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Capillary electrochromatography in anion-exchange and normal-phase mode using monolithic stationary phases

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Abstract

Hydrophilic macroporous weak and strong anion-exchange stationary phases have been prepared in a monolithic format within untreated fused-silica capillaries by the simple thermally or UV-initiated polymerization of 2-dimethylaminoethyl methacrylate, 2-hydroxyethyl methacrylate and ethylene dimethacrylate in the presence of a binary porogenic mixture of dodecanol and cyclohexanol. The tertiary amino functionalities were then alkylated in situ to afford strong anion-exchangers. These new monolithic stationary phases with optimized porous properties were used for the CEC separation of various organic anions. Thus, a mixture of 2-substituted propionic acid drugs (profens) was separated in 13 min and high column efficiencies of up to 231 000 plates/m were achieved. The separation of substituted benzoic acids indicates that the selectivity results primarily from the anion-exchange interactions, while electrophoretic migration contributes only slightly. In addition, these hydrophilic anion-exchangers are also able to separate weakly acidic, neutral and basic compounds such as phenols, xanthines and aromatic amines in normal-phase electrochromatographic mode.

Keywords: Stationary phases, electrochromatograpy; Electrochromatography; Monoliths; Profens; Benzoic acids; Porous polymers

1. Introduction

Rapid progress in decoding the human genome, the advent of proteomics, and the new approaches to drug discovery have accelerated the development of separation techniques that use the separation devices in capillary formats. These techniques, including electrophoresis, liquid chromatography, and capillary electrochromatography (CEC), are attractive alternatives to typical HPLC as they require small amounts of samples and do not generate vast amounts of waste solvents. CEC is the youngest member of this family of separation techniques and includes some features typical of both electrophoresis and chromatography. For example, it involves the use of an electric field as the driving force to transport the mobile phase and analytes through the column, while it also achieves separation in a column containing a stationary phase. Most of the current CEC columns are fused-silica capillaries

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packed with silica beads [1]. However, the fabrication of packed CEC columns is tedious since it includes a number of steps that may negatively affect the column-to-column reproducibility [2]. Therefore, extensive research targets both the development of new technologies for the repetitive preparation of columns and the preparation of stationary phase in novel shapes.

Monolithic stationary phases prepared directly by polymerization within the confines of the capillary are one of these alternative approaches to packed capillary columns. The monoliths are a single piece of porous polymer that fills completely the capillary column thus eliminating the need for retaining frits. The monolithic technology was originally developed for ''classical'' HPLC [3–10] and later adapted to the CEC column format [11–17].

The vast majority of reports on CEC concerns separations in reversed-phase mode. This well-known HPLC mechanism is easily amenable to CEC and requires separation media containing both hydrophobic chains interacting with the analytes and negatively charged functionalities that support the cathodic electroosmotic flow. In contrast, much less has been done with columns featuring positively charged functionalities. Most often, these capillary columns are packed with silica beads modified with typical anion-exchange groups [18–27]. However, these packings are not typical anion-exchangers since they contain both the negatively charged silanol groups and positively ionizable amine functionalities. This amphoteric character is often detrimental in achieving stable EOF. The flow direction is pH dependent with considerable instability in the pH region where EOF changes from cathodic to anodic [19]. Moreover, the EOF direction also depends on the type of silica and extent of its modification [21]. In contrast, synthetic polymer-based anion-exchangers involve only a single type of functionality and the matrix is stable even in solutions with high pH values as has been demonstrated for both beads [28] and monolithic stationary phases [15,29,30]. The use of monolithic polymer capillary columns eliminates completely both the problems of residual silanol groups and of in situ fabrication of the retention frits. Another advantage of synthetic polymers is that the percentage of ionizable monomer in the polymerization mixture can easily be controlled by changing the number of charged groups located on the pore surface. Thus, both ion-exchange capacity and EOF can be easily varied.

Monolithic weak anion-exchange stationary phases for HPLC were prepared from poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monoliths by ring-opening reaction of the pendant epoxide groups with diethylamine [31,32]. Using a similar approach, monolithic capillary columns for CEC were obtained by opening the epoxide groups with *N*-ethylbutylamine [33] or using strong anion-exchange-type monoliths based on a polystyrene backbone [29]. Horvath et al. prepared poly(chloromethylstyrene-*co*-divinylbenzene) monoliths and functionalized them on-column with *N*,*N*-dimethyloctylamine to highly hydrophobic strong anion-exchangers useful for peptide separation at acidic pH (pH 3). However, the post-functionalization strategies do not allow precise control over the number of ionizable functionalities. Two studies have reported the direct incorporation of cationic moieties into continuous polymer matrices [30,34]. In one of these studies, the incorporated ammonium groups were employed to generate anodic EOF without being directly involved in the chromatographic separation process [34]. We have demonstrated the preparation of hydrophilic chiral anion-exchange monoliths and their use for the enantioselective separations of acidic compounds. The positively charged chiral quinidine-based ligands of these monoliths served a dual function: (i) they created anodic EOF and (ii) performed chiral recognition [30,35].

This report demonstrates the preparation of both hydrophilic weak and strong anion-exchange monoliths by direct copolymerization of 2-dimethylaminoethyl methacrylate with 2-hydroxyethyl methacrylate and ethylene dimethacrylate. This approach simplifies the control of the anion-exchange capacity of the separation media and also enables adjustment of parameters such as surface polarity, extent of crosslinking, and porous properties that all have a significant effect on the performance of a stationary phase in the CEC mode.

2. Experimental

2.1. Materials

2-(*N*,*N*-Dimethylamino)ethyl methacrylate (**1**) was purchased from Polysciences (Warington, PA, USA).

