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Review

Development and application of polymeric monolithic stationary phases for capillary electrochromatography

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Abstract

Monolithic columns for capillary electrochromatography are receiving quite remarkable attention. This review summarizes results excerpted from numerous papers concerning this rapidly growing area with a focus on monoliths prepared from synthetic polymers. Both the simplicity of the in situ preparation and the large number of readily available chemistries make the monolithic separation media a vital alternative to capillary columns packed with particulate materials. Therefore, they are now a well-established stationary phase format in the field of capillary electrochromatography. A wide variety of synthetic approaches as well as materials used for the preparation of the monolithic stationary phases are presented in detail. The analytical potential of these columns is demonstrated with separations involving various families of compounds and different chromatographic modes.

Keywords: Reviews; Monolithic columns; Electrochromatography; Stationary phases, electrochromatography; Proteins; Peptides; Saccharides

Contents

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1. Introduction

Capillary electrochromatography (CEC) may be defined as liquid chromatography (LC) conducted in packed capillary columns across which a high electric field is imposed. The movement of the mobile phase occurs through electroosmotic flow (EOF). CEC was first introduced by Pretorius in 1974 who demonstrated the feasibility of performing electrodriven separations by applying an electric field across a glass chromatographic column [1]. This concept was extended by Jorgensen and Lukacs in 1981 to use packed capillary columns [2]. Since this time CEC has developed rapidly as an exciting alternative separation technique, especially due to the recent high demand for new miniaturized separation methods having vastly enhanced efficiencies and peak capacities compared with traditional LC techniques. Although a variety of approaches have been described for the preparation of capillary columns for CEC, the majority of these mimics in one way or another standard HPLC column technology. However, aspects of this technology have proven difficult to implement on the capillary scale, particularly because of the technical challenges associated with packing and retaining beads in narrow-bore capillary columns.

These limitations have thus spurred the development of various alternative approaches. For example, columns containing in situ polymerized organic separation media, adopted from a concept developed for much larger diameter HPLC columns, have already proven to be a viable option [3,4]. As a result of their unique properties, these monolithic materials have attracted considerable attention and have been the subject of several reviews in recent years [5–9].

Perhaps the most appealing aspect of the monolithic materials is the ease of their preparation. The simple polymerization process starts from liquid precursors (polymerization mixture) and is performed directly within the confines of a capillary or a microfluidic chip. This avoids the problems related to both frit formation and packing. Additionally, columns of virtually any length and shape are easily accessible. The polymerization mixture can be prepared from a wide variety of monomers, allowing a nearly unlimited choice of both matrix and surface chemistries. This flexibility enables the easy tailoring of both the interactions that are required for specific separation modes and the level of EOF generated by the support. Finally, the control that can be exerted over the polymerization process enables the facile optimization of the porous properties of the monolith, and consequently the flow rate and chromatographic efficiency of the system.

2. Early developments in monolithic separation media

The first monolithic CEC columns were similar to those used for capillary gel electrophoresis and contained swollen hydrophilic polyacrylamide gel [10]. Typically, the capillary was filled with an aqueous polymerization mixture containing monovinyl and divinyl (crosslinking) acrylamide-based monomers as well as a redox free radical initiating system, such as ammonium peroxodisulfate and tetramethylethylenediamine (TEMED). Initiation of the polymerization process begins immediately upon mixing of all the components at room temperature. Therefore, the reaction mixture must be used immediately. The polymerization process is normally allowed to proceed overnight to afford a capillary filled with a continuous bed of gel. It should be noted that this gel is very loose, highly swollen material that usually contains no more than 5% solid polymer.

For example, Fujimoto et al. [11] polymerized an aqueous solution of acrylamide, methylenebisacrylamide, and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) within the confines of a capillary. Despite the lack of chemical attachment to the inner wall of the capillary, these crosslinked gels showed fair physical stability. Although column efficiencies of up to 150 000 plates/m were observed for acetophenone, retention times on these columns were prohibitively long. This behavior was probably due, in part, to the relatively high background buffer concentration of 0.1 mol/L, which is at least one order of magnitude higher than that typically used in recent CEC studies. Based on his results, Fujimoto concluded that the mechanism of separation that prevailed in his system was sieving rather than an interaction of the solutes with the matrix [12].

Replacement of the hydrophilic acrylamide with the more hydrophobic *N*-isopropylacrylamide (NIPAAm), in combination with the pre-functionalization of the capillary internal surface with 3-(trimethoxysilyl)propyl methacrylate, afforded a monolithic gel covalently attached to the

capillary wall. The electrochromatographic elution of hydrophobic analytes from this column required the use of aqueous buffer/acetonitrile mixtures [13]. Improvements in the separations were observed using these "fritless" hydrogel columns leading to both shorter retention times and column efficiencies as high as 160 000 in the analysis of various steroids. The separation of hydrophobic compounds obtained using this polymer gel stationary phase exhibits many of the attributes typical of reversed-phase chromatography, including a linear dependence of the retention factor k' on the composition of the mobile phase. This led to the conclusion that, in contrast to the original polyacrylamide-based gels, size-exclusion was no longer the primary mode of separation.

3. Rigid porous monoliths for capillary electrochromatography

3.1. Imprinted monolithic columns

In recent years, molecular imprinting has attracted considerable attention as an approach to the preparation of polymers containing recognition sites with predetermined selectivity. The history and specifics of the imprinting technique pioneered by Wulff in the early 1970s [14] have been detailed in several excellent review articles [15–17]. These imprinted monoliths have also received considerable attention as stationary phases for capillary electrochromatography.

The imprinting process typically involves the pre-organization of functional monomer molecules such as methacrylic acid and/or vinylpyridine around a template molecule and subsequent copolymerization of this complex in the presence of a large amount of crosslinking monomers such as ethylene dimethacrylate and trimethylolpropane trimethacrylate [18]. Under ideal conditions, imprints possessing both a defined shape and a specific arrangement of chemically interactive functionalities matching those of the templated molecule remain in the polymer after extraction of the template.

In the early days of this technique, the imprinted materials possessed pores too small to support flow. Therefore, these polymers could only be used as crushed and sieved irregular particles. Truly monolithic technology was directly employed for CEC application only after the introduction of "superporous" imprinted monolithic capillaries by Nilsson in 1997 [18–20]. Isooctane was used as a porogen in order to produce a macroporous structure with large pores without interfering with the imprinting process. The imprinted polymers were prepared within capillaries having a vinylized inner surface using both thermally and photoinitiated polymerizations [18–21]. Using a short 8.5 cm column, the separation of propanolol enantiomers was achieved in less than 30 s with good reproducibility [22]. This ability to maintain separation power for enantiomers in short capillaries represents potential for future application in microfluidic systems.

In following this group investigated the crucial role of the carboxylic acid moieties by preparing a series of columns in which no methacrylic acid was included and which failed to afford any enantiomer separation, thus demonstrating that strong electrostatic interactions with the analyte are necessary to help form molecular imprints [23]. Furthermore, addition of surfactants was found to be effective in achieving resolution of propanolol enantiomers in water-rich electrolytes, which alone do not promote enantiomeric separations, thus opening novel ways to optimize such systems.

Using a similar process and employing mixtures of ethylene dimethacrylate with methacrylic acid and/or 2-vinylpyridine, Lin et al. developed imprinted monolithic columns for the CEC separation of racemic phenylalanine [24–26]. They also investigated a composite approach toward imprinted monoliths. A non-porous polymer imprinted with L-phenylalanine anilide was first prepared via UV initiated polymerization within a glass ampoule. This bulk polymer was ground into small irregular particles, sieved, and suspended in a solution of acrylamide and methylenebisacrylamide containing a redox initiator. This heterogeneous dispersion was then drawn into the capillary and the polymerization completed affording a gel-type monolith with immobilized solid particles. The capillary column was then used for the separation of D,L-phenylalanine [24]. Such approaches to monolithic imprinted stationary phases for CEC have recently been reviewed in greater detail [17].

Yan et al. used mixtures of methacrylic acid and EDMA for the preparation and evaluation of a 4-aminopyridine imprinted, polymer-based monolithic capillary column [27]. By varying the amount of acetonitrile in the mobile phase and the pH, it was possible to demonstrate that the separation of 4-aminopyridine and 2-aminopyridine is based on the interplay of molecular imprinting recognition, ion-exchange retention and electrophoretic migration. For example, by varying the pH from 3 to 7.5 as shown in Fig. 1, the contribution due to the molecular imprinting process could be evaluated. At low pH 2-aminopyridine is partially ionized and hence migrated more slowly than thiourea as a neutral marker. However in the pH range of 5–7.5 both analytes remained unprotonated and the migration time for 2-aminopyridine was almost identical to that of thoiurea, whereas 4-AP had a longer migration due to the selective molecular imprinting interactions.

3.2. Monoliths prepared from aqueous monomer solutions and dispersions

The preparation of continuous CEC beds involving highly crosslinked acrylamide polymers was first reported by Hjertén et al. in 1995 [28]. The original approach was complex, requiring several steps including the modification of the capillary surface with 3-(trimethoxysilyl)propyl methacrylate, two individual polymerizations, and a chemical functionalization [29]. The initial polymer matrix was

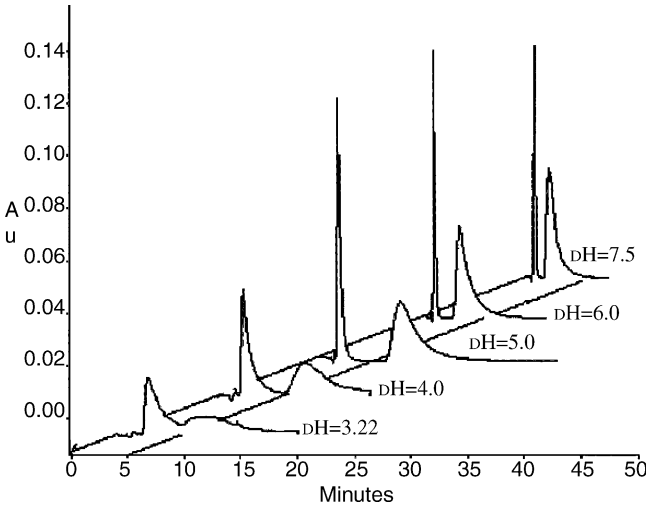

Fig. 1. Effect of pH on the separation of 2-aminopyridine (less retained peak) and 4-aminopyridine (more retained peak) at various pH values using a 4-aminopyridine molecular imprinted polymer-based capillary column (from [27] with permission). Column: 31.2 cm (10 cm packed) 100 μm i.d., electrolyte: 10% acetic acid–sodium acetate (0.01 mmol/L) in acetonitrile; temperature: 25 °C; voltage: 15 kV; UV detection: 246 nm; injection: 3.5 kPa, 3 s (reverse); sample concentration: 2 mg/mL.

formed by copolymerizing a dilute aqueous solution of 2-hydroxyethyl methacrylate (HEMA) and piperazine diacrylate using a standard redox initiation system in the presence of a high concentration of ammonium sulfate. The pores of this matrix were then filled with another polymerization mixture containing allyl glycidyl ether and dextran sulfate, and the second polymerization proceeded within the pores of the initial matrix leading to the "immobilization" of the charged dextran within the newly formed composite. Eventually, reaction of both epoxide and hydroxyl functionalities with 1,2-epoxyoctadecane led to the covalent functionalization of the matrix with a number of C_{18} chains. Several chromatographic measurements were performed using these capillaries, with retention times in excess of 20 min being required for the elution of aromatic hydrocarbons [29].

