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Review

Design of the monolithic polymers used in capillary electrochromatography columns

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Abstract

Monolithic columns for capillary electrochromatography (CEC) are receiving quite remarkable attention. 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. This review summarizes the current state-of-the-art in this rapidly growing area of CEC with a focus on monolithic capillary columns prepared from synthetic polymers. Recent achievements in column technologies for both high-performance liquid chromatography and capillary electrophoresis are used as the starting point to highlight the influence of these well established analytical methods on the development of monolithic capillary columns for CEC. The effects of individual variables on the separation properties of monolithic capillaries are discussed in detail. The analytical potential of these columns is demonstrated with separations involving various families of compounds in different chromatographic modes. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Reviews; Monolithic columns; Electrochromatography; Polymers, monolithic

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1. Introduction

After more than 30 years of study, high-performance liquid chromatography (HPLC) column technology has been optimized to a very high degree. Although new columns possessing specific selectivities, drastically reduced non-specific interaction, and improved longevity continue to be developed, increases in the plate counts per column – the measure of column efficiency – have resulted almost exclusively from the single strategy of decreasing the particle size of the stationary phase. These improvements were made possible by the rapid development of technologies that produced well-defined beads with an ever smaller size. For example, while 150–300 mm long columns packed with 10 μm particles were the standard just a few years ago, shorter column packed with 5 or even 3 μm diameter beads have become very popular [1]. Although further decreases in bead size are technically possible, the lowered permeability of columns packed with these smaller particles leads to a rapid increase in flow resistance and a larger pressure drop across the column. Accordingly, only very short columns may be used with current instrumentation and the overall improvement, as measured by the efficiency per column, is not very large. In addition, the effective packing of such small beads is also a serious technical problem. Therefore, the use of sub-μm-sized packings in ''classical'' HPLC columns is not practical today and new strategies for increasing column efficiency must be developed.

Another current trend in HPLC development is the use of mini- and microbore columns with small diameters, as well as packed capillaries that require much smaller volumes of both stationary and mobile phase. This miniaturization has been driven by environmental concerns, the steadily increasing costs of solvent disposal, and, perhaps most importantly, by the often limited amounts of samples originating from studies in such areas as the life sciences. The trade-off between particle size and back pressure is even more pronounced in these miniaturized columns. For example, MacNair et al. had to use specifically designed hardware that enabled operating pressures as high as 500 MPa in order to achieve the HPLC separation of a tryptic digest in a 25 cm long capillary column packed with 1 μm silica beads [2]. This pressure is one-order of magnitude higher than the typical 40 MPa limit of current HPLC instrumentation.

In contrast to mechanical pumping, electroendoosmotic flow (EOF) is generated by applying an electrostatic potential across the entire length of a device, such as a capillary or a flat profile cell. While Strain [3] was the first to report the use of an electric field, in the separation of dyes on a column packed with alumina, the first well documented example of the use of EOF in separation was the ''electrokinetic filtration'' of polysaccharides published in 1952 [4].

In 1974, Pretorius et al. realized the advantage of the flat flow profile generated by EOF in both thin-layer and column chromatography [5]. Although their report did not demonstrate an actual column separation, it is frequently cited as being the foundation of real electrochromatography. It should be noted however that the term electrochromatography itself had already been coined by Berraz in 1943 in a rather obscure Argentine journal [6].

The real potential of electrochromatography in packed capillary columns (CEC) was eventually demonstrated in the 1980s [7–9]. However, serious technical difficulties have slowed the further development of this promising separation method [10,11]. Since the revival of interest in CEC in the mid 1990s resulting from a search for new separation methods with vastly enhanced efficiencies, peak capacities and selectivities, research activity in this field has expanded rapidly and the number of published papers has grown exponentially.

2. Flow in CEC capillary columns

The electroosmotically driven flow of an aqueous electrolyte in an open fused-silica capillary is generated at the interface between the inner capillary wall and the liquid. The silica surface carries a number of silanol functionalities that deprotonate under conditions typical of CEC to form an immobilized layer of negatively charged species. To retain the electroneutrality of the system, these charges are counterbalanced by a ''cloud'' of cations in the solution residing in the nearby Stern layer, forming an electrical double-layer structure. Upon application of an electrical field across the capillary, the cations are driven towards the cathode, sweeping along molecules of the bulk solvent.

The thickness of the double layer depends on the zeta potential (ζ), that itself is a function of the surface charge density and ionic strength of the electrolyte. These two variables may be changed in order to effect the flow. In capillary columns packed with silica beads, the silanol groups present on the external surface of the stationary phases also deprotonate, providing the primary contribution to the overall EOF. Recent results demonstrate that even silanol moieties located within the pores of silica-based beads may also support flow, but only if the pore size is large enough to avoid overlap of the double layers [12–14].

Unlike the parabolic flow profile obtained for pressure driven flow through a column, the flow profile across a column that makes use of electroosmotic force is expected to be almost flat, since the dragging effect of friction along the wall is minimized. There is no general consent that this prediction is completely correct for the flow through filled columns. However, the recent direct visualization of flow profiles through packed capillaries driven both by pressure and by EOF indicated that the EOF driven flow profile is much flatter than that observed for the pressure driven flow [15–17]. The improved flow profile leads to a substantial reduction in the axial dispersion of separated compounds, enabling much higher column efficiencies to be achieved in CEC when compared to traditional HPLC [12,18–23].

3. Specifics of CEC

CEC is often presented as a hybrid method that combines the capillary format and electrostatic potential typical of high-performance capillary electrophoresis (HPCE) with the interactions of solutes and solid stationary phase characteristic of the HPLC separation mechanism. As such, CEC can simply be thought of as HPLC in packed microbore columns performed using capillary electrophoresis equipment. This definition not only reflects the current state-of-the-art, but also reveals the most common implementation of the method. Both capillary columns and chromatographic instrumentation developed specifically for CEC remains scarce.

CEC is still an emerging technique. Therefore, most of its current practitioners have entered this field either from an HPLC or a capillary electrophoresis background. This is often reflected in the approaches adopted, such as the development of separation media, the families of molecules separated, and the equipment employed. Since a significant proportion of the current research in CEC is concerned with column technology, it will be interesting to monitor how both of the ''parent'' sepa-

ration techniques influence the development of future separation media for CEC.

4. The heritage from HPLC

Although numerous groups the world over prepare capillary columns using a variety of approaches, the vast majority of these efforts mimic, in some manner, standard HPLC column technology. For example, HPLC-like ''hardware'', such as frits and packed columns are employed. In addition, the stationary phases packed in CEC capillaries are usually standard commercial HPLC-grade beads. Such separation media are tailored for regular HPLC modes, and their surface chemistry is optimized accordingly. However, this approach incorrectly treats CEC as simply a subset of HPLC since CEC packings must instead play a dual role: In addition to providing sites for the required interactions (as in HPLC), the packing is also involved in electroosmotic flow. As a result, even excellent HPLC packings may offer limited performance in the CEC mode. This realization of the basic differences between HPLC and CEC [12] has stimulated the development of both specific particulate packings with properties tuned for the needs of CEC as well as alternative column technologies.

4.1. Alternatives to packed capillary columns

Numerous papers have demonstrated the success of packed capillary columns in CEC-based separations. These capillaries are reviewed in detail elsewhere within this issue. The preparation of these columns includes two key steps: (i) the fabrication of retaining frits within a capillary and (ii) the packing of small diameter particles into the narrow-bore tubes. Both of these steps require considerable experimental skill and experience in order to obtain stable columns with reproducible properties. Additionally, frits constructed in situ often serve as nucleation sites for gas bubbles that are detrimental to the continuity of the mobile phase necessary to preserve conduction of the electrical current. Although bubble formation can be suppressed by pressurizing the mobile phase compartments at both ends of the capillary column, this requirement contributes to the overall complexity of the equipment.

The technical difficulties associated with packed columns have led to the development of various alternative approaches. For example, one of these competing technologies – in situ polymerized organic separation media – was adopted from a concept developed for much larger diameter columns for use in HPLC. Due to their unique properties, these monolithic materials have recently attracted considerable attention from many different research groups. The ease of preparation of monolithic materials is of particular importance. As will be shown later in this review, the production of monolithic columns is often a single-step process amounting to polymerization in a mold that avoids the problems of both frit formation and packing. Additionally, columns of virtually any length are easily accessible. The polymerization mixture may also be prepared using a wide variety of monomers, allowing a nearly unlimited choice of both matrix and surface chemistries. This flexibility enables the easy tailoring of interactions required for specific separation modes as well as EOF supported by the packing. The control exerted over the polymerization process also allows optimization of the porous properties. This, in turn, directly affects the flow-rate and chromatographic efficiency of the system.

Analogous approaches resulting in inorganic-based monolithic capillary columns by direct condensation of silicon oxide [24], entrapping of inorganic particles in inorganic gels [25–28], and sintering of silica beads [29,30] have emerged recently, and are detailed in other parts of this issue.

5. Towards monolithic polymer columns

5.1. Early developments of monolithic separation media

Kubin et al. were most likely the first who published the preparation of a continuous polymer matrix for chromatography while attempting to replace natural polysaccharide gel beads with a highly swollen poly(2-hydroxyethyl methacrylate) gel for the low pressure size-exclusion chromatography of proteins [31]. However, the permeability of this

continuous bed gel was far too low to make the material useful. The first operating monolithic columns were prepared from open-pore polyurethane foams in the early 1970s and used as stationary phases for both liquid and gas chromatography. These materials were found to suffer from excessive swelling and softening in some solvents [32–35].

Successful approaches towards continuous media for ''classical'' liquid chromatography emerged only in the late 1980s, and included macroporous disks, rolled woven matrices, compressed poly(acrylamide) gels forming continuous bed, and rigid polymer monolithic columns. The genesis, properties and applications of these novel separation media have recently been detailed in a series of excellent review articles [36–40]. Inorganic silica-based analogs of these columns were subsequently reported by several groups starting in 1996 [24,41–44].

5.2. First attempts to polymer monoliths for CEC

Early work on highly crosslinked acrylamide-based continuous beds for CEC was published by Hjertén et al. in 1995 [45]. At about the same time, Fujimoto described a similar approach to the production of a highly crosslinked acrylamide-based matrix [69,70]. This work will be discussed later in this review. Hjertén et al.'s original approach was complex, requiring a multiplicity of steps including the modification of the capillary surface with (3-methacryloyloxypropyl) trimethoxysilane, two individual polymerizations, and a chemical functionalization step [46]. The initial matrix was formed by copolymerizing a dilute aqueous solution containing 2-hydroxyethyl methacrylate and piperazine diacrylate initiated using a peroxodisulfate–*N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED) system in the presence of a high concentration of ammonium sulfate. The inorganic salt was added to obtain a porous structure with the desired permeability. The pores of this matrix were then filled with another polymerization mixture consisting of allyl glycidyl ether (20%), 2-hydroxyethyl methacrylate (50%) and piperazine diacrylate (30%) to which dextran sulfate had been added. Initiated again by the same redox system, the second polymerization proceeded within the pores of the matrix and led to the ''immobilization'' of the charged polysaccharide within the newly formed polymer, and activation of the surface for additional functionalization. Reaction of both epoxide and hydroxyl functionalities located within the monolith with 1,2-epoxyoctadecane catalyzed by BF_3 led to the covalent functionalization of the matrix with an unquantified number of C_{18} chains. Several measurements of the effect of flow-rate on efficiency as well as of flow velocity at different field strengths were carried out using 12.5 cm long capillaries (9.5 cm active length). An example of the separations obtained using a 55 cm long capillary column is shown in Fig. 1. Retention times in excess of 20 min were required for the elution of aromatic hydrocarbons on this column [46].