2-Hydroxyethyl methacrylate (**2**) (HEMA), ethylene dimethacrylate (**3**) (EDMA), cyclohexanol, 1-dodecanol, and azobisisobutyronitrile (AIBN) were obtained from Aldrich. Fused-silica capillaries (100 μm I.D.) with both conventional polyimide and UV-transparent fluorinated hydrocarbon polymer coatings were from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Column preparation

Monolithic columns were prepared from polymerization mixtures consisting of 40 wt% monomers and 60 wt% porogens (1-dodecanol and cyclohexanol) using AIBN (1 wt% with respect to the monomers) as an initiator. Weak anion-exchange monolithic stationary phases were obtained by copolymerization of ionizable monomer **1**, with **2** and **3** (Fig. 1a) using conditions shown in Table 1. Strong anion-exchange monoliths were obtained by alkylation of the weak anion-exchangers prepared by copolymerization of **1** using dimethyl sulfate as the alkylation agent (Fig. 1b). The polymerization mixtures were sonicated for 10 min to obtain clear solutions, and then purged with nitrogen for 10 min. Using a 100 μL syringe and a short Teflon sleeve, the deaerated mixtures were transferred in 50 cm long pieces of fused-silica capillaries. Typically, only a 35 cm long segment was filled with the polymerization mixture. The capillaries were then sealed at both ends with rubber stoppers. Polyimide-coated capillaries were used for thermally initiated polymerizations. Sealed capillaries and a glass vial containing the remaining polymerization mixture were submerged into a water bath and allowed to react for 20 h at 60°C. UV-initiated polymerizations were carried out using capillaries with fluorinated hydrocarbon coating while the remaining polymerization mixture was polymerized in a quartz glass tube sealed with a Teflon tape. Capillaries and the tube were placed in a box equipped with two 8 W UV lamps (VWR Scientific Products) and irradiated at room temperature for 16 h.

Macroscopic monolithic materials prepared by the bulk polymerization in vials or tubes were removed from the container, cut into small pieces, Soxhlet extracted with methanol for 12 h, and dried in vacuum at 60°C. These polymers were used for the porosimetric measurements and elemental analysis. The monolithic capillary columns were washed with an 80:20 acetonitrile–methanol mixture containing 0.4 mol/L acetic acid and 4 mmol/L triethylamine using a syringe-type micropump (Model 260D, ISCO, Lincoln, NE, USA) to remove unreacted

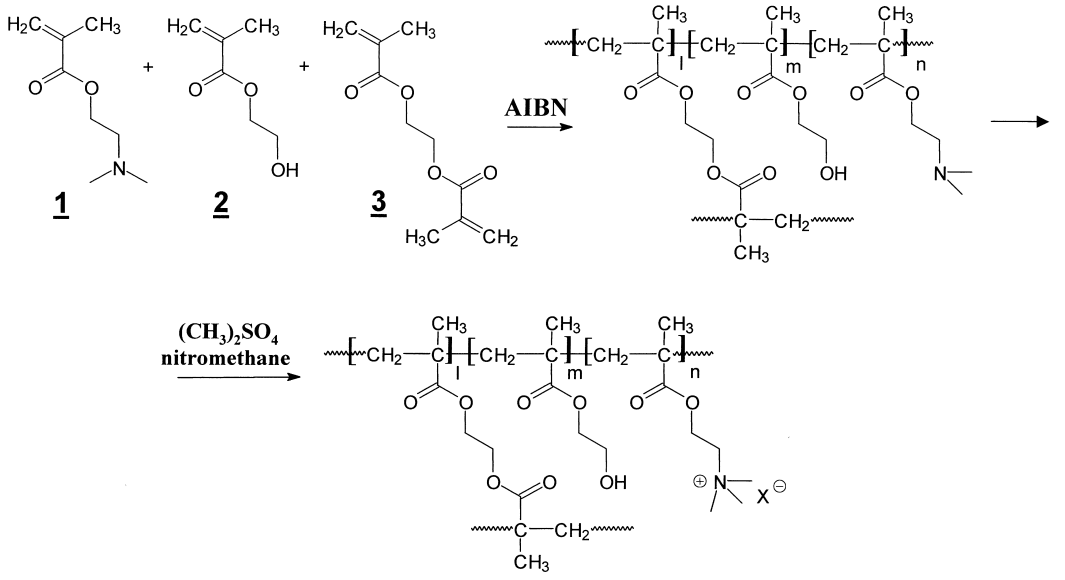

Fig. 1. Preparation of weak and strong anion-exchange monoliths by copolymerization of 2-dimethylaminoethyl methacrylate (**1**), 2-hydroxyethyl methacrylate (**2**) and ethylene dimethacrylate (**3**) followed by a quaternization with dimethyl sulfate.

Table 1
Compositions of the polymerization mixtures for the preparation of monolithic capillary columns and physical properties of the monoliths

DMAEMA[a] (wt%)	HEMA[a] (wt%)	EDMA[a] (wt%)	Cyclohexanol (wt%)	1-Dodecanol (wt%)	V_{pore}[c] (mL/g)	$d_{p,mode}$[c] (nm)	S[c] (m^2/g)	C_{tot}[c] (mmol/g)
Thermal polymerization[b]								
8.2	28.1	4.2	29.6	29.9	Nonporous	0	0	1.16
8.0	28.0	4.1	35.0	25.0	Nonporous	0	0	1.24
12.0	24.1	4.3	29.8	29.8	Nonporous	0	0	1.81
12.0	23.9	4.3	25.0	35.0	Nonporous	0	0	1.76
4.1	28.0	8.1	30.1	29.8	1.04	632	4.7	0.66
4.1	28.0	8.2	20.0	39.7	0.95	1807	<1	0.66
8.0	24.1	8.0	30.0	30.0	0.47	90	7.3	1.26
8.0	23.9	8.1	20.0	40.0	1.16	2247	<1	1.12
12.0	20.0	8.3	30.0	29.7	Nonporous	0	0.0	1.79
11.9	20.2	8.2	19.8	39.9	0.49	163	4.6	0.82
4.1	20.0	16.3	29.7	30.0	1.17	108	32.8	0.71
4.3	20.1	16.0	15.0	44.6	1.32	2093	–	0.66
7.9	16.2	15.9	30.0	30.0	0.93	54	44.0	1.26
8.3	15.9	16.2	14.9	44.7	1.44	415	11.5	1.26
Photopolymerization[b]								
8.2	28.1	4.2	29.6	29.9	0.77	1357	1.9	1.21
8.0	28.0	4.1	35.0	25.0	0.99	1574	1.8	1.17
12.0	24.1	4.3	29.8	29.8	0.64	765	1.9	1.78
12.0	23.9	4.3	25.0	35.0	1.02	1942	1.1	1.72
4.1	28.0	8.1	30.1	29.8	0.96	1298	1.0	0.63
4.1	28.0	8.2	20.0	39.7	0.91	1071	2.4	0.69
8.0	24.1	8.0	30.0	30.0	1.04	1298	1.0	1.23
8.0	23.9	8.1	20.0	40.0	1.03	1423	1.8	1.24
12.0	20.0	8.3	30.0	29.7	0.69	185	8.6	1.82
11.9	20.2	8.2	19.8	39.9	1.20	1689	1.5	1.46
4.1	20.0	16.3	29.7	30.0	1.19	1179	3.5	0.68
4.3	20.1	16.0	15.0	44.6	1.27	1569	1.7	0.72
7.9	16.2	15.9	30.0	30.0	1.23	669	6.0	1.27
8.3	15.9	16.2	14.9	44.7	1.40	2094	1.8	1.29