The same group subsequently proposed a much simpler procedure with the polymerization mixture consisting of an aqueous solution of acrylamide, piperazine diacrylamide, and vinylsulfonic acid with added stearyl methacrylate or butyl methacrylate to control the hydrophobicity of the gel [30]. Since neither of these non-polar monomers is soluble in water, a surfactant was added to the mixture, followed by sonication to form an emulsion of the hydrophobic monomer in the aqueous solution. Once initiated, the mixture was immediately drawn into an acryloylated capillary, where the polymerization was completed. The presence of the strongly acidic sulfonic acid functionalities afforded EOF that remained constant over a broad pH range. Although the initial separations performed using these continuous gel beds were good, the addition of sodium dodecyl sulfate at levels below the critical micellar concentration was reported to substantially improve isocratic CEC separations and further improvements were possible by employing a simple step gradient to enhance the resolution of various polycyclic aromatic hydrocarbons (PAHs) [30].

Yet, another method for the preparation of a monolithic capillary column that for the CEC gradient separation of proteins was later described with the first step involving a polymerization initiated by ammonium peroxodisulfate–TEMED system in a system consisting of two phases: an aqueous phase typically a solution of acrylamide and piperazine diacrylamide in a mixture of a buffer solution and dimethylformamide, and an immiscible, highly hydrophobic phase consisting of octadecyl methacrylate [31]. Continuous sonication (40 min) was required in order to emulsify the octadecyl methacrylate and form a dispersion of fine polymer particles. Following this, another portion of initiator was added to the system to restart the polymerization of two newly added monomers, dimethyldiallylammonium chloride and piperazine diacrylamide. The resulting partly polymerized dispersion was then forced into a methacryloylated capillary using pressure and, finally, the polymerization process was carried out to completion. This allowed the excellent separation of proteins in either the co-EOF or counter-EOF mode.

Hoegger and Freitag used a similar procedure to that described by Hjertén for the preparation of acrylamide monoliths and performed a systematic evaluation of the preparation and chromatographic behavior of these monoliths [32–34]. Initially, a polymerization mixture consisting of *N*,*N*-dimethylacrylamide (DMAA), methacrylamide (MAA), and vinylsulfonic acid (VSA) was used and butyl acrylate or hexyl acrylate were added to control the hydrophobicity of the gel. The retention mechanism for a series of neutral aromatic compounds was found to be neither pure reverse-phase nor pure normal-phase, even when monoliths containing large percentage of C_6 ligand were used, suggesting that in this case the separation mechanism is not solely controlled by differences in hydrophobicity.

Recently, these authors prepared similar monoliths from mixtures of piperazine diacrylamide, VSA and DMAA for the separation of charged biomolecules by CEC using a mixed-mode separation mechanism [33]. The effect of increasing the concentration of ammonium sulfate in the polymerization mixture was investigated, and resulted in an increase in the mean pore diameter as determined from both mercury intrusion porosimetry data and decrease in column back pressure. Surprisingly however no difference in EOF was observed for the three different columns prepared. The effects of variations in mobile and stationary phase composition on the separations were also considered. In the case of histidine illustrated in Fig. 2, the mixed-mode retention mechanism is clearly demonstrated by changes in the mobile phase composition. In this case, the best peak shape results when the buffer concentration is high enough to reduce electrostatic interactions with the stationary phase and the aqueous content of the mobile phase is sufficient to

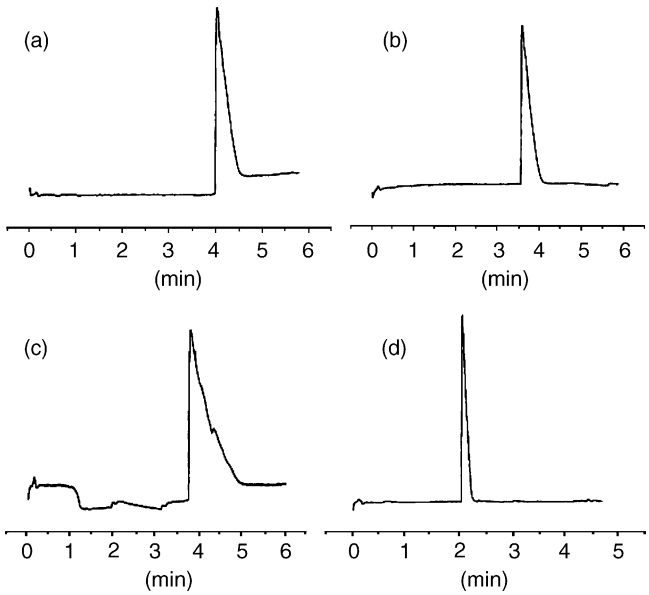

Fig. 2. Effect of the mobile phase composition on the peak shape of histidine (from [33] with permission). Stationary phase: dimethylacrylamide-based column: (a) 20 mmol/L ammonium acetate–800 mmol/L acetic acid in (acetonitrile–methanol, 8:2)–water (6:4); (b) 20 mmol/L ammonium acetate–800 mmol/L acetic acid in (acetonitrile–methanol, 8:2)–water (4:6); (c) 13.3 mmol/L ammonium acetate–800 mmol/L acetic acid in (acetonitrile–methanol, 8:2)–water (2:8); (d) 40 mmol/L ammonium acetate–800 mmol/L acetic acid in (acetonitrile–methanol, 8:2)–water (2:8).

reduce deleterious hydrophilic interactions. Changes in the stationary phase composition were also investigated with DMAA replaced by MAA, 2-hydroxyethyl methacrylate or 2-hydroxyethyl acrylate in the polymerization mixture. Retention in each case followed the expected trends based on the relative monomer hydrophilicity.

3.3. Porous polymer monoliths prepared in the presence of organic solvents

Despite the success of the use of purely aqueous polymerization systems, the poor solubility of a number of monomers in water, such as those used for the preparation of monolithic capillaries for reversed-phase CEC, led to the development of polymerization systems containing various organic solvents. In contrast to the “fixed” solubilizing properties of water, the wealth of organic solvents possessing polarities ranging from highly nonpolar to extremely polar allows the formulation of mixtures with solvating capabilities that may be tailored over a very broad range. An additional feature of organic solvents is their ability to control the porous properties of the monoliths.

3.3.1. Acrylamide-based monoliths

Palm and Novotny substantially simplified the incorporation of highly hydrophobic ligands into acrylamide-based matrices [35]. Rather than forming a dispersion by sonication, mixtures of aqueous buffer and *N*-methylformamide were used to prepare homogeneous polymerization solutions containing acrylamide, methylene bisacrylamide, acrylic acid, and C_4, C_6, or C_{12} alkyl acrylate, with the overall concentration of the monomers in solution was kept constant at 5%. The monomer mixture consisted of 60% bisacrylamide and 10% acrylic acid, while the remaining 30% were acrylamide and different proportions of the hydrophobic monomer. The composition of the mixed solvent depended on the type of alkyl methacrylate used, and ranged from 50% *N*-methylformamide for butyl acrylate to 95% for dodecyl acrylate [36,37]. Columns with high efficiency required the presence of poly(ethylene glycol) (M_r = 10 000) dissolved in the polymerization mixture which is known to induce the lateral aggregation of acrylamide chains and to contribute to the formation of more porous structures [38]. Polymerization was again achieved using the peroxodisulfate–TEMED initiating system within the acryloylated capillaries, affording monoliths possessing the opaque appearance characteristic of macroporous polymers. However, no detailed characterization of the pore structures was performed. Once the polymerization was completed, the poly(ethylene glycol) and other low molecular weight compounds were washed out of the column using electroosmotic flow.

Column efficiencies calculated for phenylketones and carbohydrates used as model analytes with on-column detection were in a range of 300 000–400 000 and 190 000–230 000 plates/m, respectively. These monolithic columns easily tolerated rather high loading levels without a concomitant loss in efficiency, though excessive tailing of the peaks was observed under overload conditions.

A monolithic CEC column incorporating dodecyl acrylate was also successfully used for the isocratic separation of ionized di-, tri, penta-, and hexapeptides [35]. The elution pattern and the efficiency of the separation were found to strongly depend on both the percentage of acetonitrile and the pH of the mobile phase, suggesting that a gradient elution method would perhaps have been more appropriate.

Zhang and El Rassi demonstrated the dual role that may be played by the charged groups incorporated primarily to support EOF on the CEC separation of some neutral, moderately polar compounds [39]. Due to the polarity of the analytes, a mixed-mode retention mechanism based on both interaction with the hydrophobic dodecyl ligands and also hydrophilic interaction with the sulfonic acid groups was observed. These columns exhibited excellent performance with separation efficiencies of up to 418 000 plates/m achieved for some urea herbicides and carbamate insecticides.

3.3.2. Polystyrene-based monolithic capillary columns

Horváth first reported the preparation of polystyrene-based porous rigid monolithic capillary columns for CEC by polymerizing mixtures of chloromethylstyrene and divinylbenzene in the presence of various porogenic solvents such as methanol, ethanol, propanol, toluene, and formamide [40].The reactive chloromethyl moieties incorporated into

the monolith served as sites for the introduction of quaternary ammonium functionalities with the pores of the monolith filled with *N*,*N*-dimethyloctylamine, and after a suitable reaction period, the column was washed with methanol and equilibrated with the mobile phase. Unfortunately, only very limited information concerning the nature and extent of modification was presented.

These capillary columns possessing positively charged surface functionalities were then used for the reversed-phase separations of basic and acidic peptides, demonstrating the excellent separation of three angiotensins and insulin with column efficiencies as high as 200 000 plates/m using acetonitrile and phosphate buffer pH 3. Good separation of chemically similar tripeptides (Gly–Gly–Phe and Phe–Gly–Gly) was also observed in a buffer pH 7 using non-functionalized "plain" poly(styrene–*co*-divinylbenzene) monoliths devoid of ionizable functionalities. In this case, the driving force for movement of the analytes through the column is their electrophoretic migration, while separation results from their interactions with the stationary phase. However, the addition of acetonitrile to the mobile phase significantly decreases the mobility of these analytes, making this approach less attractive.

