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Organic monoliths for hydrophilic interaction electrochromatography/chromatography and immunoaffinity chromatography

Dilani N. Gunasena and **Ziad El Rassi***
Department of Chemistry, Oklahoma State University, Stillwater, OK 74078-3071

Abstract

This article is aimed at providing a review of the progress made over the past decade in the preparation of polar monoliths for hydrophilic interaction liquid chromatography (HILIC)/capillary electrochromatography (HI-CEC) and in the design of immuno-monoliths for immunoaffinity chromatography (IAC) that are based on some of the polar monolith precursors used in HILIC/HI-CEC. In addition, this review article discusses some of the applications of polar monoliths by HILIC and HI-CEC, and the applications of immuno-monoliths. This article is by no means an exhaustive review of the literature; it is rather a survey of the recent progress made in the field with 83 references published in the past decade on the topics of HILIC and IAC monoliths.

Keywords

Polar organic monoliths; Hydrophilic interaction chromatography; Immuno-monoliths; Immunoaffinity chromatography

1 Introduction

It is now widely recognized that monolithic stationary phases are suitable separation media for all kind of chromatographic and electrochromatographic modes of separation of various species ranging in size from small ions and molecules to very large molecules. The wide acceptance of monolithic separation media has been facilitated by their very favorable mass transfer properties and high permeability, which give rise to high separation efficiency and allow rapid separations, respectively. Furthermore, the availability of an assortment of functional monomers and cross linkers from a rich chemistry bank and the many possibilities of porogens that can be selected from a wide range of solvents of varying polarity allowing the tailor made of monoliths with the particular interactive surface ligands and suitable porosity to solve a wide range of separation problems.

Hjertén *et al.* reported the first polymer monolithic material using soft polyacrylamide gel in 1989, but the preparation process was rather complex [1]. Later on, the same research group developed a simple method for preparing polymeric monoliths with surface bound alkyl and sulfonate groups for capillary electrochromatography (CEC) [2]. Shortly after, Svec and Frechét developed a much easier process to produce rigid macroporous polymer monoliths [3, 4]. These early works triggered the development of a variety of monoliths, the majority of which were summarized in typical review articles [5–11].

*Corresponding Author: Professor Ziad El Rassi, Department of Chemistry, Oklahoma State University, OK 74078; Tel: (405) 744-5931; Fax: (405) 744-6007; elrassi@okstate.edu.

This review article is concerned with polar monolithic stationary phases for hydrophilic interaction chromatography (HILIC)/capillary electrochromatography (HI-CEC). Some of these hydrophilic monoliths form the ideal support precursors to which enzymes [12] and antibodies [13] and other affinity ligands [14] or functionalities [15] can be readily covalently attached, thus providing surfaces at reduced nonspecific interactions [16, 17]. Therefore, it is imperative that a review article on hydrophilic monoliths for HILIC will be supplanted by a critical review on the use of these precursor monoliths in the preparation of affinity monoliths for affinity chromatography. Since the field of affinity/bioaffinity monolith chromatography has been recently reviewed [18–20], the current article will only include a review of immunoaffinity monoliths. It should be noted that review articles on hydrophilic monoliths are not many [21], and therefore, it is the aim of this review article to focus on the preparation and applications of hydrophilic monolithic materials in HILIC and immunoaffinity chromatography (IAC) over the past decade.

2 Hydrophilic interaction chromatography with polar monoliths and their applications

Although normal-phase chromatography (NPC) is the initial and traditional nomenclature designating the chromatographic technique that uses relatively polar stationary phases and significantly less polar mobile phases, the term hydrophilic interaction chromatography (HILIC) that was coined by Alpert in 1990 [22] became more and more accepted as the nature of the stationary phases used recently have evolved to encompass a wide range of functional groups on their surfaces. For instance, organic monoliths for use in the HILIC mode either by high performance liquid chromatography (HPLC) or CEC have been designed to bear also some charged groups in addition to the polar ligands in order to induce a mixed mode retention mechanism, e.g., hydrophilic interactions (HI) and electrostatic interactions. Also, for CEC, fixed charges were introduced intentionally into monolithic stationary phases in order to support a relatively strong electroosmotic flow (EOF). This led to monoliths of different kinds including neutral (void of charges), anionic, cationic, zwitterionic and amphoteric monoliths. The following sections discuss these variants of HILIC monoliths and their applications.

2.1 Neutral monoliths

Neutral monoliths have been widely used in HILIC based separations. In one study Holdšvendová *et al.* prepared a monolith for the separation of oligonucleotides by capillary liquid chromatography (cLC) [23]. The polymerization mixture contained the functional monomer *N*-(hydroxymethyl)methacrylamide, the cross linker ethylene dimethacrylate (EDMA), the binary porogen mixture consisting of propane-1-ol and butane-1,4-diol and the initiator 2,2′-azobisisobutyronitrile (AIBN). Three different polymerization mixtures with different ratio of porogenic solvents were used to synthesize three monoliths. It was observed that the higher the percentage of the porogenic solvent over that of monomers the higher was the porosity of the resulting monolith. These monolithic columns were successfully applied for the separation of mixed sequence oligonucleotides. In fact, oligonucleotides differing in sequence and length by as little as a single base were separated with gradient elution within 35 min. In addition, silanization of the inner wall of capillaries was not necessary to prepare these monolithic materials.

Recently, Zhong and El Rassi prepared a neutral polar methacrylate monolith for HILIC and HI-CEC [17]. Four different diol methacrylate based monoliths were introduced by using either glyceryl monomethacrylate (GMM) or glycidyl methacrylate (GMA) as the functional monomer. The cross linker used was either EDMA or trimethylolpropane trimethacrylate (TRIM). Among the four monoliths, GMM/EDMA showed the best retention, separation

efficiency and EOF in CEC. In nano-LC the same monolith showed the best linear flow velocity. Using the best monolith (GMM/EDMA), the authors successfully separated polar compounds including the 2-aminobenzamide derivatized *N*-glycans derived from the standard α_1-acid glycoprotein. Although the four different polar monoliths were void of fixed charges on their surface, they exhibited a relatively modest cathodic EOF in CEC due to the adsorption of mobile phase ions to the polar monolithic surface, thus forming the electric double layer necessary for producing a bulk mobile phase flow or EOF. The mobile phase ions adsorption to the surface of neutral monoliths was first exploited and evaluated by Okanda and El Rassi as early as 2005 for producing the necessary EOF for CEC separations on a neutral, nonpolar C17-monolith [24]. The phenomenon of mobile phase ions adsorption to neutral monoliths was further demonstrated by Karenga and El Rassi in RP-CEC with a variety of neutral, nonpolar monoliths [25–28].