6. Rigid porous polymer monoliths for reversed-phase CEC

The experience acquired during the early years of monolithic materials for HPLC [54] greatly facilitated the process for the preparation and development of chemistries for capillary CEC columns. Specifically, efforts have been directed towards improving both reproducibility and suitability for commercial manufacturing. Since the studies of monolithic columns for HPLC to date have only involved a limited number of polymers [i.e., polyacrylamides, poly(methacrylate esters) and polystyrenics], these chemistries have also become the focus of current research in the area of monolithic columns for CEC.

6.1. Monoliths prepared from aqueous monomer solutions and dispersions

In order to simplify the preparation described previously, a simpler procedure was developed by Hjertén et al. [47]. The polymerization mixture consisted of an aqueous solution of acrylamide, piperazine diacrylamide and vinylsulfonic acid. The desired hydrophobicity of the gel was achieved by admixing a hydrophobic monomer such as stearoyl methacrylate or butyl methacrylate. Since neither of these non-polar monomers is soluble in water, a surfactant was added to the mixture, followed by sonification to form an emulsion of the hydrophobic

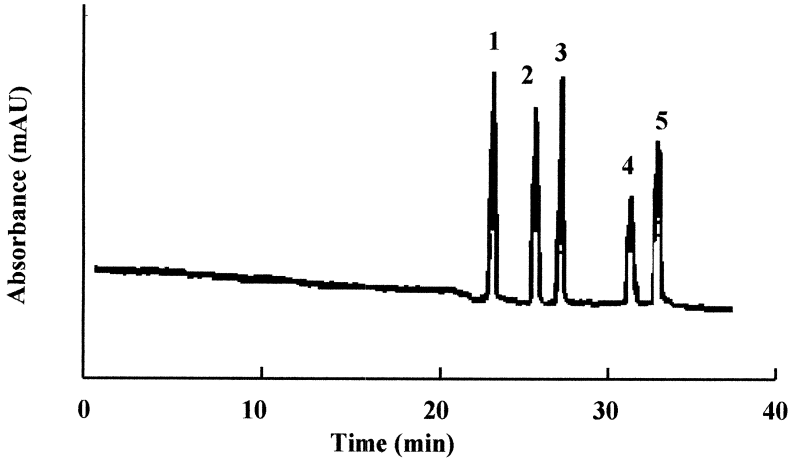


Fig. 1. Electrochromatogram of polycyclic aromatic hydrocarbons on a continuous bed with C_{18} ligands and immobilized dextran sulfate (reprinted with permission from Ref. [46]. Copyright 1996 Elsevier). Capillary column: 55 cm (52.5 cm active length)×25 μm I.D.; voltage, 26 kV, mobile phase, 62% acetonitrile in 5 mmol borate buffer, pH 8.7. Peaks: naphthalene (1), 2-methylnaphthalene (2), fluorene (3), phenanthrene (4), anthracene (5).

monomer in the aqueous solution. Once initiated, the mixture was immediately drawn into an acryloylsilanized capillary and the polymerization was completed. The presence of the strongly acidic sulfonic acid functionalities affords an EOF of a magnitude that remains constant over a broad pH range.

Although preliminary separations using these continuous gel beds were good, an elegant trick involving the strength of the mobile phase enabled a much better resolution of five polycyclic aromatic hydrocarbons (PAHs) to be achieved. A solution containing the analytes dissolved in acetonitrile–aqueous buffer (50:50) was injected electrophoretically into the capillary column, and the separation was started. After a short period of time, the solvent in the inlet vial was replaced with an acetonitrile–buffer (70:30) mixture, and the elution was completed under these conditions. A comparison of the standard isocratic run with the stepwise gradient elution (Fig. 2) shows that the latter procedure led to a significant sharpening of the peaks. The authors assumed that this improvement resulted from the gradient of the mobile phase generated by diffusion across the strong solvent–weak solvent interface [47]. In their recent study, Moffatt et al. [48] presented new experimental evidence documenting the strong effect of the composition of the solvent in the injection zone on ''peak focusing'' that clearly supports Hjertén's early hypothesis.

Addition of a surfactant such as sodium dodecyl sulfate at levels below the critical micelle concentration was also reported to improve isocratic CEC separations [47]. In explaining this phenomenon Hjertén assumed that the C_{12} alkyl groups of the surfactant interact with the C_{18} chains of the stationary phase, thus increasing both the density of the interacting hydrophobic moieties at the surface as well as the number of sulfonic acid groups that support the EOF. While the higher hydrophobicity led to stronger retention, the increase in EOF counteracted this effect by accelerating flow. Thus, the net migration time for retained PAHs did not change, but the resolution improved significantly. However, these assumptions have not been confirmed unambiguously, although the accelerating effect of the increased number of charged functionalities could easily be demonstrated using a non-retained marker.

6.2. Porous polymer monoliths prepared using organic solvents and their mixtures

Despite the undeniable success, the use of aqueous-based polymerization systems for the prepa-

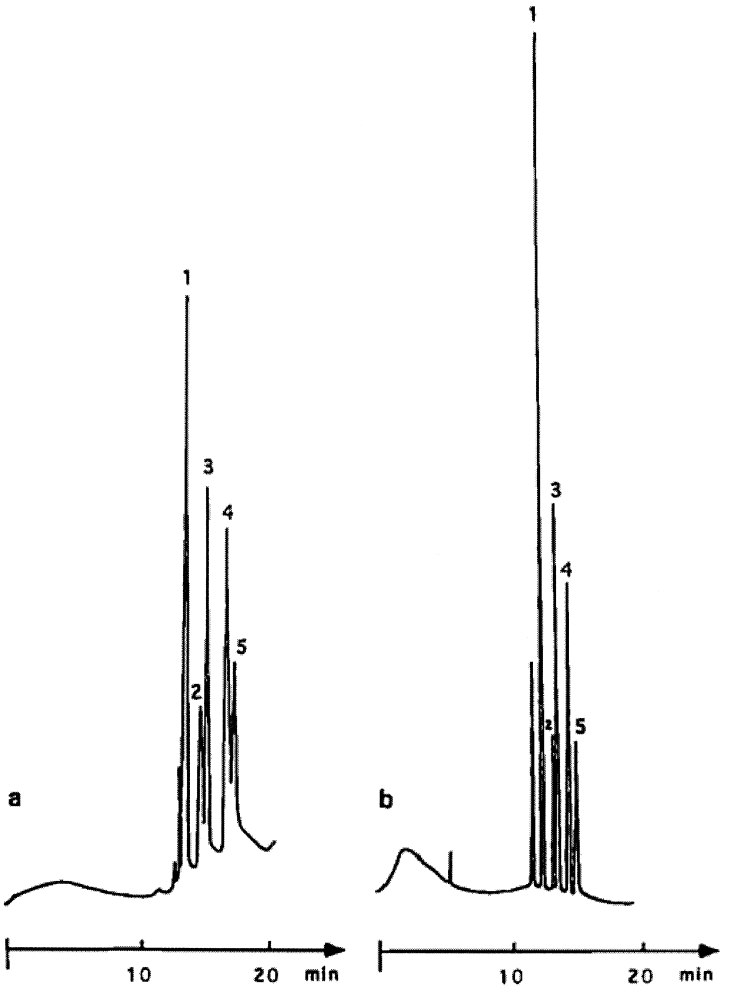


Fig. 2. Effect of gradient elution in CEC on the peak sharpening of polycyclic aromatic hydrocarbons in a C_{18} continuous gel bed capillary column (reprinted with permission from Ref. [47]. Copyright 1996 American Chemical Society). Conditions: capillary, 20 cm (16.0 cm active length)×75 μm I.D.; voltage, 3.0 kV, mobile phase at the injection, 50% acetonitrile in 4 mmol sodium phosphate buffer, pH 7.4. (a) Isocratic separation in acetonitrile–buffer (60:40). (b) The original 60:40 mobile phase was exchanged after 6 min for a 70:30 mixture of acetonitrile–buffer. Peaks: naphthalene (1), 2-methylnaphthalene (2), fluorene (3), phenanthrene (4), anthracene (5).

ration of monolithic capillaries for CEC also has some limitations. Perhaps the greatest limitation is the poor solubility of a number of polymerizable monomers in water. For example, the nonpolar monomers required to achieve the necessary hydrophobicity for a reversed-phase CEC bed are typically insoluble in water. 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.

6.2.1. Acrylamide-based monoliths

Palm and Novotny substantially simplified the incorporation of highly hydrophobic ligands into acrylamide-based matrices, and for the first time, showed reproducibility data characterizing their

preparation [49]. In contrast to the ill-defined process of sonification used previously to disperse hydrophobic monomers in an aqueous buffer [47], Palm and Novotny used mixtures of aqueous buffer and *N*-methylformamide to prepare homogeneous polymerization solutions containing acrylamide, methylene bisacrylamide, acrylic acid and C_4, C_6, or C_{12} alkyl acrylate. The overall concentration of the monomers in solution was kept constant throughout the study at the level of 5%. The monomer mixture consisted of 60% bisacrylamide and 10% acrylic acid, while the remaining 30% were acrylamide and the hydrophobic monomer present in different proportions. 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. Columns with high efficiency were only formed by polymerization in the presence of poly(oxyethylene) (M_r=10 000) dissolved in the polymerization mixture. Poly(oxyethylene) is known to induce lateral aggregation of acrylamide chains and to contribute to the formation of more porous structures [50–52]. Polymerization was achieved using the usual peroxodisulfate–TEMED initiating system within the acryloylsilylated capillaries, affording monoliths possessing the opaque appearance characteristic of macroporous polymers. However, no analytical characterization of the pore structures was performed. Once the polymerization was complete, the poly(oxyethylene) and other low-molecular-mass compounds were washed out of the column using electroosmotic flow.

This preparation method is remarkably reproducible. The column-to-column and batch-to-batch reproducibility was demonstrated for three different columns. While the reproducibility of retention factors was excellent, the variations in retention times and column efficiencies were slightly higher, but still very low.

All of the monoliths containing the alkyl acrylates behave as typical reversed-phase stationary phases, as evidenced by the linear decrease in their retention in response to increasing percentage of organic solvent in the mobile phase. Column efficiencies calculated for on-column detected peaks of phenylketones as model analytes were in the range of 300 000–400 000 plates/m. These monolithic columns easily tolerated rather high loading levels without a concomitant loss in efficiency, though an excessive tailing of the peaks was observed under overload conditions.