Horváth further reported the preparation of a porous polymer monolith for CEC of proteins and peptides by copolymerizing chloromethylstyrene and ethylene dimethacrylate in the presence of propanol and formamide [41]. The chloromethyl functionalities were subsequently modified with *N*,*N*-dimethylbutylamine to form a positively charged chromatographic surface with fixed butyl chains. In terms of separation performance such as selectivity and retention, this monolith was found to perform in a way similar to that of acrylate-based polymer monolith for the separation of basic proteins [42]. Performing the separations at elevated temperatures led to an almost two-fold increase in the speed of analysis, with a concomitant increase in the separation efficiency compared to separations performed at ambient temperature as illustrated in Fig. 3.

Xiong et al. also reported the preparation of monolithic CEC columns by polymerizing mixtures of styrene with divinylbenzene and methacrylic acid in presence of toluene as the porogenic solvent [43]. Using these monoliths, separations of phenols, chlorobenzenes, anilines, alkylbenzenes and some isomeric phenylenediamines could be achieved in less than 4.5 min.

Recently, Jin et al. expanded on this concept to demonstrate the excellent separations of a diverse series of neutral and ionic samples [44]. Mixtures of styrene, divinylbenzene and methacrylic acid were polymerized in the presence of toluene and isooctane as the porogenic solvents, varying the reaction time from 0.5 to 24 h at 70 °C. Despite not allowing complete polymerization to occur, decreasing the reaction time from 24 to 1 h still resulted in columns with both good efficiency and reproducibility. These columns were used for the separation of a wide range of analytes. For example, Fig. 4 demonstrates good separation of basic pharmaceuticals. Performing this separation at a high pH of 10.5 resulted in excellent, symmetric peaks due to ion-suppression.

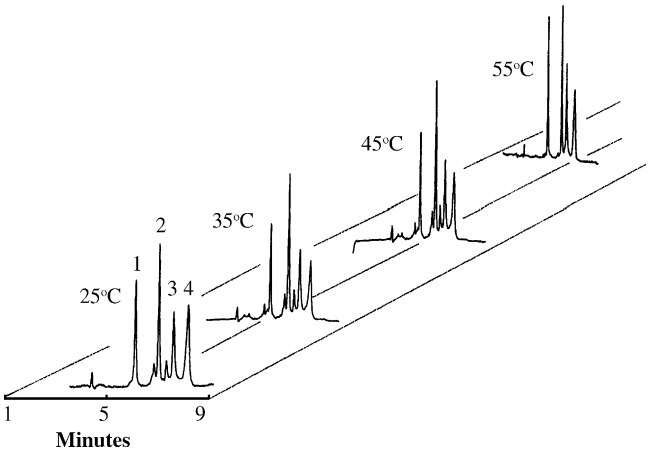

Fig. 3. Capillary electrochromatograms illustrating the effect of temperature on the separation of proteins (from [41] with permission). Column: 40 cm (30 cm effective length) × 75 mm i.d., porous styrene-based monolith with dimethylbutylammonium functionalities. Mobile phase: 30% acetonitrile in 70 mmol/L phosphate buffer, pH 2.5; reversed polarity (−30 kV), electrokinetic injection for 2 s, −2 kV. Peaks: (1) insulin; (2) α-lactalbumin; (3) myoglobin; (4) bovine serum albumin.

3.3.3. Methacrylate ester-based monolithic columns

In addition to acrylamide- and styrene-based monoliths already described, extensive work has been done regarding the materials development and optimization for monolithic CEC capillaries prepared from methacrylate ester monomers. Indeed, the number of publications describing the preparation, characterization and application of these monolithic materials in CEC has grown exponentially within the last 2–3 years.

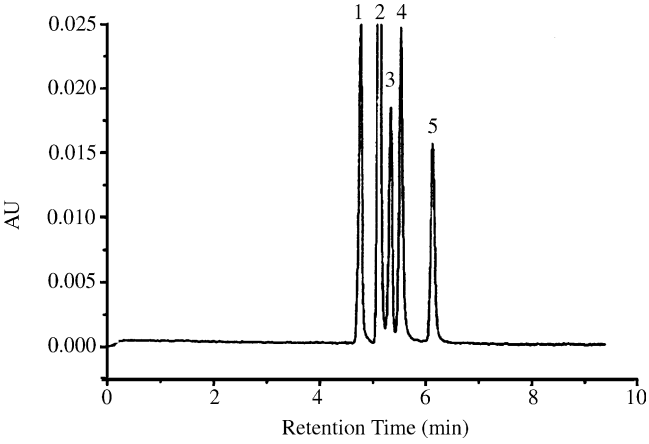

Fig. 4. Separation of basic pharmaceuticals on a monolithic column prepared from styrene, divinylbenzene and methacrylic acid (from [44] with permission). Experimental conditions: column, effective length 20.5 cm (total length 27 cm) × 100 μm i.d. × 375 μm o.d.; mobile phase, 10 mmol/L phosphate buffer containing 50% acetonitrile, pH 10.5; injection, 5 kV for 2 s; applied voltage 25 kV; detection wavelength, 214 nm. Peaks: (1) cinchonine; (2) caffeine; (3) barbital; (4) isoamyl barbital; (5) phenyl barbital.

These investigations have predominantly made use of the concepts developed from our original work with the molded rigid monolithic HPLC columns that we introduced in the early 1990s [3,45]. The experience acquired with these earlier materials proved helpful in investigating the inter-related effects of morphology and composition on the overall CEC process.

The preparation of these monolithic capillary columns is remarkably simple [46]. Either a bare or a surface treated capillary is filled with a homogeneous polymerization mixture, and radical polymerization is initiated only when desired using either a thermostated bath or UV irradiation [47,48] to afford a rigid monolithic porous polymer. Once polymerization is complete, unreacted components such as the porogenic solvents are removed from the monolith using a syringe pump or electroosmotic flow. This simple single step process has numerous advantages. For example, the fused silica tubing may often be used either directly as supplied without first performing any chemical modification of its internal surface. Most of the chemicals may be used as supplied, although careful purification contributes to better batch-to-batch reproducibility (vide infra). In addition, the final polymerization mixture contains free radical initiators such as benzoyl peroxide, azobisisobutyronitrile, or benzophenone ensuring its stability and easy handling or storage for several hours at room temperature or for days in the refrigerator without risking the onset of polymerization.

In optimizing the process, specific attention was paid to the design of the porogenic mixtures. Ideally, this system had to enable: (i) the preparation of a homogeneous, single phase polymerization mixture from the ionizable water soluble monomer that supports EOF, and the hydrophobic monomers that affect the separation without using additional compatibilizing agents, (ii) the uniform incorporation of monomers with widely differing polarities into a macroporous polymer monolith, (iii) the fine control of the porous properties of the resulting monolith over a broad range, and, finally (vi) the facile initial washing and equilibration of the capillary column resulting from its compatibility with the mobile phase used for electrochromatography. Our early study led to the development of a ternary porogenic system consisting of various proportions of water, 1-propanol, and 1,4-butanediol [46]. The monolithic capillary columns prepared using this porogen system and photochemical initiation possessed efficiencies of over 210 000 plates/m for the separation of a model mixture of aromatic compounds [47]. This approach is still the most popular employed and in recent years many other groups have also prepared methacrylate ester based monoliths based on our initial work [49–55].

The methacrylate-based polymers prepared in this way are stable even under extreme pH conditions such as pH 2 or 12 [7]. The sulfonic acid functionalities of the monolithic polymer remain dissociated over this entire pH range creating a flow velocity sufficient to achieve the separations in a short period of time. In contrast to the stationary phase, the analytes are uncharged, yielding symmetrical peaks. It should be noted that such extreme pH conditions are not tolerated by typical silica-based packings or monolithic materials.

4. Reproducibility and stability of monolithic columns

Data describing the reproducible preparation and operation of CEC columns are extremely important in terms of further stimulation of both the development and the acceptance of this technology. As this technology has matured, increasingly more groups have reported data on column-to-column, run-to-run, and day-to-day reproducibility of monolithic capillary columns [32,35,36,42,55–64]. For example, Novotny studied the reproducibility of migration times, for a series of isoflavones for acrylamide-based monoliths [36]. The average run-to-run relative standard deviations (R.S.D.s) for five analytes were 0.3%. Excellent day-to-day and column-to-column R.S.D. values were also obtained, ranging from 0.1 to 0.4% and 0.2 to 0.3%, respectively. Horváth monitored the conductivity of his modified monolithic polystyrene-based columns for over 3 months and observed no changes [40]. Similarly, the electroosmotic mobility measured over a number of days remained essentially constant [65]. A different cationic acrylate-based monolith was stable for over 300 consecutive injections of a mixture of proteins (<4% R.S.D.) [42,65]. Bandilla and Skinner compared the inter- and intra-capillary reproducibility of retention time and peak area for methacrylate ester-based capillary columns used for the separation of proteins [55]. Inter-capillary R.S.D. ranged from 5 to 12% and 7 to 16% R.S.D. for migration time and peak area respectively. The intra-capillary values were considerably lower, ranging from 1.4 to 1.7% and 7 to 10% R.S.D., respectively. Whilst these values were higher than is desirable, they could be improved by the use of thiourea as an internal standard. Additionally, the use of laboratory-built instrumentation, which was not thermostated, and differences in the amount of sample injected were both cited as probable causes for these high values.

Recently Holdšvendová et al. compared the properties of methacrylate monolithic capillary columns prepared using two different initiating systems [56]. Columns were prepared using ammonium peroxodisulfate in the presence of TEMED as the initiating system at ambient temperature or by using AIBN as the thermal initiator at 60 °C. These columns were all found to have remarkably similar properties despite the different initiation systems used. However the reproducibility (column-to-column) was better for those columns prepared by thermal initiation.

Tests of the reproducibility of retention times, retention factors, separation selectivities, and column efficiencies for our methacrylate monolithic capillary columns are summarized in Table 1. This table shows averaged data obtained for nine different analytes injected 14 times repeatedly every other day over a period of 6 days, as well as for seven

Table 1
Reproducibility of the electrochromatographic properties of methacrylate-based monolithic capillaries

Variable	R.S.D. (%)		
	Run-to-run ($n = 14$)	Day-to-day ($n = 3$)	Column-to-column ($n = 7$)
Retention time	0.18	1.19	3.50
Retention factor	0.21	0.30	1.43
Selectivity	0.05	0.10	0.11
Efficiency	1.50	4.30	7.80

Conditions: capillary columns, 30 cm active length × 100 mm i.d.; stationary phase poly(butyl methacrylate–*co*-ethylene dimethacrylate) with 0.3 wt.% 2-acrylamido-2-methyl-1-propanesulfonic acid; mobile phase, mixture of acetonitrile–5 mmol/L phosphate buffer pH 7 (80:20, v/v); UV detection at 215 nm; voltage 25 kV; pressure in vials 0.2 MPa; sample concentration 2 mg/mL of each compound; injection 5 kV for 3 s. Data shown are average R.S.D. values obtained for thiourea, benzyl alcohol, benzaldehyde, benzene, toluene, ethylbenzene, propylbenzene, butylbenzene, and amylbenzene (from [8] with permission).

different capillary columns prepared from the same polymerization mixture. As expected, both injection-to-injection and day-to-day reproducibility measured for the same column are very good. While the selectivity effectively did not change, slightly larger differences were observed for the column efficiencies [8,65].

