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Review

Porous polymer monoliths for extraction: Diverse applications and platforms

Polymer monoliths are becoming increasingly popular as sorbent materials, and, along with silica monoliths, they are sometimes touted as replacements for the particulate stationary phases used in HPLC. This critical and prospective review shows how polymer monoliths are in fact finding numerous extraction roles that do not resemble HPLC. They are showing great promise as extractors in a remarkable range of platforms, formats and hyphenated systems with functions ranging from chromatographic preconcentration to large-scale preparative extraction. Monolith surface chemistry, morphology and the approaches to monolith synthesis are discussed with regards to these emerging roles.

Keywords: Affinity chromatography / Immunoextraction / Polymer monolith / Preconcentration / Solid phase extraction

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1 Introduction

1.1 Background and scope

Since their introduction, the major application of porous polymeric monolithic stationary phases has been for HPLC and those forms of chromatography that have an emphasis on minimizing theoretical plate height and maximizing peak capacity. These applications of polymer monoliths are well described in several recent reviews [1, 2] and a book [3]. However, polymer monoliths are increasingly finding roles as sorbents for a much wider range of chromatographic applications and formats. These include SPE, preconcentration and affinity chromatography. For the purposes of this review, we have grouped all of these alternative chromatographic techniques under the term 'extraction' in order to conveniently distinguish them from the high performance separations normally associated with the term 'chromatography'.

Whilst extraction and HPLC rely on the same principles to achieve a separation, they emphasize different chromatographic properties to achieve it. HPLC typically relies on numerous cycles of sorption and desorption, with an emphasis on maximizing separation efficiency to improve separation resolution. By contrast, extraction processes are instead dependant on achieving sorption that is as strong and as selective as possible. This is typically followed by a desorption step in the form of a step gradient to facilitate rapid and complete desorption of the target species. Within the literature, such methods are described under several banners including preconcentration, SPE, sample cleanup, affinity chromatography, affinity extraction and immunoextraction.

The use of both polymer and silica monoliths for SPE and preconcentration was reviewed by Svec in early 2006 [4]. At that time, these techniques were described as less common applications of monoliths. The growing importance of monoliths for these new roles is now highlighted by the fact that roughly half of the original articles discussed in this current review were published in the past 2 years. This review therefore serves as an update to Svec's review as well as extending to discussion of a wider range of chromatographic extraction applications.

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Abbreviations: **AMPS,** 2-acrylamido-2-methyl-1-propanesulphonic acid; **BuMA,** butyl methacrylate; **CIM®,** Convective Interactive Media®; **COC,** cyclic olefin copolymer; **DVB,** divinylbenzene; **EDMA,** ethylene dimethacrylate; **GFP,** green fluorescent protein; **GMA,** glycidyl methacrylate; **IDA,** iminodiacetic acid; **IMAC,** immobilized metal affinity chromatography; **MAA,** methacrylic acid; **MAH,** *N*-methacryloyl-(L)-histidine Me ester; **META,** [2-(methacryloyloxy)ethyl] trimethylammonium chloride; **MIP,** molecularly imprinted polymer; **mRNA,** messenger RNA; **RNA,** ribonucleic acid; **SPMA,** 3-sulphopropyl methacrylate; **SPME,** solid phase microextraction; **TRIM,** trimethyloylpropane trimethacrylate; **μTAS,** micrototal analysis system

1.2 Principles of extraction

Extraction may be used to achieve various goals of analysis or purification. A range of extraction processes with terms that reflect those goals have been described in the literature, though these terms are often applied without adherence to strict definitions. Processes with the goal of increasing the local concentration of analytes in order to lower detection limits for an analytical process are often termed preconcentration and it is not unusual to report sample enrichment of several orders of magnitude. SPE is applied in a range of situations, particularly where the extraction is of a more selective nature. This also usually involves a sample enrichment effect. However, the term SPE still carries some more specific connotations associated with its original purpose to describe solvent-free sorption-based methods for sampling organic compounds in aqueous environmental samples [5].

Sample cleanup describes one of two scenarios. It may describe an extraction where the target compounds are not analytes but rather are species in the sample matrix that can interfere with the detection of the analyte. Alternatively it may describe a direct extraction of the target compound out of a matrix that could have interfered with its analysis, though such processes are also often called SPE. The term 'affinity' is applied to a range of approaches involving highly selective extractions based on complex ligands such as proteins, peptides or metal chelate moieties.

Despite the varied goals and eclectic nomenclature, the different forms of chromatographic extraction share similar requirements and give rise to common challenges. The first and most obvious is the need for an effective stationary phase. Polymer monoliths are just one of several materials that are commonly used as stationary phases for extraction. Varilova *et al*. [6] provide an excellent introduction to the numerous supports for affinity chromatography, many of which are also used for other types of extraction. They cover porous and nonporous packed particle stationary phases of silica or polymer beads as well as agarose gels, dextrans, and, of course, polymer and silica monoliths. Open-tubular formats may be used, and SPE disks of membrane-bound macroporous beads have also become popular for SPE [7]. The morphology of the stationary phase material can have powerful implications for mass transport, permeability and hydrodynamic band-broadening. It also determines the surface area, which can be a limiting factor for binding capacity. The surface chemistry of the solid support determines the binding strength and selectivity of the stationary phase. In many cases, the surface of a sorbent material needs to be modified with functionalities and ligands that are required for selective binding. Considerable effort continues to go into developing methods to impart various functionalities to the sorbent. However, many methods for the immobilization of ligands onto monoliths are based on well-established technology for the functionalization of particulate resins.

Extraction should be performed with appropriate liquid phases. The sorbent may need to be exposed to a precondition solution before the binding step. The binding step may require adjustment to the sample matrix, and the sample should be carried in a mobile phase which encourages sorption of the target molecules whilst preventing adsorption of unwanted compounds. A continued washing step is sometimes used to remove unbound or weakly bound compounds from the stationary phase. Finally, the eluting buffer needs to be chosen to ensure rapid and complete desorption of the target compound. In some cases, the extracted compounds may be fractionated by using several different desorption solutions [8, 9].

Some authors have also used the term 'solid phase microextraction' (SPME) to describe extraction onto a monolith within a capillary despite it being clear that they have intended for the extraction to be exhaustive. Pawliszyn [10] originally defined SPME as a method that relies on an equilibrium partitioning of the sample onto a stationary phase. This is quite different from the extraction techniques described in this review where the goal is total extraction of the target compounds. More recently, exhaustive extraction occurring over a defined time has also been considered to be SPME [10, 11]. However, we believe that this leads to confusion because it removes the distinction between SPE and SPME and we prefer the term 'microsolid phase extractor' [12] to describe small SPE devices. Equilibrium extraction techniques are not included within the scope of this review. We direct those interested in the technique to several recent reviews on the principles and application of SPME [10, 11, 13] or on emerging SPME technology [14, 15].

1.3 Porous polymer monoliths

In the context of functional solid materials for separation purposes, the term monolith describes a flow-porous (macroporous), highly crosslinked and therefore rigid, monolithic material that acts as a support for the stationary phase in a separation process. Such materials generally fall into one of two categories, nominally polymer and silica monoliths. Silica monoliths are inorganic materials typically prepared by thermally controlled condensation of a sol–gel of alkoxysilanes and are outside the scope of this review. Several recent reviews have focused on their synthesis and application to analytical separations [4, 16, 17].

Porous polymer monoliths are produced by polymerization of organic monomers including crosslinkers. The porosity of these materials is determined by porogenic solvents or pore-forming reagents such as PEG. A wide

range of monomers has been used for the synthesis of polymer monoliths and nearly 30 distinct monomers were encountered in the literature whilst preparing this review. However, most of these polymer monoliths can be grouped into one of several broad categories. Methacrylate and acrylate monoliths are the most widely reported type. Vlakh and Tennikova [18] have recently published an excellent review on the preparation of methacrylate monoliths. These types of monolith are usually formed by radical polymerization and are made rigid by crosslinkers such as ethylene dimethacrylate (EDMA). Styrenic monoliths are also prepared by radical polymerization and employ styrene and substituted derivatives, using divinylbenzene (DVB) as a crosslinker. The pore size of styrenic and methacrylate type monoliths can be controlled across two orders of magnitude by varying the composition of the porogenic solvent mixture [19]. Epoxy resin monoliths are prepared by condensation of epoxy resins and amines. The porous structure of epoxy resin monoliths can be controlled by pore-forming reagents such as PEG [20].

The advantages of monolithic stationary phases for high performance chromatography and electrochromatography have been well described in recent reviews [1, 21, 22]. Many of these advantages are also applicable to the use of polymer monoliths for extraction techniques. One potential advantage is that mass transport on monolithic stationary phases is dominated by convection. This means that sorption of targets onto the stationary phase is less limited by diffusion. In general, this allows the use of higher linear flow velocities, which can be a great advantage for high throughput analyses or extractions from very large sample volumes. Monoliths are also more hydrodynamically porous than packed particle beds. With the exception of perfusion chromatography, flow in ideally packed spherical sorbent particles is forced in the relatively restricted interstitial spaces. Polymer monoliths are usually at least 60% porous and it is widely assumed that this porosity is accessible to fluid flow.

One argument against polymer monoliths is that they do not have a high surface area compared to many modern sorbent materials. This is a disadvantage because binding capacity increases with surface area. However, the high surface area of materials such as porous silica beads is only accessible by diffusion, which means that separations on these materials may become poorer as flow velocity is increased. This also applies to the higher surface area of silica monoliths. Most of their surface area is found within networks of mesopores which are restricted enough to limit the sorption of slow diffusing macromolecules such as proteins. In high performance chromatography, this is observed as an increase in HETP that worsens with increased linear flow velocity. For extraction, this could be observed as a drop in binding capacity at higher flow rates, or as inefficient desorption of the target compounds. It should also be recognized that whilst the relatively small surface area of polymer monoliths appears as a significant disadvantage of these materials, it is strongly balanced by the ability to use these materials at very high flow rates and thus achieve large productivity gains.

The popularity of polymer monoliths in the literature is only partly explained by their performance advantages. An equally significant advantage, perhaps an even more important one, is their ease of synthesis. Polymer monoliths can be formed *in situ* – within a capillary, column, pipette tip or even in a microfluidic channel on a chip [23]. They can be moulded into any shape and have been demonstrated in structures as large as 8 L [24] to as small as a few nL in the channel of a microfluidic chip [25]. This capability is particularly important for micro- and nanoscale devices where the incorporation of particulate sorbent materials is difficult and plagued by poor reproducibility [26]. Some polymer monoliths can also be synthesized using UV light and masking which allows for precision control over the location of monolith within a capillary of microfluidic channel [27]. With all of these advantages, it should not be surprising that polymer monolith extractors are finding such a wide range of applications. In this review, we have focused on exploring these applications and have paid particular attention to integration into other analytical systems. Literature and discussion have been grouped primarily according to the types of roles played by the monoliths and the platforms in which they function. Monoliths have also been grouped according to their various chemistries and modes of interaction.

2 Monoliths for offline extraction

2.1 Nonspecific interactions

Hydrophobic, ion–ion and dipole–dipole forces are the least specific modes of chromatographic interaction. However, they are effective for many SPE and preconcentration applications and are most often used to increase the sensitivity of a subsequent separation.

The first report of a polymer monolith used for SPE was by Xie *et al*. in 1998 [28] and was based on hydrophobic and polar interactions. This work focused on the development of poly(DVB-*co*-ethylstyrene) monoliths with high surface area for high capacity and the authors were able to increase the surface area to an impressive 400 m^2/g by increasing the ratio of DVB to ethylstyrene, without changing the porogen composition. Similar high capacity monoliths were also prepared for extraction of polar analytes by incorporation of 2-hydroxyethyl methacrylate (HEMA) into the polymerization mixture. Since this first example there are many other examples

of polymer monoliths for SPE using nonspecific interactions and these are described in Sections 3–8 of this review.

2.2 Mixed-mode interactions

There are numerous reports from Feng *et al*. of extraction using monolithic poly(methacrylic acid (MAA)-*co*-EDMA) capillary columns [25–32]. Depending on the sample and the mobile phase, these materials are capable of interacting with the sample by hydrophobic, hydrogen bonding or ion-exchange interactions, or by any combination of these. These columns have been demonstrated for a significant range of analytical applications, most notably for the extraction of small drug molecules from human urine and plasma including for SPE pretreatment with offline coupling to CE [29–32], and HPLC [33–35] and were integrated directly into the end of the sample syringe as shown in Fig. 1. These monoliths were shown to be applicable to the extraction of a wide range of analytes including amphetamines in the urine of amphetamine users [25], angiotensin II receptor antagonists from human urine [26], opiates in urine [28], aliphatic aldehydes in human saliva [31] and antibiotics in honey, milk and eggs [30]. This same material could also be used to extract nitric oxide that had been derivatized by a fluorescent tag with 90% recovery of the tagged NO and calculated detection limits as low as 2×10^{-12} mol/L [33].

Poly(acrylamide-*co*-vinylpyridine-*co*-*N*,*N*′-methylene bisacrylamide) monoliths have also been described by Feng and coworkers [36] and applied to the determination of aspartate and glutamate in rabbit retina. Using fluorescence detection, the LODs for derivatized aspartate and glutamate were 0.14 and 0.53×10^{-9} mol/L with the SPE step providing enrichment factors of 14.1 and 14.7, respectively.

2.3 Affinity interactions

Extractions that rely on specific affinity towards biomolecules are usually described as affinity chromatography. The goals and approaches to affinity chromatography are quite diverse and the use of monolithic columns for affinity separations is a large topic in its own right, reaching beyond the scope of this review, but has been covered in several recent reviews [37–40]. Therefore, the following section is limited to a brief discussion on the chemistry and morphology of polymeric monolithic affinity extraction media. Some of the most impressive developments in this area have involved the use of Convective Interactive Media® (CIM®), and are described in Section 7 of this review.