Another research group reported a novel synthetic route to amphiphilic acrylamide-based monolithic stationary phases for CEC employing water-soluble cyclodextrins as solubilizing agents [29]. *N,N´*-Octamethylenebisacrylamide and *N,N´*-dodecamethylenebisacrylamide were synthesized in aqueous solutions in the presence of derivatized and underivatized cyclodextrins of different cavity size as the solubilizing agents. The amphiphilic stationary phases were synthesized by free radical copolymerization of the bisacrylamide (i.e., *N,N´*-alkylenebisacrylamide)–cyclodextrin host–guest complexes with hydrophilic monomers and an additional hydrophilic cross linker in aqueous solution. Free radical polymerization was started using a redox initiator containing 10% TEMED (v/v) and 10% APS (w/v) in distilled water (20–40 μL). As expected, due to their amphiphilicity, the resulting monoliths could be used for both HILIC and RPC. The elution sequence of the polar neutral solutes tested on this column followed the normal-phase mode.

Wang *et al.* prepared an HILIC polymer based monolithic stationary phase by polymerizing the neutral monomer 2-hydroxyethyl methacrylate (HEMA) and a polar cross linker pentaerythritol triacrylate (PETA) in the binary porogenic mixture cyclohexanol and dodecanol [30]. The polar sites consisting of the hydroxyl and ester groups on the surface of the monolith provided the hydrophilic sites as well as the EOF generator through attraction of ions from the mobile phase. A typical HI-CEC mechanism was observed for both neutral and charged analytes. The optimized monolithic columns were demonstrated in the separation of nucleic acids and nucleosides with > 140,000 theoretical plates/m in pressurized CEC (pCEC).

2.2 Cationic monoliths

Monoliths with surface bound positively charged groups have been used in the HILIC mode of separation as well. Que *et al.* prepared an amino phase monolith by the *in situ* polymerization of acrylamide, methylene bisacrylamide (MBA), 2-(acryloyloxy)-ethyltrimethylammonium methyl sulfate (2-AETMA) and 3-amino-1-propanol vinyl ether (APVE) in the presence of PEG as the porogen. The resulting amino monolith proved useful in HI-CEC/negative-ion electrospray ionization (ESI)-mass spectrometry (MS) [31]. The amine functionalities within the monolith imparted it with the polar character and at the same time supported an anodal EOF. This stationary phase was successfully coupled with negative-ion ESI-MS in order to separate and detect bile acids. The elution order confirmed the normal phase retention mechanism of the monolith.

Lämmerhofer *et al.* prepared a novel hydrophilic monolith by the *in situ* polymerization of 2-dimethylaminoethyl methacrylate, HEMA and EDMA in the presence of a binary porogenic mixture of dodecanol and cyclohexanol [32]. This novel monolith can be classified as an anion exchange and normal phase mixed mode separation medium. The hydrophilicity results from the hydroxyl groups of polymerized HEMA repeat units in the

monolith, which enables the separation of neutral and basic polar compounds. Weak and strong anion exchange (SAX) functionalities provide the primary interaction sites for the chromatographic separation and they support the EOF as well. Furthermore, in this investigation, the tertiary amino functionalities were alkylated *in situ* to yield quaternary amine functionalities for the CEC separation of various organic anions. In the HI-CEC mode and using these hydrophilic anion-exchanger monoliths, weakly acidic, neutral and basic compounds such as phenols, xanthenes and aromatic amines were successfully separated.

In another study, Lin *et al.* prepared a cationic HI monolithic stationary phase based on the co-polymerization of 2-(methacryloyloxy)ethyl trimethyl ammonium methyl sulfate (META) and PETA in a binary porogenic solvent consisting of cyclohexanol/ethylene glycol. META functioned as both the ion exchange sites and polar ligand provider, while PETA functioned as the hydrophilic cross linker. The hydrophilicity of this monolith increased with increasing META content in the polymerization mixture. A typical HILIC mechanism was observed when the content of ACN in the mobile phase was higher than 20% (v/v). The cationic polar monolith exhibited good selectivity for neutral, basic and acidic polar analytes. For polar-charged analytes, both HI and electrostatic interaction contributed to their retention [33].

2.3 Anionic monoliths

Monoliths containing surface bound negatively charged functional groups have also been used in HILIC. Using the similar procedures introduced by Hjertén and co-workers [34, 35], Freitag [26] and Hoegger and Freitag [27] introduced acrylamide-based monoliths for use in both CEC and nano-LC, and studied the separation mechanisms on these sorbents. Vinyl sulfonic acid (VSA) was introduced into the monoliths as the EOF generator. In one study, the authors attempted to develop CEC compatible monoliths by changing the hydrophobicity of the monoliths using MAA, dimethacrylamide (DMAA), butyl acrylate and hexyl acrylate [36]. The consistency and rigidity of the resulting monoliths were examined by varying the cross linking degree (%C) and the total monomer concentration (%T) of the DMAA/PDA monolith. As expected, the composition of the polymerization mixture greatly affected the final monolith properties and porosity. For the monoliths with MAA and DMAA as functional monomers, a transition from a gel type consistency to a hard polymer could be observed as the polymer formed when the cross linker concentration was increased [36, 37]. Additionally, the effect of added APS on the monolith consistency was also studied. This lyotropic salt (i.e., APS) affects the porosity of the monolith by the hydrophobic salting out effect. When the monoliths were produced in the absence of APS, a gel type and macroporous monolith resulted. The pore size increased with increasing the amounts of salt [36].