[a] DMAEMA, 2-dimethylaminoethyl methacrylate; HEMA, 2-hydroxyethyl methacrylate; EDMA, ethylene methacrylate.
[b] Thermal polymerization at 60°C for 20 h, photopolymerization at room temperature for 16 h.
[c] V_{pore}, pore volume; $d_{p,mode}$, pore diameter at maximum of the distribution curve; S, BET surface area; C_{tot}, total amount of amino groups incorporated in the monolith determined by elemental analysis of nitrogen.

monomers and porogens. Typically, backpressures of 13.8–20.7 MPa were observed at flow-rates of 1–3 μL/min. All capillary columns tolerated these high pressures without extrusion or visible compression of the monolith. The capillaries were then cut at both ends to a total length of 33.5 cm and a bed length of 25 cm, leaving an 8.5 cm long open segment between the detection window and the outlet end. Capillaries with the UV-transparent coating were placed directly in the alignment interface of the HP cassette, while a detection window was first created on polyimide-coated capillaries at the end of the continuous bed using a razor blade.

2.3. In situ alkylation

The monolithic poly(dimethylaminoethyl methacrylate-*co*-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) capillaries were first washed with 200 μL acetonitrile, then with 200 μL nitromethane and finally filled with 200 μL of a 25% (v/v) nitromethane solution of dimethyl sulfate using a syringe pump (kdScientific, New Hope, PA, USA). The ends of the capillary were then sealed with rubber stoppers and the alkylation reaction allowed to proceed for 12 h at room temperature followed by 30 min heating to 50°C in a water bath. The contents

of the capillary were then removed by pressurized washing with acetonitrile after the reaction time elapsed.

2.4. Characterization of porous properties

The pore size distribution of the monolithic material was determined using an Autopore III 9400 mercury intrusion porosimeter (Micromeritics, Norcross, GA, USA). Specific surface areas were calculated from data obtained by nitrogen adsorption/desorption (ASAP 2010, Micromeritics) using the BET equation.

2.5. Capillary electrochromatography

CEC experiments were carried out using a HP ^{3D}CE capillary electrophoresis instrument (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode-array detector and an external pressurization system. An equal pressure of 0.6 MPa was applied at both ends of the capillary column. The typical mobile phase consisted of acetonitrile–methanol (80:20, v/v), containing 0.4 mol/L acetic acid and 4 mmol/L triethylamine as electrolytes. The sample solutions (0.5 mg/mL) were injected electrokinetically (−5 kV for 5 s), and the separations performed at a voltage of −25 kV while the cassette compartment temperature was adjusted to 50°C. The peaks were monitored at a wavelength of 250 nm and processed by the HP ChemStation software. Acetone was used as an EOF marker.

3. Results and discussion

3.1. Preparation of monoliths with weak anion-exchange functionalities

The preparation of the monolithic capillary columns is a simple process. The polymerization mixture consisting of monomers and porogens is introduced into the capillaries with a syringe, and polymerized by applying UV light or heat. In contrast to the often recommended methacryloylation treatment of the inner walls to enhance adhesion [29], untreated silica capillaries can be used for the preparation of our monolithic CEC columns with amine functionalities. The electrostatic interactions that prevail between the silanol groups of the silica capillary and the amine functionalities of the polymer monolith appear to be sufficiently strong to negate void formation as a result of shrinkage typical of polymerizations as well as displacement during the pressurized washing. We observed that these monolithic columns can endure pressures of over 20 MPa without any damage.

The polymer matrix of monolithic CEC columns with ion-exchange functionalities described by other groups is hydrophobic, typically based on poly(styrene-*co*-divinylbenzene) [29] or polymethacrylate with lipophilic chains [33]. Our monoliths are much more hydrophilic as they mainly consist of 2-dimethylaminoethyl methacrylate and 2-hydroxyethyl methacrylate (Fig. 1). The porous structure that determines the chromatographic properties of the stationary phase is controlled by a variety of parameters such as the percentage of monomers and crosslinker in the polymerization mixture, the type of initiation (thermal or UV), and the porogens. We have found that a mixture of cyclohexanol and dodecanol is an effective porogen for the preparation of monolithic poly(2-dimethylaminoethyl methacrylate-*co*-2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) stationary phases. Examples of the pore size distribution profiles for these monoliths measured by mercury intrusion porosimetry in the dry state are shown in Fig. 2. The curves exhibit a rather narrow large peak in the range typical of macropores (pore diameter larger than 50 nm) while no appreciable volume of mesopores in the range of 2–50 nm is observed. Specific surface areas of these monoliths are typically less than 10 m^2/g (Table 1).

Both photopolymerization at room temperature and thermally initiated polymerization were used to prepare monolith from each polymerization mixture. Since the reaction temperatures are 25 and 60°C, respectively, for these two processes, monoliths with different porous properties are obtained (Table 1). For example, the thermal polymerization of a reaction mixture consisting of 4% 2-dimethylaminoethyl methacrylate (corresponding to 10% of total monomers), 20% 2-hydroxyethyl methacrylate, 16% ethylene dimethacrylate, 30% cyclohexanol, and 30% dodecanol affords a monolith with a modal pore size (pore diameter at the maximum of the distribution curve) of 108 nm (Fig. 2a, monolith 1). In contrast, UV polymerization of the same mixture

