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Review

Macroporous monolithic supports for affinity chromatography

In the early 1990s, three research groups simultaneously developed continuous macroporous rod-shaped polymeric systems to eliminate the problem of flow through the interparticle spaces generally presented by the chromatography columns that use particles as filler. The great advantage of those materials, forming a continuous phase rod, is to increase the mass transfer by convective transport, as the mobile phase is forced to go through all means of separation, in contrast to particulate media where the mobile phase flows through the interparticle spaces. Due to their special characteristics, the monolithic polymers are used as base-supports in different separation techniques, those chromatographic processes being the most important and, to a greater extent, those involving the separation of biomolecules as in the case of affinity chromatography. This mini-review reports the contributions of several groups to the development of macroporous monoliths and their modification by immobilization of specific ligands on the products for their application in affinity chromatography.

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1 Affinity chromatography

Currently, the separation of biomolecules is usually carried out with particulate media under different chromatographic principles such as ion exchange chromatography (IEC), normal and reversed phase chromatography (RPC), hydrophobic interaction chromatography and affinity chromatography (AC). In most cases, the biomolecule is retained by the media; the solutes that were not bound are removed by washing and the molecule of interest is finally eluted by a gradual change in the mobile phase composition as a gradient elution [1, 2]. The general requirements for the carriers to be used in separation of biological molecules can be summarized as follows [1]: specific surface area: 10–400 m^2/cm^3; functionality: 1–100 μmol/cm^3; porosity: 25–75% and pore size in particulates and in continuous media: 0–100 and 300–5000 nm, respectively. The supports must present structural characteristics to achieve a balance between the accessible surface area of the material and the pore sizes. An ideal support for the retention, separation and/or purification of biomolecules must show the following characteristics: high selectivity and binding capacity, lack of non-specific adsorption, mechanical and chemical stability, reusability, stability under sanitation conditions (only in the production of therapeutic proteins at industrial level) and low cost.

The AC technique was developed first by Porath et al. in the late 1960s [3, 4] and employed for protein separation. In subsequent years, other authors such as Cuatrecasas, Anfinsen and Wilchek shared their knowledge and contributed to the development of this technique [5–9]. It consists of a biospecific method of separation and purification of a biological molecule, using the properties of reversible biospecific interactions between a biological molecule and a 'specific ligand' immobilized on a polymer matrix. Different steps are involved in AC purification since during adsorption, the substrate (target molecule) is specifically

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Abbreviations: **AAm**, acrylamide; **AC**, affinity chromatography; **AGP**, α-1-acid glycoprotein; **anti-FITC**, anti-fluorescein isothiocyanate; **BPA**, bisphenol A; **CDI**, 1,1′-carbonyldiimidazole; **CIM®**, Convective Interaction Media®; **EDA**, ethylenediamine; **EDMA**, ethylene dimethacrylate; **GFP**, green fluorescent protein; **GMA**, glycidylmethacrylate; **Hep**, heparin; **HRP**, horseradish peroxidase; **hIgG**, human immunoglobulin G; **IAMC**, immunoaffinity monolithic columns; **IDA**, iminodiacetic acid; **IMAC**, immobilized metal affinity chromatography; **poly(AAm-*co*-AGE-*co*-BIS)**, poly(acrylamide-*co*-allyl glycidyl ether-*co*-*N*,*N*-methylenebisacrylamide); **poly(GMA-*co*-EGDA)**, poly(glycidyl methacrylate-*co*-ethylene glycol diacrylate); **poly(GMA-*co*-EDMA)**, poly(glycidyl methacrylate-*co*-ethylene dimethacrylate); **poly(NAT-*co*-GMA-*co*-BIS)**, poly(*N*-acryloyl-tris (hydroxymethyl) aminomethane-*co*-glycidyl methacrylate-*co*-*N*,*N*-methylenebisacrylamide); **VPBA**, 4-vinylphenylboronic acid

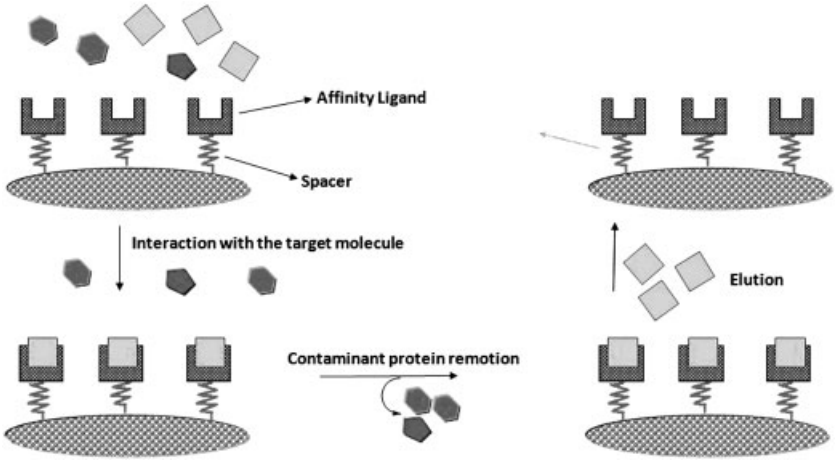

Figure 1. Different steps involved in AC purification.

bound to the ligand and the non-adsorbed material is separated by washing. Subsequently, the substrate is eluted and obtained pure after being desorbed from the media by change in the mobile phase composition. Figure 1 shows the steps involved generally in a separation of biomolecules by AC.

The ligands used in AC can be classified as follows [10]: (i) *general* ligands such as dyes, metals, protein A and G and polysaccharides, used for the retention of various biological molecules and (ii) *specific* ligands such as enzymes or substrates, antibodies or antigens, and hormones or receptors, used in the binding of a specific biomolecule. Different ligands commonly used in AC and distinct chemical reactions for the ligand immobilization were treated previously in various reviews [11–13]. Some of these have also discussed and even focused on the use of AC with macroporous monolithic materials that is the subject concerning this mini-review developed in the following sections.

2 Macroporous materials

Macroporous or heterogeneous polymeric networks, commonly used as base-supports for AC, are characterized by a porous structure formed during its preparation that is maintained even when dry. The internal structure consists of aggregates of polymeric microglobules interconnected by pores; their stiffness results from the high degree of the crosslinking they possess. They are generally particulate materials prepared from a suspension polymerization reaction [14]. Beyond the strengths of these media, particulate materials have two major limitations: the large volume interparticle and the slow diffusion of high molecular weight solutes (proteins, polysaccharides or synthetic polymers) into the pores of the particles by their low diffusion coefficients. Thus, this problem is relevant in processes where the rate of mass transfer is the rate-determining factor as in the case of chromatographic separations, catalysis, *etc.* [15]. This resistance to mass transfer in large molecules is mainly produced by the discontinuity presented by columns packed with particulate systems (due to voids between particles). The liquid phase flows mostly through the spaces between the particles where the flow resistance is lower. On the other hand, the liquid inside the pores does not practically flow, remaining 'stuck' [15]. Now, if the diffusion rate is lower than the time required for the interaction to occur between the biological macromolecule and the ligand bound to the support, the diffusion rate becomes the determining step of the process. In these cases, the solute molecules interact with the more accessible active sites within the pores of the stationary phase and not with those located in the inner zone of the stationary phase. Hence, the full potential of the separation medium is not used. For such reasons, long columns or slow flows should be employed [15]. The mass transfer is enhanced when it takes place by convection [16]. This type of transport uses the flow of the mobile phase to increase the mass transfer of solutes. Therefore, polymeric supports with large pores are necessary for solute molecules of high molecular weight to be transported by convection. Theoretical studies indicated that the maximum effect of convective transport could be achieved if the mobile phase is forced to pass through a porous medium [17, 18]. Thus, in the early 1990s, continuous macroporous polymeric systems were developed (macroporous disks [19], gels of poly(acrylamide) (poly(AAm)) compressed into a column [20], and porous monolithic polymer rods prepared within the chromatographic column [21]) to solve the problem of flow through the interparticle spaces. The key advantage that these new materials showed was the increase of the mass transfer by convective transport, since the mobile phase is forced to go through all means of separation, whereas in particulate media, the mobile phase flows in the interparticle spaces [15].