In another study, Hoegger and Freitag [38] investigated the consistency and texture of the monoliths resulting from DMAA and MAA as the second functional monomer besides VSA. PDA was used as the cross linker and the redox system consisting of 10% (w/v) APS/10% (v/v) TEMED was used for the initiation. It could be observed that a transition from gel-like structure to a more rigid polymer occurred as %T was increased. Moreover, the presence of larger amount %C was favorable since a more granular structure resulted. The effect of other lyotropic salts such as Na_2SO_4, NaCl, NH_4Cl, $MgCl_2.6H_2O$, $MgSO_4.7H_2O$ and APS on the DMAA polymer was also compared [38]. The authors did not observe a significant difference in the polymer consistencies, except in the case of magnesium chloride. Studies on the effects of solvent system showed that the polymerization could be carried out in a mixture of water and dimethylformamide (DMF) or formamide but not in pure methanol. But the resulting monoliths were harder than those prepared in pure water. The influence of functional monomers on forming hydrophilic stationary phases using a wide range of other monomers (i.e., APVE, allyl amine, HEMA, 2-hydroxyethyl acrylate and *N*-

(hydroxymethyl)-acrylamide) was also investigated. Two monomers, APVE and allyl amine both failed to produce the necessary porous monoliths, due to the low reactivity of allyl monomers compared to vinyl monomers. The monomers HEMA, 2-hydroxyethyl acrylate and *N*-(hydroxymethyl)-acrylamide containing carbonyl groups produced monoliths with the necessary rigidity and granular texture. In another study, using the optimized DMAA/PDA/VSA monolith, Hoegger and Freitag investigated the separation of positively charged amino acids and peptides by CEC [39]. The monolith was able to exhibit both electrostatic and hydrophilic interactions.

In another investigation, Que and Novotny prepared a cyano-phase hydrophilic monolith for the use in CEC by replacing 2-AETMA and APVE in the polymerization mixture with 2-cyanoethyl acrylate and VSA [40]. This column and the previously prepared amino column were both used in HI-CEC. It was reported that the cyano columns are more stable than the amino columns presumably due to the on column Schiff base formation. Both the cyano or amino derivatized polyacrylamide based monolithic columns proved useful in the analysis of complex oligosaccharide mixtures using CEC tandem MS system [40]. Low-femtomole sensitivities with the use of an ion trap mass spectrometer were obtained for the detection of neutral saccharides. The columns provided a nearly universal selectivity for a wide range of carbohydrates including mono- and oligosaccharides with the intact reducing end as well as saccharide alditols.

Later on, Tegeler *et al.* prepared the same cyano hydrophilic monolith and used it to separate complex glycan mixtures by CEC-MALDI MS [41]. In this investigation, a special sample deposition device was constructed and optimized for interfacing CEC and cLC columns to MALDI-MS. The glycans released from selected glycoproteins were first separated by a HI-CEC and deposited onto standard MALDI plates together with a suitable matrix as densely spaced sample dots. The monolithic CEC columns were run isocratically under the conditions of an MS-compatible mobile phase. The same report described a successful separation of the tryptic digest of ovalbumin using cLC-MALDI MS based on a polyacrylamide organic monolithic columns [41].

Dong *et al.* prepared a strong cation exchange and hydrophilic monolith by *in situ* co-polymerization of acrylamide, MBA and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) in the presence of dimethyl sulfoxide (DMSO) and dodecanol as porogens [42]. The sulfonic acid groups provided by AMPS monomer serve as strong cation exchange sites and cathodal EOF generating sites at the same time. The stationary phase could provide a significant and stable EOF over a wide range of pH of the mobile phase. This monolith showed NPC behavior for neutral analytes and electrostatic interaction/repulsion for charged analytes.

Wang *et al.* prepared a HILIC monolithic stationary phase by one-step *in situ* co-polymerization of HEMA, EDMA, and MAA in a binary porogenic solvent consisting of toluene and 1-dodecanol [43]. The resulting monolith was evaluated as a HI-CEC stationary phase using pCEC. It was found that the separation of charged solutes was based on a mixed mode mechanism of HI, weak electrostatic interaction and the effect of electrophoretic migration. As expected, the separation of neutral solutes was based on HI at high ACN content in the mobile phase.

A novel polar monolithic stationary phase was prepared by Lin *et al.* by the *in situ* polymerization of 3-sulfopropyl methacrylate (SPMA) and PETA in the presence of cyclohexanol and ethylene glycol as the porogens [44]. The monolith exhibited both HI and strong cation exchange characteristics. The sulfonate and hydroxyl groups on the monolithic surface could provide HI and strong cation exchange interaction sites for small polar neutral

and charged solutes. The possibility of using the monolith for both cLC and pCEC was explored. A typical HI mechanism was observed at higher organic solvent content (>70% (v/v) ACN) for polar and neutral analytes. For polar charged analytes both HI and electrostatic interaction were thought to be involved in solute retention. At optimum conditions for pCEC, the plate count reached > 170,000 plates/m and for cLC it was 105,000 plates/m. In the case of pCEC of nucleic acid bases and nucleosides, the separation of these compounds was primarily based on HI and electrostatic interaction mechanisms. No peak tailing was observed with the use of this monolith. In the cLC mode, the monolith offered the possibility of strong electrostatic interactions with analytes carrying positive charges. Four representative nucleotides were successfully separated with 70% ACN and pH 2.5 in 20 mM triethylamine phosphate as the mobile phase.

A double mixed mode of HI/cation-exchange and RP/cation-exchange monolith was prepared by co-polymerization of GMA, SPMA potassium salt and EDMA in a binary porogenic solvent consisting of DMF and 1, 4-butanediol [45]. The epoxy groups were then hydrolyzed to diol groups using HCl. The monolithic surface consisted of sulfonic groups and diol groups, which could be successfully used in CEC. By simply changing the ACN content of the mobile phase two mixed mode characteristics could be achieved on the same monolith. HI mode or HI/cation exchange was observed at higher ACN content (ACN >80% (v/v)) in the mobile phase. Alkaloids were effectively separated using the HI/cation exchange mixed mode.

