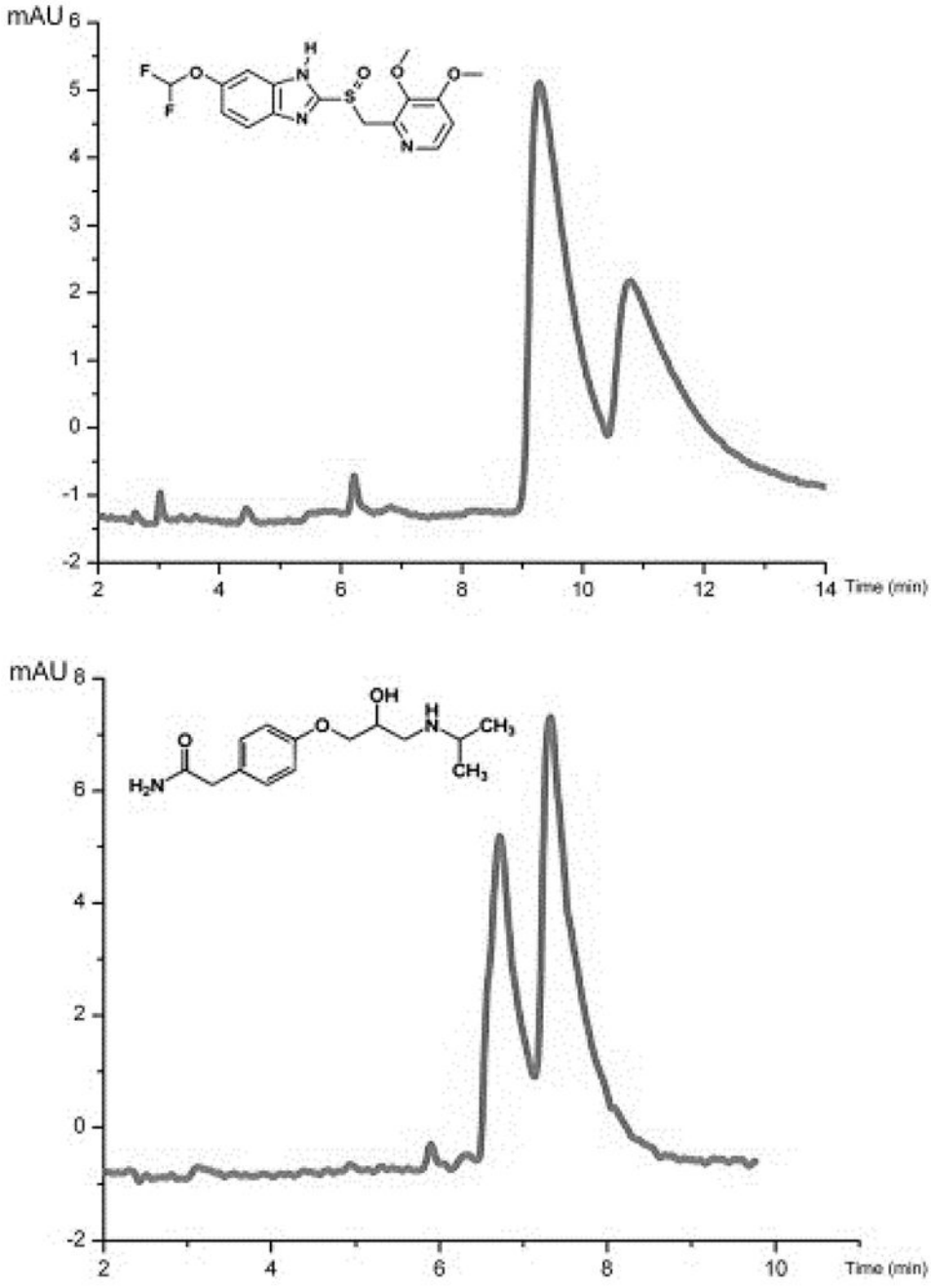

Figure 8.

Use of BSA in a silica monolith for the chiral separation of racemic pantoprazole (top) and atenolol (bottom) by capillary electrochromatography. Reproduced with permission from Ref. [318]. Copyright 2014, with permission from Elsevier.