Fig. 3 illustrates the performance of these CEC columns in the rapid separation (<5 min) of aromatic ketones. Statistical evaluation of the results of repeated injections gave average relative standard deviations (RSDs) of 0.8 (retention time), 2.55 (retention factor), and 4.4 (column efficiency) for run-to-run reproducibility, and 2.1, 6.1 and 4.6 for day-to-day reproducibility of the same set of variables [49].

6.2.2. Methacrylate ester-based monolithic capillary columns

In contrast to the characterization of the acrylamide-based monoliths that has largely been limited to studies of their chromatographic properties, extensive materials development and optimization has been performed with monolithic CEC capillaries prepared from methacrylate ester monomers. These investigations made use of the concepts established with the original molded rigid monolithic HPLC columns we introduced in the early 1990s [53,54]. The experience acquired earlier with those monoliths helped to quickly assess the interrelated effects of morphology and composition on the overall CEC process.

The production of these monolithic capillary columns is amazingly simple [55]. The bare, untreated, capillary is filled with the polymerization mixture using a syringe, and the radical polymerization is initiated thermally using an external heated bath to afford a rigid monolithic porous polymer. A typical monomer mixture contains 40% ethylene dimethacrylate, 59.7% butyl methacrylate and 0.3% 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS). Once the polymerization is complete, the unreacted components, such as the solvents that constitute the porogenic system, are removed from the monolith using either a syringe pump or electroosmotic flow (Fig. 4). This simple method for preparing monolithic capillary columns has some advantages. For example, the fused-silica tubing may be used directly without first performing any chemical modification of its internal surface. However, the methacroylsilylation can help to increase the adhe-

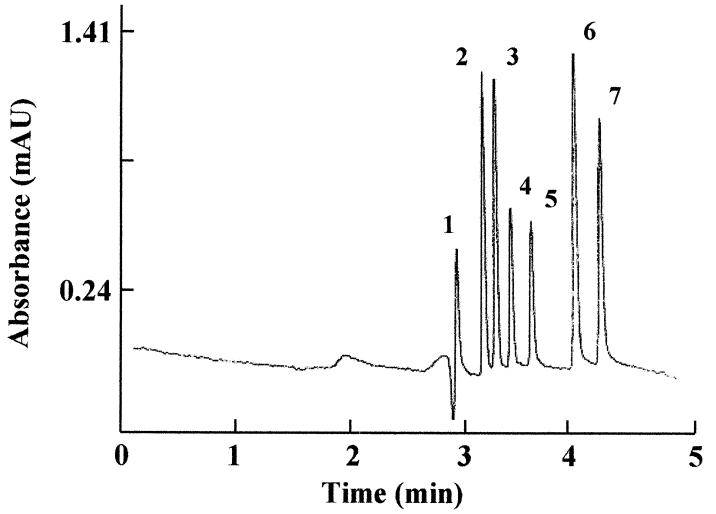


Fig. 3. Isocratic electrochromatography of alkyl phenones in a capillary filled with a macroporous polyacrylamide–poly(ethylene glycol) monolith derivatized with C_4 ligands and containing acrylic acid (reprinted with permission from Ref. [49]. Copyright 1997 American Chemical Society). Conditions: gel composition, 5.0% T, 60% C consisting of acrylic acid, butyl acrylate and methylenebisacrylamide and 3% (w/v) poly(ethylene glycol); capillary, 25 cm (20.5 cm effective length)×100 μm I.D.; injection, 0.5 kV, 1 s; detection, UV absorbance at 260 nm; temperature, 22°C; mobile phase, 20% (v/v) acetonitrile in 10 m*M* Tris–15 m*M* boric acid (pH 8.2) buffer; applied voltage, 22.5 kV (900 V/cm). Peaks; unretained marker vitamin B_{12} (1), aniline (2), acetophenone (3), propiophenone (4), butyrophenone (5), 2′,5′-dihydroxyacetophenone (6), 2′,5′-dihydroxypropiophenone (7).

sion of the monolith to the wall. Although all of the chemicals may be used as supplied, their careful purification contributes to better batch-to-batch reproducibility. In addition, the final polymerization mixture containing free radical initiators such as benzoyl peroxide or azobisisobutyronitrile can easily be handled for several hours at room temperature or left in the refrigerator for days without risking the

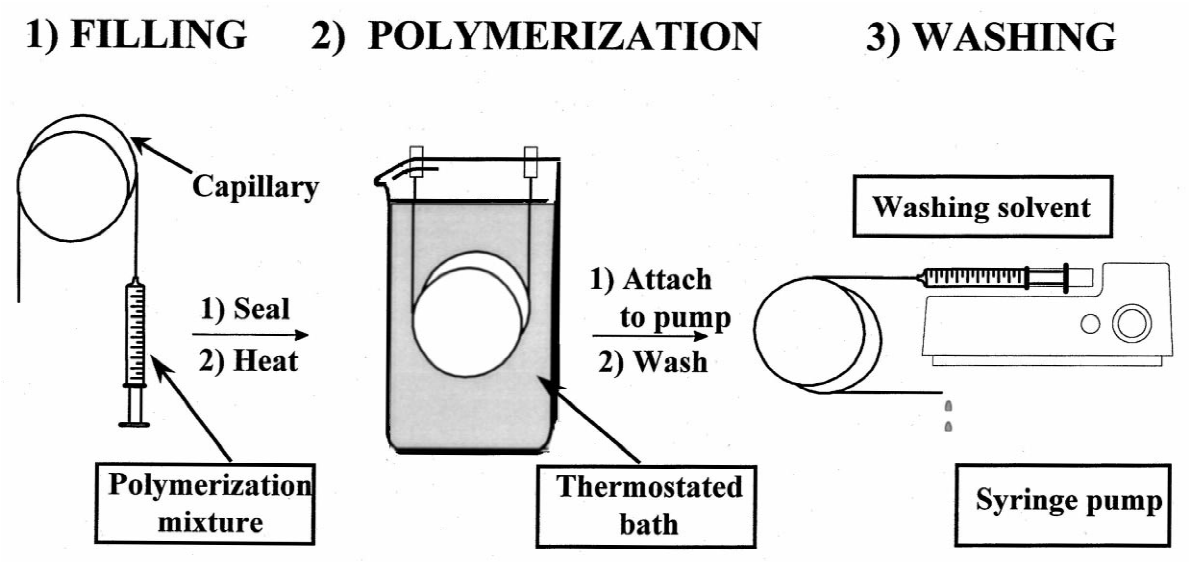


Fig. 4. Schematics for the preparation of monolithic capillary columns.

onset of polymerization. In optimizing the process, specific attention was paid to the design of the porogenic mixtures that represents 60% of the entire polymerization mixture. Ideally, this system had to enable (i) the preparation of a homogeneous polymerization mixture from the solid, hydrophilic AMPS monomer and the liquid hydrophobic butyl methacrylate and ethylene dimethacrylate monomers without using additional compatibilizing agents; (ii) the direct incorporation of monomers with such 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 final capillary column, through miscibility of the porogenic mixture with the mobile phase used for electrochromatography. An extensive study led to the development of a ternary porogen system consisting of water and various proportions of 1-propanol and 1,4-butanediol. The unoptimized monolithic capillary columns prepared using this simple process had efficiencies of over 150 000 pl/m. Fig. 5 shows the model separation of a mixture of aromatic compounds [56].

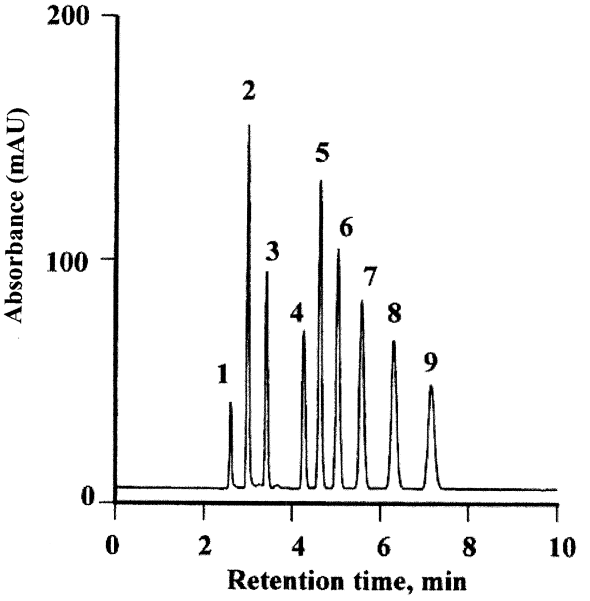


Fig. 5. Electrochromatographic separation of benzene derivatives on monolithic capillary column (reprinted with permission from Ref. [56]. Copyright 1997 American Chemical Society). Conditions: capillary column, 25 cm active length×100 μm I.D.; stationary phase with 0.3 wt.% 2-acrylamido-2-methyl-1-propanesulfonic acid; pore size, 465 nm; mobile phase, 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. Peaks: thiourea (1), benzyl alcohol (2), benzaldehyde (3), benzene (4), toluene (5), ethylbenzene (6), propylbenzene (7), butylbenzene (8), and amylbenzene (9).

The methacrylate-based polymers are stable even under extreme pH conditions. Fig. 6 shows the CEC separations of aromatic acids and anilines at pH 2 and 12, respectively [58]. The sulfonic acid functionalities of the monolithic polymer remain dissociated under either of these conditions, 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 in their respective mobile phases, yielding symmetrical peaks. It should be noted that such extreme pH conditions cannot be tolerated by typical silica-based packings.