HILIC is also useful in pharmaceutical analysis. For instance, Lin *et al.* used hydrolyzed GMA/EDMA/SPMA monolith for the separation and determination of five major opium alkaloids (narkotine, papaverine, thebaine, codeine and morphine) in pericarpium papaveris by pCEC. The method proved successful in this kind of analysis with the limit of detection of 1.5–6.0 μg/ml and recoveries with 79.0–95.9% [46]. The result revealed that the separation of alkaloids was primarily based on the mixed mode of HI and cation exchange.

2.4 Zwitterionic monoliths

Zwitterionic stationary phases have also been used for the separation of polar compounds in the HILIC mode. Polar zwitterionic stationary phases offer a distinctive environment, which allows the separation of polar and charged compounds *via* weak electrostatic interactions. Due to this fact researchers have a larger degree of freedom when choosing buffers and ionic strengths of the mobile phase. Viklund and Irgum developed two new synthetic routes for the incorporation of zwitterionic sulfobetaine groups into porous polymeric monoliths using methacrylate-based monomers [47]. The first approach involved a one-step *in situ* photo-initiated co-polymerization of *N,N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl)ammonium betaine (SPE) and EDMA. The polymerization mixture was dissolved in methanol since it could dissolve both the water-insoluble cross linker and the water-soluble SPE. Benzoin methyl ether at 1% (w/w) was used as the radical precursor. The second approach involved thermal initiated surface grafting of electrolyte responsive poly(SPE) on a rigid porous carrier poly-TRIM. The poly-TRIM was first prepared in an identical manner to that of the above SPE/EDMA monolith. The column was then filled with a solution containing 10% of the zwitterionic monomer SPE in an aqueous solution containing 1% potassium peroxodisulfate with respect to the weight of the monomers and the reaction was allowed to proceed at 70 °C. The grafted monoliths exhibited an electrolyte responsive flow permeability, whereas the permeability of the co-polymerized monoliths was unaffected by changes in ionic strength in the interval tested. Both synthetic routes resulted in polymers that showed electrostatic interactions when tested with proteins, thus providing a novel separation medium for bioseparations. The strength of the electrostatic interactions between the monoliths and the proteins were shown to be readily controlled by

the addition of low concentrations of the chaotropic salt sodium perchlorate to the mobile phase.

In a different investigation, Jiang *et al.* reported a zwitterionic monolith using SPE and EDMA within 100 μm I.D. capillaries [48] in the presence of methanol as the porogen. A typical HILIC mechanism was observed at higher organic solvent content (>60% (v/v) ACN). The monolith proved useful in the separation of neutral (e.g., acrylamides), basic (e.g., purines and pyrimidines) and acidic (e.g., benzoic acid derivatives) polar analytes. For charged analytes both hydrophilic and electrostatic interactions were responsible for retention. No evidence of swelling or shrinking of the monolith was observed even with solvents with different polarity.

More recently, the same research group prepared a novel zwitterionic hydrophilic porous monolithic column by co-polymerizing 1-(3-sulfopropyl)-4-vinylpyridinium-betaine and *N,N′*-methylenebisacrylamide. Methanol and water were used as the porogens. Dual separation mechanism of HILIC/RPC mode was observed with change in mobile phase conditions with this monolith. The transition from the HILIC to the RPC mode was around 30% (v/v) ACN in water. Superior hydrophilicity was observed on this novel monolith than the previously described SPE/EDMA monolith [48]. The optimized monolith showed comparable selectivity to commercial zwitterionic ZIC®-pHILIC phases for polar test analytes. Due to its high permeability, it was shown that fast separation of five purines and pyrimidines could be achieved on the zwitterionic monolith in less than one minute. In addition, using pH or ACN gradient nine benzoic acid derivatives were baseline separated. However, the column-to-column reproducibility of this monolith was low because of the low solubility of the cross linker MBA in the high water content porogen [49].

Another research group prepared a monolith by the polymerization of SPE and EDMA in various binary and ternary porogenic solvent mixtures with AIBN as the initiator of the polymerization reaction for the use in cLC [50]. The monolith showed RP mechanism in water rich mobile phases and HILIC mode in the presence of high concentration of ACN (> 60%–80% (v/v)) in the hydro-organic mobile phase. The novel monolith proved useful in the separation of phenolic acids with separation selectivity similar to that of the commercial ZIC-HILIC columns.

Wang *et al.* co-polymerized SPE, PETA and VSA in a binary porogenic solvent consisting of cyclohexanol and ethylene glycol [51]. PETA was used to replace EDMA as the cross linker because it is supposed to have a higher hydrophilicity due to its hydroxyl sub-layer. SPE functioned as both the electrostatic interaction and polar ligand provider and VSA was employed to generate the EOF necessary for CEC separations. The monolith provided an adequate EOF when the VSA level was maintained at 0.6% (w/w). This monolith showed good selectivity for neutral (e.g., amides and phenols) and charged (e.g., nucleic acid bases and nucleosides) polar analytes. The separation mechanism of the charged polar solutes was due to a mixed mode of HI and electrostatic interaction as well as electrophoretic mobility in the HI-CEC mode.

As a continuation of their initial works, Jiang *et al.* reported another zwitterionic hydrophilic methacrylate monolithic column prepared through a single-step co-polymerization of the zwitterionic monomer 2-methacryloyloxyethyl phosphorylcholine (MPC) and the cross linker EDMA [52]. A methanol/tetrahydrofuran mixture was a good choice of a porogen mixture for preparing a highly cross linked MPC/EDMA monolith, since it offered very good solubility for both the water-insoluble cross linker EDMA and the water-soluble MPC. This yielded monoliths exhibiting a uniform structure and good separation selectivity. This monolith showed typical HILIC characteristics at high organic solvent content in the mobile

phase (ACN > 60 % (v/v)). Moreover, this monolith consisted of both positively and negatively charged groups hence for charged analytes a weak electrostatic interaction was observed with different mobile phase pH and salt concentrations. This column showed similar hydrophilicity to the SPE/EDMA monolithic column. A good separation of small hydrophilic peptides, which was not observed on the SPE/EDMA monolithic column, was achieved on this monolithic column.