5. Assessment of the porous structure

The ability of a liquid to flow through the network of channel-like pores that traverse the length of these monolithic materials is essential to all of their applications. In addition to providing permeability, the porous structure also accelerates mass transfer within the separation medium as a result of convection [66], since *all* of the mobile phase must flow *through* the pores [67]. The importance of this fact has meant a rapid increase in the number of studies directly assessing the effect of pore size of the monolithic CEC media [33,44,50,61,68–72]. However, these still represent a relatively small subset of the literature in this field. The absence of data for other monolithic systems is probably due both to the limited means available for the control of their porous structures during preparation as well as to difficulties in determining their actual pore structure in the swollen state. It should be emphasized that the standard methods typically used for the measurement of porous properties such as mercury intrusion porosimetry and nitrogen absorption/desorption are performed with materials in the dry state, while the columns actually operate in the presence of a solvent. As a result, data measured in the dry state may not accurately reflect the operational pore size of the capillaries during the actual chromatographic process.

Horváth used three methods to determine the porosity of monolithic capillary columns in the “solvated” state [40]. First, the elution time of a low molecular non-retained tracer in mHPLC was used to calculate the total porosity. The second method based on measurements of conductivity ratio enabled an estimation of the porosity. Monolithic and empty capillaries were filled with an electrolyte and their conductivities were measured. Although several equations relating conductivity ratio to total column porosity have been derived, Archie’s equation appeared to provide the best fit of the experimental data. The last method was gravimetric, using the weight difference between a dry and acetone filled monolithic column. Since none of these three methods affords information about pore size distribution, liquid extrusion porosimetry with hexadecane was also used to determine the integral pore volume distribution. However, since this technique requires samples larger than those available from a capillary column, measurements were performed using a monolith prepared via a larger scale bulk polymerization.

Similarly, we polymerized the same mixtures used for the preparation of capillary columns in glass vials and used the products for mercury intrusion porosimetry. Since we found that a strong correlation exists between the “dry” porous properties of the monoliths and their chromatographic performance, even “dry” values may be used to tailor column performance.

6. Effects of the properties on the separation

The ability to achieve precise and independent control over both the porous properties as well as the level of charged moieties of the rigid monolithic stationary phases opened new avenues for studies focusing on the effects of these properties on the chromatographic process. Both these variables are extremely important in controlling the flow velocity and efficiency of the monolithic capillary CEC columns. In addition to these materials properties, CEC separations are also affected by the conditions under which they are performed, including the applied voltage, and the pH as well as elution strength of the mobile phase.

6.1. Pore size and efficiency

The major advantage of CEC compared to classical HPLC is that much higher column efficiencies can be achieved using identical separation media. For columns packed with beads, the efficiency is generally particle size dependent, and increases as the size of the packing decreases [73]. Since the monolithic columns are molded rather than packed, issues

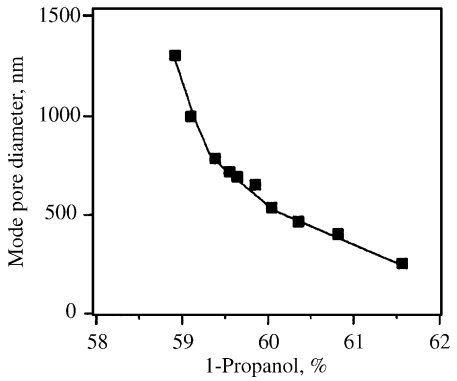

Fig. 5. Effect of the percentage of 1-propanol in the porogenic mixture on the porous properties of monolithic polymers (from [68] with permission). Reaction conditions: polymerization mixture—ethylene dimethacrylate 16.00%, butyl methacrylate 23.88%, 2-acrylamido-2-methyl-1-propanesulfonic acid 0.12%, ternary porogen solvent 60% (consisting of 10% water and 90% of mixtures of 1-propanol and 1,4-butanediol), azobisisobutyronitrile 1% (with respect to monomers), polymerization time 20 h at 60 °C.

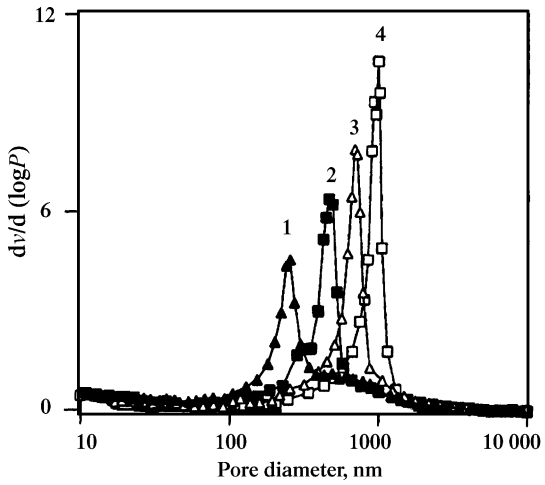

Fig. 6. Differential pore size distribution profiles of porous polymeric monolithic capillary columns with mode pore diameters of 255 nm (1), 465 nm (2), 690 nm (3), and 1000 nm (4) (from [46] with permission).

of particles size become irrelevant, and instead, the size of the pores within the monolithic material may be expected to affect the chromatographic efficiency. Indeed, initial studies have verified this strong effect [46,68].

The ternary porogenic system that we have developed enables the precise control of porous properties over a broad range [68]. For example, the percentage of 1-propanol in the porogenic solvent exerts an enormous effect on the pore diameter at the maximum of the distribution curve (mode pore diameter) as documented in Fig. 5 for a thermally initiated polymerization system. Based on these results, monoliths of any pore size within the broad range of 250–1300 nm can easily be produced by simply changing the ratio of propanol to butanediol in the porogenic mixture. It should be noted that the window of weight percentage of 1-propanol that brackets this wide range of pore sizes is sufficiently large to obtain polymers of virtually any mode pore diameter with an accuracy of ±25 nm with respect to the targeted value. Despite the fact that these monoliths are prepared from a polymerization mixture containing monomers of very different polarities, all of the mercury porosimetry profiles seen in Fig. 6 are similar to those found for monolithic polymers prepared in macroscopic formats [74].

An excellent study presented by Jiang et al. described the effect of varying the porous properties of methacrylate-based monolithic columns on the separation of different analytes [61]. These columns were characterized by mercury intrusion porosimetry, SEM, and nitrogen absorption/desorption. For small alkyl benzene analytes, the separation efficiency improves as the microglobule size, and consequently the size of the macropores, decreases. However, in the case of bulkier analytes such as multi-substituted benzenes, higher separation efficiencies were observed with monoliths possessing very large pore sizes in excess of 1200 nm. Furthermore, SEC experiments confirmed that the separation mechanism for these bulky analytes was based upon exclusion effects, rather than adsorption. This demonstrates the importance of tailoring the porous structure to best suit the specific type of analysis being undertaken.

Hoegger and Freitag also undertook a systematic study of the influence of percentage of crosslinking monomer, the porogen (lyotrophic salt), and the solvent type as well as the type and percentage of additional 'functional' monomers on the morphology and chromatographic properties of acrylamide-based hydrophilic monoliths [34]. Although no quantitative measurement of the porous properties were made, the polymers were subjected to a fairly rigorous qualitative analysis which involved description of the physical properties of the material as well as visual inspection of the morphology by SEM. This study enabled specification of several factors affecting the polymer morphology. For example, high initial monomer concentration was found to favor polymers with a rigid rather than gel-like structure. Furthermore, the presence of large amounts of crosslinker also resulted in a more granulous texture as illustrated in Fig. 7.

A unique approach was used by Chirica and Remcho [75] to produce a column with templated porosity. A capillary column was packed with ODS silica beads, then filled with a polymerization mixture consisting of a solution of divinylbenzene, styrene, ethylene dimethacrylate, and butyl methacrylate. Subsequent washing of the monolith with sodium hydroxide effected the removal of the silica beads. Using this process, it appeared that the surface chemistry of the silica beads used in the templating process controlled the surface chemistry of the final monolith by dictating the arrangement of the hydrophobic moieties of the monomers in the resulting polymer similar to the molecular imprinting (vide infra). More recently, they used a similar approach however this time polyamidoamine (PAMAM) dendrimers of the fourth and half generation were used as the templating

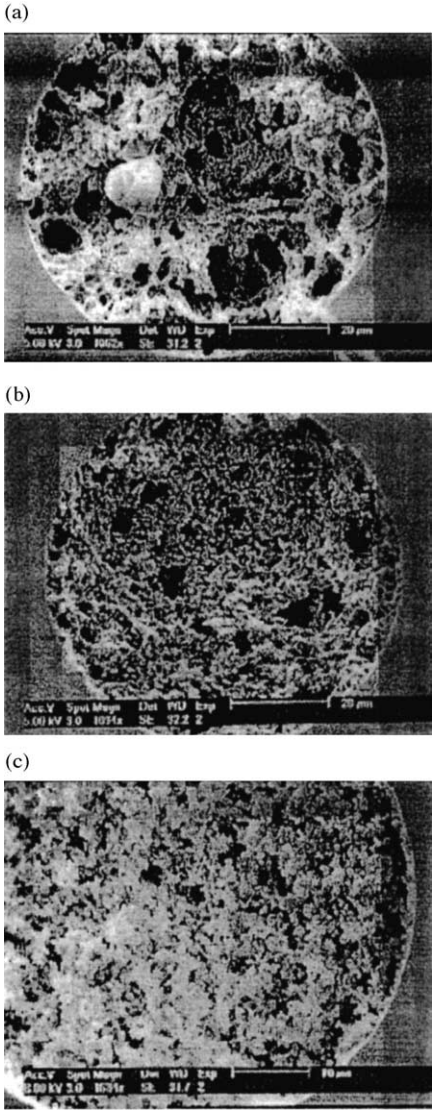

Fig. 7. Effect of crosslinking with on the structure of the poly(piperazine diacrylate-*co*-*N*,*N*-dimethylacrylamide) monoliths prepared in the presence of 50 mg/mL ammonium sulfate: (a) $T = 15\%$, $C = 30\%$; (b) $T = 15\%$, $C = 40\%$; (c) $T = 15\%$, $C = 52\%$ (from [34] with permission). T refers to the total monomer concentration in the polymerization mixture while C is the concentration of crosslinker related to the total monomers.

agent [70]. As the dendrimer concentration in the polymerization mixture was increased from 0 to 100 μmol/L, the mode pore diameter increased from 600 to 800 nm. The separation efficiency for toluene and the resolution of peaks for acetone and toluene also depended on the dendrimer concentration, thus indicating that inclusion of the dendrimer in the polymerization mixture did affect the porous structure.