6.2.2.1. Control of porous properties

The ability of a liquid to flow through the network of large canal-like pores that traverse the length of the macroporous monoliths is essential to all of their applications. In addition to providing permeability, this porous structure also accelerates the rate of mass transfer within the separation medium as a result of convection. In the early 1990s, Regnier demonstrated that convective flow through the pores of specially modified macroporous beads could be achieved to the extent of a few percent of total flow. This enabled the HPLC separations to be performed at substantially increased speeds [57]. The fundamental limitations of this hybrid bead system are removed with a monolithic column, for which *all* of the mobile phase flows *through* the pores [36]. Despite this important fact, only one study assessing the effect of this variable for monolithic CEC media has been published [56]. The absence of data for other monolithic systems is probably due both to the limited means available to control 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, such as mercury intrusion porosimetry and nitrogen absorption/desorption methods, typically used for the measurement of porous properties, are performed on materials in the

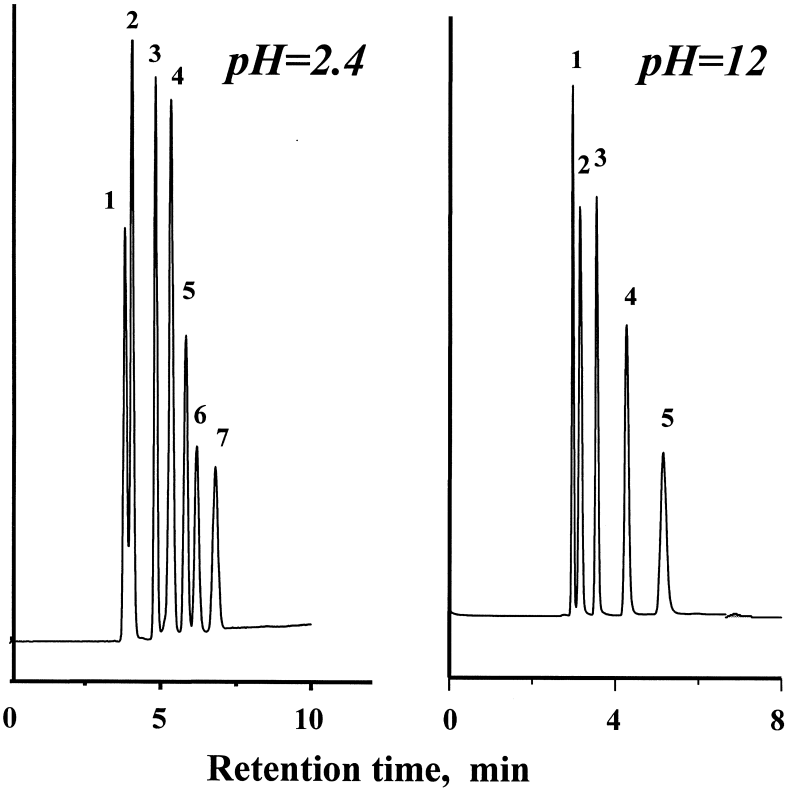


Fig. 6. Electrochromatographic separation of aromatic acids (a) and anilines (b) on monolithic capillary columns [58]. Conditions: butyl methacrylate–ethylene dimethacry late stationary phase with 0.3 wt.% 2-acrylamido-2-methyl-1-propanesulfonic acid; pore size, 750 nm; UV detection at 215 nm; voltage, 25 kV; pressure in vials, 0.2 MPa; injection, 5 kV for 3 s. (a) Capillary column, 30 cm (25 cm active length)×100 μm I.D.; mobile phase, acetonitrile–5 mmol/l phosphate buffer, pH 2.4 (60:40, v/v). Peaks: 3,5-dihydroxybenzoic acid (1), 4-hydroxybenzoic acid (2), benzoic acid (3), 2-toluic acid (4), 4-chlorobenzoic acid (5), 4 bromobenzoic acid (6), 4-iodobenzoic acid (7). (b) Capillary column, 28 cm (25 cm active length)×100 μm I.D.; mobile phase, acetonitrile–10 mmol/l NaOH, pH 12 (80:20, v/v). Peaks: 2-aminopyridine (1), 1,3,5-collidine (2), aniline (3), *N*-ethylaniline (4), *N*-butylaniline (5).

dry state, while the columns operate in the presence of a solvent. As a result, the data measured in the dry state may not actually represent the operational pore size of the capillaries during the actual chromatographic process.

Although several methods have been developed for the ''in situ'' porosity analysis of HPLC materials and columns, there are no simple and reliable methods to determine the porous properties of the monolithic capillaries in their ''swollen'' state. For example, inverse size-exclusion chromatography (ISEC) is an excellent technique for the determination of pore size distribution [59]. However, the working range of ISEC is dictated by the availability of soluble monodisperse calibration standards, possessing known hydrodynamic sizes. Even standards with molecular masses of several million are not sufficiently large to measure pores larger than 100 nm. Thus, pore sizes in the range of hundreds of nanometers to well over one micrometer – typical of rigid polymer monoliths – cannot be measured directly by ISEC. However, since a strong correlation exists between the ''dry'' porous properties of the monoliths and their chromatographic performance (see below), ''dry'' porosity measurements may still be used to tailor column performance.

The ternary porogenic system that we developed allows control of porous properties over a broad range [56]. For example, Fig. 7 shows scanning electron microscopy (SEM) micrographs of three

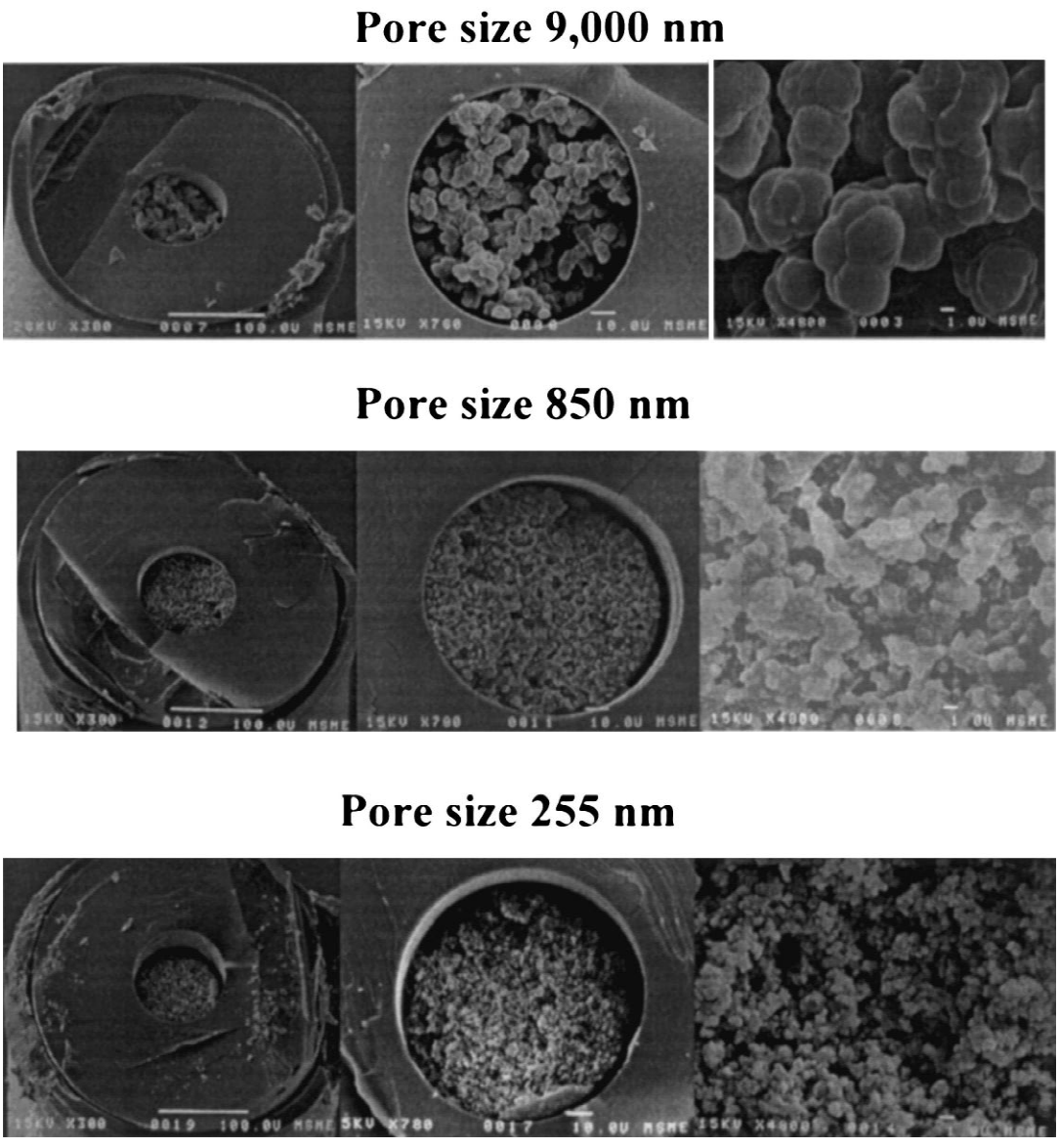


Fig. 7. SEM micrographs of monolithic capillary columns with different pore sizes [58].

monolithic capillaries in which pore sizes differ by nearly two-orders of magnitude. It should be noted that the monoliths had to be dried before the SEM measurement. In particular, the proportion of 1-propanol in the porogenic solvent has an enormous effect on the pore diameter at the maximum of the distribution curve (mode pore diameter), that was also obtained for the completely dry monolithic material, is clearly documented in Fig. 8. Using such data, monoliths of any ‘‘dry’’ pore size in the broad range of 250–1300 nm can easily be produced simply by changing the ratio of 1-propanol to 1,4-butanediol in the porogenic mixture. It should be noted that the relatively small window of 2.5% in the weight percentage of 1-propanol that brackets this wide range of pore sizes is still sufficiently large to

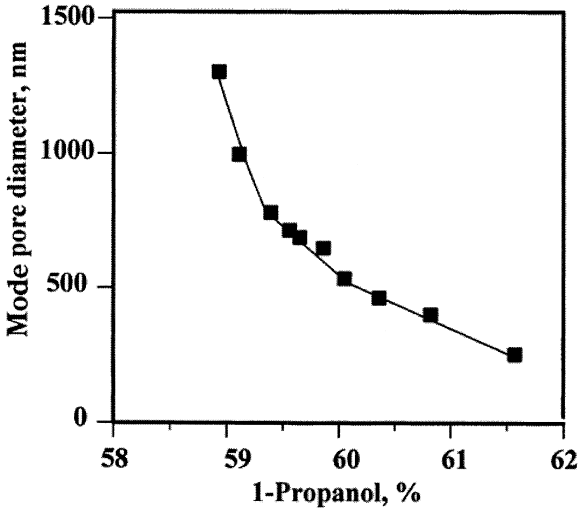


Fig. 8. Effect of the percentage of 1-propanol in the porogenic mixture on the porous properties of monolithic polymers (reprinted with permission from Ref. [56]. Copyright 1997 American Chemical Society). Reaction conditions: polymerization mixture: ethylene dimethacrylate 16.00 wt.%, butyl methacrylate 23.88 wt.%, 2-acrylamido-2-methyl-1-propanesulfonic acid 0.12 wt.%, ternary porogen solvent 60.00 wt.% (consisting of 10 wt.% water and 90 wt.% of mixtures of 1-propanol and 1,4-butanediol), azobisisobutyronitrile 1 wt.% (with respect to monomers), polymerization time 20 h at 60°C.

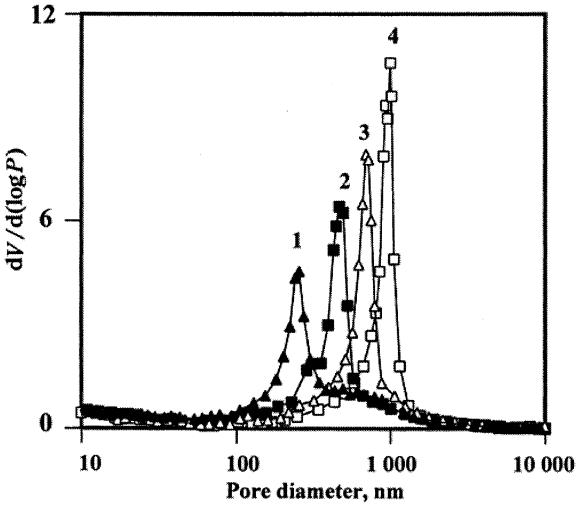


Fig. 9. Differential pore size distribution profiles of porous polymeric monolithic capillary columns with mode pore diameters of 255 (curve 1), 465 (2), 690 (3), and 1000 nm (4) (reprinted with permission from Ref. [56]. Copyright 1997 American Chemical Society).

obtain polymers of any desired pore size with an accuracy of ±25 nm with respect to a targeted value. Despite the fact that these monoliths are prepared from a polymerization mixture containing monomers of very different polarities, the mercury porosimetry profiles seen in Fig. 9 all exhibit distribution curves similar to those found for polymers prepared from mixtures of fully miscible monomers [60].