3 Monolithic materials used in AC

Macroporous monolithic materials constitute an important generation of polymers used in several fields since they

adjust to the characteristics of a single and porous particle. The continuous porous structure commonly found in polymeric monoliths made them excellent materials used in the fast separation of different compounds.

In this section, the characteristics of monolithic materials used in AC, including organic-based monoliths, cryogels and silica-based monolithic media, are presented.

3.1 Organic-based monolithic polymers

These monolithic columns introduced by Svec and Fréchet in the 1990s [22] were characterized as highly crosslinked polymers having open porous structure which can be maintained in solvents with different polarities even in dry state [23]. These supports are synthesized in a very simple way from a homogeneous polymerization mixture, containing the monomers (monovinyl monomer and agent crosslinking), the radical initiator and the porogen mixture [24]. The polymerization reaction takes place within a mold (which determines the form of the support) without stirring. Various reviews report the challenge posed in their preparation and application [12, 13, 15, 16, 22, 25–30].

An important advantage of the organic-based monoliths involves the fact that a wide variety of monomers can be employed to fabricate the final monolith. As outstanding, it may be mentioned that various monolithic columns presenting different chemistry have been prepared including AAm-based [31, 32], methacrylate-based [33–35] and styrene-based polymers [36]. The existence of certain functional groups on the surface of organic-based monolithic polymers depends on the class of monomer/s used for the preparation. The functional groups then serve for the immobilization of ligands on supports, to be used as a separation medium by AC of large and small molecules. For this reason, a greater diversity of surface chemical structures could be obtained and, according to the application of the yielded polymer support, a specific chemical functionality in the structure of the polymer rod will be required.

Sometimes, its immobilization must be carried out through a functional group not provided by the monomers used but performed through chemical modification reactions on the surface of the material [37] or through grafting reactions [38], which can increase considerably the number of active sites of the support. However, the use of monomers with reactive functional groups such as glycidylmethacrylate (GMA) or chloromethylstyrene (CMS) is optimal since the functional groups can be employed directly without chemical modification. In this sense, the frequent use of GMA as co-monomer derives from its excellent features due to the presence of epoxy groups which can rapidly react with various reagents. As an example, the epoxy groups can be hydrolyzed by the formation of a *vic*-diol and oxidized with $NaIO_4$ to create aldehyde groups which can be easily coupled to amine-containing ligands as proteins followed by reduction with $NaBH_4$ or $NaCNBH_3$.

In this manner, Zou et al. [39] immobilized protein A on modified poly(glycidyl methacrylate-*co*-trimethylolpropane trimethacrylate) (poly(GMA-*co*-TRIM)) and poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (poly(GMA-*co*-EDMA)) monoliths for AC performing the assays on a capillary instrument. The column was used for analyzing human immunoglobulin G (hIgG) in a microliter of sample.

Once the epoxy groups are hydrolyzed, 1,1′-carbonyldiimidazole (CDI) can be used in an activation step followed by immobilization of antibodies or, as described above, they can be converted to aldehyde groups for the reaction with adipic dihydrazide and $NaBH_4$ to create amine groups ready to react with aldehyde-containing ligands [40].

Hahn et al. [41] developed an affinity monolithic support with a novel immobilization strategy that consisted in the *in situ* polymerization of the ligand. In this study, the model ligand (a peptide directed against lysozyme (Lys)) was reacted to the GMA monomer prior to polymerization. Afterwards, with the conjugate, GMA and ethylene dimethacrylate (EDMA), a monolith was formed and tested against Lys. The results showed a higher affinity constant compared with that obtained with conventional sorbent.

3.2 Cryogels

As stated by Jain et al. [42], cryogels are supermacroporous gel networks formed by the cryogelation of appropriate monomers or polymeric precursors at subzero temperature using the system TEMED/ammonium persulfate (APS) to initiate the polymerization reaction. The notable feature of this system involves a unique combination of high porosity, minimal non-specific interactions due to the hydrophilic nature of polymers with adequate mechanical strength and osmotic stability. The main disadvantage of these materials is the generally low surface area they present. The diversity of its application stems from the fact that cryogels can be subjected to surface chemical modifications for the coupling of different ligands and by grafting of polymeric chains on their surfaces.

The combination of the properties of cryogels [43] allows specific applications such as direct capture of extracellularly expressed histidine-tagged protein from the fermentation broth and separation of different cell types. Kumar and Srivastava [44] prepared monolithic poly(AAm) and poly(dimethylacrylamide) cryogel affinity matrices to be used as a generic type-specific cell separation process. The monolithic cryogels were functionalized to immobilize protein A as ligand by a two-step derivatization of epoxy-containing cryogel monolith (reaction with ethylenediamine (EDA) and glutaraldehyde). Target cells were labeled with specific antibodies and then captured in the cryogel through affinity with protein A. These cells specifically captured were recovered in high yields, retaining their viability by mechanical squeezing of the spongy and elastic cryogel matrices.

Mattiasson et al. [45] prepared Con A-cryogel monoliths for separating a mixture of *Saccharomyces cerevisiae* and

E. coli cells using a chromatographic procedure. An efficient chromatographic separation of the cell mixture containing equal amounts of cells of both types was performed in a column under the optimal conditions determined, resulting in a quantitative capture of applied *S. cerevisiae* cells, whereas *E. coli* passed through the column. Bound *S. cerevisiae* cells were released by flow-induced detachment and by compression of the adsorbent in the presence of methyl α-D-manno-pyranoside. The flow through fraction contained *E. coli* cells with nearly 100% purity, whereas the fraction eluted by compression of the adsorbent contained viable *S. cerevisiae* cells with 95% purity. In another study [46], the same research group presented the preparation of a macroporous poly(dimethylacrylamide) monolith as a matrix for the direct immobilization of phages selected against human lactoferrin and von Willebrand factor (vWF). The macroporous monolithic columns were successfully used for the direct affinity capture of target proteins from particulate containing feeds like milk containing casein micelles and fat globules (1–10 μm in size) or even whole blood containing blood cells (up to 20 μm in size). Therefore, the newly developed platform based on selected bacteriophages immobilized within the macropores of the monolithic cryogels provided a convenient alternative to antibodies for the fast and selective development of the specific adsorbent.