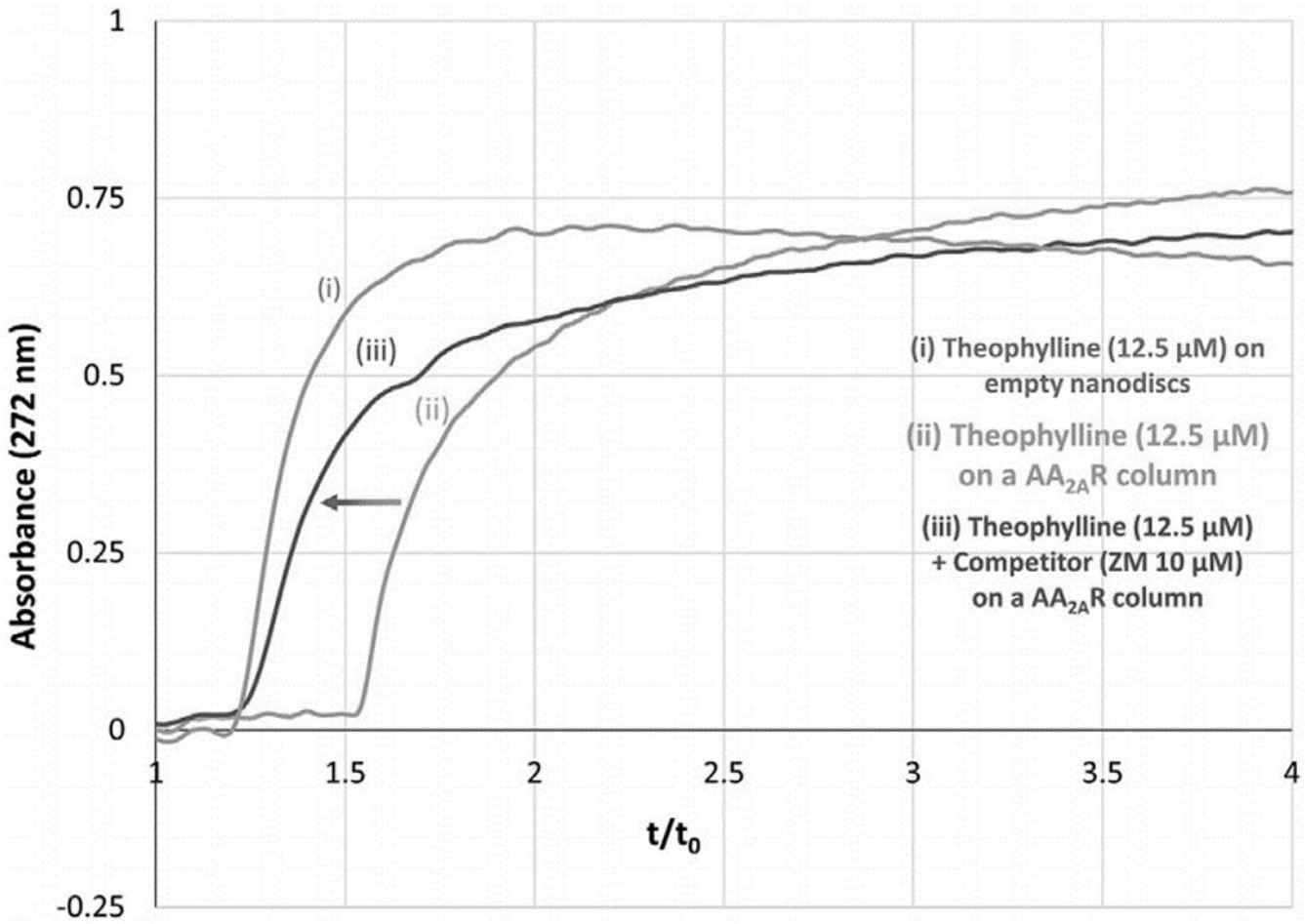

Figure 9.

Frontal analysis of the binding and competition of theophylline with a potential competing agent (ZM) for adenosine A_{2A} receptor ($AA_{2A}R$) nanodiscs that were immobilized within a monolith capillary column. Reproduced with permission from Ref. [200]. Copyright 2020, with permission from Elsevier.