6.2.3. Polystyrene-based monolithic capillary columns

Gusev et al. have recently reported the preparation of porous rigid monolithic capillary columns for CEC by polymerizing mixtures of chloromethylstyrene, divinylbenzene and azobisisobutyronitrile in the presence of various porogenic solvents such as methanol, ethanol, propanol, toluene and formamide within a silanized capillary [61]. The resulting monolith exhibited a ''classical'' macroporous structure ''wrapped'' in a thin outer layer of apparently nonporous polymer. The reactive chloromethyl moieties incorporated into the monolith served as sites for the introduction of quaternary ammonium functionalities using reaction with *N*,*N*-dimethyloctylamine. These capillary columns possessing positively charged surface functionalities were able to separate basic and acidic peptides. Surprisingly, a good separation of chemically similar tripeptides (Gly–Gly–Phe and Phe–Gly–Gly) was observed in a pH 7 buffer using unfunctionalized poly(styrene–co-divinylbenzene) monoliths devoid of charged functionalities. In this case, the driving force for movement of the analytes through the column appears to be their electrophoretic migration, while separation results from their interactions with the stationary phase [61]. However, the addition of acetonitrile to the mobile phase significantly decreases the analytes mobility, making this approach less attractive.

Huang et al. also produced macroporous polymer layer open tubular (PLOT) capillary columns for CEC similar to those employed in gas chromatography [62]. On several examples of peptide and protein separations using the PLOT capillary columns, he demonstrated the complex nature of CEC separations that often involve the interplay of EOF,

electrophoretic migration, and chromatographic retention through several examples.

7. Effects of the properties of the monoliths on the separation in CEC

The ability to achieve precise and independent control over both the pore structure as well as the level of charged moieties of the rigid monolithic stationary phases opened new avenues for studies focused on the effects that these properties exert on the chromatographic process. Both of these variables were found to be extremely important in controlling the flow velocity, efficiency and selectivity 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, such as the applied voltage, and both the pH and elution strength of the mobile phase.

7.1. Pore size and efficiency

The electroosmotic flow velocity u_{eo} in CEC is given by the equation:

$$u_{eo} = \epsilon_0 \epsilon \zeta E / \eta \quad (1)$$

where ϵ_0 is the permittivity of vacuum, ϵ is the dielectric constant of the mobile phase, ζ is the zeta potential of the electric double layer, η is the viscosity of the mobile phase at the wall, and E is the strength of the electric field, defined as

$$E = V/L \quad (2)$$

where L is the column length and V is the applied voltage. Since Eq. (1) does not include a term related to particle/pore size, less attention has been paid to these issues in the CEC literature [14,63].

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 of both of these methods is particle size dependent, and increases as the size of the packing decreases [64]. Since the monolithic columns are molded rather than packed, particles size of packing becomes irrelevant, and instead, the size of the pores within the monolithic material controlled by polymerization conditions is the variable that is expected to affect chromatographic efficiency. Indeed, initial studies have shown that the size of the flow through pores affects separation efficiency [55]. However, having defined preparative parameters that yield capillaries possessing acceptable efficiencies, this effect becomes rather complex, and its subtleties remain unexplored [56].

7.2. Solvent flow through the monolithic capillary

According to Eq. (1), electroosmotic flow is deemed to be independent of the size of the packing, and consequently the size of the interstitial voids between the particles, unless this size is so small that the electrical double layers overlap [65]. However, this general perception often reported in the CEC literature may be misleading. One of the reasons flow resistance in CEC has not been previously reported may simply be that this phenomenon is difficult to observe using traditional small pore or non-porous silica-based media. Obviously, a decrease in the particle size of a stationary phase results in an increase in its total outer surface, and therefore, an increase in the overall number of surface silanol functionalities. Since electroosmotic flow velocity is directly proportional to the number of surface silanol groups in the system [66], any slowing effect due to an increase in flow resistance with decreasing particle size might be offset by the concomitant increase in the driving force for electroosmotic flow. Nevertheless, a 60% decrease in EOF has been observed in packed capillaries compared to that in open capillaries [67,68].

In contrast, the ability to easily control both the mode pore diameter and level of charged functionalities of the methacrylate ester monolithic capillaries enables the direct investigation of the net effect of transport channel size on flow velocity. Fig. 10 clearly demonstrates a two-fold increase in flow velocity through the monolithic capillaries as the pore size increases from 250 to 1300 nm [56]. A similar increase in flow velocity was observed when pore sizes were increased up to 4 μm [55]. This range of pore sizes significantly exceeds the thickness of a few nanometers at which the electrical double layers would overlap in a system utilizing a mobile phase containing 5 mmol/l buffer [56]. If it

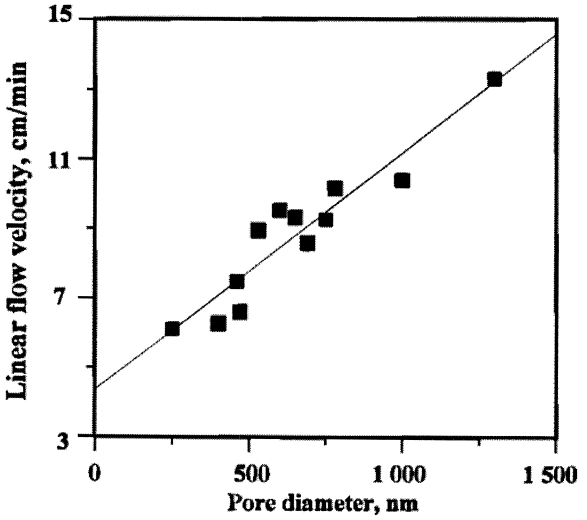


Fig. 10. Effect of mode pore diameter on flow velocity of the mobile phase through monolithic capillary columns (reprinted with permission from Ref. [56]. Copyright 1997 American Chemical Society). Conditions: capillary column, 30 cm active length×100 μm I.D.; stationary phase with 0.3 wt.% 2-acrylamido-2-methyl-1-propanesulfonic acid; pore size, 465 nm; mobile phase, acetonitrile–5 mmol/l phosphate buffer, pH 7 (80:20, v/v).

is assumed that the observed decrease in flow-rate with decreasing mode pore diameter is simply due to 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 thereafter remain constant, since the number of pores within which overlap of the electric double layer could occur decreases rapidly as the pore size increases. In practice however, this phenomenon is not observed. The fact that the overall flow velocity increases linearly over a broad range of pore sizes strongly supports the contention that this increase in flow-rate is related macroscopically to a decrease in the resistance to flow through the channels. However, the situation is complicated by additional effects that may result from microscopic variations in the strength of the electrical field in both small and large pores, zeta potential, and viscosity at the surface. The effects of the tortuosity and the variations in the cross sectional area of a packed structure on the conductance and chromatographic performance of CEC capillaries packed with beads have recently been discussed in the literature [68].

7.3. Control of surface chemistry

7.3.1. Charged functionalities

The 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 charged functionalities that support the electroosmotic flow is a major advantage of the polymeric monolithic capillaries. The surface charge density component may easily be adjusted by changing the percentage of charged monomer in the polymerization mixture. For example, linear increases in migration velocity paralleling increases in AMPS monomer contents were observed by Fujimoto in both 6% crosslinked polyacrylamide gels [69] as well as 9.7% crosslinked *N*-isopropylacrylamide polymers [70]. Similarly, increasing the content of sulfonic acid groups within the methacrylate ester monoliths was expected to significantly increase the flow velocity, and thus reduce the run time for each analysis. In order to test this hypothesis, a series of capillaries with AMPS percentages of 0.3–1.8 wt.% were prepared, keeping both the percentage of crosslinker and the porogen composition constant [56]. The first two chromatograms in Fig. 11 compare the separations of a model mixture obtained using the capillaries possessing the lowest and highest AMPS contents. As expected, the flow velocity increased with increasing level of charged monomer, as seen from the retention time of the unretained marker, thiourea. However, the separation deteriorates dramatically. While the monolithic capillary with 0.3 wt.% AMPS afforded good separation of the individual components of the test mixture, a very poor separation was observed on the column containing 1.8 wt.% AMPS, with all of the alkylbenzenes eluting as a single peak. Detailed characterization of the resulting polymers showed that an unexpected increase in pore size had accompanied the targeted increase in AMPS level. Deconvolution of these simultaneously changing variables can be performed by controlling independently the variables that affect porous properties and chemistry. Therefore, a second set of capillary columns was produced with near constant mode pore diameters within the narrow range of 690–740 nm. As shown on the third chromatogram of Fig. 11, an

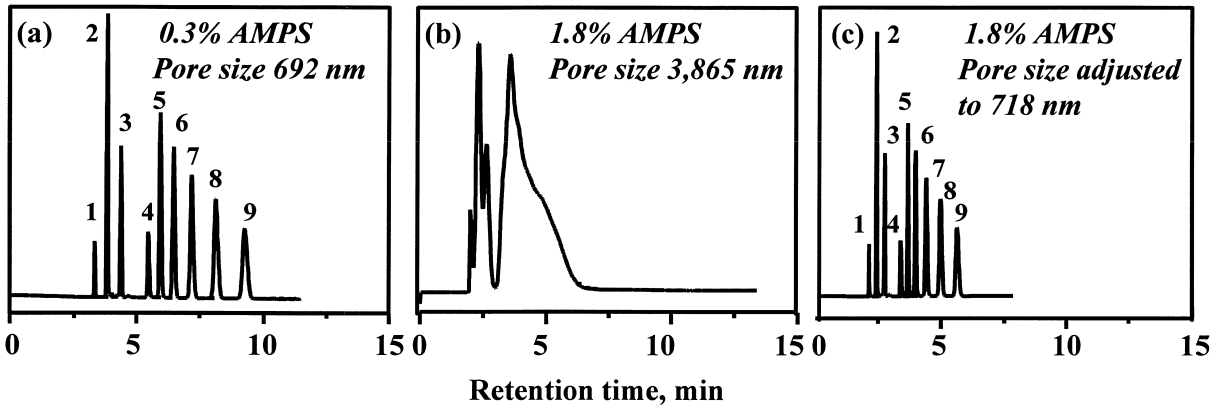


Fig. 11. Effects of percentage of 2-acrylamido-2-methyl-1-propanesulfonic acid and pore size adjustment on the electrochromatographic properties of monolithic capillaries (reprinted with permission from Ref. [56]. Copyright 1997 American Chemical Society). Conditions: capillary column, 30 cm active length×100 μm I.D.; stationary phase with 0.3 (a), 1.8 (b), and 1.8 wt.% (c) 2-acrylamido-2-methyl-1-propanesulfonic acid; mobile phase, 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. Peaks: thiourea (1), benzyl alcohol (2), benzaldehyde (3), benzene (4), toluene (5), ethylbenzene (6), propylbenzene (7), butylbenzene (8), and amylbenzene (9).

excellent separation was obtained even using a capillary with 1.8 wt.% AMPS in only half of the time needed for the separation using a capillary with only 0.3% AMPS.