3.3 Silica-based monolithic columns

Silica-based rod columns [47–49] are prepared by sol-gel process of sequential hydrolysis and polycondensation of organo-silicium compounds. Their preparation started in the early 1990s, and their study on silica-based monoliths was stimulated by the introduction of the first commercially available product, the Chromolith™ columns, on the market in 2000 [50]. These monoliths exhibit high porosity, which allows them to be available for convection; the average size of the macropores leads to a column permeability comparable to a conventional bed-packed one [51]. In a review [50], Cabrera offered an overview covering various aspects of this column type for the high-throughput analysis of drugs and metabolites, chiral separations, in the analysis of pollutants and food-relevant compounds, and in bioanalytical separations as in proteomics. She concluded that many applications, originally developed using packed columns, could be performed with a monolith while reducing the analysis time by a factor of 5–10. All studies that compared monolithic silica columns and conventional packed silica columns in terms of their physical and chromatographic properties concluded that both types of silica-based columns are comparable regarding performance, selectivity and reproducibility. In another review [52], Guiochon identified the characteristic differences between monolithic silica standard- and narrow-bore columns and those of traditional packed columns, their advantages and drawbacks, the preparation methods of monoliths of different forms and the current status of the field.

Mallik et al. [53] developed an α-1-acid glycoprotein (AGP) containing-silica monolith and evaluated its use in chiral separations in terms of its binding, efficiency and selectivity. They compared the results with data obtained for this protein when it was used as a chiral stationary phase (CSP) with HPLC-grade silica particles or poly(GMA-*co*-EDMA). The surface coverage of AGP in the silica monolith resulted higher than that obtained with the other supports and much better when it was evaluated from two chiral analytes (*R*/*S*-warfarin and *R*/*S*-propranolol). The higher protein content of AGP silica monolith gave higher retention toward both chiral analytes tested, contributing to greater resolution. In addition, the AGP silica monolith gave the lowest backpressures and best separation impedances for the assayed compounds.

Hage and Yoo [54] examined the development and use of affinity microcolumns containing HSA silica monoliths for the high-throughput analysis of drug–protein interactions. It was then concluded that apart from the smaller amount of protein required for column preparation, the advantages gained by using smaller columns involved lower retention times and lower backpressures as compared with those obtained by traditional HPLC affinity columns. However, the disadvantage observed in decreasing column length implied lower precision in retention factor and plate height measurements. In addition, they compared microcolumns containing silica particles versus silica monoliths.

Svec et al. [55] reported the preparation of brush-type CSP from a silica monolith and, separately, from porous silica beads via a process of in-column modification including attachment of the chiral selector via copper-catalyzed azide–alkyne cycloaddition. The surface of both silica monoliths or packed beads of the particles was modified with azide groups by treatment with 3-(azidopropyl)-trimethoxysilane followed by immobilization of a proline-derived chiral selector containing an alkyne moiety. The separation performance of these triazole-linked stationary phases was demonstrated in enantioseparations of *p*-acidic amino acid amide derivatives, which afforded high separation factors.

4 Special applications of monolithic columns in AC

4.1 Immunoaffinity chromatography

Immunoaffinity chromatography is a process in which the binding affinity of an antigen to a parent antibody is used as a basis of separation. This procedure employs antibodies immobilized on the solid support over which biological samples are passed, and the antigen, specific for the immobilized antibody can be isolated. It can also be used to purify or enrich antibodies, the antigen being the specific immobilized ligand.

Sometimes, the binding of a biomolecule (used as ligand) to a chromatographic support could alter its active site and consequently reduce (or eliminate) its activity. In this manner, Brne et al. reported the oriented immobilization of two antibodies (IgY and mAb 69.26 anti-HSA antibodies with affinity for HSA and inter-α-inhibitor protein (IaIp), respectively) in convective interaction media (CIM®) monoliths containing hydrazide groups in their surface [56]. Firstly, they modified the surface of the epoxy-CIM® monoliths with adipic acid dihydrazide to obtain hydrazide groups in the monolithic surface. The conversion of epoxy groups for this reaction was very low (below 7.7%) compared with other reactions with epoxy groups. The oxidized antibodies were then covalently immobilized on the modified monolith through the aldehyde groups present on the Fc region of the antibody structure. The affinity columns showed good selectivity against HSA and IaIp in human serum although the dynamic binding capacity reached was low.

Recently, Kasper et al. immobilized anti-V5 antibody on CIM® epoxy discs for the immunoaffinity isolation and purification of blood group antigens from cell culture supernatant [57]. The target proteins presented both a V5-tag (for the purification using CIM® media with the immobilized antibody) and a His-tag (for the conventional purification using HiTrap® metal affinity column). With both purification methods, it was possible to isolate the target proteins with high purity. However, the purification process using CIM monoliths was more efficient and faster (three times) than those using the conventional purification with a packed column. The same affinity process was also adapted at the preparative scale using a CIM® 8 mL tube. In this case, the recovery of eluted proteins was above 90%. This recent publication further exemplifies the advantages of the use of monolithic supports for the purification of large biomolecules from complex samples.

Tscheliessnig and Jungbauer used CIM® Protein A HLD disks as affinity supports for the fast evaluation and quantification of IgG from crude supernatant of Chinese hamster ovary cells and purified samples [58]. They tested different equilibration buffers to reduce the non-specific adsorption of host cell proteins which could interfere in the quantification of IgG. Using this method, the authors reported the quantification of IgG in a concentration range of 23–250 μg/mL within 5 min.

4.2 Lectins as affinity ligands

Lectin AC is commonly used for the purification and profiling of glycoproteins and their fragments. The interest in the study of glycoproteins centers on the fact that alterations during glycosylation processes are related to different diseases such as cancer and inflammation processes. The most common lectins used as affinity ligands are Con A and wheat germ agglutinin (WGA). Feng et al. published the chelation of Con A on a Cu^{2+}-iminodiacetic acid (IDA)-poly(GMA-*co*-EDMA) capillary column [59]. In comparison with other approaches where Con A was covalently bound to the chromatographic support, in this study the authors contend that this non-covalent immobilization of Con A presents some advantages such as the stability of the chelated ligand, a higher number of disposable active sites to interact with the glycoprotein and the possibility of using ammonium buffers in the elution steps, avoiding the use of high sugar concentrations. This affinity column, compared with conventional Con A lectin chromatography, showed a greater capability of enrichment using a minimal amount of sample. Figure 2 depicts the difference in the number of identified glycoprotein (from normal and bladder cancer patients) using these two approaches. Finally, to prove the application of these affinity columns in the profiling of low amounts of complex sample, these capillary columns were successfully implemented in the elucidation of glycoprotein from mouse urine samples. Zhong and El Rassi reported the immobilization of Con A and WGA on silica-based monolithic supports [60]. The silica monolith was prepared by the sol-gel process, and γ-glycidoxypropyltrimethoxysilane was further grafted to obtain epoxy groups in the monolithic surface. The epoxy groups were then opened with sulfuric acid, and the resulting diol groups were oxidized with $NaIO_4$ to obtain aldehyde groups. Finally, the immobilizations of lectins were performed by reductive amination reaction using $NaBH_3CN$. The capillary columns with immobilized lectins were tested in the affinity separation by nano-liquid chromatography (nano-LC) and CEC of some monosaccharides and standard glycoproteins (transferring, ovalbumin, AGP and κ-casein).