Whilst a variety of polymer materials have been used to create monoliths with affinity interactions, this field has been heavily dominated by the use of glycidyl methacrylate (GMA), and in particular poly(GMA-*co*-EDMA) monoliths. This trend may be partly explained by the ease of *in situ* synthesis of this type of monolith and the availability of well-characterized procedures in the literature. The relatively low surface area of methacrylate type monoliths is not of such a great concern in the case of affinity chromatography because the target analytes are often slow-diffusing macromolecules which cannot efficiently access the higher surface area of materials with hierarchical porous structures.

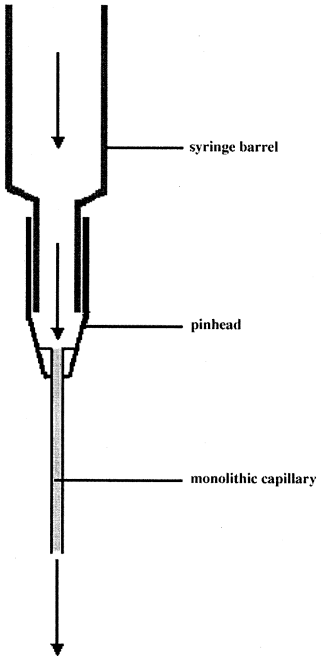

Figure 1. Scheme of the novel polymer monolith microextraction (PMME). Reproduced from ref. [30] with permission.

The almost exclusive use of GMA as an attachment monomer may not be entirely justified on chemical grounds. There is at least one other monomer, namely 4,4-dimethyl-2-vinylazlactone that may provide a more convenient and reactive functional group for attaching proteins and peptides to the monolith surface [41, 42]. The paucity of reports that use alternative reactive monomers such as 4,4-dimethyl-2-vinylazlactone may be partly explained by the fact that alternative monomers are not as widely available as GMA.

GMA monoliths are typically prepared by thermally initiated copolymerization of GMA with EDMA. However, trimethyloylpropane trimethacrylate (TRIM) is an interesting alternative to EDMA. As a trifunctional crosslinker, it has the potential to create highly rigid structures. Pan *et al*. [43] prepared and compared monoliths made of poly(GMA-*co*-TRIM) and poly(GMA-*co*-EDMA) and found that the TRIM monolith had better mechanical stability than its EDMA counterpart. The surface epoxide

groups of these monoliths were converted to aldehyde groups and used for covalent attachment of Protein A which was used for extraction of IgG from human serum without observing any nonspecific adsorption of BSA. The TRIM monolith had a good combination of surface area and permeability and compared favourably against the poly(GMA-*co*-EDMA) monolith in terms of mechanical stability and having a narrow pore size distribution. This clearly showed that poly(GMA-*co*-TRIM) is a good base material for affinity monolith chromatography despite EDMA remaining the preferred crosslinker for most researchers.

The key advantage of GMA-based monoliths is the reactivity of the surface epoxide groups, particularly towards amine nucleophiles. Epoxide groups provide a convenient point of covalent attachment for a virtually endless variety of affinity ligands. The simplest approach to covalent attachment is to allow a nucleophile on the affinity ligand, typically an amine from an amino acid residue, to attack the epoxide group. However, it is common to first modify the epoxide group itself in order to better control the reaction [44] or to introduce a spacer arm [45]. An objective comparison of several immobilization methods was done by Mallik *et al*. [46] by attaching HSA to a poly(GMA-*co*-EDMA) monolith. In the simplest approach, they allowed the amine residues on the protein to react directly with the epoxide groups. For three other methods, they began by hydrolysing the epoxy groups to diols using dilute sulphuric acid. Following this, three alternative reactions were used to convert the diols to either aldehydes, succinimidyl carbonate groups or imidazolyl carbamate groups. These intermediate functional groups were then used to attach the HSA. They observed that the direct reaction of protein with the epoxy group provided the lowest conversion of functional groups, whilst the reaction using the aldehyde as an intermediate, known as the Schiff base method, gave the highest loading of HSA. The Schiff base method also yielded the monolith with the greatest performance for affinity chromatography, which compared favourably against a silica-based HSA monolith. It should be noted that at the maximum of the kinetic curve the degree of conversion is approximately equivalent for both the epoxy and aldehyde groups but that this observed difference in conversion is primarily due to the poorer reaction kinetics for the epoxy group, which is a recognized disadvantage of using this reactive group.

Hahn *et al*. [47] showed that it was feasible to create an affinity monolith using preconjugated GMA. In this approach, GMA was reacted with a peptide directed against lysozyme prior to the monolith polymerization process. The peptide–GMA conjugate had a strong interfering effect on the morphology of the monolith, however they were able to reoptimize the polymerization conditions and create an effective affinity monolith with

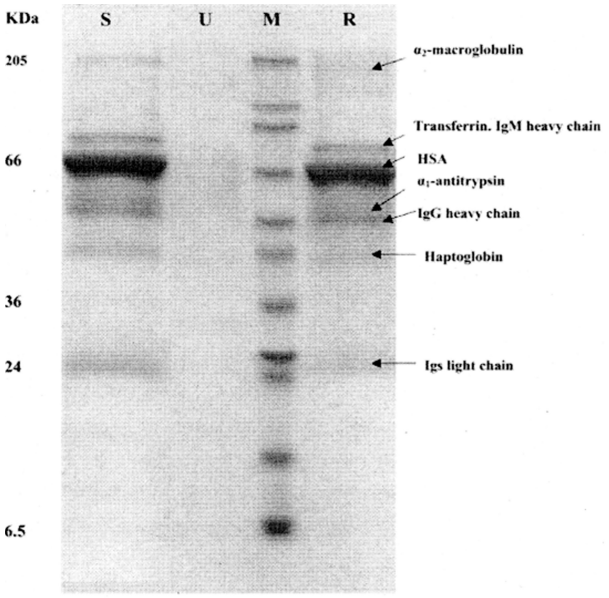

Figure 2. SDS-PAGE of 1:10 diluted serum (S), unretained fraction of 1:10 diluted serum (U), SigmaMarker (M) and retained fraction of 1:10 diluted serum (R). Conditions: 10% Tris-glycine gel (14×16 cm^2, 1.5 mm thickness) for 80 min at 200 V. Reproduced from ref. [50] with permission.

30% conjugated GMA. Despite their success, this approach has not been popular because it is more complicated and does not offer a clear advantage over the more widely accepted method of reacting onto the surface epoxide groups of a poly(GMA-*co*-EDMA) monolith.

Bedair and El Rassi [48, 49] introduced an ionizable monomer, [2-(methacryloyloxy)ethyl] trimethylammonium chloride (META) to produce poly(GMA-*co*-EDMA-*co*-META) monoliths that could generate a stable EOF and thus be operated in electrochromatography mode without a pump. These monoliths were used to bind mannan and lectins to perform affinity separations, achieving significant enrichment of protein samples. This approach was taken further by coupling poly(GMA-*co*-EDMA) monolithic capillary columns with different immobilized lectins in tandem [44]. This scheme was successfully used to resolve α_1-acid glycoprotein into two glycoform fractions. This approach was extended in subsequent work in which they coupled eight different monoliths in tandem for microscale depletion of the top eight most abundant proteins in human serum in a single run (Fig. 2) [50]. The tandem affinity columns were also integrated with immobilized trypsin monolithic columns to achieve simultaneous depletion and digestion of proteins.

Denizli and coworkers [51, 52] used the functional monomer *N*-methacryloyl-(L)-histidine Me ester (MAH) to create a monolith for immobilized metal affinity chromatography (IMAC). MAH was copolymerized with HEMA and EDMA to create a relatively hydrophilic structure

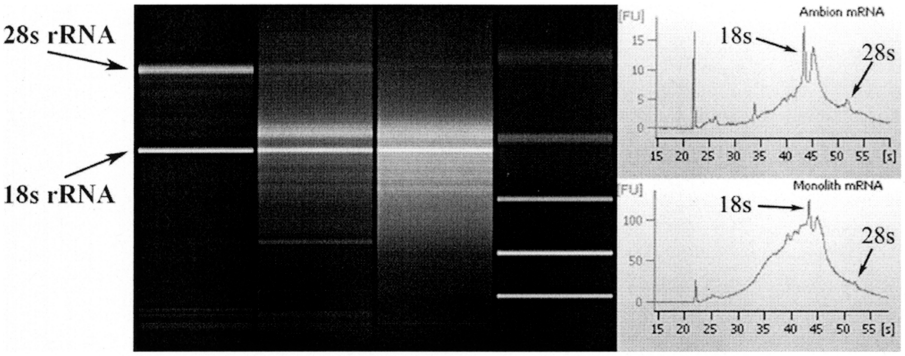

Figure 3. Enrichment of mRNA trapped on monoliths (lane 3) from Ambion mRNA (lane 2) next to total RNA (lane 1) and an RNA ladder (0.2, 0.5, 1.0, 2.0, 4.0 kb, lane 4). Figures on the right show electropherograms of lane 2 (top) and lane 3 (bottom). Reproduced from ref. [53] with permission.

with the aim to avoid nonspecific interactions. This approach resulted in monoliths with relatively high surface area of 215 m^2/g. The histidine groups were able to capture Cu(II), which, in turn, acted as a ligand for adsorption of antibody IgG. The monolith, created in a glass tube of 100 mm × 10 mm id, was able to extract an impressive amount of IgG from human serum with high purity – up to 104 mg/g of sorbent.

In addition to proteins, peptides and small metabolites, affinity chromatography can be used to extract the nucleic acid-based polymers which are the most important analytes for molecular biologists. Satterfield *et al*. [53] used photopolymerized poly(GMA-*co*-EDMA) capillary monoliths for microscale extraction of eukaryotic messenger RNA (mRNA) from a matrix that included a large amount of ribosomal RNA (rRNA). This separation took advantage of the fact that mRNA has a so-called 'polyA tail', meaning that it has a block of adenosine nucleotides at one end. A 30-mer of dT with an amine spacer group was reacted with the epoxide groups to create ligands with strong selective affinity for the mRNA as illustrated in Fig. 3. The monolith was able to extract at least 16 μg of mRNA from 315 μg of total RNA and was resistant to buffers at least up to pH 9 and showed excellent stability without loss of performance after drying or storage for several months.

In our own research [54], we prepared poly(GMA-*co*-EDMA) monoliths in capillaries for microscale boronate affinity chromatography. The monoliths were functionalized by *p*-hydroxyphenylboronic acid to provide the phenylboronate groups that are necessary for boronate affinity. The monoliths demonstrated very high selectivity for nucleosides over 2-deoxynucleosides as shown in Fig. 4 and are theoretically capable of affinity towards any molecule with a *cis*-vicinal diol group, which includes many classes of biomolecules with sugar moieties. By photografting a branched layer of poly(GMA) onto the pore surface of the monoliths prior to the functionalization reaction, we were able to increase the affinity of the material as measured by the retention factors of the nucleosides. If the retention of these materials could be increased further, perhaps by increasing the surface area, then they would be good candidates for online MS extraction phases or as SPE modules on microfluidic chips.

More unusual approaches have also been taken to the preparation of monoliths for affinity extraction. Sun and Chai [55] described a urea-formaldehyde monolith, with bimodal pore structure for separation of proteins by affinity interactions. Cibacron Blue F3GA was covalently attached by nucleophilic reaction between the chlorine groups in the dye and the imino groups of the monolith pore surface, though ligand density was significantly lower than for comparable silica or agarose materials. This material was shown to be effective for affinity extraction of several proteins using a pH or salt gradient for elution. In another interesting approach, Davidson *et al*. [56] synthesized a 'pseudo' molecular imprinted monolithic polymer bearing covalently bound 'tweezer' receptors specific for cholesterol. The relative amount of functional monomer (MAA) in the polymerization mixture was shown to be important for selectivity towards cholesterol over several structurally related alkaloids.

Polymer monoliths with specific affinity for particular metals have also been investigated by Wang and Zhang [20, 57]. Epoxy-based monoliths were synthesized from Bisphenol A diglycidyl ether and ethylene diamine using PEG (PEG-1000) as a porogen in an approach similar to that used to prepare silica monoliths. The porosity of the monolith was 76% which is higher than the typical methacrylate and styrene-based monoliths and could allow greater flow rates at lower pressures. This work suggested that the slow step-addition reaction by which this monolith forms facilitates a more heterogeneous monolithic structure than can be achieved *via* vinyl radical polymerization monoliths primarily due to the slower rate of reaction for the condensation reaction allowing more effective heat dissipation. Using this approach selective extraction and preconcentration of Pb(II) from aqueous samples for analysis by flame atomic absorption spectroscopy (FAAS) [20]. Cu(II) was also extracted from aqueous samples in a similar manner [57].