In a study by Skeríková and Jandera, a zwitterionic polymethacrylate monolithic column was reported for the separation of strong polar phenolic acids in μHPLC mode [53]. The monomer and cross linker chosen were SPE and EDMA, respectively. 1-Propanol, 1,4-butanediol and water were selected as the porogens. The authors demonstrated that the monolith could be used for both HILIC and RPC separation modes. Strongly polar phenolic acids, which are weakly retained and often poorly separated in RPC, were separated on the zwitterionic polymethacrylate monolithic column under various elution conditions. The best separation efficiency was achieved using a relatively high ionic strength acetate buffer (20–30 mM) and gradient elution with alternating increasing (HILIC mode) and decreasing (RPC mode) concentration of aqueous buffer in aqueous ACN. The novel sulfobetaine monolith proved effective in the separation of phenolic acids in a beer sample.

A novel monolithic stationary phase with mixed mode of hydrophilic and SAX interactions based on the *in situ* co-polymerization of PETA (a hydrophilic cross linker), SPE (a zwitterionic monomer) and a selected quaternary amine acrylic monomer was designed as a multifunctional separation column for CEC. Although the zwitterionic functionalities of SPE and hydroxyl groups of PETA on the surface of the monolithic stationary phase functioned as the HI sites, the quaternary amine acrylic monomer was introduced to control the magnitude of the EOF and provide the SAX sites at the same time. Three different quaternary amine acrylic monomers were tested to achieve maximum EOF velocity and highest plate count. The optimum monolith (designated as HI and SAX stationary phase) was obtained when [2- (acryloyloxy)ethyl]trimethylammonium methylsulfate was used as the quaternary amine acrylic monomer. This HI/SAX monolith showed good separation performance for a range of polar analytes including nucleotides, nucleic acid bases and nucleosides, phenols, estrogens and small peptides. Column efficiencies greater than 192,000 theoretical plates/m for estriol and 135,000 theoretical plates/m for charged cytidine were obtained [54].

2.5 Amphoteric monoliths

Although classified by the authors as having zwitterionic surface, the monolith introduced by Guerrouache *et al.* is rather amphoteric in nature with primary amine functionalities and carboxylic acid groups. The amphoteric monolith was prepared by a two-step approach [55], which involved the UV-initiated free radical co-polymerization of *N*-acryloxysuccinimide and EDMA in the presence of AIBN as the initiator and toluene as the porogen. In a subsequent step, polymer backbone hydrophilization was carried out to improve the HILIC behavior of these monolithic CEC columns. This involved first the grafting of an alkyldiamine incorporating a short aliphatic segment *via* the ester groups of the *N*-acryloxysuccinimide moieties (i.e., aminolysis) followed by converting the remaining ester groups to carboxylic acid groups *via* hydrolysis. Typical HILIC behavior was observed on this column in the CEC mode.

3 Immunoaffinity chromatography (IAC)

Using monolithic stationary phases for IAC is becoming very popular due to the attractive characteristics of monoliths. This review article summarizes the research efforts using polar

monoliths in IAC published in past decade, and it is not concerned with other bioaffinity monoliths.

As other types of affinity chromatography, the ideal support for IAC should have good mechanical stability, ease of attaching the desired antibodies, negligible non-specific binding and good efficiency. The support material should be as hydrophilic as possible to decrease non-specific hydrophobic interactions of the biological agents present in the sample. In addition, the size and distribution of pores of the porous stationary phase should also be considered. Despite their favorable high specific surface area for high density ligand immobilization, supports with small pores are not suitable for antibody immobilization since the tiny pores are not accessible by the bulky antibody molecules [18]. On the other hand, macroporous supports offer small specific surface area, and consequently, the immobilized ligand surface density will be reduced. A pore size compromise for antibodies immobilization has been formulated to be in the range of 300–500 Å pore size [56]. Based on this short discussion of the ideal support for IAC, monolithic supports seem to be a good choice given their reasonable rigidity, favorable pore size distribution and high permeability.

3.1 Immuno-monoliths

Several different stationary phases have been utilized as IAC supports since the early days of this technique. Researchers have been using very low performance materials as IAC stationary phase such as agarose, cellulose, acrylamide polymers, polymethacrylate derivatives and polyethersulfone matrices. The reason for the popularity of such materials is that, they are economical for applications such as purification and extraction of biologically related compounds. However, the major disadvantage of such supports is that they are normally suitable for work under gravity flow and they cannot withstand high backpressures.

Using monolithic stationary phases for affinity chromatography is a fast developing area of research for the past few years [19]. The most frequently used monolith for the IAC is GMA/EDMA monolith in the shape of rods and disks. This also includes several supports available under the name convective interaction media (CIM) that are commercially available. Typically, the monomer GMA is polymerized with the EDMA cross linker in a binary porogenic mixture consisting of cyclohexanol and dodecanol in the presence of the free radical initiator AIBN. The resulting monolith contains epoxy groups that can be used readily to immobilize affinity ligands. In addition, the same monolith can be treated with acids to hydrolyze the epoxy groups to diol functionalities on the surface of the monolith. The diol form gives a hydrophilic surface that is similar to that used with more traditional affinity supports such as agarose or diol bonded silica [18]. More hydrophilic surfaces are very suitable for affinity chromatography since it helps to reduce non-specific binding. In addition, the diol form can later be converted into many functional groups through which several different ligands can be attached [18, 57–59].

There are several advantages of using GMA/EDMA monolith for affinity chromatography. One such advantage is that it allows the readily binding of ligands and other spacer arms having different functional groups through its epoxy groups on the surface. Also, it is easily prepared in various pore sizes by simply changing the ratios among the monomers and/or between monomers and porogens. In addition, it is thought to have reduced non-specific binding for biological agents than other affinity support materials. However, in a recent work, Svec *et al.* reported that the hydrophilicity of diol functionalities originating from the hydrolyzed GMA/EDMA monolith was not sufficient to avoid adsorption of hydrophobic albumin in a highly aqueous mobile phase [12]. Furthermore, compared to silica supports or silica monoliths the GMA/EDMA monolith tends to have low surface area, limiting the amount of ligands that can be bound to the monolith [57].