4.3 Boronate AC

Boronate AC is an important tool for the specific extraction and separation of *cis*-diol-containing compounds such as catechols, nucleosides, nucleotides, nucleic acids, carbohydrates, glycoproteins and enzymes. The first paper reporting the use of monolithic polymers in boronate AC was carried out by Hilder et al. [61]. They performed two approaches for the covalent immobilization of *p*-hydroxyphenylboronic acid as ligand on a poly(GMA-*co*-EDMA) capillary column. The first method consisted of a nucleophilic substitution reaction between the epoxy groups on the monolith and the boronic compound. The second was carried out using the nucleophilic reaction but on a poly(GMA-*co*-EDMA) capillary column previously photografted with a layer of GMA. These affinity columns were tested in the separation of ribonucleosides as model mixture by micro-LC and CEC. Higher retention factors were yielded with the support modified via photo-grafting of poly(GMA). This was attributed to the presence of higher amount of functional groups on the polymeric surface apt to react with the boronic acid.

Ren et al. published two papers [62, 63] reporting the *in situ* preparation of monolithic columns using

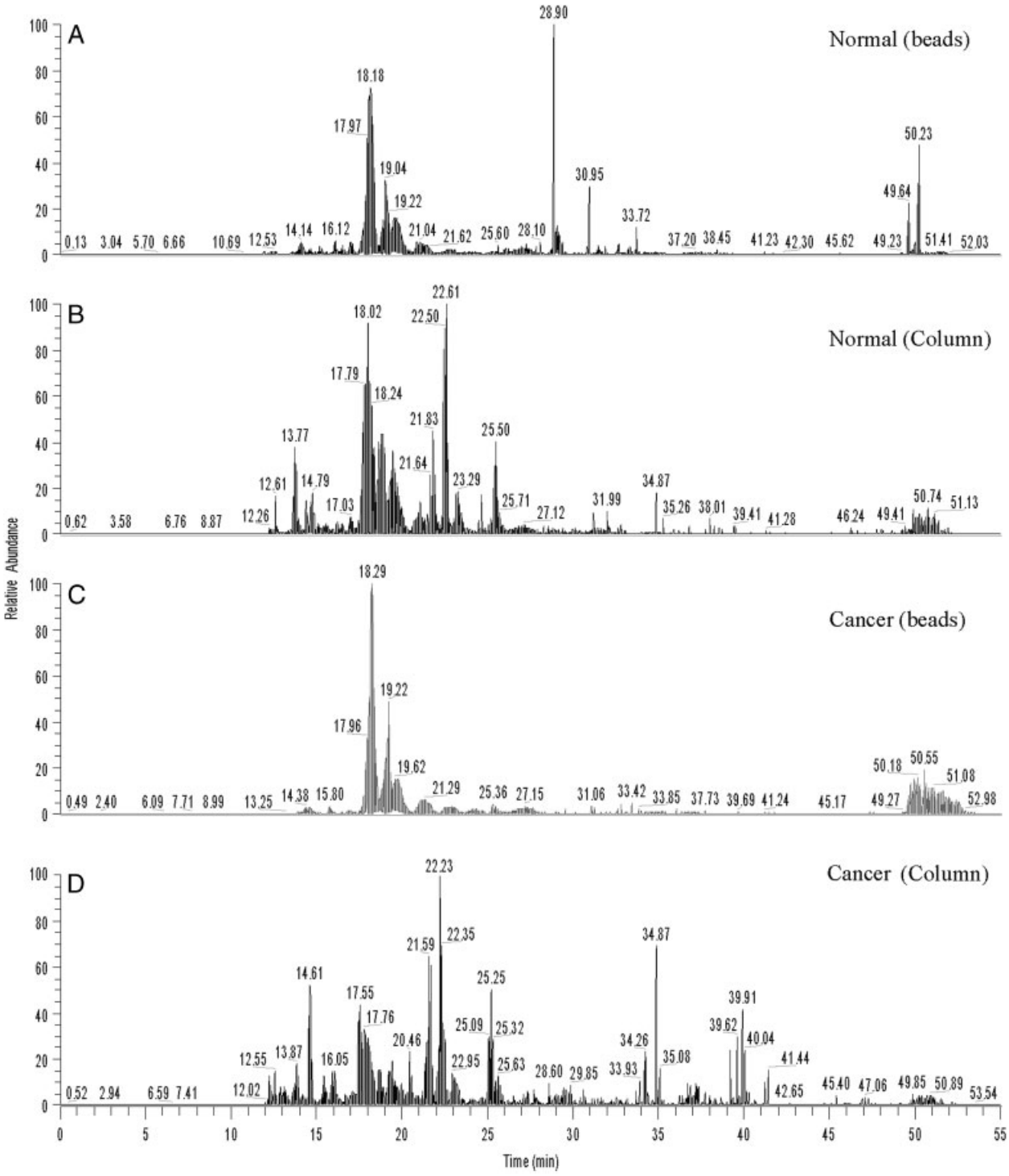

Figure 2. Base peak chromatogram of the glycoprotein enrichment of urine specimen from a bladder cancer patient, the initial protein amounts of sample are 10 μg. (A and B) Normal sample, enrichment using Con A beads and monolith, respectively; (C and D) bladder cancer sample, enrichment using Con A beads and monolith, respectively (from [59] reproduced with permission from American Chemical Society).

4-vinylphenylboronic acid (VPBA) as monovinyl monomer and different crosslinkers. In a paper [62], the authors reported the synthesis of poly(VPBA-*co*-EDMA) at 75°C using a mixture of diethylene glycol and ethylene glycol as porogenic mixture. Besides boronate affinity interactions with the analytes, these monolithic columns showed different retention mechanisms such as reversed phase and cation exchange interactions. The authors demonstrated

that the use of a Lewis base (ion F^-) in the mobile phase enhances the interaction between the boronate groups in the monolith and the diol groups in the target analyte. Then, these monolithic columns were used in the capture of adenosine and flavin adenine dinucleotide. In other paper [63], in order to avoid the non-specific interactions above mentioned, the same research group prepared a more hydrophilic monolithic column consisting of VPBA cross-linked with Bis. In this case, the authors used porogenic mixture formed by dimethylsulfoxide, dimethylformamide and water as good solvents and dodecanol as poor solvent. These capillary columns were applied in the specific capture of glycoproteins (lactoferrin and horseradish peroxidase (HRP)).

Chen et al. [64] reported the one-step preparation of poly(3-acrylamidophenylboronic acid-*co*-ethylene dimethacrylate) (poly(AAPBA-*co*-EDMA)) monoliths inside 530 μm id capillaries using PEG with different molecular weights as porogens. These monolithic columns were positively used in the selective enrichment of glycopeptides (from tryptic digest of HRP) and glycoproteins (from a mixture of HRP and BSA).

It is important to mention that in all cases where monolithic polymers were prepared using monomers containing boronic acid moieties [62–64], the yielded materials presented very low pore diameter (<11 nm) which would cause low permeability from these chromatographic supports. Therefore, optimization of the polymerization conditions in the preparation of these functionalized monoliths could improve their hydrodynamic properties.