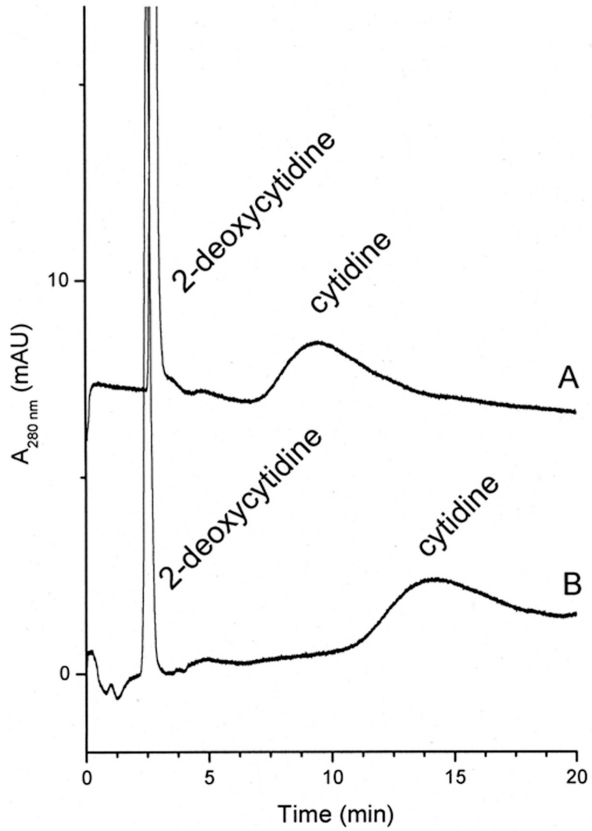

Figure 4. Separation of 2-deoxycytidine and cytidine using *p*-phenylboronate modified monoliths. (A) Micro-LC separation mode, 8 cm surface modified monolith. Column, 33 cm × 100 μm id (8.5 cm to the detector); BGE, 100 mM ammonium acetate, pH 9 with 100 mM $CaCl_2$; sample, 200 ppm of each ribonucleoside in BGE; conditions, 9 bar; injection, 18 s at 8 bar. (B) CEC separation mode, 6.5 cm poly(GMA) grafted monolith; column, 33 cm × 75 μm id (8.5 cm to the detector); BGE, 50 mM HEPES, pH 8.7; sample, 100 ppm 2-deoxycytidine and 500 ppm cytidine in BGE; conditions, –10 kV with 8 bar pressure on both vials; injection, 18 s at 8 bar. Reproduced from ref. [54] with permission.

2.4 Cell capture

The capture and separation of cells can be an unusual concept for those who are accustomed to separating individual molecules. However, these methods rely on familiar affinity type interactions such as those described previously in this review. In this case, however, the targets of specific adsorption are moieties which are expressed on the surface of cells. Cells are obviously much larger than molecules. Bacteria have lengths in the range of micrometers and would not permeate through most of the monoliths described elsewhere in this review. Therefore, work in this area has described monoliths with much larger pores. Dainiak *et al*. [58] selectively captured *Escherichia coli* cells using polyacrylamide cryogel monoliths. This type of monolith is prepared by cooling the polymerization solution. Water-ice crystals provide the spaces that become the pores whilst the polymer-forming reagents are activated by becoming concentrated in the intercrystal zones with pores in the range of 10–100 μm. The monoliths were modified with iminodiacetic acid (IDA) and Cu^{2+} to give them an IMAC interaction. Furthermore, it is likely that this monolith was quite fragile and therefore it is possible that this large pore diameter was necessary to allow a reasonable flow rate. The *E. coli* cells were adsorbed onto the cell surface by the complexation of histidine residues on the cell-surface peptides with the chelated Cu^{2+} ligands on the monolith pore surface. In contrast, the cell surface of different bacteria, *Bacillus halodurans*, is dominated by acidic and hydrophobic amino acid residues that do not contribute to binding with the chelated copper ligands. Based on this difference it could be demonstrated that the monolith could fractionate a mixture of the bacterial cells into their two groups quite effectively. The unretained fraction contained *B. halodurans* whereas the imidazole-eluted fraction contained around 95% *E. coli* cells and 7% *B. halodurans*. In subsequent work, attempts were made to optimize the protein binding and morphology of the macroporous polyacrylamide cryogel monoliths and a range of approaches to creating IDA-functionalized monoliths that gave materials with different morphologies were demonstrated [59]. The acrylamide-based cryogel monoliths were also demonstrated for lectin affinity separations using Con A as the affinity ligand [60]. In this case, the cryogel monoliths were produced in the 96-well mini-column plate format which allowed for parallel runs for rapid optimization of the adsorption and elution conditions. They also prepared a similar monolith as a large column with dimensions of 113 × 7.1 cm^2 which was used to separate a mixture of *E. coli* and *Saccharomyces cerevisiae* cells. The *S. cerevisiae* cells were retained by the affinity ligands with high selectivity. Interestingly, the desorption of the *S. cerevisiae* cells was aided by a compression step in which the monolithic column was squeezed. The authors suggest that this causes conformational changes to the monolith at the microscopic level that facilitate more rapid desorption of the bound cells. In similar work, Noppe *et al*. [61] used epoxy-activated acrylamide cryogel monoliths to immobilize phages. The immobilized phages expressed a peptide that acted as an affinity ligand for lactoferrin, a human protein found in milk. The lactoferrin could then be eluted with concentrated NaCl in greater than 95% purity.

Williams *et al*. [62] showed that a similar approach could be used for the capture of tagged lipid-enveloped viruses. They prepared cryogel monoliths of poly(acrylamide-*co*-*N*,*N*′-methylene bisacrylamide) and GMA and modified them with Streptavidin. This material could selectively capture biotin-tagged Moloney Murine Leukemia Virus from crude cell culture supernatant. Whilst

they were only able to recover a small portion of the virus, this still resulted in a sample enrichment factor of more than 400.

2.5 Molecular imprinting

Molecular imprinting is the process of forming a polymer in the presence of a template molecule. When the template molecule is removed, the cavities left behind can have an arrangement of functional groups that allows cooperative binding of the template molecule and molecules with similar structure. Molecularly imprinted polymers (MIPs) may act as highly selective extraction phases with selectivity for a chosen compound. Enantioselectivity is also possible by imprinting with an optically pure chiral compound. MIP materials may be compared to affinity sorbents in the sense that they can have very high specificity towards target compounds. They typically demonstrate longer shelf life and chemical resistance to a wider range of liquid phases than affinity sorbents [63], which are often dependant on easily degradable biomolecules. On the downside, MIP stationary phases are notorious for poor kinetics giving rise to very broad peaks and excessive tailing for the target compounds [64]. It is largely for this reason that MIP sorbents have been largely confined to academic research. This may explain the fact that there is a wide variety of approaches used to synthesize them. Numerous MIPs have been demonstrated as SPE sorbents over the past decade and Ye [63] provides an efficient overview of these in a book chapter published in 2005. Recently, MIPs have been receiving increased attention and therefore we would also direct readers to some more recent reviews that cover their synthesis [65, 66], application to separations [67] and chromatographic properties [64]. It is very difficult to draw a line between MIP monoliths for extraction and high performance chromatography, therefore the following is concerned primarily with the major developments related to MIP monoliths for chromatographic extraction and is not intended to be comprehensive.

Until very recently, molecular imprinted macroporous polymer monoliths have only been a very small branch of the MIP field. The most common type of MIPs that have been reported for SPE applications were formed as bulk polymers that were fractured and sieved to create irregular sorbent particles that could be packed into columns [63]. A good solvent such as ACN is sometimes added to aid the formation of small pores and cavities. These irregular particle beds are poor stationary phases in terms of eddy diffusion, permeability and reproducibility. One way to address this concern is to form spherical MIP particles by suspension polymerization or related techniques [68]. Another potential solution to this problem is to produce the MIP in the form of macroporous polymer monoliths, and this has been one of the greatest motivations behind developing MIP monoliths. However, the polymer monolith format offers several other advantages as well as some potential disadvantages that may be equally significant.

Matsui *et al*. 1993 [69] were the first to report a porous monolithic MIP stationary phase, imprinting a monolith of the type that had only very recently been introduced by Svec and Fréchet [70]. They prepared three porous poly(acrylic acid-*co*-EDMA) polymer columns by thermally initiated polymerization in the presence of three different isomers of diaminonaphthalene. Comparison of the retention factors of the isomers on each column demonstrated that in all three cases the columns showed stronger retention for their respective template isomers. Chiral selectivity was also demonstrated by preparing the same type of monolith using either L-phenylalanine anilide or its enantiomer as templates [69]. The columns produced by this method gave separation factors (α) of around 1.5 for their respective templates *versus* the enantiomers, indicating that there was significant selective adsorption as a result of the imprinting effect. This work also highlights some of the potential advantages and disadvantages of the MIP monolith synthesis as compared to the more conventional approaches. The most obvious advantage is the ease of preparation, *in situ* within the steel column. The resulting monolith was a more regular stationary phase than the alternative of packed irregularly shaped particles. Furthermore, the MIP monolith approach may be more economical because the entire polymer was used as a stationary phase rather than a portion of the fractured particles that fit into a certain size range. After synthesis, much of the template molecule can be recaptured by flushing the column. These economic advantages are significant because in some cases the template molecule can be extremely valuable. On the other hand, the polar porogenic solvents that are needed to make this type of monolith could reduce the imprinting effect by competing with the monomers for interaction with the template molecule. Despite this early report by Matsui *et al*. molecular imprinting was sidelined during the early years of monolithic stationary phase development because of the focus on developing monoliths with more conventional modes of interaction. However, MIP monoliths have received renewed and continuously increasing interest during the past 5 years.

In 2003, Zhang *et al*. [71] prepared a monolithic MIP from a surprisingly complex mixture of styrene, GMA, MAA, DVB and triallylisocyanurate. The monolith was imprinted with ceramide III, an amide of fatty acids that is of interest to the pharmacological and cosmetics industries. The monolith was prepared in an HPLC column and was successfully used to enrich a sample of ceramides in yeast lipids. The durability of this material was evident in that no obvious changes in adsorption behaviour or back-pressure were observed after 6 months

of use. As a control, a nonimprinted monolith was prepared by repeating the polymerization in the absence of the ceramide III template. The morphologies of the two monoliths were shown to be very different by SEM and mercury intrusion porosimetry. The nonimprinted control monolith had a modal pore size of approximately 2000 nm whereas the addition of ceramide III resulted in a far smaller size of approximately 300 nm for the imprinted monolith.

This finding highlights a very important concern regarding the design of MIP monoliths. The morphology of a methacrylate or styrenic type polymer monolith is strongly dependant on the solvation and phase separation of polymer chains at the early stages of polymerization [18]. Therefore, the addition of any molecule to the polymerization mixture including a template molecule, has the potential to dramatically affect such properties as pore size distribution and surface area. Since the imprinting and morphology cannot be separated, the design of effective MIP monoliths for new targets can require optimization of both chemistry (imprinting effect) and morphology. By contrast, monoliths with affinity ligands only require optimization of the binding process because a standard monolith 'recipe' can be used.

The potential of the template molecular to change the morphology also raises another concern. It can complicate the evaluation of a molecularly imprinted monolith. Zhang *et al*. [71] assessed the imprinting effect on their monolith by showing that ceramide III was significantly retained on the imprinted monolith whereas there was no observable retention on the control monolith. However, given the very large difference in morphology between the control and imprinted monoliths, this is not a fair comparison. The imprinted monolith had far smaller flow pores and is likely to have had a higher surface area and these properties may in fact be partly responsible for the difference in retention. Whilst this issue was highlighted by Zhang *et al*. as they examined the morphology of the control monolith, it may also be an issue in some of the other MIP monolith work in which the control monolith was not structurally characterized.

A good approach was taken by Ou *et al*. [72] to demonstrate that effects were indeed due to selective binding. They developed a MIP monolith extraction column for the analysis of Bisphenol A which is an important environmental pollutant at trace concentrations. The column was prepared with EDMA and 4-vinylpyridine using Bisphenol A as the template. Bisphenol A was shown to be more strongly retained on the imprinted monolith compared to the retention on a control (nonimprinted) monolith, whereas several analogous substituted phenols did not show a significant increase in retention on the monolithic column. This demonstrated effectively that the increased retention of Bisphenol A on the MIP monolith was the result of selective binding rather than morphological differences between the monoliths and the authors attributed the selectivity of the monolith to formation of imprinted sites that gave synergistic binding of the two hydroxyl groups in Bisphenol A. The column was demonstrated in a SPE-LC format by hyphenating it to an analytical RP-HPLC column.

Recent developments in the MIP monolith literature indicate that the field has moved beyond its proof-of-principle stage and on to a more exploratory phase. Imprinted monoliths have now been applied to SPME [73] and have been used as stationary phases for electrochromatography [74, 75]. For example, Zheng *et al*. [74] demonstrated electrochromatography on a poly(MAA-*co*-EDMA) monolith. The MAA provided carboxylic acids that facilitated an EOF by providing fixed negative surface charges. However, these groups also played a crucial role in the binding sites, and it was therefore possible that their ionization state would effect the specific binding of the target. This would conceivably lead to a dilemma in this type of monolith in those cases where the target-binding interaction requires a low pH which cannot support EOF. In this case, this was not an issue and the target, oxytocin, could be selectively retained, whilst generating an appropriate EOF. The oxytocin was eluted in a remarkably narrow peak.

Contributions due to electrophoresis and EOF must be considered and controlled when working in electrochromatography mode, and this can create additional demands on the chemistry of the monolith and on the composition of the mobile phase. Therefore, electrochromatography is only viable in situations where it offers a clear advantage. It might prove to be most useful for a MIP monolith in a micrototal analysis system (μTAS) device, where the EOF could remove the need for a hydrodynamic pump.