Monoliths with two significantly different percentages of dimethyldiallylammonium chloride were recently prepared in order to control the EOF component of the overall migration rate of proteins [71]. These charged moieties were incorporated into the monolith during a late stage of the preparation process, and appeared to be extremely well suited for the desired separation (see below).

7.3.2. Retention and selectivity

Most CEC separations reported to date have been performed in the reversed-phase mode. The hydrophobicity of the stationary phase determines the selectivity of the separation, and retention may easily be controlled by adjusting either the composition of the mobile phase or the hydrophobicity of the surface. The first option is easiest to implement. However, in contrast to the rich variety of solvents used in HPLC, acetonitrile-based solvent systems are used in most CEC applications, due to their high dielectric constant and low viscosity [15,47,49,56]. Little has been done to date to tailor the surface chemistry of the stationary phase. Palm and Novotny [49] prepared a series of monolithic columns incorporating a variety of monomers that differ in the length of their pendant alkyl chains. However, the performance of these monoliths containing butyl, hexyl and dodecyl methacrylate moieties has not been demonstrated in comparative separations, preventing an assessment of the effect of such changes in surface chemistry for a homologous series of monoliths.

The effect of surface polarity is even more important in separations where two or more simultaneous interactions must occur to achieve the required 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 have to be run under reversed-phase conditions as opposed to the normal-phase mode typically used in chiral HPLC. In addition, non-specific interactions in reversed-phase mode may be highly detrimental to the discrimination process involving subtle differences between the enantiomers.

The importance of tailoring surface chemistry is demonstrated by three different monolithic capillary columns that were prepared by direct copolymerization of the chiral monomer 2-hydroxyethyl methacrylate (*N*-L-valine-3,5-dimethylanilide) carbamate **1** with ethylene dimethacrylate **2**, AMPS (or its sodium salt) **3**, and butyl- **4** or glycidyl methacrylate

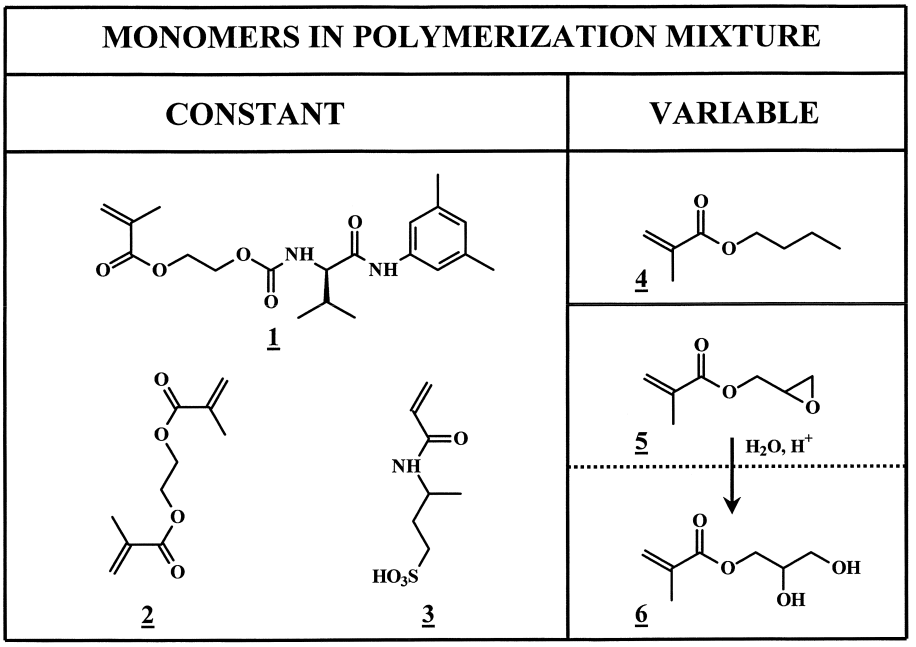


Fig. 12. Chemical composition of capillary columns for chiral CEC.

5 (Fig. 12) [72]. These columns were subsequently used for the enantioseparation of racemic *N*-(dinitrobenzoyl)leucine diallylamide **7** that is shown in Fig. 13. The chiral separations achieved using the various columns are compared in Fig. 14. Although the column containing butyl methacrylate as a non-chiral component did resolve the racemic analyte, the peaks were very broad and tailed severely. The efficiency of this system was poor, as plate counts of only 600 and 160 plates/m were obtained for the respective enantiomers (Fig. 14a). However, when pure acetonitrile was used as the mobile phase, a narrowing of both peaks and a concomitant increase in column efficiency to 2500 and 540 plates/m were observed. These improvements indicate that the tailing observed previously may have resulted from non-specific hydrophobic interactions between the chiral analyte and the relatively hydrophobic surface of the separation medium. Therefore, new capillary columns were prepared substituting the more hydrophilic glycidyl methacrylate for the highly hydrophobic butyl methacrylate. Although this capillary column exhibited a surprisingly high efficiency of 210 000 plates/m for the unretained peak of thiourea under reversed-phase conditions, the incorporation of the more hydrophilic monomer resulted in a substantial decrease in hydrophobic selectivity (α_{CH_2} = 1.08), as seen in the poor separation of alkylbenzene derivatives. However, the chiral separation was clearly improved (Fig. 14b). The peaks for the enantiomers were sharper, and the column efficiencies calculated for this separation increased to 8100 and 1900 plates/m.

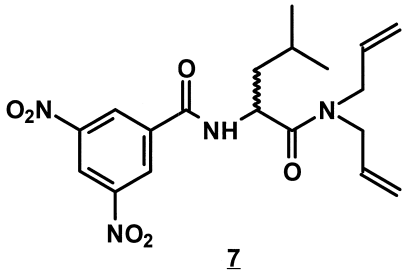


Fig. 13. Racemic analyte for the chiral CEC separations.

Instead of defining and optimizing new conditions for the direct incorporation of highly hydrophilic monomers into a monolith, the epoxide rings of the glycidyl methacrylate-based monolith were hydrolyzed using dilute aqueous sulfuric acid to afford the

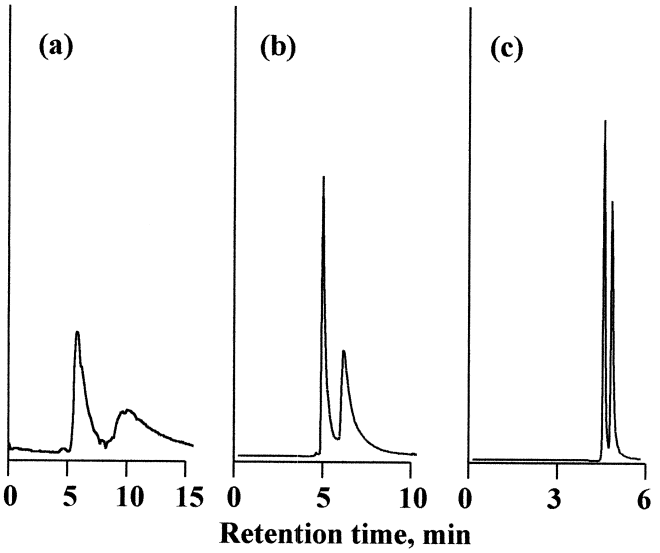
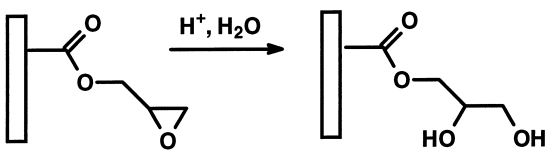


Fig. 14. Effect of the hydrophilicity of chiral monolithic columns on the electrochromatographic separation of *N*-(3,5-dinitrobenzoyl)leucine diallyl amide enantiomers (Modified from Ref. [72]). Conditions: monolithic column, 30 cm active length×100 μm I.D.; mobile phase, acetonitrile–5 mmol/l phosphate buffer, pH 7 (80:20, v/v); UV detection at 215 nm; voltage, 25 kV; pressure in vials, 0.2 MPa; injection, 5 kV for 3 s. Stationary phase with butyl methacrylate (a), glycidyl methacrylate (b), and hydrolyzed glycidyl methacrylate (c).

very hydrophilic diol functionalities. This hydrolytic reaction shown in Scheme 1 was easily performed in situ within the pores of the monolithic capillary column. After hydrolysis, the diol-functionalized capillary did not separate the alkylbenzenes in the reversed-phase mode at all. However, this monolithic column afforded a significantly improved separation of the enantiomers (Fig. 14c). The peaks in this separation were narrow and well resolved ($R_s = 2.0$). Column efficiencies (61 000 and 49 500 plates/m) were rather high, and even peak tailing was greatly reduced, suggesting that few undesirable interactions remained.

This example demonstrates clearly the power of the monolithic technology that enables easy fine tuning the surface chemistry. The wealth of commercially available monomers possessing a variety of functionalities, together with the extreme simplicity of the preparation of the monolithic columns, makes this approach an appealing option for the design of capillary columns with high selectivities.



Scheme 1.

8. The legacy of capillary electrophoresis

In contrast to HPLC, the influence of high-performance capillary electrophoresis (HPCE) on the development of separation media for CEC is less pronounced. In light of the prevailing use of fused-silica capillaries in HPCE, these materials have also been investigated for use in CEC. Since solute–stationary phase interactions are key to the CEC process, appropriate moieties must be bound to the capillary wall. However, the wall surface available for reaction is severely limited. For example, a 100 μm I.D. capillary only has a surface area of $3 \cdot 10^{-4}$ m^2 per meter of length, with a density of functionalization sites of approximately $3.1 \cdot 10^{18}$ sites/m^2. Moreover, surface modification cannot involve all of the accessible silanol groups, since some must

remain to support the EOF. As a result, the use of bare capillaries in CEC has been less successful. In contrast, chemical etching of the inner capillary surface increases the overall surface area affording capillaries with a much greater number of interacting sites that can be used for modification without sacrificing EOF. This results in the magnification of the column loadability and improvement in the overall separation [73]. Such open tubular electrochromatography columns (OTECs) are described elsewhere in this issue.