4.4 Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC), based on the specific coordinate covalent bond of amino acids (particularly histidine) to immobilized metals (such as cobalt, nickel, copper) on supports, is commonly used for the purification of histidine containing proteins or peptides. These affinity modes have been applied to methacrylate-based monoliths, cryogels and silica-based monolithic columns.

Cheeks et al. reported the separation of histidine-tagged lentiviral vectors from crude cell culture using poly(AAm-*co*-AGE-*co*-Bis) cryogels and CIM® discs [65]. IDA was used as chelating ligand, and bivalent ions (Cu^{2+}, Ni^{2+} and Co^{2+}) were used as metals to interact with the target analytes. Both stationary phases showed viability in the purification and concentration of lentiviral vectors with some advantages and disadvantages related to the structure of each material (cryogels or rigid CIM® supports). On the one hand, CIM® disc showed better results in terms of capture, concentration and elution capacity though these supports needed a sample pre-clarification step to avoid clogging of the porous matrix. The better performance of these monoliths is based on the fact that CIM® discs present a higher surface area than that of typical cryogels supports. On the other hand, the characteristic high permeability found in monolithic cryogels allowed them to be used directly in one-step bioprocessing since no sample pre-treatment was needed.

CIM-IDA® discs with Cu^{2+}, Ni^{2+} and Zn^{2+} were positively used in the purification of polyclonal and monoclonal IgG [66]. The authors studied the influence of different buffer systems and flow rates over the binding and elution of IgG from the monolithic discs; a dynamic binding capacity up to 16 mg/mL was reached for the support CIM-IDA-Cu^{2+} disc, whose value was in the same order than that obtained for CIM® discs with specific ligands like Protein A and Protein G. Therefore, CIM® discs with immobilized metals as pseudo-affinity ligands could prove an interesting alternative for the purification of immunoglobulins.

Recently, Erzengin et al. published the preparation of poly(2-hydroxyethyl methacrylate (HEMA)-*co*-Bis) cryogels with embedded Cu^{2+}-attached sporopollenin particles [67]. These monolithic cryogels were used in affinity adsorption of HSA from aqueous solutions and human plasma. Under optimized adsorption conditions, the authors reported high adsorption capacity values (27.1 mg HSA/g support) possibly due as a consequence of the large surface area reached by the embedding process.

Currivan et al. prepared capillary monolithic columns with a gradient of IDA ligand along the column length [68] and the gradient ligand coverage was studied by scanning capacitively coupled contactless conductivity detection (sC^4D) [69]. Although the application of these novel monolithic columns has not been demonstrated, it is important to mention that the characterization technique used in this work could be a useful non-invasive method to gain information on the longitudinal homogeneity of capillary columns used in IMAC.

4.5 Use of aptamers as affinity ligands

Other ligands used in AC are aptamers, i.e. synthetic nucleic acids (ssDNA or RNA) with a specific and complex 3-D structure allowing them to interact specifically with a wide variety of target molecules [70]. Zhao et al. reported the preparation of affinity columns using an aptamer (61-mer aptamer) with affinity toward cytochrome *c* [71]. Over a streptavidin-modified poly(GMA-*co*-TRIM) monolithic column, the biotinylated aptamer was covalently bound. This monolithic column was tested in the separation of mixtures of cytochrome with abundant non-specific proteins, showing a specific interaction for the target biomolecule. Since this aptamer had a G-quartet structure, this column was also used in the separation of thrombin from non-specific proteins. In order to show the application of these columns to complex samples, cytochrome *c* and thrombin were positively separated and detected from spiked samples of human serum and rat liver tissue. Following a similar approach, the same research group reported the separation of thrombin using two aptamers (29- and 15-mer aptamers) which interacted with the different binding sites of the thrombin molecule [72]. The

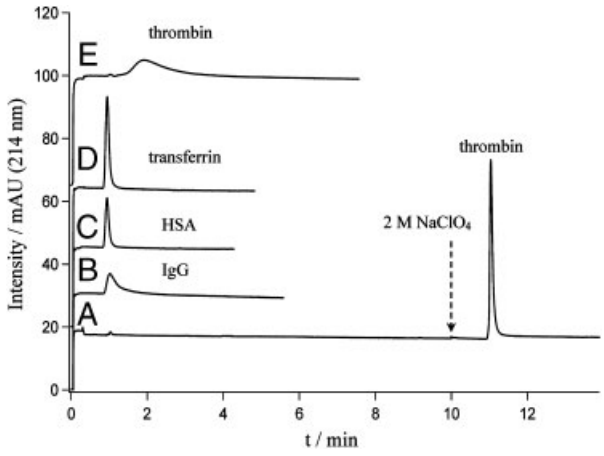

Figure 3. Chromatograms showing strong retention of thrombin (A) and little retention of IgG (B), HSA (C) and transferrin (D) on a monolithic column modified with Apt 29. There was only weak retention of thrombin on a monolithic column without immobilized aptamer (E). Curve A: 0.50 mg/mL thrombin was injected onto the Apt 29-modified monolithic column. The mobile phase for the first 10 min was the binding buffer consisting of 20 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (pH 7.4). The mobile phase was then switched to 2 M $NaClO_4$ (indicated by the arrow) in order to elute the captured thrombin. Curves B–D were from the injection of 0.50 mg/mL IgG (B), 0.25 mg/mL HSA (C) and 0.25 mg/mL transferrin (d) and running with the binding buffer as the mobile phase. There was no need for elution with 2 M $NaClO_4$ because these proteins were not retained. Curve E: The 0.50 mg/mL thrombin was injected onto the monolithic column with no aptamer and the mobile phase was the binding buffer as in (A–D). Samples were injected at a pressure of 8 bar for 5 s (from [72] reproduced with permission from American Chemical Society).

separation of the target molecule was carried out by both a direct detection method (using one aptamer and UV or fluorescence detection) and a sandwich detection assay. The latter approach was implemented first by binding one aptamer on the monolithic column to interact with thrombin. In Fig. 3, the chromatograms show strong retention of thrombin and little retention of IgG, HSA and transferrin on a monolithic column modified with aptamer 29. The other labeled aptamer (with tetramethylrhodamine) was used as the reporter probe. With this method, the selectivity was improved and there was no need of labeling the target biomolecule, an important procedure in the case of diluted samples. These affinity monolithic columns showed good stability and reproducibility (the RSD for the run-to-run and column-to-column analysis was 2 and 3%, respectively). Since aptamers interact specifically with the target molecule, these affinity ligands have potential application as pre-concentrators to improve the detection limit and also clarify complex samples.