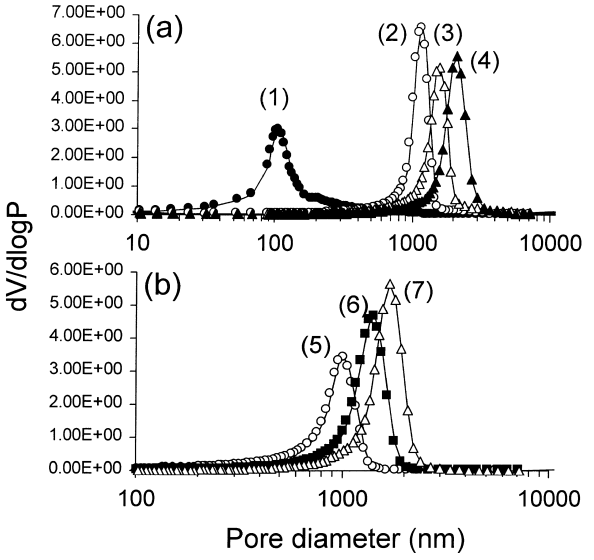

Fig. 2. Pore size distribution profiles of poly(2-dimethylaminoethyl methacrylate-*co*-2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) and blank poly(2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) monoliths as measured by mercury intrusion porosimetry. Polymerization mixtures: (a) 2-dimethylaminoethyl methacrylate 4%, 2-hydroxyethyl methacrylate 20%, and ethylene dimethacrylate 16%, cyclohexanol 30 (curves 1 and 2) or 15% (curves 3 and 4), 1-dodecanol 30 (curves 1 and 2) or 45% (curves 3 and 4). Thermal polymerization (curves 1 and 4): 20 h at 60°C; UV-initiated polymerization (curves 2 and 3): 16 h at room temperature. (b) 2-Dimethylaminoethyl methacrylate/2-hydroxyethyl methacrylate 0/32 (curve 5), 8/24 (curve 6), and 12/20% (curve 7), ethylene dimethacrylate 8%, cyclohexanol 20%, 1-dodecanol 40%, UV-initiated polymerization at room temperature for 16 h.

leads to a monolith with modal pore size of 1179 nm (Fig. 2a, monolith 2) as a result of the lower temperature used for the polymerization [36]. Using a higher percentage of dodecanol (more than 40 wt%) in the polymerization mixture compensates for this effect. Under these conditions, the pore size distribution curves for both monoliths 3 and 4 shown in Fig. 2a prepared by UV and thermally initiated polymerization, respectively, are similar. Fig. 2 once again confirms our earlier observation that the higher the percentage of dodecanol in the polymerization mixture, the larger the pores regardless of the initiation mechanism [37].

The direct preparation of functionalized monoliths allows broad variations in the contents of ionizable groups and good control over both the ion-exchange capacity and the magnitude of the electroosmotic flow. Nitrogen elemental analysis can be used to confirm that the functional monomer 2-(dimethylamino)ethyl methacrylate has been completely incorporated into the monolith. For example, monoliths with 0.7–2.0 mmol/g of amine functionalities are easily prepared using a polymerization mixture containing 4–12% **1** (Table 1). Obviously, changes in monomer composition trigger changes in porous properties of the monolithic material. To demonstrate this we used a polymerization mixture containing 40% dodecanol, 20% cyclohexanol, 8% ethylene dimethacrylate, and 32% monovinyl monomers **1** and **2** in various proportions. Fig. 2b shows that the modal pore size of a monolith prepared from a mixture containing 32% **2** is 1025 nm while this size is 1423 and 1689 nm for polymerization mixtures consisting of 8% **1**+24% **2** and 12% **1**+20% **2**, respectively.

Table 1 also demonstrates the considerable effect of the level of crosslinking on porous properties of the monoliths. While a macroporous structure is not obtained in the thermal polymerization of reaction mixtures containing only 4% ethylene dimethacrylate (10% with respect to total monomers), UV polymerization of the same mixture does afford monoliths with macropores allowing flow-through even from such mixtures poor in crosslinker. As expected from our previous studies [37], the pore size decreases as the percentage of crosslinker in the polymerization mixture increases.

Table 1 also demonstrates the effect of the composition of the polymerization mixture on pore volume. In theory, the overall pore volume should closely match or even exceed that of the porogenic solvents, which, in this study, represent 60% of the original mixture. This is generally true for monoliths prepared from polymerization mixtures within the range that safely affords macroporosity. However, the phase separation and development of the porous structure during polymerization of mixtures laying at the borderlines, such as those containing only 10% EDMA and/or high contents of the ionizable monomer, is less than ideal and the complete separation of the ''gel'' phase from the solvent phase may occur. This results in the creation of monoliths with pore volumes smaller than the theoretical value or even completely nonporous monoliths.

The porous properties are measured in the dry state and may not fully correspond to those of the

monoliths upon conditions of their use in CEC column. Unfortunately no better method for the determination of porous properties of monolithic polymers within the capillary column is currently available. Despite this drawback, mercury intrusion porosimetry affords values that can be easily visualized and compared.

3.2. Preparation of monoliths with strong anion-exchange functionalities

The monoliths with weak anion-exchange functionalities can also serve as precursors for the monolithic strong anion-exchangers. Using the on-column alkylation reaction shown in Fig. 1, monolithic columns with quaternary ammonium groups are easily obtained that have porous properties identical to those of the weak anion-exchangers from which they originated. This approach enables a direct comparison of monolithic columns with both types of anion-exchange functionalities and identical porous properties.

Stable electroosmotic flow (EOF) affording sufficient flow-rate is the key requirement for the application of monolithic columns in CEC. The magnitude of EOF depends on the ζ-potential of the stationary phase, which is determined by properties of the mobile phase such as the dielectric constant-to-viscosity ratio, ionic strength, and pH as well as the surface charge density of the stationary phase. Our preparation method enables a facile control of the number of ionized surface functionalities. We have shown previously that only 0.3% of 2-acrylamido-2-methylpropanesulfonic acid (AMPS) units incorporated into the polymer is sufficient to afford the EOF required for very efficient CEC separations in reversed-phase mode [14]. In contrast to the AMPS units that only provide the driving force affording EOF, the anion-exchange functionalities of the present monolithic capillary columns have a dual function: (i) they support the electroosmotic flow and (ii) are the primary interaction sites for the chromatographic separation. Therefore, a much higher coverage of their surface with ionogenic sites is required. Our chromatographic results (see below) with the monoliths shown in Table 1 indicate that the optimum content of 2-dimethylaminoethyl methacrylate in the monolith is 10–20%. These columns exhibit reasonable EOF, good selectivity, and acceptable retention times. Any higher percentage of **1** leads to excessively long retention times and decreases the throughput of the separation system.

Strong selector–analyte interactions typical of ion-exchange CEC mode are often accompanied by relatively long run times for columns with the standard bed lengths of 25 cm which is dictated by requirements of the common HP ^{3D}CE instrumentation. Since the resolution of our monolithic columns is sufficiently high, the long run times can be decreased by simply reducing the length of the monolithic segment while increasing the length of the open segment as has been demonstrated in our previous work [30].