The most interesting development in recent MIP monolith literature is a new approach to forming MIP monoliths based on surface grafting. This approach was demonstrated by Courtois *et al*. in 2006 [76] and was achieved using a TRIM monolith. This material provided a stable support surface for the formation of a porous photografted layer of molecular imprinted polymer. The excess of vinyl groups from the trifunctional TRIM monomer ensured that there were plenty of sites for grafting on the monolith surface by radical polymerization. The grafting solution was a mixture of MAA and EDMA in toluene, with a small amount of template molecule and 2,2′-azobis(2-methylpropionitrile) (AIBN) initiator which was initiated by UV exposure. For the template molecule, Courtois *et al*. worked with one of their three targets: bupivacaine and two related small anaesthetic drugs. Examination by SEM revealed that this photografting process produced a layer of numerous small globules,

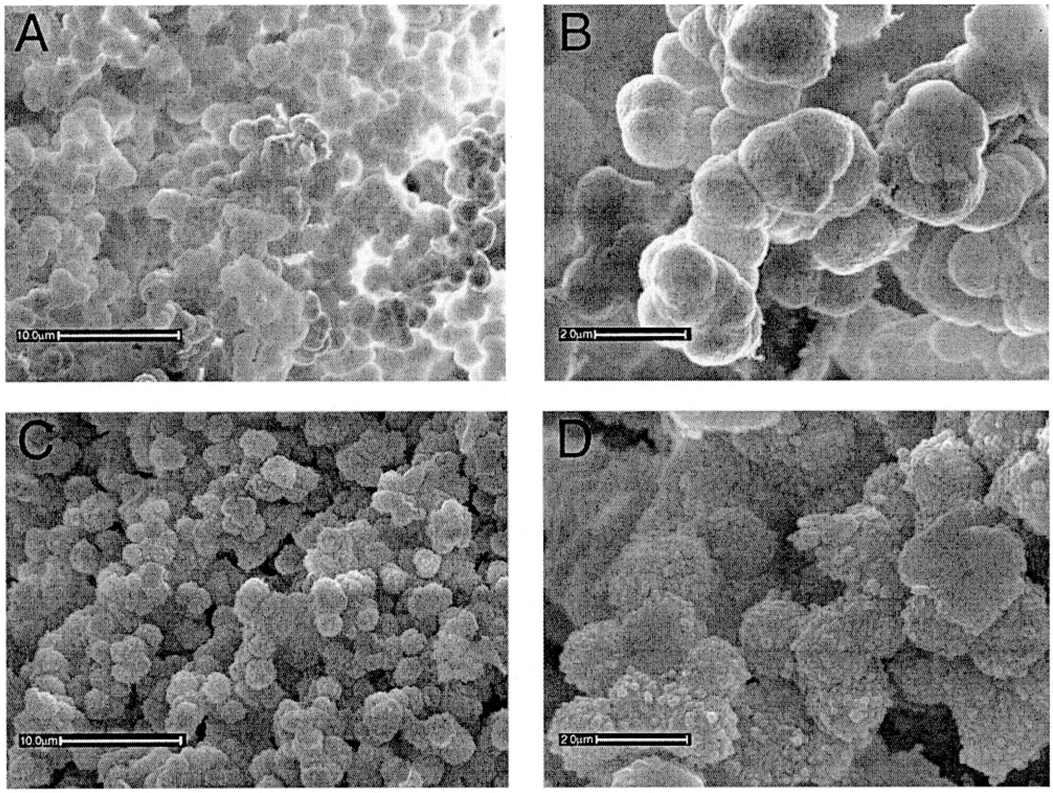

Figure 5. Scanning electron micrographs of (A) nongrafted core monolith at magnification 3000 ×, (B) nongrafted core monolith at magnification 10 000 ×, (C) grafted BV-mMIP at magnification 3000 × and (D) grafted BV-mMIP at magnification 10 000 ×. Reproduced from ref. [76] with permission.

themselves attached to the larger globules of the original TRIM monolith as shown in Fig. 5. The MIP monoliths formed in this way demonstrate good selectivity. A similar approach has since been used by Ou *et al*. [77] to graft a layer of MIP onto a silica monolith scaffold. The main advantage offered by this surface grafting approach is that the macroporous structure of the monolith and the surface chemistry can be controlled independently. This significantly simplifies the design and evaluation processes. It may also be more economical because the template molecule can be more easily recovered after synthesis due to it being present only in the thin, surface-grafted layer of the monolith.

Recent studies have directly compared the properties of monolithic MIPs with other types of MIP sorbent. Kim and Guiochon [78] performed insightful experiments in 2005 that compared the thermodynamic properties of these two types of MIP stationary phase material. They prepared both monolithic and fractured bulk polymer type stationary phases that were imprinted with Fmoc-L-tryptophan. Whilst the preparation procedure for the monolith was by far the more convenient, Kim and Guiochon found a reduced enantioselectivity in the monolithic MIPs compared to the fractured, bulk type. They attribute this to the polar porogens used in the preparation of the monolith. It was possible that the porogens could compete with the monomers to complex with the template molecule during the polymerization, as suggested earlier by Matsui *et al*. [69]. Oxelbark *et al*. [79] also recently conducted a very thorough experimental comparison of various MIP stationary phase types including polymer monoliths and fractured bulk polymers. They concluded that the monolithic phases were superior in terms of analysis speed and reduced nonspecific binding. However, in terms of binding capacity and the efficacy of the imprinting process (imprinting factor), the more traditional fractured bulk polymers were more effective. Oxelbark *et al*. identified that the simplicity of the monolith preparation process was a significant advantage in favour of the polymer monolith MIP format, even though it is unrelated to chromatographic performance. This ease-of-preparation is a particularly significant advantage in the case of molecular imprinting polymer sorbents because a new stationary phase must be prepared for each new application. This ease-of-preparation stands in contrast to difficulty of designing a monolithic MIP with appropriate morphology and chemistry. However, the difficulties of MIP monolith design and synthesis

may be circumvented by the surface-grafting method that was demonstrated by Courtois *et al*. [76], and we expect this approach to gain popularity.

3 Polymer monoliths for online SPE-HPLC

Although HPLC is typically a very sensitive technique that can handle a relatively large sample volume, especially when a gradient elution is employed, an SPE step can improve sensitivity by washing away interfering compounds and concentrating the analyte from a very large sample into a small plug that can be introduced into the column. In this context, monoliths offer the advantage of high linear flow rates without sacrificing adsorption capacity or desorption efficiency. This may have the direct benefit of cutting analysis time. Furthermore, the possibility of high linear flow rates allows relatively fast extraction from large volumes of very dilute sample, even in an SPE monolith with very small bed volume.

SPE-HPLC using a polymer monolith was first introduced by Xie *et al*. in 1998 [28] as described already in Section 2.1 and since this time there have been many developments in this field. Schley *et al*. [80] used a 10×0.20 mm id poly(styrene-*co*-DVB) monolith for desalting and preconcentration of proteins and peptides. This procedure was coupled online to HPLC with a Dionex Ultimate 3000 capillary LC system and an FLM-3100 microcolumn switching unit. Up to 0.5–1.6 μg of peptides and proteins could be adsorbed onto the column using a 0.10% heptafluorobutyric acid adsorption/washing solution [80]. The preconcentrated sample was eluted onto a 60 mm analytical poly(styrene-*co*-DVB) monolithic capillary column for high-resolution separation. Reducing the concentration of protein by a factor of 100 accompanied with an increase in sample injection volume had no significant effect on the separation. In terms of capacity, a quick calculation shows that this monolith [80] had a specific protein/peptide capacity comparable to that of the highly successful Millipore C_{18} or C_4 Zip-Tip sample preparation columns (http://www.millipore.com/faqs/tech1/4xmtxh). Swart and Dragan [81] also demonstrated that the poly(styrene-*co*-DVB) monolithic preconcentration columns do not have a negative effect on the HPLC separation of peptides and proteins.

Feng's group have described the use of poly(MAA-*co*-EDMA) monoliths for sample extraction coupled online with HPLC systems in a number of publications [82–88]. The offline use of these monoliths has already been described in Section 2.2. Whilst these monoliths were capable of hydrophobic, hydrogen bonding or even coulombic interactions (at low pH), the choices of washing and desorption buffers suggests that they have primarily been used as both hydrophobic and also as mixed-mode stationary phases. In all this work, the monoliths were prepared in fused-silica capillaries which have a very small bed volume compared to the analytical columns in which they were coupled. The specific SPE-HPLC configuration used was first introduced by Fan *et al*. [83] and consisted of two six-port valves and two pumps, as shown in Fig. 6. The first valve and pump controlled the introduction of sample solution onto the SPE capillary column at 0.04 mL/min and the position could then be switched to flush the SPE column with a phosphate buffer washing solution to remove proteins and other contaminants. The second valve and pump controlled the introduction of a methanol/ammonium acetate desorption solution into the SPE capillary at 0.02 mL/min, eluting the sample onto an analytical column packed with a RP silica sorbent in which the desorption solution functioned as a mobile phase. The small-diameter monolithic SPE column had to be bypassed during the separation on the analytical column so that the flow rate could be increased to 0.5 mL/min. This configuration has been used for a range of applications including the determination of methylxanthines in human serum [82], ketamine [83] and amphetamine class drugs [84] in urine samples, camptothecin in human plasma [85], angiotensin II receptor antagonists for the treatment of hypertension [86, 87] and antibiotics in chicken eggs [88]. One of the specific advantages of this material for these kinds of analysis is that the biocompatibility of the polymer means that is does not irreversibly bind proteins and thus does not foul easily. For example, one SPE column could be used over 200 times for the analysis of human plasma without loss of efficiency [85].

The same group have also used a slightly different approach for the online extraction of small acidic analytes with mixed mode (RP/cation-exchange) monoliths based on poly(acrylamide-*co*-vinylpyridine-*co*-*N*,*N*′-methylene bisacrylamide) [89]. This method has been applied to the determination of Bisphenol A and ethynlestradiol, two endocrine disrupters in environmental water samples with detection limits of around 0.1 ng/mL [90]. The method was described as SPME. However that the column required more than 27 min of sample flow at 40 μL/min to reach equilibrium but was instead operated for 10 min in order to reduce analysis time suggests that this is more akin to SPE rather than a true microextraction technique. Using a larger format monolith prepared in 0.76 mm id PEEK tubing allowed the use of higher flow rates and greater sample throughput specifically for the determination of estrogens in environmental waters [91]. In this case, the mesopore structure of the monolith was also investigated and was shown by a N_2 sorption method to have mesopores in the range of 3–20 nm. Despite these small pores, the monolith had a relatively low surface area of just 4.89 m^2/g in the dry state. The columns also showed somewhat disappointing intra- and

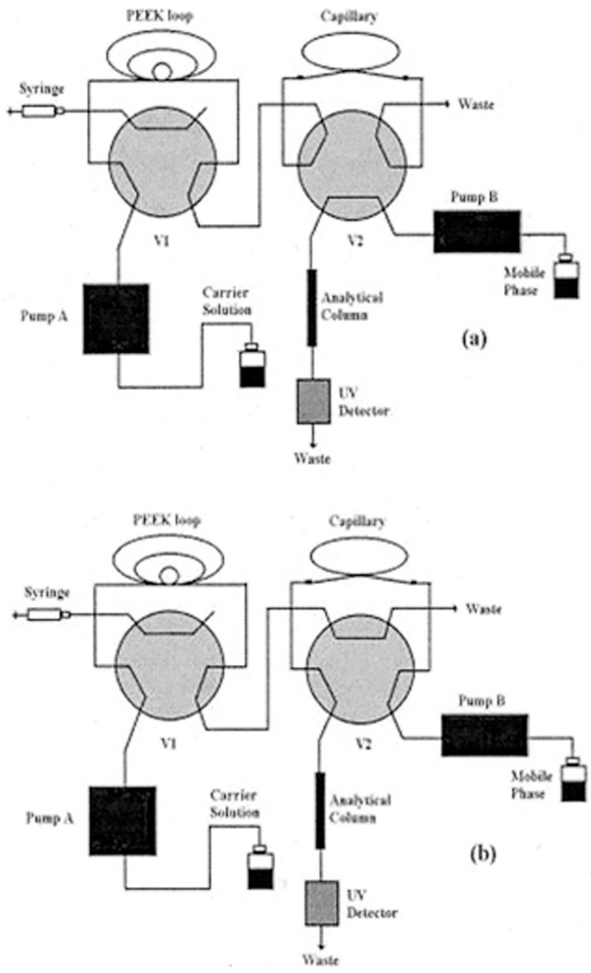

Figure 6. Construction of in-tube SPME-HPLC. (a) Extraction mode; (b) desorption mode. Reproduced from ref. [83] with permission.

interday reproducibility of 12 and 9.8%, respectively. However, the sensitivity of the method was very good, with detection limits of estrogens in spiked lake water samples ranging from 0.02 to 0.35 ng/mL.

Poly(GMA-*co*-EDMA) type monoliths have also been described by Wen and Feng [92] for online capillary SPE-HPLC. In this case, the epoxide groups on the surface of the monolith were hydrolysed to diols and these were used for the selective extraction of small polar analytes. The addition of small amounts of organic solvent to the mobile phase buffer actually reduced the extraction efficiency, and demonstrated that the polar interactions were more important than nonspecific hydrophobic interactions. The monolith was coupled online to an HPLC system in the same fashion as described previously by Feng's group. In this system, the extraction allowed quantification of small polar compounds such as nitrophenols at concentrations down to 2 ng/mL. A similar approach was taken by Wei *et al*. [93], however in this case the epoxide groups were converted to carboxylic acids to provide weak cation-exchange sites. The epoxide groups were first reacted with diethylamine to create a reactive spacer followed by reaction with chloroacetic acid to afford the carboxylic acid. Proteins were able to be washed through the column by aqueous buffers whilst the target molecules, small basic drugs from the a1-adrenergic receptor antagonist class, were retained and preconcentrated. Yang *et al*. [94] demonstrated a similar monolith for the removal of HSA.

4 SPE coupled online to CE

SPE-CE is a particularly compelling hyphenated technique. This is because SPE offers a solution to one of the greatest limitations of CE – poor concentration sensitivity. There are two very recent reviews on SPE-CE, covering both online and offline methods [95, 96] but this section of this review will only focus on online coupling of SPE to CE.