4.6 Use of other ligands

Peskoller et al. used the antibiotic polymyxin B as affinity ligand for the capture of Gram-negative bacteria (*E. coli*) [73]. The authors reported the fast preparation (only 1 h) of a novel monolithic polymer obtained through a polyaddition reaction polymerization of poly(glycerol-3-glycidyl ether) using BF_3 as initiator and mixtures of organic solvent as porogenic agent. The polymer presented porous sizes between 1 and 22 μm. For the binding of the ligand, the CDI method was implemented. Firstly, the epoxy groups were opened to diol functionality with sulfuric acid. These hydroxyl groups then reacted with CDI to obtain the active imidazolyl carbamate groups which finally reacted with the amine groups on polymyxin B. The application of these monoliths was demonstrated in the enrichment of aqueous samples with spiked *E. coli* cells at high flow rates obtaining a recovery of 97% in only 200 μL elution volume. Tennikova's research group published the use of CIM® disks monolithic columns with different functionality (epoxy and CDI activated) for the isolation of influenza virus [74]. As ligands, sialic acid derivatives were used by its affinity to hemagglutinin found on the virus membrane. These ligands were immobilized by different synthetic pathways and different molecular size proteins (soybean trypsin inhibitor and BSA); a synthetic polymer and natural polysaccharides (chitosan and heparin (Hep)) were used as intermediate spacers. The performances of the supports prepared were assayed with virus-like particles (nanoparticles of poly(Sty) containing hemagglutinin) and real influenza virus. From all the supports tested, the affinity disk with chitosan as spacer evidenced the higher adsorption capacity (6.9×10^{12} virions/mL support). Chromatograms for influenza A virus are shown in Fig. 4; the chromatogram obtained using the disk containing chitosan as spacer demonstrated the difference in the adsorption.

Compared with other biomolecules employed as affinity ligand, the use of peptides presents some advantages such as its relatively easy large-scale production and low cost. Recently, Neff and Jungbauer reported the use of peptide affinity discs for the rapid quantification of both purified IgM and IgM from cell culture supernatant [75]. The peptide ligand was screened by epitope mapping using peptide SPOT synthesis and then covalently bound on an epoxy-activated CIM monolithic disc pretreated with iodoacetic anhydride (spacer). Using these affinity discs, the limit of detection for IgM from cell culture supernatant was 48 μg/mL. In another work, Han and Forde used a 16-mer peptide as ligand for the affinity purification of pDNA from clarified lysate of *E. coli* [76]. The authors stated that the direct pDNA isolation using affinity monolithic columns could eliminate the several steps needed for the purification of DNA using other commercial purification methods. The authors first prepared poly(GMA-*co*-EGDA) inside a poly(propylene) tube (12×1.5 cm), and the ligand immobilization reaction was then carried out. Using this monolithic column, it was possible to isolate pDNA from a clarified lysate containing other impurities such as RNA, gDNA and proteins. The recovery for the isolation process was 81% of pDNA with a purity of 92%.

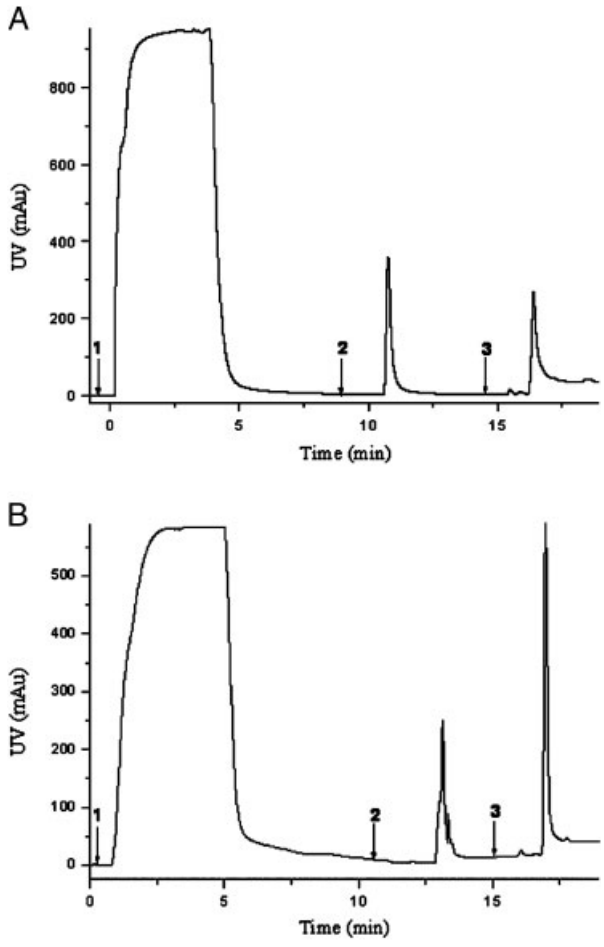

Figure 4. Chromatograms of influenza A virus (frontal analysis) on the following: (A) disk, sialyllactose, (B) disk–chitosan–sialyllactose; (1) sample loading (0.05 M acetate buffer, pH 6.2), (2) washing by 0.4 M NaCl in 0.01 M PBS (pH 6.5) and (3) virus elution with 0.2 M NaOH (pH 14). Flow rate was 2.0 mL/min (from [74] reproduced with permission from American Chemical Society).

Delattre et al. used CIM®-EDA monolithic disks with histidine as pseudoaffinity ligand for the purification of oligouronides [77]. The ligand was immobilized through its amine group, and the interactions with the target molecules were mainly of ionic type between the positive charges on the ligand and the negative charges of the carboxylate groups in the oligouronides. The purification of oligosaccharides was performed with different chromatography supports such as Sepharose, silica-based gels and CIM® disks. From the different purification processes, the same oligosaccharide fractions were obtained with all supports though in the case of purification with CIM® disks, the total experiment time was <5 min while the time needed for soft gels was 30 min.

Our research group [78, 79] reported the preparation of poly(*N*-acryloyl-tris (hydroxymethyl) aminomethane-*co*-glycidyl methacrylate-*co*-*N*,*N*-methylenebisacrylamide) (poly(NAT-*co*-GMA-*co*-Bis)) discs using different porogenic solvents for the retention of antithrombin III (AT-III). From all the porogens assayed, the macroporous rod-shaped polymer obtained with PEG 6000 as co-porogen showed the best porous properties. The products obtained reacted with EDA and hexamethylenediamine (HMDA) in order to introduce amine groups and to analyze the spacer length on the immobilization of the ligand Hep, which was covalently bound through reductive amination reaction. Finally, the amount of ligand coupled to the discs was not influenced by the two length spacers assayed. The amount of Hep coupled on discs [591.50 and 489.90 μg Hep/g dry polymer for poly(NAT-*co*-GMA-*co*-Bis)-EDA-Hep and poly(NAT-*co*-GMA-*co*-Bis)-HMDA-Hep, respectively] was similar to that obtained using macroporous polymer beads previously reported by other authors. The supports yielded showed high retention capacity for AT-III.

4.7 Use of AC in the characterization of biological interactions

Since AC is based on the specific interaction between the immobilized ligand and a target molecule, this type of chromatography is also used to perform a quantitative characterization of the complex formed between biomolecules and compounds with pharmaceutical interest [80]. In this manner, Platonova et al. used monolithic disks to study the interactions between a polynucleotide bounded in the polymeric surface and different polycations (which differ in structure and charge type) with potential application as non-viral vectors for gene delivery [81]. Two synthetic polynucleotides (poly(rA) and poly(rC)) were used to mimic the DNA structure and bound to the monolithic support via epoxy reactive groups. Using these modified disks and frontal elution method, the authors reached different parameters related to the complex formation between these macromolecules; they therefore selected the most promising candidates to be used for gene delivery.