Cameron et al. also described the preparation of monolithic stationary phases for CEC from the polymerization of bicontinuous microemulsions [72]. The porous structure was examined by both mercury intrusion porosimetry and SEM and this process was shown to produce monoliths with narrow pore size distribution. Despite this promising result, poor peak efficiencies of 84 000 plates/m for thiourea and only 6000 plates/m for dipropylphthalate were obtained, suggesting that these materials require further investigation before they may be considered suitable as CEC stationary phases.

6.2. Solvent flow through the monolithic capillary

Electroosmotic flow is generally reported to be independent of the size of the packing, and consequently the size of the interstitial voids between the particles, unless this size is too small and the electrical double layers overlap [76]. The ability to independently control both the pore size and the level of charged functionalities of the methacrylate ester monolithic capillaries has enabled the direct investigation of the net effect of transport channel size on flow velocity. As the pore size increased from 250 nm to 4 mm, a two-fold increase in flow velocity was observed for monolithic capillaries with the same level of charged moieties prepared using thermal initiation [68]. A similar increase in flow velocity was also observed for monoliths prepared by UV initiated polymerization [47]. This range of pore sizes significantly exceeds the thickness of a few nanometers at which the electrical double layers would overlap for a system utilizing a mobile phase containing 5 mmol/L buffer [76]. If it is assumed that the observed decrease in flow rate with decreasing pore size simply results from the increasing percentage of pores within which overlap of the electric double layers occurs, then the flow velocity should reach a maximum value for those monoliths having sufficiently large pores, and remain constant thereafter, since as the pore size increases, the number of pores within which overlap of the electric double layer can occur decreases rapidly. In practice however, this phenomenon is not observed. The fact that the overall flow velocity increases linearly over a broad range of pore sizes may support the contention that this increase in flow rate is macroscopically related to a decrease in the resistance to flow through the channels. Since this is not likely in EOF driven systems, additional effects such as microscopic variations in the strength of the electrical field in both the small and large pores, the effects of tortuosity, and conductance variations in the cross sectional area of the structure can be held responsible for these changes in chromatographic performance [77].

The dependence of the magnitude and direction of the apparent EOF on the mobile phase can be rather complex, as recently illustrated by Bedair and El Rassi [78]. For a monolith containing the ionizable monomer, [2-(acryloyloxy)ethyl]trimethylammonium methylsulfate, the direction and magnitude of the EOF depended largely on the nature of the electrolyte used in the mobile phase as shown in Fig. 8. This is most likely due to the adsorption of electrolyte ionic components onto the solid stationary phase, thus becoming the zeta potential determining ions. More recently, we have also observed similar effects in our own columns [79].

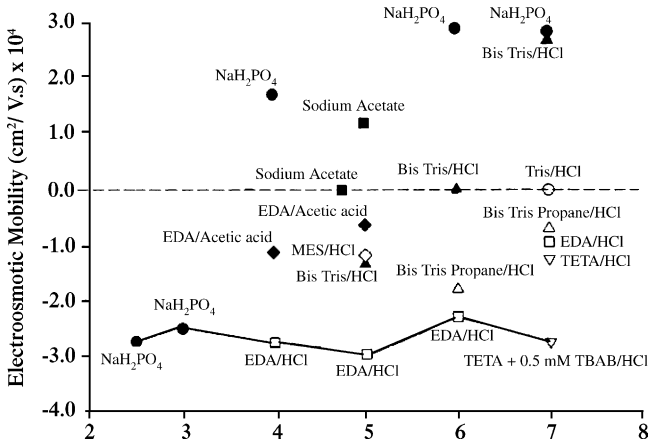

Fig. 8. Effect of the pH value of the mobile phase on the apparent EOF velocity for poly(pentaerythritol diacrylate monostearate-*co*-[2-(acryloyloxy)ethyl]trimethyl ammonium methylsulfate monolith (from [77] with permission). Capillary column: 33.5 cm (25 cm effective length) × 100 μm i.d., mobile phases, 1 mmol/L buffer at 80% (v/v) acetonitrile; running voltage ±25 kV.

6.3. Control of ionizable surface chemistry

Electroosmotic flow velocity is directly proportional to the zeta potential that, in turn, is directly related to the surface charge. In contrast to silica-based CEC media, the ability to easily control the level of the charged functionalities that support the electroosmotic flow is a major advantage of the polymeric monolithic capillaries. This variable can easily be adjusted by changing the percentage of ionizable monomer in the polymerization mixture. For example, Fujimoto observed that migration velocity increased linearly with AMPS content for both 6% crosslinked polyacrylamide gels [11] and 9.7% crosslinked *N*-isopropylacrylamide polymers [13]. Similarly, increasing the content of sulfonic acid groups within the methacrylate ester monoliths [46] significantly increased the flow velocity, thus reducing the overall analysis time. Desirable chromatographic performance was maintained in these high flow capillaries by making concomitant changes in the composition of the porogenic mixture in order to keep the pore sizes of the monoliths virtually constant.

In addition to the ability of ionizable surface functionalities to control the EOF, changes in surface chemistry can also be used to control both the nature and strength of the chromatographic interactions. Considering methacrylate-based monoliths as an example, simple changes in functionality of the monomers facilitate control of the relative hydrophobicity of the monolith. In the case of ionic analytes, ion-exchange or ion-exclusion effects are also significant, due to the dual function of ionizable groups introduced to control the EOF. Similarly, the use of reactive monomers offers other possibilities for controlling the exact nature of the surface chemistry [42].

However, as already noted, any change in the surface chemistry resulting from changes in the composition of the polymerization mixture requires a corresponding adjustment in the composition of the porogenic solvents in order to maintain optimal pore size. In order to avoid the time-consuming process of re-optimizing each novel separation medium, we developed an alternative approach for the preparation of porous polymer monoliths for CEC. In this approach generic, well-defined porous monoliths with precisely controlled pore size are first prepared using UV initiated polymerization of polymerization mixtures consisting of butyl methacrylate, ethylene dimethacrylate, 1-decanol and cyclohexanol or 1,4-butanediol. The desired surface chemistry is then introduced in the generic monoliths by the photoinitiated grafting of suitable polymer chains onto the surface of the pores [80,81]. The desired degree of incorporation of surface functional groups is obtained within a very short period of time. Using this approach, a wide variety of different surface chemistries is readily accessible without the need to re-optimize the composition of the polymerization mixture of the bulk monolith. For example a column grafted with poly(AMPS) could be used for the separation of four peptides in under 40 s [81]. We further extended this concept by using a series of photografting reactions to polymerize several layers on top of each other. The major advantage of this approach is the ability to generate polymer shells and thus shield functionalities in the lower layer from unwanted interactions with the analytes. For example, the sulfonic acid groups of AMPS are required to generate EOF, but they can also adsorb proteins and peptides via strong Coulombic interactions. Thus, covering the grafted AMPS layer with another layer of polymer with desirable properties can provide steric shielding. The proof of concept of this is demonstrated in Fig. 9 with alternating layers of AMPS (A) and butyl acrylate (B) photografted for 5 min on silicon wafer spin coated with cyclic olefin copolymer (COC) [80]. Since the thickness of the grafted polymer layer is less than the sampling depth of X-ray photoelectron spectroscopy (XPS), sulfur is detected in each layer, however its content is significantly higher when poly(AMPS) forms the top layer. Utilizing this approach capillary columns photographed with a layer of poly(AMPS) followed by poly(BuA) were prepared that for the first time

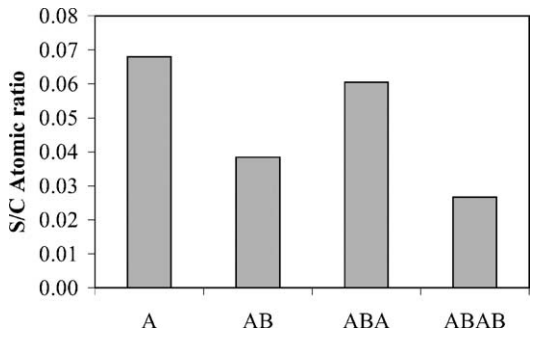

Fig. 9. Atomic ratio of sulfur to carbon (S/C) for subsequently grafted "block-like" layers using 2-acryalmido-2-methyl-1-propanesulfonic acid (A) and butyl acrylate (B) (from [79] with permission).

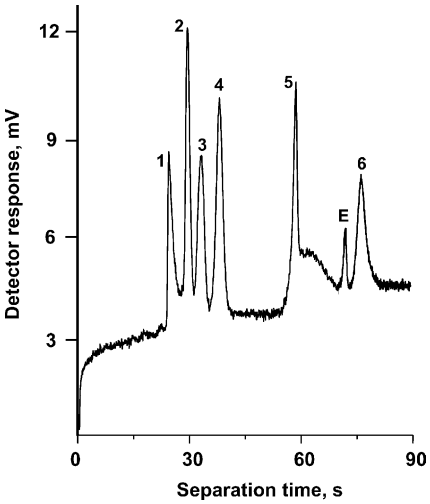

Fig. 10. Separation of a mixture of basic and acidic peptides using a monolithic capillary column with layered chemistries. Column: poly(butyl methacrylate–*co*-ethylene dimethacrylate) monolith, 8.5 cm × 50 μm i.d., pore size 1.6 μm; mobile phase: 20 mmol/L ammonium acetate in water–acetonitrile (1:1); 30 kV, 25 °C; injection 5 kV for 3 s. Peaks: substance P (1), [Arg^8]-vasopressin (2), bradykinin potentiator B (3), bradykinin fragment 1–5 (4), oxytocin (5), Gly–Tyr (6), EOF (E).

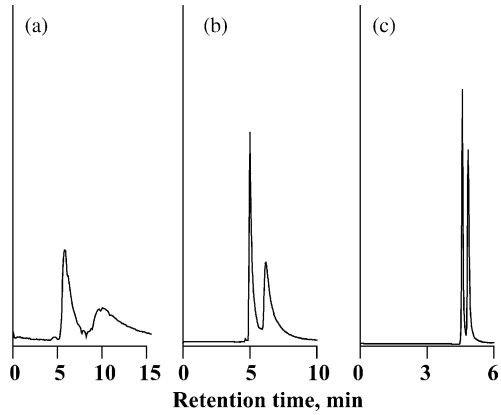

Fig. 11. Effect of the hydrophilicity of chiral monolithic columns on the electrochromatographic separation of *N*-(3,5-dinitrobenzoyl)leucine diallylamide enantiomers (from [8] with permission). Conditions: monolithic column, 100 mm i.d. × 30 cm active length; mobile phase, 80:20 mixture of acetonitrile and 5 mmol/L phosphate buffer pH 7; UV detection at 215 nm; voltage, 25 kV; pressure in vials, 0.2 MPa; injection, 5 kV for 3 s. Stationary phase containing butyl methacrylate (a), glycidyl methacrylate (b), and hydrolyzed glycidyl methacrylate (c) units.

enabled rapid and efficient CEC separation of a variety of highly basic peptides and proteins at neutral pH shown in Fig. 10.