Several research groups have now demonstrated online coupling of polymer monolith extraction to CE. This has been achieved by forming short monolithic columns at the injection end of capillaries. Intuitively, this would appear to be a relatively simple and elegant type of online preconcentration system. A group of similar compounds can be preconcentrated up to detectable levels, separated by CE and detected all in the same instrument. This entire analysis would be faster and would require less moving parts. Also, in contrast to the offline methods, all of the extracted sample will be used in the CE separation and none will be wasted. Those who are familiar with CEC will already be aware that this is not so simple; the online SPE-CE approach can be a reasonably difficult approach. The composition of the loading buffer and eluent and their mode of introduction into the capillary must be compatible with their role as electrolytes in controlling electrophoresis and EOF within the capillary. The polarity, ionic strength and pH must all be considered, along with any compounds that are needed to displace the analytes from the stationary phase after preconcentration. There is also the potential for the sample plug, which may be of a much higher or lower ionic strength than the BGE, to complicate the separation. Furthermore, the surface charge of the monolith is crucial [97]. As we have previously shown, even a small (5 mm) section of stationary phase within a capillary will dominate the EOF generated in the entire capillary [98]. An additional obstacle is that flow through a monolith can sometimes create pressure differences leading to gas bubbles which are problematic for CE. We suspect that this phenomenon is significantly under-reported by researchers in this field despite being a major source of difficulty. However, if an appropriate scheme of electrolytes/eluents and careful procedures can be devised then there is

the possibility of both excellent sample enrichment and high resolution separation. This approach also has the potential for very fast analyses with very small sample volumes.

Baryla and Toltl [27] were the first to demonstrate CE with an online preconcentration using a polymer monolith. They prepared a poly(butyl methacrylate (BuMA)-*co*-EDMA) monolith at the inlet end of a capillary by photoinitiated radical polymerization and demonstrated preconcentration of S-propranolol, achieving detection limits in the low nanomolar range. This employed a rather elegant procedure involving several steps. First, the sample is pumped through the monolithic column using external pressure. Unbound sample is then flushed from the column with water. Next, separation/elution buffer is introduced into the capillary *from the outlet end* by EOF, with an applied negative voltage. This is continued up until the point at which the full length of the capillary (including the preconcentration column) was filled with the separation/elution buffer, as determined by monitoring the current. This means that the bound sample was initially eluted back towards the inlet of the capillary. This required a careful approach so as to ensure that the sample did not go so far as to exit the capillary through the inlet end. Finally, the positive voltage was applied and the S-propanol migrated along the capillary towards the UV-detector. The authors claim that there was no loss of efficiency as a result of the preconcentration. However, as there was only one analyte in the system, they did not demonstrate a true CE separation.

We have since extended this concept for the separation of a range of antidepressants [97]. In part due to difficulties in achieving a stable EOF we added a small amount of an ionizable monomer, 3-sulphopropyl methacrylate (SPMA), to the monolith. This increased and stabilized the EOF, therefore enabling greater control over the loading and eluting process. This control was crucial and allowed the combination of the preconcentration effect of the monolith with field-enhanced sample stacking. With the combined effect of these two preconcentration techniques, we were able to demonstrate sample enrichment factors of up to 500 coupled with a well-resolved CZE separation. An important factor in achieving this separation was careful control of the amount of SPMA incorporated in the monolith and hence the EOF generated, as illustrated in Fig. 7.

Lee's group [99] have also developed a polymer monolith for immunoextraction which was coupled online with CE. Protein G was immobilized on a poly(GMA-*co*-TRIM) monolith and its capability to extract IgG in a formate/formic acid buffer demonstrated [99]. The adsorbed IgG was released by injecting a plug of 50 mM formic acidic. By this method, they were able to detect IgG in samples with estimated concentrations as low as ~1 nmol/L, representing a very high enrichment factor. This method

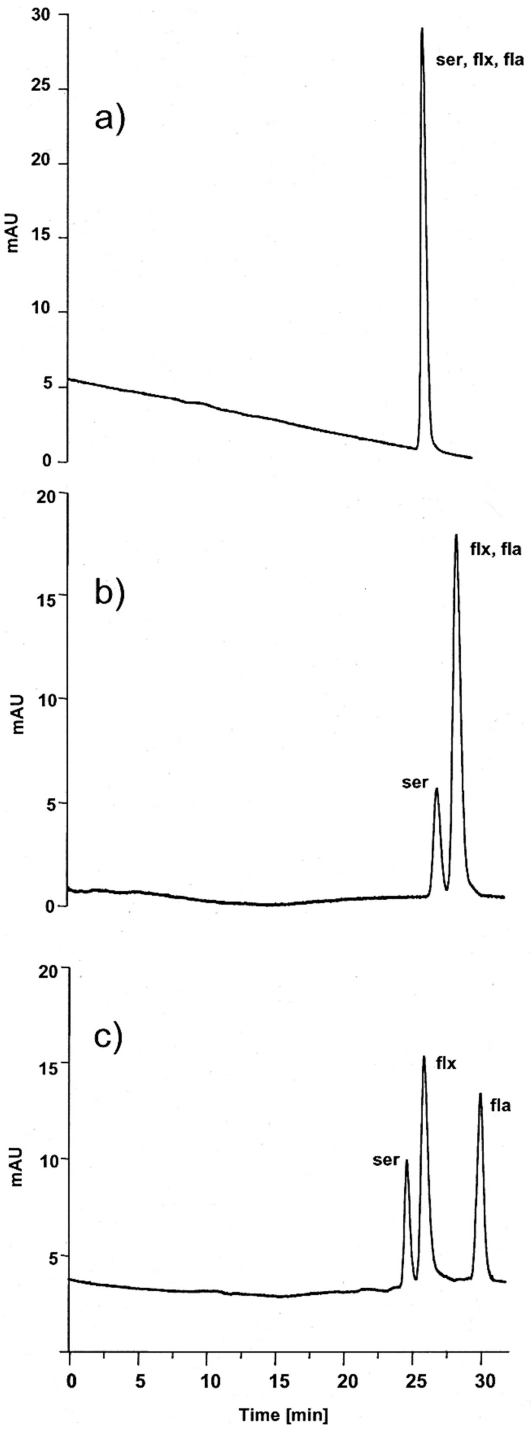

Figure 7. Comparison of the elution/separation of sertraline, fluoxetine and fluvoxamine by SPE-CE using monolithic SPE materials containing varying amounts of SPMA as the ionizable monomer; (a) 1% SPMA (w.r.t. total monomers) with elution/separation using 2 M acetate buffer, pH 3.5/ACN (10:90 v/v), (b) 0.5% SPMA (w.r.t. total monomers) with elution/separation using 1 M acetate buffer, pH 3.5/ACN (10:90 v/v) and (c) 0.1% SPMA (w.r.t. total monomers) with elution/separation using 200 mM acetate buffer, pH 3.5/ACN (10:90 v/v). Reproduced from ref. [97] with permission.

relied heavily on external pressure and did not apply an electrical potential to their capillary until after the analyte was eluted beyond the extraction/preconcentration column. Again, there was only one analyte in the system, so there was no true CZE separation. The authors stress that their technique is applicable to any protein for which an antibody is available, and later [100] demonstrated that the binding of IgG was very specific. Lee's group also prepared a poly(BuMA-*co*-EDMA) monolith at the inlet end of a polyvinyl alcohol-coated silica capillary [100]. This capillary column was demonstrated as a hydrophobic preconcentration module, extracting lysozyme, cytochrome c and trypsinogen A standards from a dilute solution. The capillary was first equilibrated in a solution of 50 mM formate/formic acid at pH 7.6, which served as the binding buffer. The protein standard was then injected at 1 bar for 12–40 min before washing with the formate/formic acid buffer for 7 min to elute any unbound protein. The bound proteins were then eluted by a plug of 70% ACN in 0.1% TFA solution with the elution efficiency reaching a plateau with an elution buffer plug of 700 mbar for 0.1 min. Good resolution of three proteins was achieved by this method, with RSD of below 3% for peak area and around 1% for migration time.

The versatility of these new extraction materials was demonstrated by directly coupling the Protein G monolith [99] upstream of the preconcentration monolith [100]. From an injection of a sample of 100 μg/L IgG, 5 μg/L lysozyme and 5 μg/L of cytochrome *c*, high concentration of IgG was captured on the Protein G column whilst the other proteins passed through and were extracted/concentrated by the poly(BuMA-*co*-EDMA) monolith. This technique then required that the protein concentrator capillary be disconnected from the immunoextraction column at this stage, to be installed in a CE. Following washing, preconditioning and elution steps as described earlier [100], the two low-concentration proteins were successfully separated by CE, free from interference by the high abundance protein. This research hints at an exciting possibility. The selective removal of all high abundance proteins from a complex sample such as serum would allow preconcentration of very low abundance proteins. Coupled directly to fast, high-resolution CE, this could be a very powerful new approach to proteomics. However, as conceded by Lee *et al*. in this work, there is a long way to go. Such a system would require affinity for a wide range of high abundance proteins. Furthermore, the capacity of the affinity extraction would need to be thousands of times that of the preconcentration phase so that it could remove these proteins from the sample stream before they could block adsorption of the low abundance proteins on the preconcentration monolith. Nevertheless, this is an excellent example of combined sample cleanup and preconcentration and the integration of two types of polymeric monolithic extraction materials further demonstrates the potential of monoliths for sample cleanup and preconcentration. In particular, this demonstrates the versatility of the methacrylate ester type monoliths. The application of monoliths to the removal of signal-obscuring high abundance proteins is further elaborated in a recent review by Josic and Clifton [38].

Vizioli *et al*. [101] demonstrated coupling of an IMAC porous polymer monolith to a CE separation. This monolith was prepared by irradiation (γ-rays) of a mixture of GMA and diethylene dimethacrylate. This preparation technique has the disadvantage in the sense that the monolith was not truly prepared *in situ*. Rather, a section of the capillary in which this monolith was prepared was cut out and fixed between pieces of open capillary using PTFE sleeves and epoxy resin. IDA, attached by reaction with the epoxy groups on the monolith surface in a solution of DMSO, was used as a metal-chelating ligand. By treating the IDA-reacted monolith with $CuSO_4$ solution, a Cu(II) loading of 1.55 μg/g of monolith was achieved. The IMAC columns were characterized and were shown to be very effective at selectively retaining peptides with histidine residues. In this sense, this material was applied for sample cleanup, and sample enrichment capability was not investigated despite this being another potential application of the monolith.

Zhang *et al*. [102] also prepared a Cu(II)-type IMAC column for direct coupling with CE separation. A 1 cm section of poly(GMA-*co*-EDMA) monolith that was prepared *in situ* by thermally initiated free-radical polymerization then reacted with IDA and loaded with Cu(II) ions. This reaction was achieved in aqueous solution, whereas Vizioli *et al*. had shown that the reaction in DMSO was more efficient. Zhang *et al*. focused on the sample enrichment capability of their monolith and working with a sample solution of four synthetic peptides, they were able to demonstrate sample enrichment to factors of at least several hundred. Their procedure required just two separate electrolytes/eluents and all steps were controlled by voltage rather than pressure. This approach would be helpful if this procedure were to be ported to a μTAS format or to any platform where a pump is not typically included. Both Zhang *et al*. and Vizioli *et al*. achieved similar RSD values for migration time and peak area: generally less than 5%. However, Vizioli *et al*. reported greater column–column reproducibility.

In a different approach Yone *et al*. [103] prepared an IMAC monolithic column by attaching iron protoporphyrin IX to monoliths which they prepared by γ-irradiation of a solution of GMA and diethylene glycol dimethacrylate. These monoliths were able to extract angiotensin I by either coordination of histidine groups with the iron chelate or alternatively by π–π stacking of tyrosine or phenylalanine residues with the protoporphyrin itself. This was confirmed by the fact that an alternative pep-

tide that had no histidine or aromatic groups was not able to be extracted by the monolith. The angiotensin I sample was introduced into the capillary by pressure and the monolith was then washed for 4 min in the separation buffer which consisted of 50 mM phosphate buffer adjusted to pH 7 with HCl. The peptide was released from the monolith by pressure-driven introduction of a plug of low pH buffer with 25% ACN, followed by a CE step. The system gave a 10 000-fold improvement in the LOD compared to a standard hydrodynamic injection. However, they did not demonstrate the separation of angiotensin I from any other analytes during the CE step.

Breadmore [104] described an interesting approach in which he synthesized a poly(BuMA-*co*-EDMA-*co*-SPMA) monolith at the inlet end of a fused-silica capillary by photoinitiated free-radical polymerization. This monolith was demonstrated as an online preconcentration module for amino acids. In this approach, large volumes of sample could be injected and extracted onto the monolith surface by means of mixed mode (hydrophobic and cation exchange) interactions. Desorption was realized by an approach termed 'frontal electroelution', using a strong electrolyte that was also a strong eluent by inclusion of ethylenediamine and 60% ACN. In this approach, a boundary is set up between the two electrolytes as it moves through the monolith. The amino acids are then desorbed from the monolith in the presence of the strong electrolyte. They begin migrating towards the detector by electrophoresis, but they move faster than the boundary and become readsorbed onto the monolith once they re-enter the weak electrolyte. In this way, the amino acids become concentrated into a sharp zone around the boundary between the two electrolytes. Once the boundary has moved beyond the monolith, the analytes are free to be separated by electrophoresis. Breadmore reported a linear response for increased injection times for up to the equivalent of almost 15 capillary volumes of sample, which is 300 times greater than the typical 5% of capillary volume used in CZE. Unfortunately, the CZE separation of the amino acids could not be achieved because the UV-detection window was positioned the point in the capillary just downstream of the monolithic column, before they had a chance to separate. This was clearly a very successful demonstration of online SPE-CE with a polymer monolith. However, the complex requirements of this approach are a perfect example of how the methods described in this section will not be viable for many separations and sample matrices.