As previously mentioned, Hage's research group reported the use of silica monolithic columns with a length as short as 1 mm for the rapid analysis of the interaction between HSA (used as model protein) and carbamazepine and *R*-warfarin (used as model pharmaceutical compounds) [54]. For the HSA immobilization, the silica columns were first modified to obtain diol groups and the protein was then covalently bound by reductive amination reaction. With *R*-warfarin, it was demonstrated that both types of supports could be used in HSA microcolumns for the determination of the retention factors or the plate heights in drug–protein binding studies. Comparing the results obtained with those reached from 7 μm silica particles, silica monoliths presented some advantages such as lower retention time, lower back pressure and higher efficiency. However, the authors mentioned the lower precision in the different measurements performed as possible drawbacks in the use of these micro-columns. The information supplied in this

paper should be useful in creating and adapting affinity microcolumns that contain HSA or other proteins for drug-binding studies.

4.8 Use of monolithic AC coupled to other analytical techniques

An important trend in the use of affinity monoliths in separation science is their use in tandem analytical techniques. Therefore, affinity columns are being used to selectively extract a target compound or group of compounds of complex samples before their study with a second analytical separation.

Immunoaffinity monolithic columns (IAMC) have been recently used in the on-line clean-up of compounds with environmental interests from complex samples [82, 83]. Li et al. prepared IAMC for the extraction of bisphenol A (BPA) from water samples followed by quantification with liquid chromatography electrospray ionization–tandem mass spectrometry (LC-ESI/MS/MS) [82]. The use of this system should reduce the risk of sample contamination since the sample is directly loaded to the system without previous pre-treatment. Poly(GMA-*co*-EDMA) monolithic columns were pre-treated to get aldehyde groups; the antibodies against BPA were covalently bound through reductive amination reaction. These affinity columns were used in the extraction of BPA from environmental real samples showing better results compared with those of an off-line SPE technique using commercially available supports. The same research group recently reported the preparation of an IAMC for the on-line clean-up of pyrethroid insecticides in tandem with a reverse-phase HPLC analysis [83]. Poly(GMA-*co*-EDMA) was polymerized at 80°C inside a stainless steel cartridge using an original approach to reduce the shrinkage of the polymeric network during the polymerization reaction. Specific antibodies against pyrethroids were then covalently bound onto the monolithic columns via Schiff-base reaction and this column was used in the enrichment of four pyrethroid insecticides which were further separated by RPC. Figure 5 shows the enrichment of the insecticides using the monoliths.

Although these affinity columns were tested with standard mixture, further studies with real samples should be performed to show the real clean-up capacity of these columns.

Monolithic columns in tandem with other analytical methods are helpful for the analysis of diluted samples as in phosphorylated proteins and peptides. In this manner, Feng et al. reported the use of Fe^{3+}-IDA silica monolithic columns for the phosphoproteome analysis of tryptic digest [84]. First, the IMAC capillary column was used for the enrichment of phosphopeptides from tryptic digest of biological samples and then coupled with a μRPLC-nano ESI MS system for the phosphopeptide identification. Using this coupled system, the authors reported the identification of peptides even with injections of low amount of sample (1 fmol).

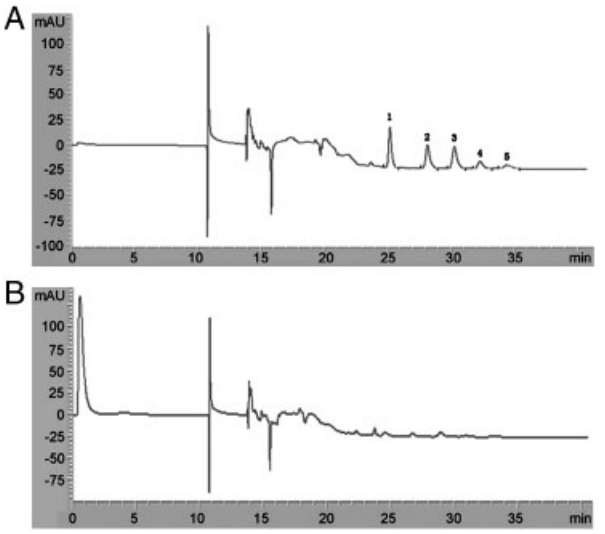

Figure 5. Comparisons of the binding desorption of the pyrethroids on (A) IAM and (B) control column. Peaks 1–5: flucythrinate, deltamethrin, *trans*-permethrin, *cis*-permethrin, flumethrin (from [83] reproduced with permission from Elsevier).

A promising direction in the use of affinity monolithic columns suggests their integration with CE in microdevices. The main advantages of using miniaturized systems include the low amount of sample needed and the integration of two processes (sample pretreatment and CE separation) in a single microdevice. Woolley's research group published several works in this field. Sun et al. prepared a ~2 mm immunoaffinity poly(GMA-*co*-EDMA) monolith coupled with CE in a microchip [85]. The porous polymer was photopolymerized inside the channel of the microdevice, and anti-fluorescein isothiocyanate (anti-FITC) was used as affinity ligand.

This microchip was used in the separation of a mixture of FITC-tagged proteins in the presence of an interfering protein (untagged green fluorescent protein (GFP)). Figure 6 shows the purification and separation of FITC-tagged IgG and HSA from GFP. Therefore, this kind of microdevices could be used in the simultaneous analysis of other biomolecules relevant in clinical diagnosis. In another study, Yang et al. reported the immobilization of anti-FITC antibodies on poly(GMA-*co*-EDMA) monoliths for the enrichment and further micro-CE separation of a mixture of FITC-tagged amino acids [86]. For the antibody immobilization, the monolithic polymer was first treated with EDA and then with (sulfosuccinimidyl-4-N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) (a cross-linker) to achieve maleimide groups on the polymeric surface which further reacted with the thiol entities of the reduced antibodies. The authors stated that this approach offers some advantages over other immobilization reactions of antibodies, such as lower reaction times and lower amount of antibody used. After the antibody immobilization, lysozyme solution was flushed through the affinity column in order to

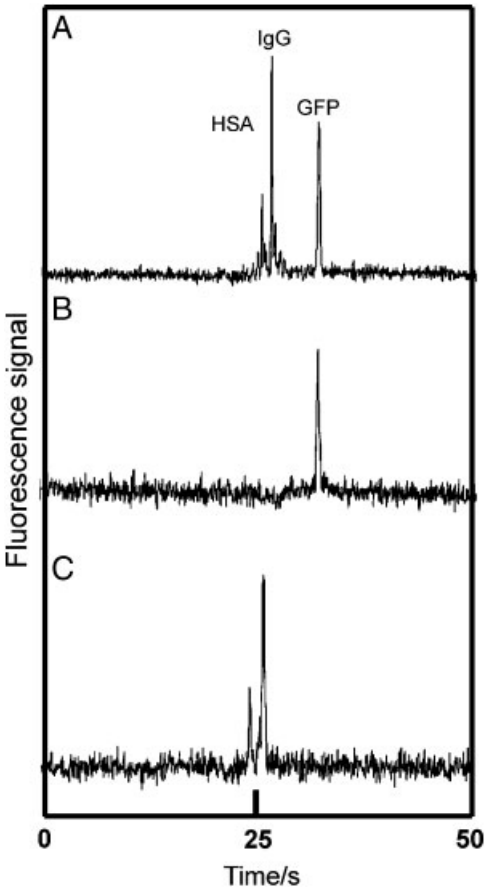

Figure 6. Purification and separation of FITC-tagged IgG and HSA from GFP. (A) Microchip CE of 20 μg/mL FITC HSA, 50 μg/mL FITC IgG and 300 μg/mL GFP. (B) Microchip CE of the rinse solution after loading 20 μg/mL FITC HSA, 50 μg/mL FITC IgG and 300 μg/mL GFP on the monolith. (C) Microchip CE of the eluate from the anti-FITC monolith in (B) (from [85] reproduced with permission from American Chemical Society).