Chen *et al.* introduced an immunoaffinity capillary column made with poly 2-vinyl-4, 4-dimethylazlactone (VDA)/HEMA/EDMA monolith as a support for antibody immobilization [60]. The incorporation of VDA into the monolith appeared to be advantageous, since it can react easily with amino or thiol groups of biomacromolecules to form stable covalent bonds at room temperature [61]. The polymerization was initiated by AIBN, while the use of functional monomers VDA, HEMA, and EDMA enabled the preparation of monolith with reactive, hydrophilic and cross linking functionalities, respectively. 1-Decanol was used here as the porogen. The median pore diameter of the through pores in poly VDA/HEMA/EDMA supports measured by mercury intrusion porosimetry in the dry state was 1.02 μm. In this work, anti-testosterone polyclonal antibody was immobilized, and the resulting immuno-monolith was used for the extraction of testosterone by competitive immunoassay and on-line laser induced fluorescence (LIF) detection. High sensitivity (limit of detection lower than 70 nM) was achieved through enrichment and LIF detection. The column was found to be reusable even after exposure to 80% methanol, with a RSD of 7.3% ($n = 4$). Further optimization of antibody immobilization was needed to achieve the utmost benefits of this novel monolith.

Recently, Gunasena and El Rassi developed a novel hydrophilic monolithic material for use in AC in general and in IAC in particular [16]. The polymerization mixture consisted of GMM as the functional monomer and PETA as the cross linker in a ternary porogenic solvent mixture consisting of cyclohexanol, dodecanol and water. The GMM/PETA monolith with surface immobilized anti-haptoglobin was evaluated in IAC. The diol functionality of the monomer and the hydroxyl group of the cross linker made the monolith very hydrophilic thus exhibiting negligible non-specific binding towards blood serum proteins while capturing the specific antigen haptoglobin. The results were promising in terms of finding an ideal support for affinity chromatography with minimal non-specific binding.

Other important types of monoliths used in IAC are cryogels. In the preparation process the monomers acrylamide, allyl glycidyl ether and MBA are dissolved in an aqueous phase with TEMED and APS as catalysts. Allyl glycidyl ether provides the monolith with the epoxy active groups through which ligands could be immobilized. The mixture is then cooled to −10 °C, which allows ice crystals to form in the solution while the polymerization is initiated. Polymerization occurs around the ice crystals and after polymerization the monolith is allowed to thaw in order to remove water from the monolith. This procedure allows creating macropores that are typically 10–100 μm in diameter. Ice crystals act as porogens in these types of monoliths and the size and the shape of ice crystals determines the pore dimensions of the final monolith. The pores in cryogels are comparatively larger than in other gels and they are highly hydrophilic in nature. These characteristics together with lower backpressure make cryogels useful for many different applications. The large pores can allow the passage of large proteins, cells and microbes without being concerned about clogging pores. On the other hand, the large pores means less surface area for the ligands to bind, which could lead to diminished binding capacity. Several reviews and protocols have been published discussing the preparation of cryogels and their applications over the past decade [62–66]. Cryogels have been used so far for the immobilization of concanavalin A and protein A in the field of affinity chromatography. Some applications of cryogels are discussed in the following section.

3.2 Applications of immuno-monoliths

Immuno-monoliths have been used in different applications of IAC such as isolation/purification, extraction, preconcentration, analysis, etc. These applications are reviewed in the following sections.

3.2.1 Isolation/purification/depletion—There are several examples in the scientific literature about isolation and purification of biological substances using immuno-monoliths. High-performance monolithic disk chromatography, including its affinity mode, represents an efficient method for the fast separations of biological molecules of different sizes and shapes. In one study, Mönster *et al.* developed an IAC technique using CIM based disks to isolate and purify three blood group antigens from cell culture supernatant. The CIM used in the study was of epoxy type [67], and the proteins employed were derived from blood group antigens Knops, JMH and Scianna, equipped both with a His-tag and with a V5-tag by which they could be purified. The epoxy CIM disks were covalently coupled with monoclonal antibody directed against the V5-tag, and the resulting immuno disks were able to isolate the indicated antigens at a higher flow rate and efficiency than the conventional bed-packed columns. Using monolithic support, with all the analyzed proteins, the eluted amount was up to 50% higher than using gel beads.

In another study, GMA/EDMA-based CIM monolithic disks were used for the rapid, quantitative affinity fractionation of pools of polyclonal antibodies from blood sera of rabbits, immunized with complex protein-peptide conjugates. The ligands used were bovine serum albumin and related derivatives and bradykinin [68]. The combination of several disks with different affinity ligands in the same cartridge allowed the separation of different antibodies to be achieved within a few minutes. The highly reproducible results of this immunoaffinity technique were compared with data obtained by the widely used enzyme-linked immunosorbent assay (ELISA). ELISA experiments revealed that the antibodies isolated by this monolith keep their biological activity while their purity is more than one order of magnitude. This method enabled direct determination of antibodies even in complex biological matrices.

In a series of investigations from our laboratory Jmeian and El Rassi reported immuno-monoliths based on GMA/EDMA monolithic columns for the depletion of eight top high abundance proteins from human serum [13, 69, 70]. In one investigation, an integrated microcolumn-based fluidic platform was introduced for the simultaneous depletion of high-abundance proteins and the subsequent on-line concentration/capturing of medium- and low-abundance proteins from human serum [69]. The platform consisted of on-line coupling of tandem affinity microcolumns to an RPC microcolumn to achieve first the depletion of the high abundance proteins by the tandem affinity microcolumns followed by the concentration and capturing of medium and low abundance proteins by the RPC microcolumn. The tandem affinity monolithic microcolumns, which are based on GMA/EDMA macroporous monoliths, are characterized by their relatively high permeability in pressure-driven flow and consequently short processing time of serum samples prior to analysis by 2-DE. The microcolumn-based fluidic platform was applied to serum samples from osteoarthritis donors before and after soy protein supplementation, and from healthy donors, and the resulting depleted serum samples from high-abundance proteins were profiled for protein expression by 2-DE. In a more recent investigation, Jmeian and El Rassi [70] reported an expanded integrated platform whereby tandem affinity monoliths for depletion of high abundance proteins were coupled to immobilized metal affinity chromatography columns and an RPC column to achieve the subsequent fractionation/concentration of medium and low abundance proteins from human serum. This platform allowed the subsequent in-depth proteomics profiling of human serum by 2-DE and LC-MS/MS.