The pH value of the mobile phase affects the ionization of the ion-exchange functionalities and therefore also the electroosmotic flow. Both types of ion-exchangers are quaternized in acidic solutions. Therefore, both types of monolithic columns support anodic EOF. However, Fig. 3 shows that the EOF created by the weak anion-exchanger is rather unstable and varies widely with pH. In contrast, the EOF generated by the strong anion-exchangers is always higher and the working range of these capillary columns even extends to alkaline pH values (Fig. 3).

3.3. Effect of stationary phase on CEC performance

The strong effect of pore size on the EOF created within the monolithic columns with positively charged fixed ions is similar to that observed in our previous studies with reversed-phase and enantioselective monolithic columns [15,30]. For example, Fig. 4a shows that a moderate increase in the modal pore diameter from 1071 to 1298 nm leads to a considerable 25% increase in the linear flow velocity from 1.5 to 2 mm/s at an electric field strength of 845 V/cm (curves 3 and 2). This effect likely reflects changes in the morphology of the monoliths. Assuming identical ζ-potential for both monoliths, changes in EOF result from differences in conductivity. The monolith with smaller pores and higher tortuosity has lower permeability which affects EOF velocity. This effect can be quantified in terms of electrokinetic permeability, which is proportional to the slope of the plot of flow velocity vs. field strength (Fig. 4a) [38]. In addition, the path

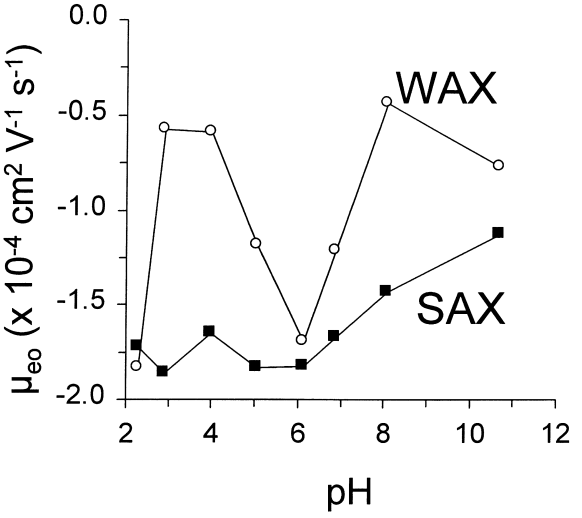

Fig. 3. Effect of pH of the aqueous buffer on the electroosmotic mobility in weak and strong anion-exchange-type monoliths. Conditions: weak anion-exchange monolith, 2-dimethylaminoethyl methacrylate 4%, 2-hydroxyethyl methacrylate 20%, and ethylene dimethacrylate 16%, cyclohexanol 15%, 1-dodecanol 45%; UV polymerization 16 h at room temperature; $d_{p,mode} = 1569$ nm; strong anion-exchange monolith was prepared by on-column alkylation of the above monolith. Column dimension: inner diameter 0.1 mm, total length 335 mm, active length 250 mm. Mobile phase: 5 mmol/L phosphate–acetonitrile (20:80), pH of phosphate buffer adjusted with 5 mmol/L phosphoric acid or concentrated sodium hydroxide, voltage −15 kV, injection −5 kV for 5 s, separation temperature 50°C, UV detection at 250 nm.

length through the more tortuous flow channels of the monolith with smaller pores is longer. Therefore, the electric field strength, which is the driving force of EOF, defined as the applied voltage divided by the length of the path, is lower and, consequently, the flow velocity is slower.

Flow velocities also are significantly affected by the percentage of crosslinking monomer in the polymerization mixture. Fig. 4a also shows the effect of ethylene dimethacrylate on the linear flow velocity in two monolithic capillary columns with a modal pore size of 1100 nm as measured in the dry state. At an electric field strength of 845 V/cm, the flow velocity is 2.55 mm/s for the monolith with 40% ethylene dimethacrylate (plot 1) while it is only 1.56 mm/s once the percentage of crosslinking monomer is decreased to 20% (plot 3). Since the crosslinking density affects swelling, the material of the less crosslinked monolith swells to a larger extent. As the monolith is enclosed in the capillary, it cannot increase its radial size and the swollen polymer fills the macropores thus decreasing their volume. The lower the crosslinking, the smaller the pore size in the swollen state. This decrease in permeability also results in lower flow-rate.

Van Deemter plots for a retained solute, *p*-toluic acid, document significant effects of both crosslinking and pore size on the column efficiency (Fig. 4b). Although both monoliths 2 and 3 contain 20% ethylene dimethacrylate, they differ in their pore size distribution. Clearly, a decrease of only about 200 nm in mode pore size from 1298 nm (monolith 2) to 1071 nm (monolith 3) is accompanied by a dramatic increase in column efficiency. Contributions of both flow maldistribution due to bed non-uniformities (*A* term) and mass transfer resistance (*C* term) to chromatographic band spreading decrease significantly affording 110 000 theoretical plates/m with the plate height at the minimum of the curve being 9 μm. Comparison of the chromatographic properties for monoliths featuring the same ''dry'' pore size of about 1100 nm (monoliths 1 and 3) proves the poorer performance of the more crosslinked monolith (monolith 1). Similar effects were also observed in our previous study of a monolithic enantioselective CEC column [35]. This suggests that the presence of a less crosslinked, perhaps gel-like, layer of swollen polymer lining pores of the monolith enhances the chromatographic performance. The theoretical considerations presented recently by Liapis support our hypothesis [39].

3.4. Effect of the mobile phase

The choice of mobile phase in CEC is primarily determined by the chromatographic requirements of retention, selectivity, efficiency, and flow velocity. With our anion-exchange stationary phases non-aqueous mobile phases are preferred due to their lower conductivity enabling use of the higher buffer concentrations required to balance the strong ionic interactions between solutes and sorbent, without creating excessive Joule heating. Since EOF is directly proportional to the ratio of dielectric constant/viscosity, organic solvents with lower viscosities also sustain higher flow velocities. Table 2 summarizes the results of CEC separations achieved using different mixtures of acetonitrile–methanol. We have also observed in our previous studies