In 2005, Hutchinson *et al*. [105] described a novel monolithic extraction material and demonstrated it as a preconcentration module for CE. Monoliths based on poly(BuMA-*co*-EDMA-*co*-2-acrylamido-2-methyl-1-propanesulphonic acid (AMPS)) were prepared with the small amount of ionizable monomer providing charged sites for binding of cationic latex particles bearing quaternary ammonium groups. This innovation opens up the possibility of combining the convective mass transfer and *in situ* synthesis advantages of polymer monolith stationary phases with the durability and high binding capacity normally associated with state-of-the-art ion-exchange chromatographic column technology. Successful binding of the latex particles to the monolith surface was inferred by the reversed EOF, resulting from positive surface charge provided by the quaternary ammonium groups. The EOF was found to be very stable from run to run. The latex particles were also directly observed to be attached to the monolith pore surface using SEM (Fig. 8). These columns were tested initially in CEC mode as well as CE mode. Preconcentration and separation of a range of inorganic ions was achieved using a transient isotacophoretic boundary formed between two electrolytes, resulting in sample enrichment by both adsorptive preconcentration and electrophoretic sample-stacking effects. Following on from this work, Hutchinson *et al*. [106] demonstrated the same latex-coated monoliths for the preconcentration of organic anions ions in CE. The preconcentration process was similar to that in their previous paper, and allowed improvements in LOD, depending on the analyte, by factors of up to 10 400. The latex coating of the monoliths in these reports was only sparse, and we expect that better results will be achieved in the future with an improved monolith with complete monolayer latex coverage [107].

Most recently, our colleagues Thabano *et al*. [108] demonstrated an effective and relatively simple SPE-CE approach for preconcentration and separation of neurotransmitters in urine. UV-initiated poly(MAA-*co*-EDMA) monoliths were synthesized in the first 8 cm of 75 μm id fused-silica capillaries. The neurotransmitters were all positively charged in the presence of a pH 7 phosphate buffer binding/washing solution and were adsorbed by the negatively charged carboxylic acid provided by the MAA monomer. Urine samples needed to be diluted to give a sodium concentration close to 50 mM which was the optimum for binding. The neurotransmitters were strongly retained and it was possible to inject up to 15 column volumes of 1 μg/mL standard dopamine or epinephrine solution using external pressure with little effect on peak width and without exceeding the binding capacity of the poly(MAA-*co*-EDMA) monolith. For elution, a low pH phosphate buffer was introduced into the inlet of the column by electroosmosis and electrophoretic migration. This formed a moving pH boundary which desorbed the neurotransmitters from the monolith. The neurotransmitters were then separated in the open section of the capillary by differences in their electrophoretic mobility. The method resulted in an improvement in sensitivity of 462 times for dopamine. The robustness of the column was demonstrated by the fact that it was used for over 700 runs over the course of the experiment.

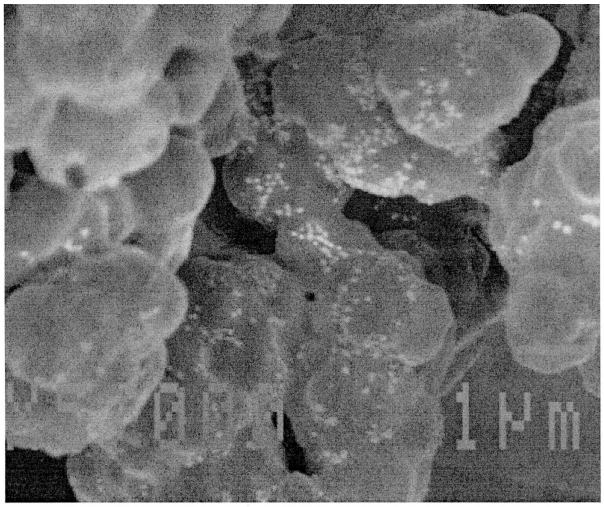

Figure 8. SEM of the latex-coated polymer monolith within a fused-silica capillary. Reproduced from ref. [105] with permission.

In summary, SPE-CE with polymer monoliths can yield excellent preconcentration whilst maintaining the high separation resolution for which CE is renowned. Offline methods, whilst slower and less integrated, can be used for excellent analyses for a wide range of samples. Online hyphenation of SPE and CE clearly has great potential, with a possible advantage in that all of the enriched sample is used in the CE separation. This is best achieved with planned and thoughtful consideration of the processes that can occur on the monolith and in the electrolyte, such as was demonstrated by Breadmore [104]. However, the approach can be very difficult, as demonstrated by the lengths that some researchers have had to go to just get reasonable separations under *ideal conditions* with *simulated* sample solutions. This will discourage all but the most experienced CE operators from undertaking the method development required for practical applications of this approach. We predict that if the monolithic SPE-CE approach ever becomes a common analytical tool then it will necessarily be in the form of commercial, ready-made capillary columns with out-of-the-box methods for well-defined niche applications.

5 SPE coupled online to CEC

Many of the same considerations that must be taken into account when coupling SPE online with CE also hold true for SPE coupled to CEC including careful choice of electrolytes and buffers and exact adsorption and elution conditions. However in the case of CEC where the column extends throughout the entire capillary, problems associated with the interface between the SPE and separation dimensions can be avoided. A significant portion of the previous work in this general area has been performed using silica-based monoliths and is well described in another recent review by Svec [4].

Palm and Novotny [109] first described this general approach using an acrylamide-based monolithic column in 1997 in which portions of the acrylamide in the polymerization mixture were substituted with other functional monomers. An optimized monolith of this type was used for SPE-CEC by Starkey *et al*. in 2002 for SPE-CEC [110]. This monolith had a very high percentage crosslinker and included vinylsulphonic acid to provide negatively charged sites for generating EOF even at very low pH. Lauryl acrylamide was included to provide the monolith with hydrophobic interaction capability. Using this approach on-column preconcentration of isoflavones was achieved, followed by separation in CEC mode. The column was preconditioned in a mobile phase consisting of 30% ACN prepared in 2.4 mM ammonium formate buffer at pH 2.7. The same mobile phase was used during the electrochromatography. For preconcentration, a long 90 s injection was used, allowing a large amount of sample to become adsorbed onto the front of the monolith. These samples had a lower ACN content than the mobile phase, and this allowed them to be better adsorbed on the front of the monolith as they were injected. When the mobile phase was reintroduced for the CEC step, they were swept up and concentrated into a sharp peak by the effect of the higher ACN content of the mobile phase. This resulted in a sevenfold improvement in sensitivity, with detection limits of 100 ppb. To enhance the preconcentration further, a water plug was injected prior to sample introduction. The water plug increased the ability of the monolith to adsorb the isoflavones, and allowed detection of concentrations as low as 15 ppb. After preconcentration, the isoflavones, uncharged in the pH 2.7 buffer, were separated by chromatographic interaction with the monolith. Plate counts as high as 245 000 were achieved for separating a standard solution of isoflavones without preconcentration, reducing slightly to 210 000 when the enhanced preconcentration procedure was applied. The method was demonstrated for determination of isoflavones in human breast milk and serum.

Although CEC separation was not demonstrated, Ping *et al*. [111] achieved sensitivity improvements of a factor of 22 000–24 000 for benzoin, alkylbenzenes and caffeine using an SPE-CEC approach with a thermally initiated poly(BuMA-*co*-EDMA) CEC capillary column. In this case, a very small EOF was generated by the monolith that could be presumed to the effect of surface charges arising from carboxylic acid groups present on the monolith due to hydrolysis of ester groups in the methacrylate backbone or adsorption of ions onto the polymer surface [112].

Augustin *et al*. [113] demonstrated a poly(hexyl acrylate-*co*-1,3-butanedioldiacrylate-*co*-AMPS) monolith for

CEC, formed by UV-initiated radical polymerization using AIBN as initiator. The latter monomer was included in order to promote the EOF. The capillary was completely filled with the polymerization mixture; however the final 9 cm were masked by aluminium foil so that that section could serve as a detection window and as a path to the outlet. Samples were prepared in water with only a very small amount of ACN and online preconcentration was achieved on the monolith by introducing the sample by either electrokinetic injection or by hydrodynamic injection using 0.8 MPa pressure for up to 128 min. The preconcentrated analytes were able to be eluted by a solution composed of 20% 5 mM acetate buffer at pH 4.6 and 80% ACN, which was pumped through the capillary using an EOF generated by 20 kV. Using these methods, 200-fold enrichment was possible for polyaromatic hydrocarbons and enrichment factors of up to 3500 were achieved for chlorophenols with chlorophenol detected at a concentration of 0.4 ppb which corresponds to environmental levels. The same monolith was demonstrated for a similar procedure in a microfluidic chip.

6 Polymer monoliths for online SPE-MS

Since this approach was first described by Moore *et al*. in 1998 [114] there have been several papers describing polymer monoliths for online SPE coupled directly with mass spectrometric (MS) detection. With the sensitivity improvements offered by lower column flow rates and online sample handling, monolithic SPE devices offer clear advantages for this application. This is reflected in recent developments within this field.

Marcus *et al*. [115] compared the effectiveness of a commercially available Dionex LC Packings monolithic SPE device for the identification and characterization of proteolytic digests of proteins. Using glucose oxidase, lipase and cytochrome c as reference proteins the performance of the monolithic SPE device was compared to a particle packed device (PepMap, Dionex LC Packings) of similar dimensions. The separation system was coupled offline to MALDI-TOF-MS *via* automatic fractionation and direct spotting of the peptides onto an anchor chip target. In this format, the monolithic SPE device was shown to not negatively influence chromatographic performance and in combination with MALDI-TOF-MS the method was shown to be fast and highly efficient for protein characterization and identification. However, using the monolithic SPE device the sample capacity was reduced.

Following from previous work in this area [12, 114], Zhang *et al*. [116] recently demonstrated a different approach to a bifunctional monolithic column for preconcentration and digestion of proteins. Using a combination of BuMA and GMA in the polymerization mixture, followed by subsequent immobilization of trypsin both hydrophobic C_4 groups and digestion sites were incorporated into the final monolith. Including the C_4 groups was beneficial as it increased the S/N and the number of peptides that could be identified from a given sample by using a combination of enrichment and digestion. These columns also showed excellent tolerance to organic solvent and maintained reproducible enzymatic activity for at least 30 days.

Bedair and Oleschuk [117] have also demonstrated lectin affinity chromatography. Using what by this time has become a well-established approach, they formed a poly(GMA-*co*-EDMA) monolith by photoinitiated polymerization directly within an electrospray emitter and the monolith was then functionalized with Con A using a Schiff base method. In this way in addition to serving as stationary phases for preconcentration, the monolith also served as nanospray interface for sheathless coupling to MS/MS detection with zero dead-volume. This was applied to preconcentration of glycopeptides from a tryptic digest of bovine pancreatic ribonuclease B for structural elucidation by MS/MS. Preconcentration was not performed online, but rather the capillary column was removed for the preconcentration loading step and reconnected to the MS/MS before elution by acetic acid in 50% ACN solution. No glycopeptides were detected in the nonenriched sample. After preconcentration of 20 μL of sample on the column they were able to detect five different glycoforms of the glycosylated peptide that were known to be in the tryptic digest of the protein. These had been selectively retained by the lectin affinity monolithic stationary phase. The enrichment of the sample allowed structural determination of the glycopeptides by MS/MS.

Another novel approach was reported by Altun *et al*. [118] who demonstrated microextraction using a monolith directly polymerized within a syringe which was then directly coupled to a triple quadrupole MS. The performance of a methacrylate-based monolith formed from poly(GMA-*co*-BuMA-*co*-EDMA) was compared to a syringe packed with either silica or polymer-based extraction phases for the extraction of lidocaine, ropivacaine and related metabolites from plasma. Whilst the monolithic material was shown to have low binding capacity compared to the particulate-based materials the considerably lower back-pressure was advantageous for working with plasma samples. Using this method it was possible to detect the analytes spiked in plasma and directly in patient urine samples.

The direct coupling of on-chip SPE with an IT ESI-MS has also been shown using an integrated electrospray tip [119]. Using the method described by Stachowiak *et al*. [120] the channel of a cyclic olefin copolymer (COC) polymer microdevice was modified by photografting and a hydrophobic poly(BuMA-*co*-EDMA) monolith formed

using UV-initiated polymerization at 365 nm. A 5 mm section of this monolith was shown to have a capacity of 81 ng for imipramine. To allow sufficient flow for MS analysis, make up flow was also applied through the chip using a side arm channel. This approach was shown to be viable for the simple determination of imipramine spiked into urine samples, thus demonstrating the potential for using this approach for both sample cleanup and preconcentration of drugs from biological fluids.

7 Convective interactive media and related formats

Whilst several groups have now synthesized polymer monoliths for preparative scale extraction, this field has been dominated by a successful commercial venture. BIA separations of Ljubljana, Slovenia, introduced a commercial range of short polymer monolith columns called CIM in the late 1990s. They introduced the materials as short monolithic columns that have all the familiar advantages of monolithic supports, emphasizing this by the nominal reference to convective fluid flow [121]. CIM are described as high-throughput media; capable of preparative as well as analytical scale separations. It is perhaps in the former application, particularly for the preparative purification of proteins that they have been most successful.

CIM come in two families: thin disks for axial flow and hollow cylinder columns for inward radial flow. Both are designed to be operated in specially designed housing. Desorption/separation is typically by gradient elution, and a large variety of applications and modes of interaction have been presented. The actual thickness of the stationary phase material in all CIM is quite small, from 1.5 mm in the disk format up to 40 mm for the largest of the preparative scale radial flow columns (http://www.biaseparations.com/documents/TechLibrary/brochures.asp). This is to allow high flow rates at relatively low pressure. The types of interaction typically occurring on CIM are strong, such that greater thickness is unnecessary and would merely lead to greater band-broadening and increased flow-resistance [121]. The greater thickness of the larger radial flow columns (operated at flow rates as high as 2 L/min) is used to allow collection of a greater quantity of the target biomolecule rather than to increase separation resolution. It should be noted that, in spite of the frequent references to the CIM trademark and their convenient packaging, these are neither unique nor special types of monolithic material. CIM are primarily poly(GMA-*co*-EDMA) and poly(styrene-*co*-DVB) monoliths with flow pores of around 1500 nm. Therefore they are comparable to many of the in-house prepared monoliths described elsewhere in this review.