Poly(hydroxyethyl methacrylate) (PHEMA) cryogel with surface immobilized protein A was introduced for the removal of IgM-antibody from human plasma [71]. The immobilization of protein A onto the PHEMA cryogel surface was carried out *via* cyanogen bromide activation. The supermacroporous structure of the PHEMA cryogel made it possible to process blood cells without blocking the cryogel column. The authors observed

that the IgM-antibody adsorption capacity decreased significantly with increasing the plasma flow-rate. The maximum IgM-antibody adsorption amount was 42.7 mg/g. In addition, it was demonstrated that IgM-antibody molecules could be repeatedly adsorbed and eluted without noticeable loss in the IgM-antibody adsorption amount.

Delmotte *et al.* prepared a miniaturized immunoadsorber by the immobilization of anti-myoglobin- and anti-NT-pro-BNP antibodies onto epoxy-bearing methacrylate-based 2.0 × 6.0 mm i.d. monolithic CIM disks. They successfully coupled the antibodies to the GMA/EDMA polymeric disk material using two different methods. Antibodies specific to biomarkers were either directly bound to the monolithic support or immobilized *via* the formation of a streptavidin-biotin complex. They have demonstrated that the novel disks are suitable for the isolation of cardiac biomarkers from blood serum samples. Loading capacities in the high picomole range for immunoadsorbers with a bed volume as little as 22.4 μL are more than sufficient to isolate the proteins present at biologically relevant concentrations [72].

Rucevic *et al.* prepared a CIM monolithic columns for isolation of low abundance membrane proteins [73]. In this study, monoclonal antibody (mAb) against the integral plasma membrane protein carcinoembrionic antigen cell adhesion molecule (CEACAM 1) was cross linked to protein A or protein G CIM affinity columns with a bed volume of only 60 μL. The antigen was eluted with 0.1 M citric acid, pH 2.3, containing 1% octylglucoside. The fraction eluted was collected and separated by SDS-PAGE. It was found that the majority of membrane proteins do not bind to the column. CEACAM1 was specifically bound and eluted in high concentrations. Only a small amount of this protein was found in unbound fraction.

Brne *et al.* used a CIM epoxy monolith which is a monolith that resulted from GMA/EDMA in the oriented immobilization of antibodies [74]. CIM monoliths are very effective in fast affinity binding. In this work, adipic acid hydrazide was covalently attached the to the epoxy groups. The highest achieved conversion of epoxy groups to hydrazide functionalities was 7.7%. The carbohydrate groups of antibodies were then oxidized to aldehyde groups and the antibodies were attached through the hydrazide groups. Two different antibodies were immobilized and the columns were tested for their selectivity. IgY developed against HSA and a mAb 69.26 specific towards lalp proteins were immobilized onto the columns. These columns were tested with human serum and a good selectivity was observed as proved by SDS-PAGE. Dynamic binding capacity of both columns was low and nonspecific binding of other proteins could also be observed.

Kumar *et al.* introduced a novel continuous supermacroporous monolithic cryogel affinity adsorbent for the specific fractionation and separation of human peripheral blood lymphocytes. [75]. The strategy of the affinity adsorbent is based on the interaction of protein A from *Staphylococcus aureus* with cells bearing IgG antibodies on the surface. After treating lymphocytes with goat anti-human IgG (H+L), the IgG-positive B-lymphocytes were efficiently separated from T-lymphocytes. Protein A carrying DMAA cryogel matrix specifically bound IgG-bearing B-lymphocytes through the F_c region, while non-bound T-lymphocytes passed through the column. More than 90% of the B-lymphocytes were retained in the column while the cells in the pass through fraction were enriched in T-lymphocytes (81%). The viability of the T-lymphocytes isolated was greater than 90%. They demonstrated that the technique can be applied in general to cell separation systems where IgG antibodies against specific cell surface markers are available.

Ahlqvist *et al.* developed a chromatographic method based on affinity supermacroporous monolithic for binding and analyzing inclusion bodies during fermentation [76]. The

antibody treated inclusion bodies from lysed fermentation broth can be specifically retained in protein A and pseudo-biospecific ligand sulfamethazine modified supermacroporous cryogels. Optimum binding of 78 and 72% was observed on both protein A and sulfamethazine modified cryogel columns, respectively, using IgG labeling of the inclusion bodies. Unlabeled inclusion bodies did not show non-specific binding to the gel and passed through the gel unretained. This investigation also showed the potential of using antibody binding cryogel affinity matrices in developing general binding and separation system.

The just preceding research group also developed a method to monitor the production of inclusion bodies by ELISA using cryogel mini-column plates [77]. In one method inclusion bodies were labeled with polyclonal antibodies and labeled particles were bound to macroporous monolithic protein A cryogel adsorbents inserted into the 96-well plate. Alternatively, inclusion bodies immobilized on phenyl-cryogel mini-column plates were used in indirect ELISA.

3.2.2 On-line preconcentration/immunoextraction—In one study, Liang *et al.* used GMA/EDMA monolith to prepare class-specific immunoaffinity monoliths (IAM) for efficient on-line clean up of pyrethroids [78]. The GMA/EDMA monolith was synthesized in a 50 mm × 4.6 mm i.d. stainless steel cartridge with two auxiliary pipette tips. Antibodies against pyrethroids were immobilized onto the monolith *via* reductive amination through the epoxy groups of the monolith. The amount of antibody immobilized was found to be 0.20 ± 0.02 μmol IgG per gram of the dry monolith. With a column switching valve system, the IAM could be readily adapted to an RPC system. Under optimum conditions IAM specifically retained deltamethrin, flumethrin, flucythrinate and cis/trans permethrin, which were further separated by RPC-18 column. This work claims to have high throughput and high efficient online clean up of pyrethroids in various samples.