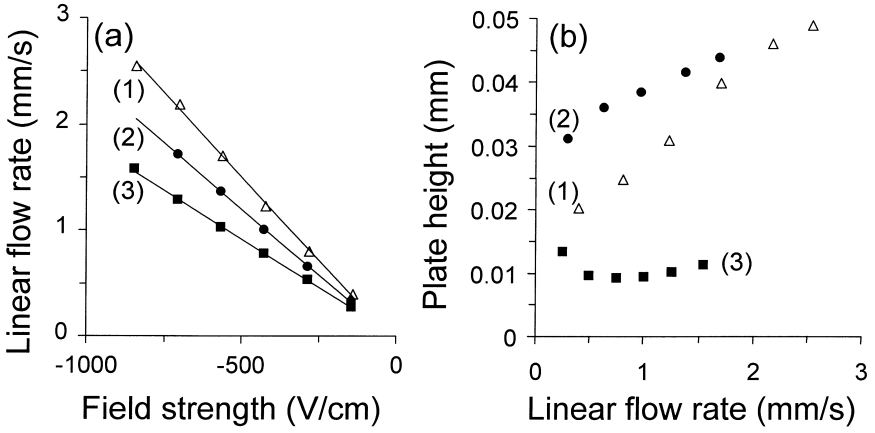

Fig. 4. Effect of crosslinking and pores size of strong anion-exchange monoliths on linear flow velocities (a) and theoretical plate heights for *p*-toluic acid (b). Conditions: on column alkylated monolith prepared from mixtures consisting of 2-dimethylaminoethyl methacrylate 4 wt%, 2-hydroxyethyl methacrylate 20 (1), and 28% (2,3), and ethylene dimethacrylate 16 (1), and 8% (2,3), cyclohexanol 30 (1,2), and 20% (3), 1-dodecanol 30 (1,2), and 40% (3); UV-initiated polymerization at room temperature for 16 h; $d_{p,mode}$ = 1179 (1), 1298 (2), 1071 (3) nm. Column dimensions: inner diameter 0.1 mm, total length 335 mm, active length 250 mm. Mobile phase: 0.4 mol/L acetic acid and 4 mmol/L triethylamine in acetonitrile–methanol (80:20), injection −15 kV for 5 s, temperature 50°C, void marker acetone, UV detection at 250 nm.

[20,30] that the composition of the mobile phase has a paramount effect on the electroosmotic flow velocity, retention, as well as column efficiency. The solvent affects the physico-chemical properties of both solutes and stationary phase through ion-solvation, pK_a shifts of mobile and fixed charges, changes in dielectric constant, and viscosity. As a consequence, these effects play a significant role in electroosmotic, electrophoretic, and chromatographic processes. Since the dielectric constant/viscosity ratio of methanol–acetonitrile mixtures seems to reach its maximum in a mobile phase containing

Table 2
Effect of methanol in methanol/acetonitrile mobile phase on chromatographic properties of strong anion exchange monolithic CEC columns[a]

Methanol %	I[b] μA	pH_a[c]	u[d] mm/s	Acetone		Phenol			4-Toluic acid		
				t_0, min	N^e, m^{-1}	t_r, min	k_{app}[f]	N^e, m^{-1}	t_r, min	k_{app}[f]	N^e, m^{-1}
0	−4.0	4.7	0.94	4.78	79 210	8.93	0.87	62 010	15.42	2.22	58 550
20	−10.1	6.2	0.97	4.62	81 380	7.41	0.60	104 300	13.49	1.92	85 080
40	−19.4	5.6	0.81	5.58	70 740	7.67	0.38	86 600	11.29	1.03	96 540
60	−22.5	5.4	0.68	6.59	78 510	8.61	0.31	92 460	11.51	0.75	95 180
80	−21.2	5.2	0.58	7.74	89 580	10.06	0.30	79 090	13.35	0.73	83 280
100	−14.4	4.9	0.47	9.67	73 110	12.48	0.29	64 650	15.47	0.60	78 420

[a] Conditions: Monolithic column prepared from polymerization mixture consisting of 2-(dimethylamino)ethyl methacrylate 8%, 2-hydroxyethyl methacrylate 24%, ethylene dimethacrylate 8%, cyclohexanol 20%, 1-dodecanol 40% and alkylated on-column; UV polymerization at room temperature for 16 h, d_p, mode = 1423 nm. Column dimension: inner diameter 0.1 mm, total length 335 mm, active length 250 mm.

[b] Electrical current.

[c] Apparent pH measured in the non-aqueous solution.

[d] Flow velocity.

[e] Column efficiency.

[f] Apparent retention factor $k_{app} = (t_r - t_0)/t_0$ where t_0 is the retention time of acetone and t_r are the retention times of specific analytes.

20% methanol, EOF reaches its maximum (Table 2). At higher methanol contents, the linear flow velocities decrease as the viscosity of the mobile phase increases significantly. Although more polar phases with higher methanol contents have higher elution strength and enable significant reduction in apparent retention factors k_{app}, the shortest elution times are mostly achieved with the mobile phase containing 20% methanol since flow velocity is highest with this mixture (Table 2). The column efficiency varies with the type of analyte. The optimal mobile phase (20–40% methanol) simultaneously affords high flow-rates, short run times, and column efficiencies in the range of 100 000 plates/m.

Fig. 5 compares the separation of a mixture of substituted benzoic acids in two different mobile phases containing 20 and 40% methanol. Although the retention times are significantly shorter in the solvent mixture containing the higher percentage of methanol and even enable elution of 3,5-dihydroxybenzoic acid in less than 60 min, the concomitant changes in selectivity may preclude using this approach to accelerate the analysis. Therefore, other means such as the use of columns with a reduced length of the monolithic segment are better suited to achieve faster separations [30]. The high resolving power characteristic of this monolithic stationary phase reflects both its high efficiency and its good selectivity.

3.5. Effect of temperature

Temperature affects a number of system variables such as viscosity of the mobile phase, ζ-potential, and strength of the solute–stationary phase interactions. For example, Fig. 6 shows an increase in the electric current from −13 μA at 20°C to −20.5 μA at 30°C (0.7 μA/°C), indicating lower flow resistance at elevated temperatures. Since the viscosity of the mobile phase is lower while the ζ-potential of the stationary phase is higher at higher temperatures, temperature can be used to modulate flow velocity. Thus, an increase in temperature from 15 to 60°C almost doubles the flow velocity from 0.78 to 1.30 mm/s (Fig. 6a). In addition, solute–sorbent interactions are also weaker at elevated temperatures, which