6.4. Retention and selectivity

The majority of CEC separations reported to date have been performed in the reversed-phase mode. Under these conditions, the hydrophobicity of the stationary phase determines the selectivity of the separation, and retention can easily be controlled by adjusting either the composition of the mobile phase or the hydrophobicity of the surface, with the first option being easier to implement. However, in contrast to the rich variety of solvents available for use in HPLC, acetonitrile-based mobile phases are employed in most CEC applications due to their high dielectric constant and low viscosity [30,35,68,82].

The effect of surface polarity is enhanced in separations where two or more simultaneous interactions must occur in order to achieve the desired selectivity. This is particularly true in molecular recognition processes such as chiral separations. Since aqueous buffer systems are almost universally used as CEC mobile phases, enantioseparations are often attempted under reversed-phase conditions as opposed to the normal-phase mode typically used in chiral HPLC. Therefore, non-specific hydrophobic interactions would be highly detrimental to the discrimination process that involves subtle differences between the enantiomers.

The importance of tailoring surface chemistry was demonstrated using three different monolithic capillary columns that were prepared by direct incorporation of the chiral monomer—2-hydroxyethyl methacrylate (*N*-L-valine-3,5-dimethylanilide) carbamate [83]. These columns were tested for the enantioseparation of a model racemic compound, *N*-(dinitrobenzoyl)leucine diallylamide. Fig. 11 compares the chiral separations achieved using the various columns. Although the column containing butyl methacrylate as a hydrophobic comonomer did resolve the racemic analyte, albeit with very low plate counts, the peaks were very broad and tailed severely (Fig. 11a). Replacing the highly hydrophobic butyl methacrylate with the more hydrophilic glycidyl methacrylate resulted in a significantly improved chiral separation as non-specific interactions with the highly hydrophobic stationary phase were avoided (Fig. 11b). The peaks for the enantiomers were sharper, and column efficiencies calculated for this separation increased to 8100 and 1900 plates/m. Instead of defining and optimizing new conditions for the direct incorporation of an even more hydrophilic monomer into the monolith, the epoxide rings of the previous monolith were hydrolyzed using dilute aqueous sulfuric acid to afford very hydrophilic diol functionalities. This hydrolytic reaction was easily performed in situ within the pores of the monolithic capillary column. After this hydrolysis, the diol-functionalized hydrophilic capillary was unable to effect any separation of alkylbenzenes in the reversed-phase mode. However, this monolithic column afforded a significantly improved separation of the enantiomers (Fig. 11c). The peaks in this separation were narrow and well resolved ($R_s = 2.0$), column efficiencies were improved to 61 000 and 49 500 plates/m, and peak tailing was greatly reduced, suggesting that few undesirable interactions remained. Unfortunately, this substantial increase in column efficiency was accompanied by a decrease in selectivity.

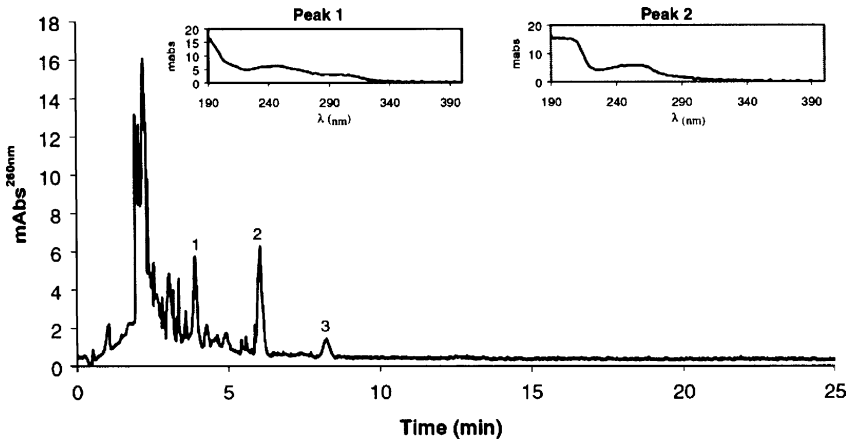

Fig. 12. Electrochromatogram of human blood serum after 4 days consumption of a soy-based product (from [36] with permission). Inset spectra correspond to the components diadzein and genistein. Conditions: voltage; 800 kV/cm; column, 33 cm column with 25 cm effective length; mobile phase, 30% acetonitrile prepared in 2.4 mmol/L ammonium formate (pH 2.7); injection, water plug injection for 30 s at 3 kV, followed by sample injection for 150 s at 20 kV. Peaks: (1) genistein; (2) daidzein; (3) apigenin.

7. Applications

7.1. Reversed-phase separations of small molecules

The separation of neutral, organic molecules under conditions of reversed-phase chromatography or normal-phase chromatography have received much attention as these are often used as test solutes to evaluate column performance. Other small neutral molecules such as polyaromatic hydrocarbons [84–86], steroids [13,59], hormones [44], bile acids [58], humic degradation compounds [87], pesticides and herbicides [39,88,89], various acids [50] and bases [44] isoflavone phytoestrogens [36] and various drugs [32,44,54,90] have also been separated by CEC using porous polymer monoliths. While many publications have reported the analysis of standard if often complex mixtures, the analysis of "real" samples has also been attempted [36,59,87,91]. For example, Novotny et al. recently described the excellent separation of isoflavone phytoestrogens in human blood serum following consumption of a soy-based product as shown in Fig. 12 [36]. Using the same monolithic column, on-line preconcentration could also be achieved. The results obtained using this rapid CEC method were in agreement with those obtained using GC/MS thus demonstrating the feasibility of this method for use in clinical studies.

7.2. Ion-exchange

Increasingly more papers have appeared describing ion exchange CEC separations using monolithic columns. As discussed earlier, the ionic nature of the stationary phase, required to support EOF can be readily used for separations in the ion exchange mode. Additionally, the separation of ionic analytes also offers the opportunity to obtain unique separation selectivity, based on the competing electromigration and chromatographic separation processes that prevail. Efficient separations of acidic analytes such as non-steroidal anti-inflammatory drugs (profens)[92] and aromatic acids [92,93] as well as basic analytes such as tricyclic antidepressants [94] and aromatic bases [93] have been reported.

7.3. Size-exclusion

The precise control of porous properties for monolithic CEC columns is very useful in the design of specialized CEC columns for separations in the size-exclusion chromatography (SEC) mode. SEC is an isocratic separation method that relies on differences in the hydrodynamic volumes of the analytes. Because all solute-stationary phase interactions must be avoided in SEC, solvents such as pure THF are often used as the mobile phase for the analysis of synthetic polymers, since they dissolve a wide range of polymers and minimize interactions with the chromatographic medium. Despite the reported use of entirely non-aqueous eluents in both electrophoresis and CEC, we observed no appreciable flow through the methacrylate-based monoliths using pure THF as the mobile phase. However, a mixture of THF with 2% water was still capable of dissolving polystyrene standards with molecular weights as high as 980 000 and substantially accelerated the flow velocity. Fig. 13 shows the first SEC separation of polystyrenes in the CEC mode using a methacrylate-based monolithic capillary column. The molecular weights of the individual peaks were assigned by injections of the individual standards. The elution order of the polystyrene standards and toluene confirms that size exclusion is the prevailing separation mechanism. Although the porous properties of the monolithic column used for this experiment were not optimized for SEC separations, these results demonstrate that CEC is not limited to the reversed-phase mode of chromatography. Several groups are also involved in studying the potential of size-exclusion CEC for the determination of molecular weight distribution

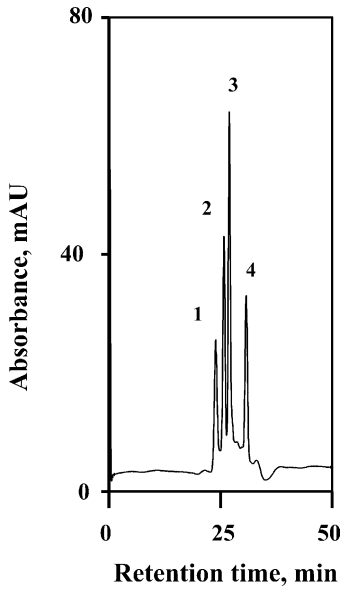

Fig. 13. Electrochromatographic size-exclusion chromatography of polystyrene standards (from [95] with permission). Conditions: monolithic capillary column, 30 cm active length × 100 mm i.d.; stationary phase, monomer mixture 59.7% butyl methacrylate and 40% ethylene dimethacrylate, 0.3 wt.% 2-acrylamido-2-methyl-1-propanesulfonic acid; pore size 750 nm; mobile phase, tetrahydrofuran containing 2% (v/v) of water; UV detection at 215 nm; voltage, 25 kV; pressure in vials, 0.2 MPa; sample concentration, 2 mg/mL of each compound; injection, 5 kV for 3 s; peaks, polystyrene standards, molecular mass 980 000 (1), 34 500 (2), 7000 (3), and toluene (4).

of synthetic polymers [96–99]. An extensive review of these studies is published by Kok elsewhere in this issue.

7.4. Normal phase

Although reversed-phase CEC is technique of choice for separation of a wide range of hydrophobic to slightly polar compounds, the separation of very polar compounds is problematic. Normal phase liquid chromatography, using polar stationary phases and less polar mobile phases has proven to be extremely efficient for the separation of polar species by HPLC and as a result there are now several examples of normal phase stationary phases that have been developed for CEC [58,62,92].

Novotny et al. prepared an amino phase acrylamide based monolithic column by copolymerisation of [2-(acryloyloxy)-ethyl]trimethylammonium methyl sulfate, 3-amino-1-propanol vinyl ether, acrylamide and *N*,*N*′-methylene-bis-acrylamide [58]. Using this column in conjunction with a hydrophobic CEC column allowed more complete detection of both the hydrophobic and hydrophilic bile acids in biologically important mixtures.

Glycosylation is the major post-translational modification of proteins, thus the details of the oligosaccharide structures attached to the protein backbone are essential to understanding the function of glycoproteins. Indeed, these oligosaccharides are often the selectivity determinants through various sugar–sugar and sugar–protein interactions. For this reason, Novotny has invested significant efforts into the development of novel acrylamide based, hydrophilic monolithic stationary phases tailored for the efficient analysis of different carbohydrates [35,37,62]. The use of a tailored polymeric monolithic column facilitated coupling to mass spectrometric (MS) detection, which is difficult using alternative approaches. For example, most silica-based hydrophilic interaction columns suffer from a lack of reproducibly, while HPLC methods require strong buffers and alkaline conditions that are not easily adjustable to MS operation.