block the non-specific adsorption sites. Using this affinity microcolumn, the authors reached an enrichment of 20 times, a recovery of 91% and a 25 000-fold concentration decrease in a contaminant protein. Figure 7 shows a microchip CE of FITC-labeled amino acids and GFP, before and after affinity column extraction. The results demonstrated that these microchip affinity monoliths can selectively enrich desired species from biological mixtures through specific antibody–antigen interactions.

Recently, the same research group reported the use of integrated microdevices to isolate and quantify four protein biomarkers with importance in cancer diagnosis [87]. Although in this study, the affinity columns consisted of a layer of poly(GMA-*co*-EDMA) inside the micro-channel walls, the authors mentioned that the use of porous monolithic polymers as affinity columns could increase the binding capacity of the affinity support and therefore increase the number of biomarkers to be detected.

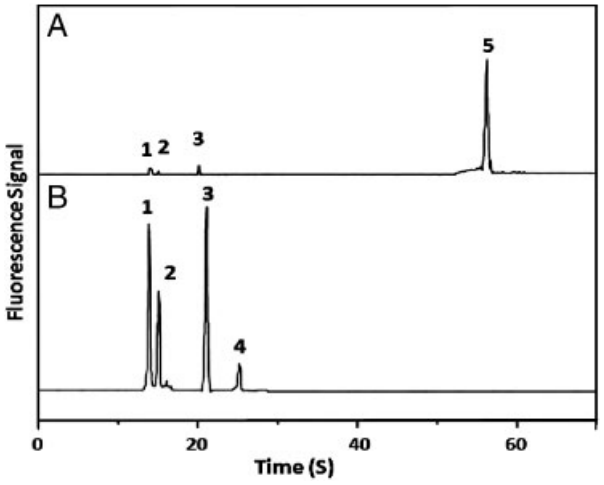

Figure 7. Microchip CE of amino acids and GFP (A) before and (B) after affinity column extraction. Peaks 1–5 are Gly, Phe, Arg, FITC and GFP, respectively (from [86] Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission).

5 Concluding remarks

Rod-shaped macroporous monolithic polymers have been developed as a useful generation of materials to be tested and applied in AC since they meet important requirements for acting as ideal supports for the retention, separation and/or purification of biomolecules. These supports can reach important characteristics including high selectivity and binding capacity, mechanical and chemical stability, reusability and stability under sanitation conditions.

The different papers discussed in this work have documented and explained how the particular architecture of the macroporous monolithic polymers creates unique and important separation characteristics. The great potential of these separation media was so confirmed.

Looking the different examples summarized in Table 1, it is clear that organic-based monolithic columns are the most commonly used as affinity media. The possible explanation of this trend is the conjunction of the advantages found for these materials such as their open porous structures in different solvents and in a broad pH range, the rigidity and the possibility to use monomers with different functional reactive groups which could be further used for the ligand immobilization. The possible drawbacks of silica monoliths could be the larger number of steps needed in their preparation and the numerous chemical reactions for the ligand immobilization. About cryogels, their low specific surface area could be the main reason for which these are less used in AC.

Future development in the use of monolithic columns in AC could be related with their application in sample preparation and in the enrichment of target analytes in trace concentrations for complex mixtures. Therefore, these materials could be coupled to other analytical techniques such as CE, GC and HPLC.

Table 1. Applications of monolithic polymers in AC

Monolith	Ligand	Application	Ref.
Epoxy CIM®	Antibodies	Depletion of HSA from human serum	[56]
	Antibody	Purification of blood group antigens from cell culture supernatant	[57]
	Protein A	Quantification of IgG from crude cell supernatant	[58]
	Peptide	Quantification of IgM from cell culture supernatant	[75]
	Poly(rA) and poly(rC)	Study of the interactions between polynucleotideolycations	[81]
Epoxy and CDI CIM®	Sialic acid derivates	Isolation of influenza virus	[74]
EDA CIM®	Histidine	Purification of oligouronides	[77]
IDA CIM®	Cu^{2+}, Ni^{2+} and Zn^{2+}	Purification of polyclonal and monoclonal IgG	[66]
Poly(GMA-*co*-EDMA)	Con A	Enrichment of glycoprotein	[59]
	Peptide	Purification of pDNA	[76]
	Antibody	Extraction of BPA from water samples	[81]
	Antibody	Clean-up of pyrethroids	[83]
	Antifluorescein isothiocyanate	Separation of a mixture of FITC-tagged proteins	[85]
	Antifluorescein isothiocyanate	Separations of a mixture of FITC-tagged amino acids	[86]
	Boronic acid	Separation of ribonucleosides	[61]
Poly(GMA-*co*-TRIM)	61-mer aptamer	Separation of cytochrome *c* and thrombin	[71]
	29- and 15-mer aptamers	Separation of thrombin	[72]
Poly(VPBA-*co*-EDMA)	Boronic acid	Capture of adenosine and flavin adenine dinucleotide	[62]
Poly(VPBA-*co*-BIS)	Boronic acid	Capture of glycoproteins	[63]
Poly(AAPBA-*co*-EDMA)	Boronic acid	Enrichment of glycopeptides and glycoproteins	[64]
Poly(glycerol-3-glycidyl ether)	Polymyxin B	Capture of bacteria cells	[73]
Poly(NAT-*co*-GMA-*co*-BIS)	Heparin	Purification of AT-III	[78]
Cryogels			
Poly(AAm-*co*-AGE-*co*-BIS)	Cu^{2+}, Ni^{2+} and Co^{2+}	Lentiviral vectors from crude cell culture	[65]
Poly(HEMA-*co*-BIS)	Cu^{2+}-attached sporopollenin particles	Adsorption of HSA	[67]
Silica	Con A and WGA	Separation of glycoproteins	[60]
	HSA	Study of the interaction between HSA and pharmaceutical compounds	[54]
	Fe^{3+}	Enrichment of phosphopeptides from tryptic digest	[84]

In addition, it is important to mention the fast development of new ligands-containing monoliths as aptamers, peptides, antibodies, etc. to be used in AC. However, these ligands are not found in commercially available monolithic columns. At the moment, columns containing Protein A, Protein G and Protein L as specific ligands (manufactured by Bioseparations) and ProSwift columns containing Con A as ligand (manufactured by Dionex) are available in the market. Therefore, the development in the future of new commercial available monolithic columns containing new ligands should be expected.

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