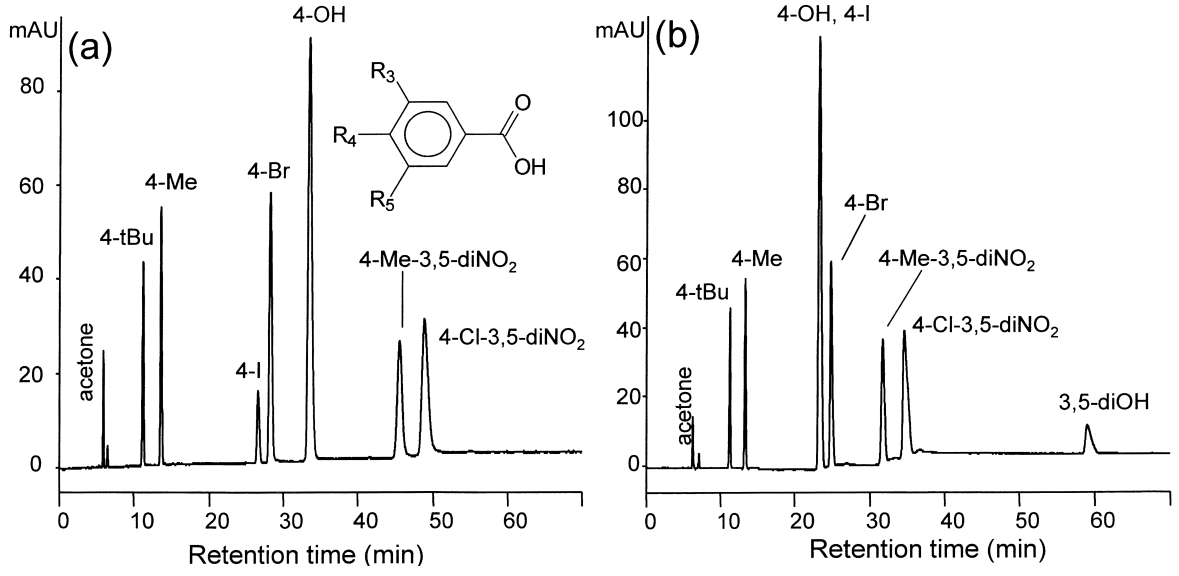

Fig. 5. Effect of methanol content in the non-aqueous polar organic mobile phase on the CEC separation of substituted benzoic acids. Conditions: on column alkylated monolith prepared from mixtures consisting of 8% 2-dimethylaminoethyl methacrylate, 24% 2-hydroxyethyl methacrylate, 8% ethylene dimethacrylate, 20% cyclohexanol, 40% 1-dodecanol; UV-initiated polymerization at room temperature for 16 h; $d_{p,mode}$ = 1423 nm. Column dimensions: inner diameter 0.1 mm, total length 335 mm, active length 250 mm. Mobile phase: 0.4 mol/L acetic acid and 4 mmol/L triethylamine in acetonitrile–methanol (80:20) (a) and (60:40) (b), voltage −25 kV, injection −5 kV for 5 s, temperature 50°C, UV detection at 250 nm.

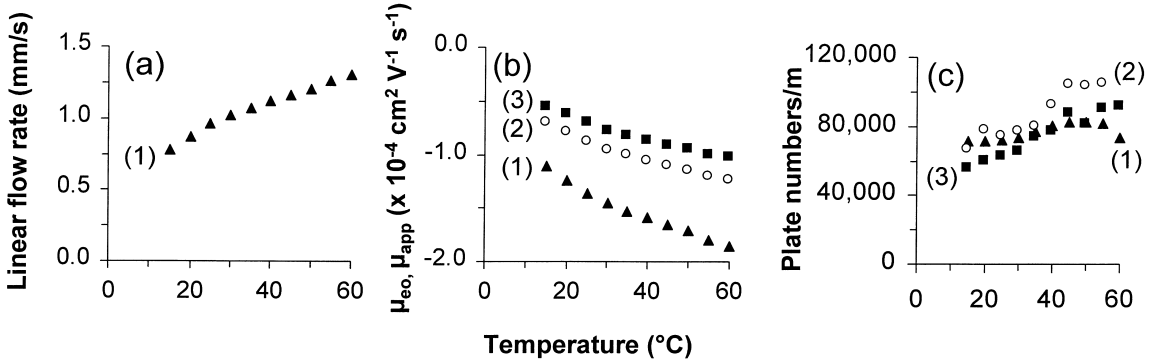

Fig. 6. Effect of temperature on linear flow velocities (a), electroosmotic μ_{eo} and apparent mobilities μ_{app} (b), and theoretical plate numbers (c). Conditions: monolithic column as in Fig. 5. Mobile phase: 0.4 mol/L acetic acid and 4 mmol/L triethylamine in 80:20 acetonitrile–methanol, voltage −25 kV, injection −5 kV for 5 s, UV detection at 250 nm. Analytes: acetone (1), phenol (2), and *p*-toluic acid (3).

results in reduced retention times and therefore higher apparent mobility μ_{app} (Fig. 6b). Similarly, the column efficiency improves significantly at a higher temperature as a result of the increase in mass transfer kinetics. This is demonstrated by the larger slopes of the plots for retained compounds phenol and *p*-toluic acid in contrast to non-retained acetone (Fig. 6c). These results suggest that higher temperatures are useful to achieve good separations.

3.6. Separations in anion-exchange and normal-phase CEC modes

The potential spectrum of applications of these monolithic anion-exchange stationary phases for the separation of ionizable compounds can range from biologically active acidic biopolymers such as nucleic acids, peptides, and proteins, to low-molecular-mass compounds such as drugs. Fig. 7 shows as an example the separation of a mixture of four non-steroidal anti-inflammatory drugs (profens) — ibuprofen, naproxen, ketoprofen, and suprofen. This baseline separation, which is achieved within less than 8 min with an efficiency of 231 000 plates/m for ibuprofen, demonstrates the separation power of our monolithic stationary phase for compounds with closely related structures.

The polar functionalities of quaternized poly(2-dimethylaminoethyl methacrylate-*co*-2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate) monoliths can also be used for the separations of neutral and even basic compounds in the normal-phase-like mode. For example, substituted phenols can be separated according to their hydrophilicity (Fig. 8a). 4-Iodoanisole, which does not contain hydroxyl groups, elutes first followed by vanillin and phenol with their single phenolic hydroxyl group, while 4-methylcatechol with two phenolic hydroxyl functionalities is the most polar, hence the most retained component of the mixture. The column efficiencies achieved in the non-aqueous organic mobile phase exceed 80 000 plates/m.