These authors also demonstrated the application of this and a similar monolith containing cyano functional groups for the separation of saccharide mixtures [62]. In order to couple the CEC separation directly to a mass spectrometer, they chose a polar mobile phase together with an acetonitrile–aqueous volatile buffer. Both the cyano and amine columns separated a range of carbohydrates according to a normal phase mechanism, with the cyano column exhibiting far greater stability through the course of the study. Using his new approach, Novotny separated several saccharides derived from glycoproteins using MS detection. The separation of a complex fraction of the O-linked glycans chemically released from bile-salt-stimulated-lipase, a relatively large glycoprotein, consisting of 722 amino acid residues is shown in Fig. 14 [37]. Although the analysis of this fraction was previously achieved using matrix-assisted laser desorption ionization (MALDI) MS, the CEC separation also permits some isomeric separation, which would not otherwise be observed.

We have also prepared hydrophilic macroporous weak and strong anion-exchange monoliths and demonstrated their use in both anion-exchange and normal phase mode [92]. Hydrophilic, strong anion-exchange monoliths were prepared by copolymerisation of 2-(*N*,*N*-dimethylamino)ethyl methacrylate, 2-hydroxyethyl methacrylate, and ethylene dimethacrylate. Their subsequent alkylation using dimethyl sulfate led to strong anion-exchanger. These monoliths

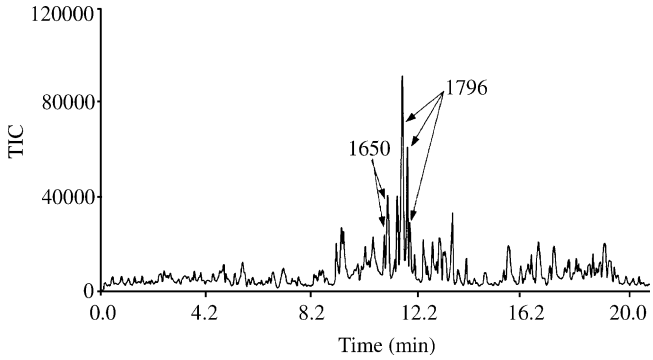

Fig. 14. Mass electrochromatogram of a complex fraction of the *O*-linked glycans chemically released from bile-salt-stimulated-lipase (from [37] with permission). Experimental conditions: amino column 28 cm, field strength 500 V cm^{-1}, mobile phase acetonitrile–water–ammonium formate buffer (240 mmol/L, pH 3.0, 55:44:1, v/v/v), injection 1 kV, 10 s.

separated neutral and even basic compounds such as substituted phenols and xanthines in the normal phase mode.

7.5. Enantioselective separations

Several approaches to enantioselective CEC monoliths have already been described. For example, Koide and Ueno prepared a monolithic gel for chiral resolution by polymerizing acrylamide, bisacrylamide, and AMPS in the presence of polymeric carboxymethyl-β-cyclodextrin [100,101]. Baseline separation of terbutaline enantiomers was possible even with a separation factor α of only 1.03. The same group also separated dansyl derivatives of amino acids and some other acidic compounds using positively charged polyacrylamide gels with covalently attached allylcarbamoyl-b-cyclodextrin. The column efficiencies claimed in this work reached 150 000 plates/m with selectivity factors of 1.2 and resolutions of up to 7 [57]. Separations of different chiral cationic, neutral, and primary amino compounds were achieved using these columns [60]. For example, the resolution was sufficient to determine the optical purity of L-alanine-2-naphthylamide containing only 0.1% of the minor enantiomer.

Hjertén used a similar approach involving the copolymerization of methacrylamide, piperazine diacrylamide, and vinylsulfonic acid in the presence of a L-4-hydroxyproline derivative to afford monolithic CEC columns that could be used for ligand-exchange CEC of underivatized amino acids [102] or hydroxy acids [103]. Similar polymerizations in the presence of 2-hydroxy-3-allyloxy-propyl-β-cyclodextrin led to monoliths that enabled enantiomeric resolution of acidic, neutral and basic drugs [104]. Interestingly, the resolution was found to be independent of the EOF and fast separations could be achieved without loss of resolution. Positively charged monoliths affording reversed EOF exhibited similar or better selectivity than negatively charged gels.

A polymer-based monolithic chiral stationary phase involving a macrocyclic antibiotic as chiral selector was reported by Maruška et al. [105]. The columns were prepared by copolymerization of *N*-(hydroxymethyl)acrylamide, allyl glycidyl ether, and piperazine diacrylamide with vinyl sulfonic acid used to introduce ionic functionality. Following polymerization, the epoxy groups of allyl glycidyl ether units were converted to aldehyde groups and the chiral selector, vancomycin was immobilized via reductive amination. The enantiomers of thalidomide, warfarin, coumachlor and felodipine could be separated with good both resolution ($R_s = 2.50$) and efficiency (120 000 plates/m). The same group also demonstrated another approach to the preparation of a stationary phase for CEC by polymerization of methacrylamide, *N*-isopropylacrylamide and piperazine diacrylamide in the presence of functionalized cyclodextrins [106]. Although the claimed formation of polyrotaxane structures is not unambiguously confirmed and remain highly speculative, these columns were found to be useful for the separation of several enantiomers, as well as various alkyl benzoates.

More recently, Maruška reported the synthesis of monolithic chiral stationary phases with immobilized human serum albumin (HSA). In order to protect the specific binding sites of HSA during the required allyl-activation step, additives known to interact strongly with HSA were used. Of these, L-tryptophan was found to provide the most efficient improvements in the enantioseparations. Baseline separations of D,L-kynurenine were achieved in about 5 min with increased separation resolution and efficiency compared to the separations in the typical liquid chromatography mode using the same stationary phase.

We demonstrated the preparation of acrylate-based monolithic CEC columns containing an ionizable chiral monomer, *O*-[2-(methacroyloxyethyl)carbamoyl]-10,11-dihydroquinidine [69,92,107]. Separations using this stationary phase are controlled by an anion-exchange mechanism. Additionally the use of an ionizable chiral monomer eliminated the need for the addition of a charged comonomer that was required in the previous studies [83]. Monoliths with highly hydrophilic surfaces were prepared directly from polymerization mixtures involving hydrophilic 2-hydroxyethyl methacrylate comonomer. Surprisingly, monoliths comprising 10 and 20 wt.% of ethylene dimethacrylate (crosslinker) exhibited better mass transfer characteristics and thus higher column efficiencies than those with a 40% (w/w) crosslinking despite the equal ‘dry state’ pore size adjusted to 1000 nm. This was attributed to swelling and to better homogeneity of the solid–liquid interface. Since the less crosslinked materials swell while the overall space available within the column is fixed, the pores are partly filled with the swollen polymer chains. This process is thought to improve the chromatographic properties of the monolith. Since this decrease in pore size also leads to a concomitant decrease in flow velocity, this finding is consistent with earlier results [95].

Very high column efficiencies of 242 000 and 194 000 plates/m were obtained for the separation of *N*-2,4-dinitrophenyl valine enantiomers using a 25 cm long capillary column [107]. Similarly high efficiencies were also obtained for other chiral acids such as *N*-benzoylleucine and α-aryloxycarboxylic acid herbicide Fenoprop. Moreover, due to the high enantioselectivity and resolution, the column length could be reduced to only 8.5 cm while still enabling baseline separation of a wide variety of chiral acids [108].

Recently, a new chiral monomer derived from cinchona alkaloid, *O*-9-(*tert*-butylcarbamoyl)-11-[2-(methacryloyloxy) ethylthio]-10,11-dihydroquinine was prepared [71]. The previous studies with the similar chiral monomer described in the preceding paragraphs helped to significantly reduce the number of experiments required to arrive at optimum polymerization mixture. Direct comparison of analogous monoliths confirmed the superiority of the new monomer, due to its more bulky carbamate residue. In particular,

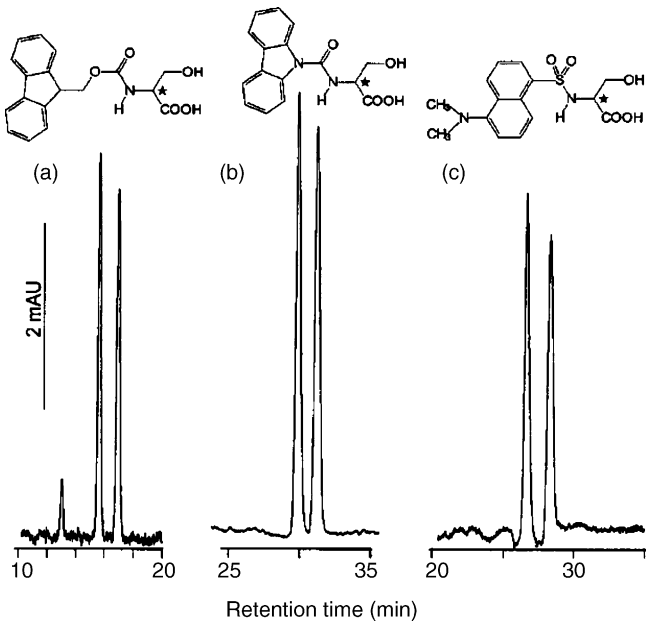

Fig. 15. CEC separation of serine enantiomers labeled with different fluorogenic reagents: (a) FMOC-Ser, (b) CC-Ser and (c) DNS-Ser (from [71] with permission). Monolithic column: polymerization mixture, 8% chiral monomer, 28% 2-hydroxyethyl methacrylate, 4% ethylene dimethacrylate, 15% cyclohexanol, and 45% 1-dodecanol. UV-initiated polymerization 16 h at room temperature. Column dimensions: total length 45 cm, effective length 36.5 cm, length of the monolith 20 cm, i.d. 0.1 mm; mobile phase: 0.4 mol/L acetic acid and 4 mmol/L triethylamine in acetonitrile–methanol (80:20); capillary temperature, 50 °C; voltage, −25 kV; injection, −15 kV for 5 s.

higher selectivity for a variety of acidic chiral analytes demonstrated in Fig. 15 broadened the application range.

Using methacrylate ester based monolithic columns, Pumera et al. prepared chiral stationary phases for CEC by physical or chemical bonding of β-cyclodextrins [109]. Chemical incorporation of the β-cyclodextrin into the polymer matrix was found to be far more effective than physical adsorption for the separation of enantiomers of ibuprofen as well as (−)-ephedrine and (+)-pseudoephedrine.

7.6. Proteins and peptides

The quest for easily prepared capillary columns that enable the CEC separation of proteins and other biomolecules is the current “holy grail” of electrochromatography, drawing the interest of several research groups. Monolithic materials appear well suited to meet this goal due to the excellent control that can be exerted over both their chemical and physical properties. In contrast to the CEC separation of small model neutral molecules such as alkylbenzenes, aromatic ketones, and PAHs, the separation of ionized macromolecular analytes such as peptides, proteins, and nucleic acids is difficult as they exhibit electrophoretic migrations in the electric field. For example, proteins can migrate in either direction in the capillary columns. Depending on their net charge at the pH of the mobile phase used for the separation, they may move towards the cathode or the anode. However, the possible electrostatic interactions of the protein molecules with the charged functionalities of the packing are an even more important issue in the design of columns for CEC of proteins. This situation is quite similar to the initial stage of ion-exchange chromatography [110]. Since the charged functionalities of the packing are needed to generate EOF, they cannot be entirely excluded from the system. Therefore, either steric shielding or the use of a mobile phase with a pH value that can suppress the formation of unwanted charges on the protein is required to decrease or completely eliminate these undesired interactions.