That CIM are prepared from polymer rather than silica monoliths may be in part explained by the difficulties of preparing larger volume silica monoliths, which are notorious for shrinking away from the walls of larger volume containers during formation. The inert nature of polymer monoliths and the simplicity of the functionalization process also make the polymer chemistry very suitable for this format. Furthermore, the fact that CIM are often used for large, slow diffusing molecules such as proteins means that the mesoporous structures typically found in silica monoliths would in fact be a liability in this case rather than an advantage. Aside from their quite simple composition and convenient housing, it is the slim dimensions of CIM products along the path of fluid flow as compared to their large overall volume that is their most defining characteristic. A representative selection of research dealing with the use of CIM for extraction follows.

7.1 CIM disk for affinity extractions

The most commonly reported application of CIM is for selective biological affinity extractions. The approaches typically involve immobilization of an affinity ligand by reaction with the epoxy groups present on the surface of the poly(GMA-*co*-EDMA) CIM. In many cases, the complex affinity ligands are reacted directly with an epoxide group, whilst in other cases the epoxide groups are first used to build different functional groups for enhanced reactivity [45, 122]. BIA Separations offer CIM with preattached affinity ligands including protein A and protein G for immunoextraction (http://www.biaseparations.com/documents/TechLibrary/brochures.asp).

Berreux *et al*. [123] demonstrated the advantages of using CIM for affinity chromatography in 2000. They described an optimized procedure for binding of protein A, protein G and protein L to a CIM epoxy monolith. Such is the reactivity of the epoxy groups on the poly(GMA-*co*-EDMA) monolith that the immobilization reaction was possible under very mild conditions. The disks were immersed in a 100 mM carbonate buffer at pH 9.3. After 1–2 h, the disks were transferred to a 5.0 mg/mL solution of the appropriate affinity ligand with the maximum observable degree of ligand immobilization accomplished after 16 h at only 30°C. These affinity monoliths functioned well over a large range of flow rates and concentrations and the results demonstrated that the convective mass transfer on polymer monoliths of this type allows the separation to be performed at higher flow rates. The disk was still able to selectively extract protein when operated at 6 mL/min (equivalent to almost 18 disk volumes *per* minute) which was the maximum flow rate with the equipment available. In this situation, the speed-limiting factor may be the kinetics of the affinity interaction.

CIM are ideally suited for end-to-end coupling of multiple columns with different functionality. Ostryanina *et al*. [124] showed how this could be used to perform a very complicated separation task in a single step with four different CIM affinity disks prepared and connected in tandem. They were used to resolve a complex mixture of polyclonal antibodies into fractions that were defined by their affinity for particular regions of the antigen. The antigen, in this case, was a protein linked to a peptide by a linker group. Four different affinity ligands were immobilized on four separate CIM disks: one with the full protein–peptide conjugate, one with just the protein and a linker group, one with just the protein and one with just the peptide. The high permeability of these monolithic stationary phases made it possible to maintain flow through the series of four monolithic disks and elution of the bound antibodies was performed on individual CIM disks after dismantling the system. Specific subsets of the polyclonal antibodies (with regards to site specific affinity) could be targeted by varying the order of the CIM disks. The elution required a strong eluent (HCl solution at pH 2.0). Fortunately, the elution was complete in a matter of seconds and the pH of the eluted fraction could be promptly neutralized. As such, there was negligible inactivation of the fractionated antibodies, as confirmed by ELISA. Their results showed again that increase in flow rate did not cause a change in efficiency of the interactions on the monolith, due to the convective mass transfer on the monolithic stationary phase.

Peterka *et al*. [125] demonstrated IMAC on a CIM disk. They demonstrated very selective affinity for histidine-containing proteins with a high binding capacity of up to 20 mg/mL of monolith. They modified the epoxy groups on the CIM by a method adapted from work by Luo *et al*. [126]. Elution was achieved by displacement using imidazole and the authors were able to selectively extract protein LK-801 and green fluorescence protein with six histidine tags (green fluorescent protein (GFP)-6His) [125] as shown in Fig. 9. Both proteins had undergone partial purification by ammonium sulphate precipitation. Dynamic binding capacity (10% of breakthrough) was estimated to be around 17 mg/mL for crude LK-801, in the presence of its coprecipitates. For tagged GFP, the capacity was even higher, at 30 mg/mL. Increasing the flow rate from 1 up to 8 mL/min did not significantly affect the dynamic binding capacity, demonstrating the advantage of the convective mass transfer in the monolith. This implies that it would be possible to work at even higher flow rates. The fact that they were able to demonstrate very similar isolations of LK-801 with both small monolithic disks and larger 8 mL columns indicates that this system has great potential for upscaling. There is therefore every reason to believe that the process could be adapted for much larger scale purification, perhaps using the 800 mL epoxy CIM columns sold by BIA separations.

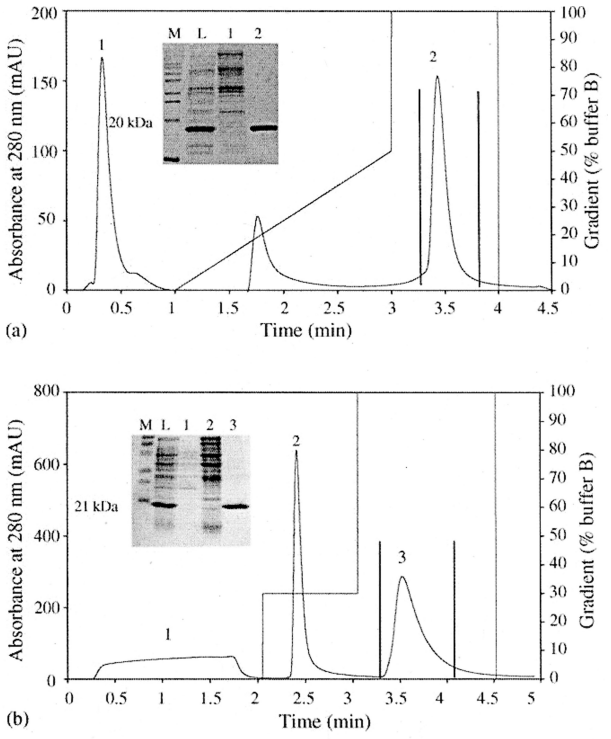

Figure 9. Purification of LK-801 on CIM disk and CIM 8 mL tube monolithic column. Conditions: (a) stationary phase, CIM IDA-Cu^{2+} disk monolithic column; mobile phase, buffer A, 20 mM phosphate, 0.2 M NaCl, pH 7.1; buffer B, 20 mM phosphate, 0.2 M NaCl, 100 mM imidazole, pH 7.1; sample, LK-801 dissolved ammonium sulphate precipitate; injection volume, 200 μL; flow-rate, 3 mL/min; gradient, see figure; detection, UV at 280 nm; collected fraction, 3.3–3.8 mL; SDS-PAGE analysis: M, molecular weight standard (10–200 kDa, Fermentas); L, load; 1, flow through; 2, elution. (b) Stationary phase, CIM IDA-Cu^{2+} 8 mL tube monolithic column; mobile phase, buffer A, 20 mM phosphate, 0.2 M NaCl, pH 7.1; buffer B, 20 mM phosphate, 0.2 M NaCl, 100 mM imidazole, pH 7.1; sample, LK-801 dissolved ammonium sulphate precipitate; loaded volume, 51 mL; flow-rate, 40 mL/min; gradient, see figure; detection, UV at 280 nm; collected fraction, 3.3–4.1 mL; SDS-PAGE analysis: M, molecular weight standard (low range, BioRad); L, load; 1, flow through; 2, impurities; 3, elution. Reproduced from ref. [125] with permission.

Calleri *et al*. [127] give an example of how CIM can be used for online SPE-LC. They used a 12 mm × 3 mm id modified epoxy CIM which had affinity for protein AFB1. The column was hyphenated to an HPLC separation using a column-switching configuration with two pumps and two six-port valves, allowing online immunoextraction and quantification of the AFB1. These reports clearly demonstrate that CIM and similar polymer monolith extraction media are suitable media for preparative scale separation by affinity extractions.

In other pioneering work in this area, Hagedorn *et al*. [128] demonstrated the first application of CIM in heterogeneous flow injection analysis (FIA). An immobilized

affinity ligand (*h*-IgG) was used for affinity extraction of Protein G from recombinant *E. coli* cell lysates. Using the CIM disk in place of typical cartridge-based systems it was possible to increase the flow rate to up to 5 mL/min and greatly accelerate the analysis, without any noticeable loss in performance.

7.2 Peptide affinity chromatography with CIM

Peptide affinity chromatography is a relatively new method of extraction with great potential advantages over its more conventional competitors [129]. CIM have been a popular media for the demonstration of this technology. Amatschek *et al*. [130] were quick to show the suitability of CIM for peptide affinity chromatography. They immobilized peptides on CIM disks, sepharose and AF epoxy toyopearl 650 M resin for affinity chromatography of a human blood-clotting protein, factor VIII (pdfVIII). It was found that peptides could be immobilized on the monolith with a density of 4–5 μmol/mL as opposed to 60 μmol/mL and 5–15 μmol/mL for the resins AF epoxy toyopearl and sepharose, respectively. Despite this much lower ligand density, they found that the peptides immobilized on the monolith were better utilized than those in the resins, probably as a result of better presentation and accessibility [129]. This may suggest that chemical structure of polymer monolithic supports of this kind gives them an advantage for the presentation of small sized affinity ligands.

Direct synthesis of peptide affinity-ligands, *in situ* on polymer monoliths, may be a viable alternative to immobilizing preprepared peptides on the monolith pore surface. The approach offers exciting possibilities which have been successfully demonstrated using CIM. For example, Pflegerl *et al*. [45] worked on *in situ* peptide synthesis and affinity chromatography on poly(GMA-*co*-EDMA) CIM disks. As in their previous work [130], they also chose pdfVIII as a model protein for their study. Initially, they worked with CIM disks that had been aminofunctionalized by reaction of ethylenediamine onto the epoxide groups. From this anchor point, they synthesized peptide no. 35, a sequence of eight amino acids known to bind to pdFVIII [130]. They were able to demonstrate selective binding of pdFVIII. However, there was some nonspecific adsorption which they attributed to the ethylenediamine spacer. Correspondingly, their next approach was amination of the monolith surface by reaction of ammonia onto the epoxide groups to yield a simpler anchor point without a spacer. The lack of a spacer was justified as the polymethacrylate chains themselves should aid in the presentation of the peptides [45]. This approach appeared to be successful in reducing the nonspecific adsorption. They achieved a peptide ligand density of 5 μmol/mL, which corresponds to the density achieved previously when they performed a conventional immobilization of the same peptide [130]. In this sense at least it would appear that the direct synthesis approach may be able to compete with the immobilization approach. This finding was given further support in studies by Vlakh *et al*. [131, 132]. Working with peptide affinity ligands for recombinant tissue plasminogen activator (t-PA), the *in situ* synthesis approach was compared to the alternative of immobilizing peptides that had been separately synthesized. CIM Epoxy Disks from BIA Separations were used as the solid supports in both cases. Performing frontal analysis experiments, they did not find any significant variation between the two types of sorbent in any of the important affinity parameters. Affinity constant, ligand density and binding capacity corresponded remarkably.

In a continuation of their earlier work, Pflegerl *et al*. [133] demonstrated how the *in situ* affinity peptide synthesis approach could be used to improve rapid screening of peptides for affinity chromatography. They miniaturized the CIM down to a volume of 10 μL and placed more than 20 of them on a 96-well Microlute Microplate over a vacuum manifold. Using their peptide synthesis technique on the monoliths on the plate, they ran a semi-automated combinatorial synthesis of 20 variations of peptide 35 in which a lysine in the middle of the peptide was replaced with each of the other proteogenic amino acids. This was designed to be a combinatorial screening approach to find active affinity peptides for pdFVIII. The peptide synthesis was nearly fully automated with the use of an Autospot robot. However, the CIM disks had to be removed from the microplate during side-chain deprotection as the sealing gaskets were not stable in TFA. This could be overcome by using a different material for the gaskets. Verification that the synthesis had been successful relied on three extra disks that were synthesized in parallel to the 20 affinity-screening disks. These three test monoliths were ground up and subject to amino acid analysis, which showed that the correct sequences had been generated, though at low yields of 0.7 μmol/mL. Immediately after preparation, the 20 different affinity peptides were screened for affinity against pdFVIII by applying simulated chromatography conditions to the disks, still working over the original 96-well plate. Analysis of the flow-through and eluent allowed them to see which of the 20 peptides had the strongest interaction [133]. The results were found to be much clearer than those attained by performing a parallel experiment that used a spot synthesis approach with a non flow-porous cellulose membrane.

Mechanistically, this approach to affinity peptide screening offers some exciting advantages over the incubation methods that would normally be used for developing peptide libraries. The more conventional cellulose membrane spot synthesis was dependant on diffusion both for the synthesis and affinity-testing steps. By con-

trast, the polymer monolith synthesis had the advantage of flow through and convective mass transfer for the synthesis step and the affinity testing step, making this a potentially faster approach for a rapid flow-through binding test as opposed to the incubation of the cellulose spots. It enabled this group to selectively identify the peptides with very high binding affinity. Furthermore, they were able to assay not just the amount of protein bound on the monolith, but also the contents of the flow-through and, later, the desorbed eluent.