8.1. Polymer gels

CEC capillary columns filled with hydrophilic polymer gels mimic those used for capillary gel electrophoresis [74]. Typically, the capillary is filled with an aqueous polymerization mixture that contains monovinyl and divinyl (crosslinking) acrylamide-based monomers as well as a free radical redox initiating system, such as ammonium peroxodisulfate and TEMED. Since initiation of the polymerization process begins immediately upon mixing all of the components at room temperature, the reaction mixture must be used immediately. Typically, the polymerization process is allowed to proceed overnight to afford capillaries filled with continuous gel beds. It should be noted, that these gels are very loose, highly swollen materials that usually contain no more than 5% solid polymer.

For example, Fujimoto et al. [75] polymerized an aqueous solution of acrylamide, methylenebisacrylamide (5%), and 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. However, 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 used in this study. This concentration is at least one-order of magnitude higher than the current CEC standard. Changes in both the AMPS content of the gel as well as the strength of the electrical field allowed the flow velocity to be modified over the range of 0.3–3.6 cm/min, somewhat accelerating the separation process. Column efficiencies of up to 150 000 plates/m were observed for slightly retained acetophenone. The good correlation of the migration times of acetone and acetophenone with the expected ''pore size'' characteristics of the gel – based on the composition of the polymerization mixture – and the lack of explicit hydrophobic interacting moieties led Fujimoto to the conclusion that the prevailing mechanism of the separation was sieving [69].

Replacement of the hydrophilic acrylamide by the more hydrophobic *N*-isopropylacrylamide, in combination with the pre-functionalization of the capillary with (3-methacryloyloxypropyl) trimethoxysilane, 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 [70]. In contrast to the previously described work, a substantial improvements in the separations of aromatic ketones and steroids was observed using these ''fritless'' hydrogel columns, as seen by the column efficiencies of 159 700 and 159 800 found for hydrocortizone and testosterone, respectively [70]. The separations of hydrophobic compounds obtained using this polymer gel stationary phase exhibited 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 mechanism was no longer the primary mechanism of separation.

8.2. Replaceable separation media

The use of capillaries filled with solutions of linear polymers is another interesting column technology originating directly from the field of electrophoresis. In contrast to the polymer gels, the preparation of these pseudostationary phases need not to be performed within the confines of the capillary. Although it may be argued that linear polymers do not belong to the category of organic monoliths, it should be noted, that as employed, these materials exist as physically entangled polymer chains that effectively resemble highly swollen, chemically crosslinked gels. Additionally, these materials effect separations in an identical manner, using EOF as the driving force. Several research groups have already reported the use of specifically designed copolymers [69,75–80] and modified dendrimers [81,82] containing both

charged and hydrophobic moieties as alternatives to micelles in micellar electrokinetic chromatography (MEKC) for the separation of neutral analytes.

Fujimoto et al. [75] realized, that in contrast to polyacrylamide homopolymers, the migration of neutral compounds through a capillary column would be achieved more easily if charged functionalities were incorporated into the neutral polyacrylamide chains. Indeed, acetone and acetophenone may be baseline separated using a 10% aqueous solution of an acrylamide copolymer containing 2.4% AMPS. However, nearly 100 min were required to effect this separation at a field strength of 260 V/cm, making this approach impractical even with the use of higher voltage.

Alternatively, Tanaka et al. [76] alkylated commercial polyallylamine with C_8–C_{16} alkyl bromides, followed by a Michael reaction with methyl acrylate and subsequent hydrolysis of the methyl ester to obtain free carboxyl functionalities. Although difficult to prepare, this polymer effected efficient separations of ketones and aromatic hydrocarbons in less than 20 min at 400 V/cm. Similarly, Potoček et al. [77] has demonstrated the separation of phenols using a partially hydrolyzed polyacrylamide solution.

Schure et al. [80] published an excellent study employing a pseudostationary phase of methacrylic acid, ethyl acrylate and dodecyl methacrylate prepared by a modified emulsion polymerization process [83], relating the physical properties of the polymer solution to the quality of CEC separation obtained. For example, increasing the concentration of the linear polymer solution increased the number of interacting moieties, improving the efficiency to a maximum of 293 000 plates/m in a 3.72% polymer solution. Increasing the polymer concentration also led to an increase in the number of carboxyl functionalities, alkylbenzenes and PAHs. Fig. 15 shows the electrochromatographic separation of aromatic antioxidants using this material.

Fig. 15. Separation of antioxidants (reprinted with permission from Ref. [80]. Copyright 1997 American Chemical Society). Conditions: 4.03% linear polymer retentive phase, mobile phase 40% acetonitrile in buffer, pH 9.1; voltage, 30 kV; injection, 1 kV for 15 s. Peaks: hydroxyquinone (1), *p*-methoxyphenol (2), phenothiazine (3), and butylated hydroxytoluene (4).

Columns filled with polymer solutions are extremely simple to prepare, and the ''packing'' can easily be replaced as often as desired. These characteristics make the pseudostationary phases excellent candidates for use in routine CEC separations such as quality control applications where analysis and sample profiles do not change much. However, several limitations limit their widespread use. For example, sample capacity is typically very low, pushing typical detection methods close to their

SEC, solvents such as pure tetrahydrofuran (THF) are often used as the mobile phase, since they dissolve many synthetic polymers and contribute to minimize their interactions with the chromatographic medium. Despite the reported use of entirely non-aqueous eluents in both electrophoresis and CEC [97], no appreciable flow through methacrylate-based monoliths was observed using pure THF as the mobile phase. However, a mixture of THF with 2% water, which still dissolve polystyrene standards with molecular masses as high as 980 000, was found to accelerate substantially the flow velocity [96]. Fig. 17 shows the first SEC separation of polystyrenes in a CEC system using a methacrylate-based monolithic capillary column. The molecular masses of the 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 were not optimized for SEC separations, this result demonstrates that CEC is not limited to the reversed-phase mode of chromatography. An extensive study of SEC separations of polystyrenes in the CEC mode using packed capillary column with dimethylformamide as the solvent has recently been published [98].

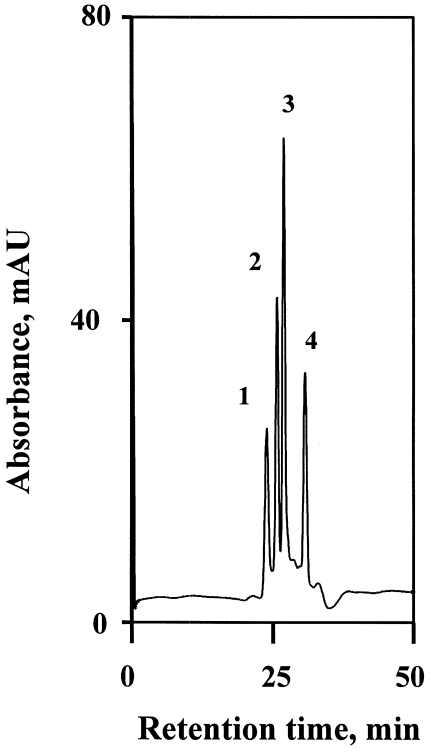


Fig. 17. Electrochromatographic size-exclusion chromatography of polystyrene standards (reprinted with permission from Ref. [96]. Copyright 1997 American Chemical Society). Conditions: monolithic capillary column, 30 cm active length×100 μm I.D.; stationary phase, 59.7 wt.% butyl methacrylate, and 40 wt.% ethylene dimethacrylate in monomer mixture, 0.3 wt.% 2-acrylamido-2-methyl-1-propanesulfonic acid; pore size 750 nm; mobile phase, tetrahydrofuran containing 2 vol.% 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).

9.3.2. Reversed-phase CEC of peptides and proteins

The quest for easily prepared capillary columns that enable the CEC separation of proteins is the current ''holy grail'' of electrochromatography, drawing the interest of many research groups. Monolithic materials appear well suited to fulfill this goal due to the excellent control that can be exerted over both their chemical and physical properties. However, very few reports of success in this area may be found. In contrast to the CEC separation of small model neutral molecules such as alkylbenzenes, aromatic ketones and PAHs, the separation of charged macromolecular analytes such as peptides, proteins, and nucleic acids is difficult as they exhibit electrophoretic migrations in the presence of an 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, an even more important issue in the design of columns for CEC of proteins concerns the possible electrostatic interactions of the protein molecules with the charged functionalities of the packing. Such interactions are likely to occur due to the very low concentration buffer solutions employed. This situation is quite similar to the initial stage of ion-exchange chromatography [92]. 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.

The first successful separation of such charged molecules was published in 1997 [49]. The isocratic separation of four oligopeptides (di-, tri, penta- and hexapeptide) was demonstrated on a monolithic capillary column prepared by polymerizing a solution of acrylamide, methylenebisacrylamide, acrylic acid and dodecyl acrylate dissolved in a mixture of 5% aqueous buffer solution and 95 *N*-methylformamide in the presence of poly(oxyethylene). Fig. 18 shows the isocratic baseline separation that was achieved in less than 5 min at 900 V/cm. The elution pattern and the efficiency of the separation depended strongly on both the percentage of acetonitrile as well as the pH of the mobile phase, suggesting that a gradient elution method would have been even more appropriate. However, larger proteins could not be eluted isocratically.

Ericson and Hjertén recently described the preparation of a monolithic capillary column that was used successfully to effect the CEC separation of proteins [71]. The first step involved a polymerization initiated by ammonium persulfate–TEMED system in a two-phase system: (i) acrylamide and piperazine diacrylamide dissolved in a mixture of a buffer solution and dimethylformamide and (ii) octadecyl methacrylate. Continuous sonication had to be applied for 40 min to emulsify the highly hydrophobic octadecyl methacrylate and form a dispersion of fine polymer particles. In the next step, solution of potassium ferricyanide was added to ''retard'' the continuing polymerization. The polymerization process was restarted using another portion of initiator after the addition of two new monomers, dimethyldiallylammonium chloride and piperazine diacrylamide to the system. This partly polymerized dispersion containing the added initiators and monomers was then forced into a methacryloylsilylated capillary and polymerized to completion.

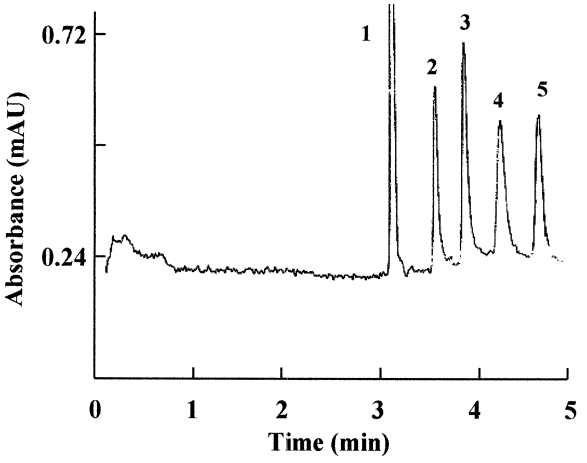


Fig. 18. Isocratic electrochromatography of peptides in a capillary filled with a macroporous polyacrylamide–poly(ethylene glycol) monolith, derivatized with C_{12} ligands (29%) and containing acrylic acid (reprinted with permission from Ref. [49]. Copyright 1997 American Chemical Society). Conditions: mobile phase, 47% acetonitrile in a 10 m*M* Tris–15 m*M* boric acid (pH 8.2) buffer; voltage, 22.5 kV (900 V/cm); sample concentration, 4–10 mg/ml; UV detection at 270 nm. Peaks: system peak (1), Tyr–Arg (2), Gly–Gly–Tyr–Arg (3), Tyr–Ala–Gly–Phe–Leu–Arg (D-Ala^3 leucine enkephalin-Arg) (4), Tyr–Gly–Gly–Phe–Leu–NH_2 (leucine enkephalinamide) (5).