Immunoaffinity chromatography is also a proved approach for analyzing biological sample components. In one study, the GMA/EDMA monolith was used for the preparation of monolithic pre-concentrators in polymer microchip capillary electrophoresis for the analysis of amino acids [79]. The epoxy groups were treated with ethylenediamine and then sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) and reduced antibodies were immobilized through the maleimide group. To enhance analyte selectivity, the antibodies were immobilized on the monoliths and subsequent lysozyme treatment blocked non-specific adsorption. The enrichment capability and selectivity of these affinity monoliths were evaluated by purifying fluorescently tagged amino acids from a mixture containing green fluorescent protein (GFP). Twenty fold enrichment and 91 % recovery were achieved for the labeled amino acids with a > 25,000 fold reduction in GFP concentration as indicated by microchip electrophoresis analysis. These devices are simple, inexpensive, and effective platform for trace analysis in complex biological samples.

Sun *et al.* developed an affinity monolith integrated poly(methyl methacrylate) microfluidic chip for on-line automated immunoaffinity extraction integrated with electrophoretic separation of fluorescein isothiocyanate (FITC)-tagged proteins [80]. Monoliths with epoxy groups for antibody immobilization were prepared by direct *in situ* photo polymerization of GMA and EDMA in a porogenic solvent consisting of 70% 1-dodecanol and 30% cyclohexanol. Anti-FITC was attached on the monolith as the affinity ligand. FITC–HSA and FITC–IgG were selectively retained by the immunoaffinity column and an elution efficiency of 92% was achieved for FITC–HSA. The developed immunoaffinity column/ capillary electrophoresis micro device showed great potential for biomolecular analysis.

Calleri *et al.* developed an immunoaffinity disk for the on-line solid-phase extraction of aflatoxin B1 (AFB1) [81]. Polyclonal anti-AFB1 was covalently immobilized on 12 mm × 3

mm i.d. monolithic CIM disk, which is a GMA/EDMA monolith, *via* its epoxy groups. The polyclonal anti-AFB1 thus obtained was successfully used to retain specifically AFB1. The immunoaffinity-disk was integrated in a chromatographic system. A column-switching configuration that requires two six-port valve and two HPLC pumping systems was employed for on-line immunoextraction and subsequent quantification of AFB1.

In another study, a highly selective and efficient method for on-line extraction of bisphenol A (BPA) from water samples followed by quantification with LC–ESI-MS/MS has been reported using GMA/EDMA monolith [82]. The surface epoxy groups of the monolith were converted into aldehyde and antibodies against BPA were covalently immobilized onto the monolithic column *via* Schiff base reaction. The IAC columns thus obtained were on-line coupled to LC–MS/MS using column-switching valves and the system was applied to analyze BPA in real environmental water samples. The method achieved a detection limit of 0.3 ng L^{-1} using a sample volume of 100 mL. The linear calibration range was 1.0–160 ng L^{-1}. Samples including tap water, lake water and effluent from municipal sewage treatment plants were all measured with satisfactory results.

Faure *et al.* prepared a GMA/EDMA miniaturized immunoextraction column in fused silica capillary [83]. The monomer mixture composition, initiation mode and porogen composition were optimized and anti-ochratoxin A antibodies were immobilized through single step on the epoxy groups. In order to evaluate the specificity of the analyte–antigen interaction on this immuno-sorbent, the retention of ochratoxin A was examined on this support but also on two matching sorbents. One of them constituted by the non-bonded monolith and the other one bonded with non-specific antibodies. Only the monolith bonded with anti-ochratoxin A antibodies lead to retention, showing the specificity of the interactions involved. This affinity phase based on a monolithic polymer support exhibits a high potential for specific preconcentration of small molecules.

Chen *et al.* used the VDA/HEMA/EDMA monolith described above for the immunoextraction of testosterone. Polyclonal anti-testosterone antibodies were covalently coupled to the monolith *via* VDA [60]. Fluorescently labeled testosterone at C_3 was designed as a tracer to estimate the extraction ability of this immunoaffinity column. The performance in a more realistic application was then demonstrated successfully for the rapid extraction of testosterone by competitive immunoassay and on-line LIF detection. Moreover, this monolith was shown as a suitable material for an immunoextractor to detect small molecular compounds specifically and rapidly with high sensitivity (sub ng/mL level).

4 Concluding remarks

As shown in this review article, polar monoliths and their use in HILIC/HI-CE and IAC have made significant progress. However, there are still an ample amount of room for further improvement specially in the design of monolithic support at reduced nonspecific interactions for affinity chromatography in general and IAC in particular. At the same time, this improvement, which mainly entails the hydrophilization of organic monoliths that are composite materials (i.e., composed of polar and nonpolar functionalities), will be beneficial for providing more hydrophilic monolithic columns for HILIC of polar compounds.

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Nonstandard abbreviations

2-AETMA	2-(acryloyloxy)-ethyltrimethyl ammonium methyl sulfate
AIBN	2,2′-azobisisobutyronitrile
AMPS	2-acrylamido-2-methyl-1-propanesulfonic acid
APS	ammonium persulfate
APVE	3-amino-1-propanol-vinyl ether
DMAA	dimethacrylamide
EDMA	ethylene dimethacrylate
GMA	glycidyl methacrylate
GMM	glyceryl methacrylate
HEMA	2-hydroxyethyl methacrylate
MAA	methacrylamide
MBA	methylene bisacrylamide
META	2-(methacryloyloxy ethyltrimethylammonium methyl sulfate
MPC	2-methacryloyloxyethyl phosphorylcholine
PDA	piperazine diacrylamide
PEG	polyethylene glycol
PETA	pentaerythritol triacrylate
PHEMA	poly(hydroxyethyl methacrylate)
SPE	*N,N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl)ammonium betaine
SPMA	3-sulfopropyl methacrylate
TEMED	*N,N,N′,N′*-tetramethyl-ethane-1,2-diamine
TRIM	trimethylolpropane trimethacrylate
VDA	2-vinyl-4,4-dimethylazlactone
VSA	vinylsulfonic acid

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