Similarly, Fig. 8b shows that theophylline (1,3-

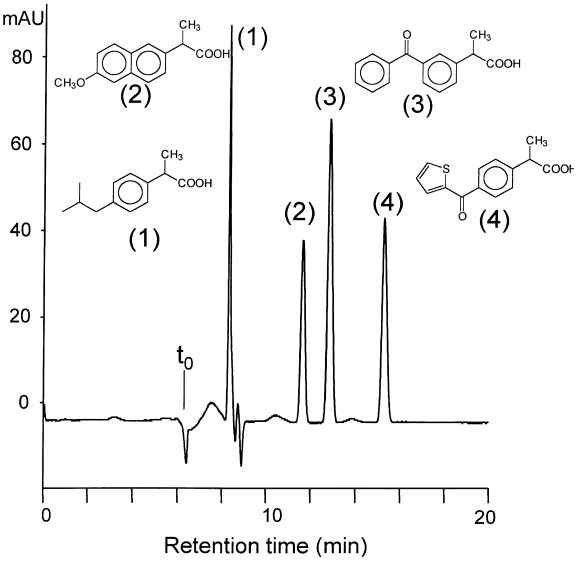

Fig. 7. Separation of the non-steroidal anti-inflammatory drugs ibuprofen (peak 1), naproxen (2), ketoprofen (3), and suprofen (4) in anion-exchange CEC mode using a strong anion-exchange monolithic column. For conditions, see Fig. 5b.

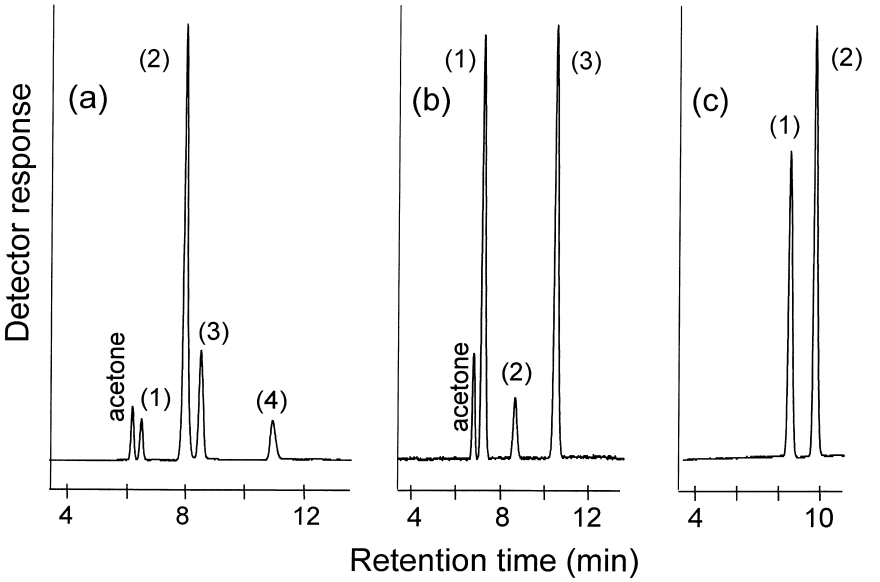

Fig. 8. Separation of phenolic compounds (a), and xanthines (b) in normal-phase CEC mode using a strong anion-exchange monolithic column. Conditions: on column alkylated monolith prepared from mixtures consisting of 8% 2-dimethylaminoethyl methacrylate, 24%, 2-hydroxyethyl methacrylate, 8% ethylene dimethacrylate, 20% cyclohexanol, 40% 1-dodecanol; UV-initiated polymerization at room temperature for 16 h; $d_{p,mode} = 1423$ nm. Column dimensions: inner diameter 0.1 mm, total length 335 mm, active length 250 mm. Mobile phase: 0.4 mol/L acetic acid and 4 mmol/L triethylamine in acetonitrile–methanol (60:40) (a), and (80:20) (b). Voltage −25 kV, injection −5 kV for 5 s, temperature 50°C (a) and 25°C (b), UV detection at 250 nm. Analytes: (a) 4-iodoanisole (peak 1), vanillin (2), phenol (3), and 4-methylcatechol (4); (b) caffeine (peak 1), theobromine (2), and theophylline (3).

dimethyl-xanthine) can be well separated from other xanthine derivatives such as caffeine (1,3,7-trimethyl-xanthine) and theobromine (3,7-dimethyl-xanthine). The advantage of this separation method is that caffeine, a stimulant that is likely to be present as an ''impurity'' in real life samples such as blood plasma, elutes close to the front, while the target analyte theophylline, a widely used bronchospasmolyticum, is retained and well separated from other xanthine derivatives. This may facilitate considerably the diagnostic determinations of theophylline.

The normal-phase separation abilities are preserved even in aqueous mobile phases. The strong hydrogen-bonding capability and high polarity of 2-aminopyridine enables its separation from the less polar *N*-ethylaniline. It is worth noting that despite the basic nature of these analytes the peaks are symmetrical. In addition, this separation was achieved in a mobile phase containing buffer with a pH value of 10.7, a value that would be prohibitive for silica-based stationary phases.

4. Conclusion

''Molded'' monolithic capillary columns prepared by the copolymerization of a solution of ionizable monomer, a crosslinker, and a polar monomer in the presence of a porogenic solvent within the confines of untreated fused-silica capillaries represent a new family of separation media for capillary electrochromatography. Columns with both tertiary and quaternary amino functionalities affording weak and strong anion-exchangers are easy to prepare by a simple ''molding'' process followed, in some instances, by a chemical modification step. This very simple approach completely avoids the fabrication of frits and the packing of small beads into capillaries. The monolithic materials may be optimized to achieve both high efficiency and selectivity in CEC separations.

In addition to this suitability for the reversed-phase and enantioselective separations that we demonstrated recently, monolithic columns prepared by the in situ polymerization of a tertiary amine-func-

tionalized monomer and subsequent quaternization are also well suited for the preparation of CEC columns for separations in both ion-exchange and normal-phase modes. The preparation technique enables easy adjustment and fine-tuning of porous properties and good control over the surface chemistry through the composition of the polymerization mixture. Remarkably high column efficiencies of over 230 000 plates/m were easily achieved for the separation of acidic analytes in ion-exchange mode with optimized monolithic columns. This efficiency vastly exceeds those of typical conventional packed HPLC columns. In addition, the hydrophilic selectivity that results from the large number of hydroxyl groups of polymerized 2-hydroxyethyl methacrylate repeat units enables the separations of neutral phenolic compounds and basic aromatic amines using the less common normal-phase CEC mode. Accordingly, this novel monolithic stationary phase can be classified as an anion-exchange/normal-phase mixed-mode separation medium.

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