Understanding and controlling the forces that drive the movement of charged molecules in general and proteins in particular during CEC separations is extremely important. Depending on the net charge carried by a protein as determined by both its isoelectric point (p*I*) and the pH value of the mobile phase, the molecules can move by electrophoresis towards either the anode or the cathode. In contrast, EOF proceeds in only one direction as determined by the charge of the mobile counterions in the Stern layer. In Ericson and Hjertén's approach, using immobilized quaternary ammonium cations, EOF always proceeds from the direction of the anode to the cathode. Electroosmotic flow was also used to transport the mobile phase gradient (generated using an HPLC instrument to mix the gradient) and introduce it into the capillary. Two overall flow scenarios shown schematically in Fig. 19 were discussed [71]:

(i) In the monolith containing a high level of charged moieties, electroosmotic flow outweighs the electrophoretic migration. In this ''normal flow gradient'' situation, both the EOF and the net migration of the protein molecules occur in the same direction, provided all of the proteins have net charges of equal sign. This was achieved by using a mobile phase consisting of 80% acetonitrile and 20% buffer with a pH value of 2. This pH is well below the p*I* of the proteins, ensuring that the biopolymers are positively

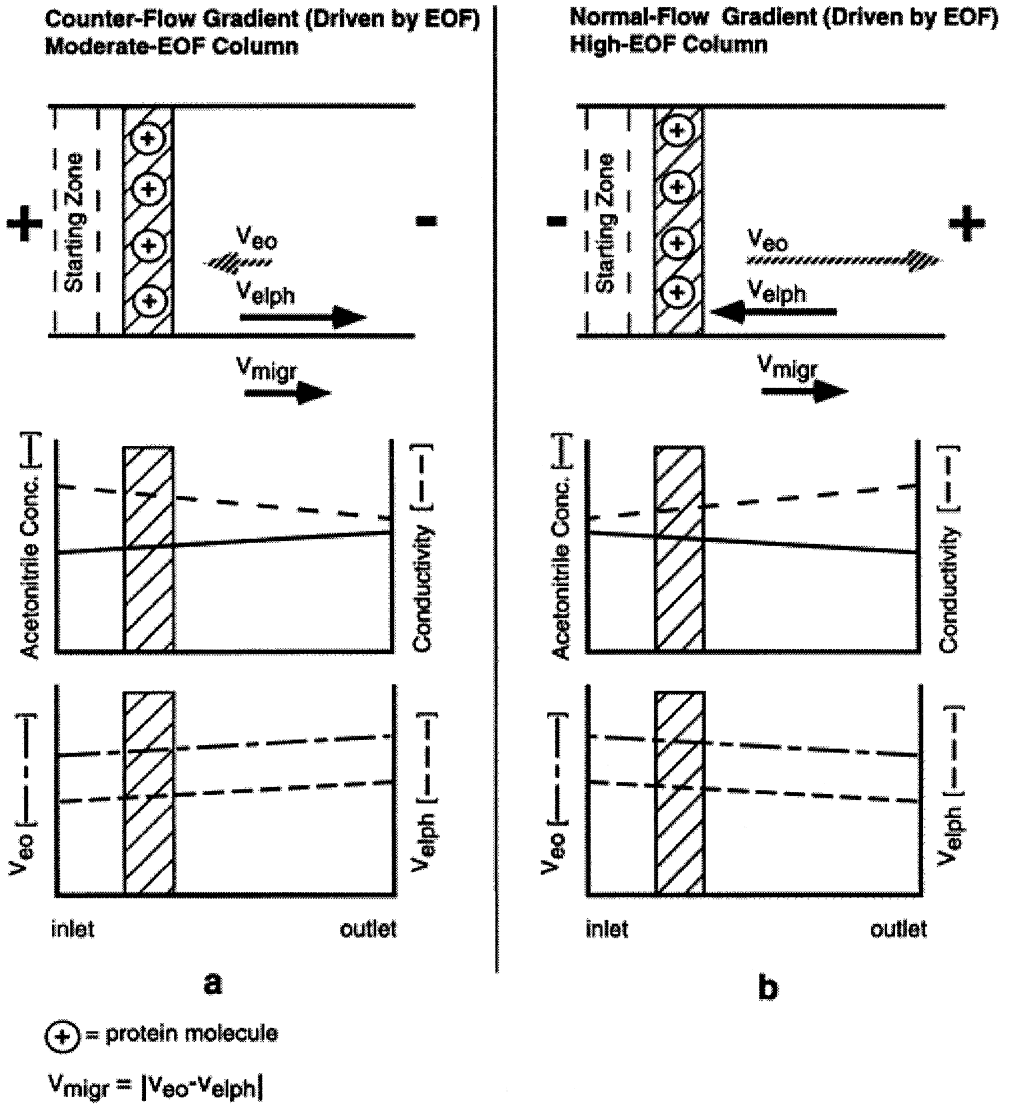


Fig. 19. Diagram illustrating the principle for counterflow (a) and normal-flow (b) gradients (reprinted with permission from Ref. [71]. Copyright 1997 American Chemical Society). The direction of electroosmotic flow is opposite to that of the electrophoretic movement in both methods and is opposite to the net migration velocity, ν_{migr} $(=\nu_{eo}-\nu_{elph})$, in (a) and coincides with the net migration direction in (b). ν_{eo} is not constant along the capillary, and ν_{elph} is higher in the direction of the electrophoretic migration.

charged. Samples were injected at the cathode, and, as with other typical gradient systems, the percentage of acetonitrile was higher at the capillary inlet (cathode) than at the outlet, with detection occurring at the anodic end of the column.

(ii) In contrast, in a monolith containing a moderate number of charged moieties, electromigration of the charged proteins was faster than the electroosmotic flow. In this ''counterflow gradient'' system, the EOF proceeded in the same direction. However, protein analytes were injected at the anode and migrated in a direction opposite to the gradient due to their electrophoretic mobilities. Accordingly, the detector was positioned at the cathode end of the capillary column.

Fig. 20 shows excellent separations of four pro-

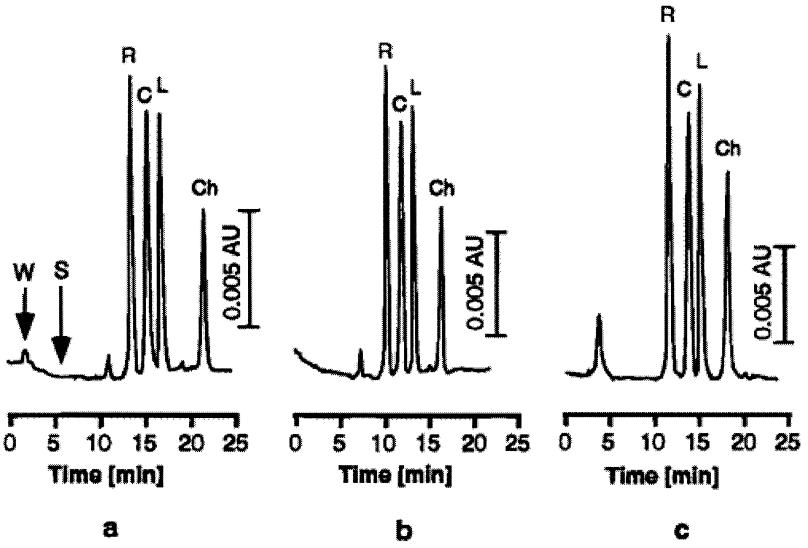


Fig. 20. Chromatograms obtained by gradient electrochromatography (a, b) and reversed-phase chromatography (c) (reprinted with permission from Ref. [71]. Copyright 1997 American Chemical Society). Sample: ribonuclease A (R); cytochrome *c* (C); lysozyme (L); chymotrypsinogen (Ch). Protein concentration: 0.6 mg/ml of each protein except for ribonuclease A (1.8 mg/ml); mobile phase: a linear gradient from 5 to 80% acetonitrile in 5 mmol/l sodium phosphate, pH 2.0; on-tube detection at 280 nm. Columns: 8.0 cm (6 cm effective length)×50 μm I.D.; voltage, 5.5 kV (700 V/cm); (a) moderate-EOF column, (b) high-EOF column, (c) conventional capillary RP-HPLC; pressure, 5 MPa.

teins in both moderate (a) and high EOF columns (b) using electrically driven flow and compares the separation with that achieved using standard HPLC methodology (c). Since the separations were primarily governed by the nature of the mobile phase gradient, all three chromatograms are very similar. This comparison also demonstrates that the separation is achieved via reversed-phase partitioning rather than electrophoresis.

It should be noted that this successful approach to monolithic CEC column for the separation of proteins employs a polymeric matrix containing positively charged moieties. Since the overwhelming majority of proteins also possess net positive charges at pH 2, the potential for undesired electrostatic interactions is greatly diminished. The occurrence of very strong electrostatic interactions most likely explains why all attempts to separate proteins on columns containing carboxylic or sulfonic acid moieties at pH values below the p*I* have failed to date [49].

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 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 side groups pK_a values of 10.07, 10.53 and 12.48, respectively, 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.

10. Conclusion

Although considerable progress has been made in the preparation of packed CEC columns, technical problems such as in situ formation of retaining frits and column packing methods continue to slow the general acceptance of the CEC technique. Consequently, monolithic stationary phases have emerged

as attractive alternatives, due to the simplicity of their preparation and the virtually unlimited choice of chemistries they offer. This technology completely eliminates the need for retaining frits and their associated problems, since the dimensional stability of the monolithic structures result from their rigidity and/or chemical attachment to the inner wall of the capillary. Additionally, these materials can easily be prepared even within channels of very narrow size by in situ polymerization, suggesting that this technology may uniquely be suited for the further development of on-chip miniaturized analytical systems.

Currently, numerous approaches to monolithic columns for CEC are under development. In addition to monoliths based on synthetic polymers presented in this review, entirely inorganic analogs have recently been reported [24,41–44]. Hybrid methods and alternative column technologies have also been described, including the immobilization of molecularly imprinted particles within a polymer gel matrix [99], the use of in situ prepared polymer monoliths as retaining frits for packed beds [100], and ''hanging'' cellulose acetate fibers anchored at one end in the porous silicate inlet frit [101].

Although much remains to be done in this area of materials research, the various monolithic approaches appear to best address the unique identity of CEC that is distinct from its parents HPLC and electrophoresis, promising the preparation of reproducible, well defined, highly efficient, and easily prepared capillary columns for CEC in a variety of chromatographic modes.

Acknowledgements

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