Together, these reports on the use of monoliths for direct peptide synthesis for affinity chromatography show some of the most exciting potential roles for polymer monolith sorbents. The CIM monoliths were shown to be effective both as substrates for a complex solid phase synthesis and as ideal materials to test the synthesized ligand for affinity. Furthermore, they were shown to have fundamental advantages over alternative methods. These advantages emerged out of a combination of the favourable properties of polymer monoliths that have been noted in other parts of this review. The poly(GMA-*co*-EDMA) monoliths were suitable for the direct peptide synthesis approach to affinity chromatography because of their convective mass transport, low flow-resistance, appropriate reactivity, mechanical strength and, not least, by the fact that they were easily prepared in the desired shapes. Polymer monoliths such as the poly(GMA-*co*-EDMA) CIM could play a crucial role in seamlessly interfacing combinatorial chemistry and biochemistry with analyses that depend on separations. This could allow more efficient and more powerful approaches to screening libraries of drugs, peptides or other molecules, with numerous potential applications for high-throughput biochemistry research.

One of the most exciting recent developments in CIM has been described by Kalashnikova *et al*. [134, 135] in the application of these supports for isolation of the influenza virus. This has been accomplished using both 3-D microarrays for quantitative detection [134] as well as in the disk format for fine purification [135]. A range of suitable affinity ligands were immobilized on poly(GMA-*co*-EDMA) monoliths and the interaction with both virus-mimicking synthetic particles and influenza A virus was investigated. A detailed optimization of the immobilization step was undertaken and from this it was shown unequivocally that sorbents bearing sialyllactose immobilized on a surface *via* low weight protein as spacer and polysaccharide demonstrated the greatest efficiency for virus isolation.

7.3 CIM with ion-exchange functionality

CIM with ion-exchange modes of interaction have been used in protein purification and analysis. Vovk *et al*. [8] demonstrated semi-preparative extraction and fractionation of isoenzymes of tomato pectin methylesterase using a cation exchange CIM bearing sulphonate groups. They injected nine disk volumes of crude tomato extract and divided the extracted compounds into several fractions by eluting with a concentration gradient of NaCl.

Josic *et al*. [9] applied a diethylaminoethanol modified CIM to their proteomics research. They used the ready-made anion exchange monolithic disks and columns by BIA Separations to enrich and fractionate proteins from rat liver plasma membranes. This quick method allowed the researchers to effectively add another dimension to their proteomic separation. Bound fractions of protein were eluted with increasing concentrations of NaCl. The fractions obtained could be separated in a further two dimensions by 2-DE. The extraction process resulted in sample enrichment that allowed detection of less abundant membrane proteins that would otherwise have been undetectable.

Despite an emphasis on larger molecules, CIM have also been used for extracting small inorganic species. Tyrrell *et al*. [136] modified CIM disks for extraction of Cu(II) from environmental water samples. They coated poly(styrene-*co*-DVB) CIM disks with three different organic acids in the hope of generating a suitable copper-chelating sorbent. The monoliths were then tested as part of a FIA system designed to detect copper in estuarine and coastal seawater. Extraction of the copper from the seawater was important for selective detection because the chemiluminescence detection method they used was sensitive to interference by several components of seawater. Dipicolinic acid was found to be the most effect ligand for copper capture. With this system, they were able to detect Cu(II) in simulated seawater with a linear response ($R^2 = 0.998$) over a range of 0–125 μg/L. This report is another example of how polymer monolith materials are highly suited to rapid screening of chromatography ligands.

8 Microfluidic chips

Microfluidic chips are still in their infancy as platforms for liquid phase analytical chemistry. They are associated with the wider aim of creating a new generation of μTAS. A key goal of this project is the capability to seamlessly integrate several analytical processes in the confines of a small device. As such, a large amount of research has been focused on the question of how to integrate solid phase functional media into chips. Monolithic stationary phases that can be formed *in situ*, within the microchannels of the device, are at an obvious advantage in terms of ease-of-fabrication [137]. This is particularly true for those types of monolith that can easily be formed by UV-initiated radical polymerization such as those prepared from methacrylate esters [138].

Another goal of μTAS is to increase analysis speed. This means that the stationary phase materials on microfluidic chips may need to perform rapid separations and operate at high linear flow velocities. Therefore, the convective mass transfer and low flow resistance common in monoliths can be especially valuable on the chip platform. Extraction is one of a growing number of processes that have been demonstrated on microfluidic chips using porous monoliths as functional media [137, 139], yet there is only a modest number of publications in this area despite the interest it receives. This is probably due to the difficulty and expense involved in fabricating or procuring the microfluidic chips needed for such research. It is likely that the rate of research output in this area will increase once more labs gain the ability to fabricate chips or gain access to prototype chips from industry. In the meantime it should be noted that many of the monoliths that have been created in capillaries, described in previous sections, have the potential to be integrated into a chip-type device. This is due to the fact that fused-silica capillaries typically have similar dimensions to the channels on microfluidic chips, and are therefore natural experimental models for the chip platform.

Extraction by a polymer monolith on a microfluidic chip was first demonstrated by Yu *et al*. in 2001 [25]. Monoliths were synthesized in surface modified glass chips with channels of dimensions 40 μm × 100 μm × 8 cm. Using the photoinitiated polymerization methods they had pioneered [138], Svec's group experimented with two types of polymer monolith in the chips. They were able to control the extent of the monoliths within the channel by photomasking. Such precise control would have been very difficult with a thermally initiated polymerization. The first monolith was a hydrophobic monolith of poly(BuMA-*co*-EDMA) whilst the second included META, which provided functionalities for anion-exchange interactions. Using a high linear velocity of 12 mm/s both types of monolith were successfully demonstrated for the preconcentration of small organic acid Coumarin 19, which could be eluted by ACN in a sharp peak, thus achieving preconcentration with an enrichment factor of 1650. The monoliths were also used to demonstrate preconcentration of GFP and a fluorescently labelled tetrapeptide. GFP could be preconcentrated up to an enrichment factor of 1000 [140].

Tan *et al*. [141] prepared similar monoliths in a chip made from COC. The channels were formed by a hot embossing process. Eight fused-silica capillaries were buried in the hot COC to act as reverse-moulds for flow channels. After the embossing process, the capillaries were removed from the COC chip whilst it was still warm, and the channels were broken in the process. However, they immediately returned to their previous shape, leaving circular channels with dimensions of 360 μm internal diameter and lengths of 85 mm. Photoinitiated synthesis of a poly(BuMA-*co*-EDMA) monolith was then performed in the channels similar to that described by Svec's group [25]. This was possible because COC is one of the few UV-transparent polymers that have been used to make microfluidic chips [141]. A small amount of AMPS was also added to the monolith polymerization mixture. This monomer may have given some mixed mode character to the stationary phase, but it was added with the intention of supporting EOF [141]. Despite this, flow through the monolith was not generated by voltage but was instead controlled by a syringe infusion pump. This system was directly coupled to ESI-MS by capillaries that connected to the outlets of the channels on the COC chip. They tested the SPE device by extracting the antidepressant drug imipramine from dilute solutions and eluting it into an ESI-MS detector. Working with spiked urine samples, linear response was achieved over the range of 25–10 000 ng. The column-to-column reproducibility was poor in terms of adsorptive capacity, with a RSD of 26% across eight monolithic columns. The re-usability of the SPE columns was not tested because it was claimed that single use columns would be acceptable, given that many contemporary SPE columns are intended for single use.

Surprisingly, some of the most impressive applications of polymer monoliths for extraction on chips have been of the more complex affinity chromatography variety. The first demonstration of this that we found was by Mao *et al*. [142]. This group demonstrated separation of glycoforms for lectin affinity chromatography. They used a commercial Microfluidic Toolkit (Micralyne, Edmonton, AB, Canada), however chose not to use commercial chips, instead fabricating their own glass chips by photolithography. Wet chemical etching was used to form small channels of dimensions 70 μm × 20 μm and poly(GMA-*co*-EDMA) monoliths were synthesized *in situ* in accordance with the methods described previously [140]. *Pisum sativum* agglutinin (PSA), a lectin, was immobilized by reaction with the epoxy groups on the monolith surface. Mao *et al*. were then able to selectively bind the different glycoforms of chicken and turkey ovalbumin. The different glycoforms of the fluorescently labelled glycoproteins could be eluted with partial resolution by introducing a step gradient of displacing sugar into the monolith using the scheme illustrated in Fig. 10. Another impressive aspect of this work was that the entire separation process operated using EOF. This is a prized achievement in the field of μTAS because it eliminates the need for a bulky or expensive external or internal pump device. The goal of increased analysis speed was well satisfied – the entire process was complete within 400s.

Li and Lee [143] prepared short sections of poly(GMA-*co*-TRIM) monoliths in etched glass chips. Once again, the use of photomasking allowed relatively simple control of

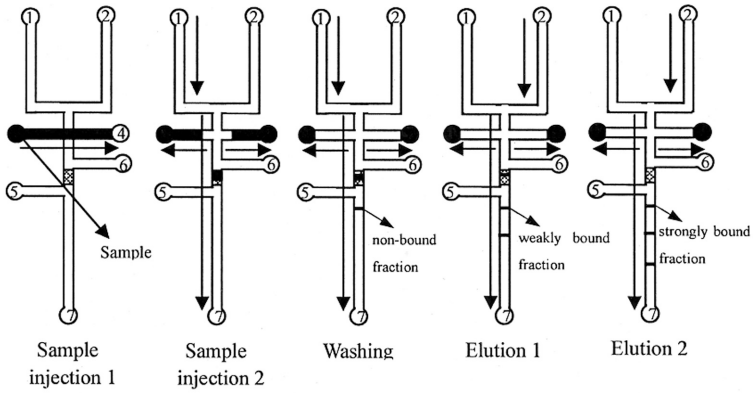

Figure 10. Operation sequence of PSA-AMC. The arrows indicate the movement of solutions. Reproduced from ref. [142] with permission.

the formation of the monolith in the channel. They immobilized Cibacron-blue-3G-A onto the monolith, using ethylene diamine as a reactive bridge. This dye was intended to act as an affinity ligand for lysozyme and human albumin. MALDI-TOF and a stereo fluorescence microscope was used to detect the labelled proteins and they found that they could selectively retain lysozyme over nonspecifically bound protein cytochrome c. They then attempted to extract human albumin from cerebrospinal fluid as a sample cleanup and were able to selectively remove albumin over another ubiquitous protein, transferrin g. However, a significant level of nonspecific adsorption was observed in both of these experiments. In related work described in Section 6, Li and Lee in collaboration with Yang *et al.* [119] prepared a methacrylate-based monolith on a chip that functioned *both* as an on-chip electrospray interface as well as SPE material.

9 Concluding remarks

Polymer monoliths are being used as chromatographic extractors in an increasingly wide variety of platforms and for a growing range of applications. Researchers frequently refer to the advantages of polymer monoliths with regards to mass transport, mechanical strength and permeability. In some cases, these advantages have been realized as impressively fast separations or as the ability to process large sample volumes with relatively small columns.

However, from a researcher's perspective, the standout feature of polymer monoliths is the convenience and scalability of the *in situ* synthesis, which facilitates preparation of columns of any shape and size. In several cases, the ease-of-synthesis was probably the *only reason* that the researchers chose to work with polymer monoliths as opposed to using other types of sorbent. This aspect of polymer monoliths has made them popular research-enabling tools; they are probably the most convenient class of sorbent for testing new separation formats and new chemistry for novel selectivity. For example, the combinatorial solid-phase synthesis and assessment of peptide affinity ligands performed by Pflegerl *et al.* [133] would have been much more difficult if they had used any other class of sorbent material.

Polymer monoliths appear to be effective extractors across the entire range of column sizes. At the large end of the scale, large-volume commercially available polymer monoliths (CIM) have been demonstrated for rapid preparative scale isolation of proteins. At the other extreme, easily prepared polymer monolith capillary columns have become favoured materials for in-line SPE-CE and online SPE-MS where they can also double as electrospray interfaces. Whilst some of these microscale SPE applications suffer from complicated operating procedures they are at least likely to find niche applications in analyses that require rapid online sample enrichment. On microfluidic chips, photoinitiated methacrylate monoliths are the most convenient due to the fact that their formation can be spatially defined within the desired section of a microchannel through simple photomasking.

The wide variety of polymer materials and approaches to monolith synthesis give chemists the ability to produce a huge array of materials with different extraction-related properties. However, from a separation scientist's perspective, this diversity is also a great challenge in the sense that it is difficult to determine the 'best' polymer monoliths for a given extraction application. The widespread use of materials such as poly(GMA-*co*-EDMA) and poly(MAA-*co*-EDMA) does not necessarily imply their superiority. Rather, their popularity may be partly the result of a self-fulfilling cycle in which they are assumed to be the most appropriate polymers due to their ubiquity in the literature. It would be helpful if there were more reports that involved direct comparison of the performance of various types of polymer monoliths as well as other classes of sorbent including silica monoliths. It would also help to see performance comparisons with the less common types of polymer monolith such as those based on poly(TRIM), epoxy resin and urea-formaldehyde. These chemistries have received surprisingly lit-

tle attention despite having been successfully demonstrated in a few reports.

Stationary phase chemistry is particularly important in the case of extraction type separations because they are dependant on achieving very high selectivity. Despite this, and despite the relative ease with which the monolith chemistry can be tweaked, most researchers have instead focused on optimizing the mobile phase for their extractions. Monoliths such as poly(MAA-*co*-EDMA) and poly(styrene-*co*-DVB) afford modes of nonspecific binding which are appropriate for some preconcentration and SPE applications. However, to produce materials with more specific interactions, researchers most often perform extra synthesis steps to immobilize ligands onto the internal surfaces of the monoliths. This enables the surface chemistry of monolith to be optimized independently of the macroporous morphology. Numerous successful approaches to ligand immobilization have been demonstrated and this is another source of confusion which would benefit from more direct experimental comparison.

Finally, the relatively low specific surface area of polymer monoliths is oft cited as a disadvantage of using these materials and may have had an impact on the adsorption capacities of some of the sorbents described in this review. However, the numerous successful application demonstrations clearly show that these materials have sufficient surface area to be effective sorbents in very many cases and this factor need not be considered an impediment to widespread adoption of this technology.

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