One can speculate on the viability of the alternative approach utilizing stationary phases having acid groups at their surface and buffers with high pH values. Such systems should also diminish the undesired electrostatic interactions, allowing the separation to be performed as required. Although this approach appears straightforward, no attempts to separate peptides or proteins in this manner have been documented. The problem lies in the basicity of certain amino acid residues such as tyrosine, lysine, and in particular arginine, with respective pK_a values of 10.07, 10.53, and 12.48, that contribute to their high positive net charge. Therefore, these functionalities cannot be deprotonated completely under the conditions available for CEC separations, preventing unwanted Coulombic interactions from being fully suppressed.

Taking these considerations into account, it is not surprising that the separation of proteins by CEC has been performed almost exclusively using monolithic columns containing positive functional groups for the generation of EOF, thus eliminating the possibility of strong

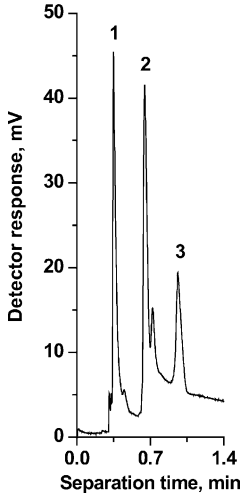

Fig. 16. Separation of a mixture of basic proteins and peptides using a monolithic capillary column with layered chemistries. Generic column: poly(butyl methacrylate–*co*-ethylene dimethacrylate), 8.5 cm × 50 μm i.d., pore size 1.6 μm; mobile phase: 20 mmol/L ammonium acetate in water–acetonitrile (1:1); 30 kV, 25 °C; injection 5 kV for 3 s. Peaks: melittin (1), cytochrome *c* (2), bradykinin fragments 1–5 (3), EOF (E).

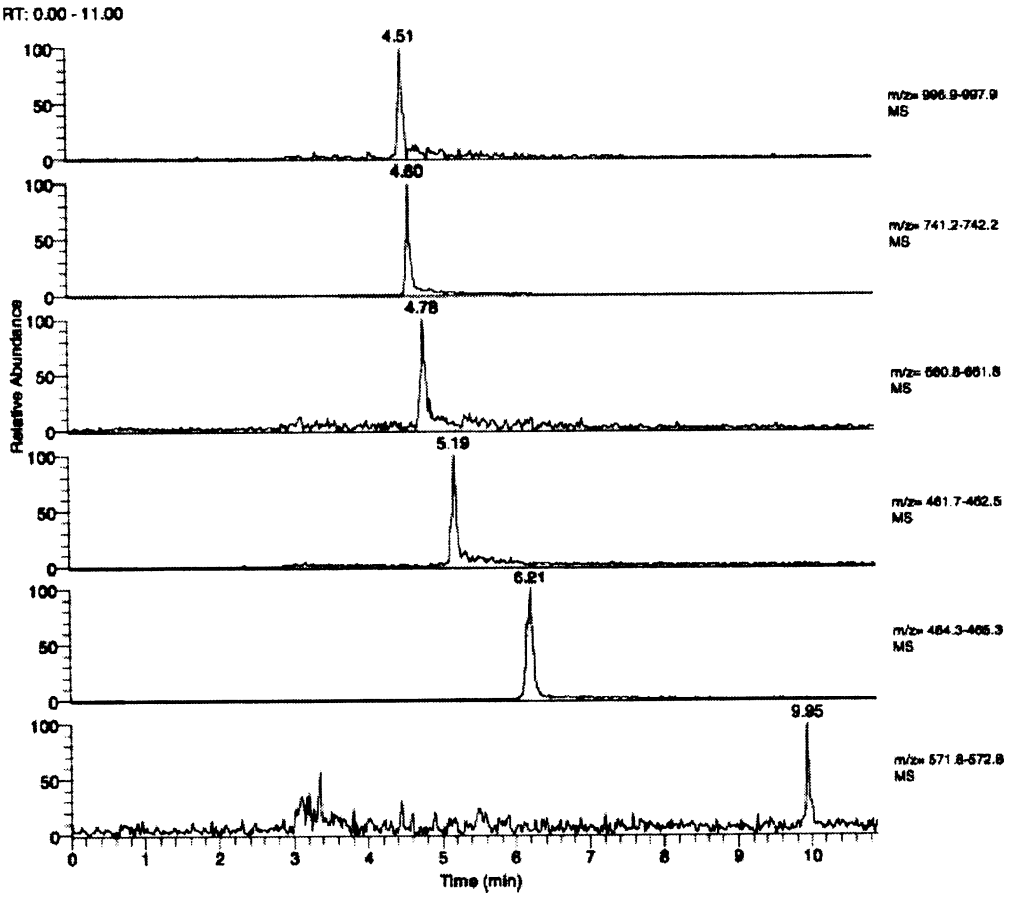

Fig. 17. Extracted ion electrochromatograms for selected peptide ions of bovine serum albumin digest (50 fmol; injection volume, 10 nL) separated under isocratic elution conditions (from [116] with permission). Column, 19 cm × 100 μm i.d. × 365 μm o.d.; flow rate, 120–130 nL/min; eluent, 25% (v/v) B. Solvents: (A) 0.1% (v/v) acetic acid in water, (B) 0.1% (v/v) acetic acid in acetonitrile; applied voltage, −10 kV on the inlet, +2.3 kV on the outlet; electric field strength, 647 V/cm. Efficiencies for peaks from top to bottom: 237 000, 185 000, 218 000, 176 000 and 802 000 plates/m. Average efficiency: 334 000 plates/m.

cation-exchange interactions with the stationary phase [31,41,42,111]. Despite these difficulties, excellent separations of various basic proteins with high separation efficiency have been achieved similar to those shown previously in Fig. 3.

To avoid the undesired interactions, Horváth et al. recently developed a new kind of monolithic capillary column for CEC with a positively charged polymer layer on the inner wall of a fused silica capillary and a neutral monolithic packing as the bulk stationary phase [112]. The fused-silica capillary was first silanized with 3-glycidoxypropyltrimethoxysilane to which polyethyleneimine was then covalently attached forming an annular positively charged polymer layer for the generation of electroosmotic flow. A neutral bulk monolithic stationary phase was then prepared by copolymerization of vinylbenzyl chloride and ethylene dimethacrylate in the presence of 1-propanol and formamide as porogens. Benzyl chloride functionalities of the monolith were subsequently hydrolyzed to benzyl alcohol groups. Mixtures of peptides were successfully separated in counter directional mode via a dual mechanism that involves a complex interplay between selective chromatographic retention and differential electrophoretic migration.

The majority of protein separations by CEC using monolithic capillary columns required the use of an acidic buffer in order to ensure that all the proteins carried sufficient positive charge. Recently however, Bandilla and Skinner reported the separation of several proteins at pH 10.0 [55].

During the last few years, we have also developed novel monolithic stationary phases that allowed the extremely rapid separation of a variety of proteins under mild conditions of low ionic strength at neutral pH. A monolithic column with zwitterionic functionality was prepared by photoinitiated grafting of poly(*N*,*N*-dimethyl-*N*-(2-methacryloyloxyethyl)-*N*-(3-sulfopropyl) ammonium betaine. Using this column the separation of four proteins in less than 1.3 min at neutral pH with column efficiencies of up to 4 million plates/m was achieved [5]. In contrast to many CEC separations exhibiting extremely high efficiencies, these separations were highly reproducible. The extremely high efficiencies suggest that a narrow migration

zone is maintained, which is likely due to a focusing mechanism. Such rapid, efficient, and reproducible separations of proteins may have important implications for the rapidly growing field of proteomics. For a detailed account on peak compression effects in CEC, see a review article by Enlund et al. published in this issue.

The second approach we used, as already described in some detail in section 6.3., was the grafting of consecutive layers of AMPS and butyl acrylate to shield the AMPS functionalities required to generate EOF from Coulombic interaction with basic proteins and peptides [79]. Using this approach allowed the separation of a series of highly basic peptides and proteins varying widely in size shown in Figs. 10 and 16.

In contrast, the separation of small to medium-sized peptides is not nearly so difficult, due to their less complex retention behavior. These separations have been demonstrated under both acidic [33,41,42,49,70,79,81,113,114] and basic [115,116] separation conditions using both positively and negatively charged monolithic columns. Most separations reported have focused on the separation of simple mixtures of only a few peptides, with other demonstrating the application to a protein digest [41,70,111]. Most recently, Karger described the application of monolithic columns polystyrene-based for high efficiency peptide analysis in CEC mode [116]. The fast and efficient separation of a digest of bovine serum albumin coupled to peptide mass fingerprint analysis by electrospray ionization (ESI) MS shown in Fig. 17 illustrates the excellent potential of such columns as efficient separation tools for proteomic applications.

8. Conclusions

In recent years, monolithic stationary phases have emerged as attractive and increasingly more popular alternatives to packed CEC columns due to the simplicity of their preparation as well as the virtually unlimited choice of chemistries they offer and the elimination of need for retaining frits and the associated problems they create. Thus, the use of monolithic columns in CEC decreases the technical barriers to the general acceptance of the CEC technique, which may have otherwise soon disappeared as a viable separation technique. In addition to the monolithic stationary phases for CEC prepared from synthetic polymers shown in this review, a wide variety of monolithic stationary phases based on both monolithic silica and “monolithized” beds formed from packed silica beads have also been developed. A detailed description of all these alternative approaches is well beyond the scope of this review and information concerning these stationary phases can be obtained from other sources [117–129].

A few examples of polymeric monoliths shown above also demonstrate the benefits of the facile tuning of porosity and surface chemistry using the direct polymerization procedure that is afforded by some of the monolithic media. The wealth of commercially available monomers possessing a variety of functionalities is further magnified by the possibility to functionalize the surface of the pores using grafting, and/or post-modification. This, together with the extreme simplicity of the preparation of the monolithic columns, makes monoliths an appealing option for the design of CEC capillary columns with high selectivities. Since monolithic materials can easily be prepared even within channels of very narrow dimensions by a single step in situ polymerization and their formation can be restricted to a specific area using photochemically initiated polymerization through a suitable mask, this technology is also uniquely suited for the further development of miniaturized analytical systems on-chip. These applications are dealt with by Stachowiak et al. elsewhere in this issue. In contrast to packed beds, monolithic structures exhibit excellent dimensional stability as a result of their rigidity and/or chemical attachment to the inner wall of the capillary.

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