Published in final edited form as:

J Chromatogr A. 2013 November 22; 1317: . doi:10.1016/j.chroma.2013.07.100.

Neutral, Charged and Stratified Polar Monoliths for Hydrophilic Interaction Capillary Electrochromatography

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Abstract

Novel polar monoliths were introduced for hydrophilic interaction capillary electrochromatography (HI-CEC). In one case, a neutral polar monolith resulted from the *in situ* polymerization of glyceryl methacrylate (GMM) and pentaerythritol triacrylate (PETA) in a ternary porogenic solvent. GMM and PETA possess hydroxyl functional groups, which impart the monolith with hydrophilic interaction sites. This monolith is designated as hydroxy monolith. Although the hydroxy monolith is neutral and void of fixed charges on the surface, a relatively strong cathodal EOF was observed due to the electric double layer formed by the adsorption of ions from the mobile phase, producing a bulk mobile phase flow. The second monolith is charged and referred to as AP-monolith that possesses amine/amide functionalities on its surface, and was prepared by the *in situ* polymerization of *N*-(3-aminopropyl) methacrylamide hydrochloride (NAPM) and ethylene dimethacrylate (EDMA) in the presence of cyclohexanol, dodecanol and methanol as porogens. Over the pH range studied a strong anodal EOF was observed. The AP-monolith was further exploited in HI-CEC by modifying its surface with neutral mono- and oligosaccharides to produce a series of the so called sugar modified AP-monoliths (SMAP-monolith), which are considered as stratified hydrophilic monoliths possessing a sub-layer of polar amine/amide groups and a top layer of sugar (a polyhydroxy top layer). The SMAP-monoliths can be viewed as a blend of both the hydroxy monolith and the AP-monolith. The polarity of the various monoliths seems to follow the order: hydroxy monolith < AP-monolith < SMAP-monolith. The novel monoliths were characterized over a wide range of elution conditions with a variety of polar solutes including phenols, substituted phenols, nucleic acid bases, nucleosides and nucleotides

Keywords

Polar Monolithic Capillary Columns; Stratified Hydrophilic Monoliths; Hydrophilic Interaction Capillary Electrochromatography

1 Introduction

Polar monolithic columns are increasingly gaining wide acceptance not only for use in hydrophilic interaction liquid chromatography (HILIC) and hydrophilic interaction capillary electrochromatography (HI-CEC) but also as precursor monoliths for the immobilization of

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a variety of enzymes, antibodies and other affinity ligands, thus providing surfaces at reduced nonspecific interactions (for a recent reviews, see refs [1–4])

Although normal-phase chromatography (NPC) is the initial and traditional terminology used to designate the chromatographic method that uses relatively polar stationary phases and significantly less polar mobile phases, the term hydrophilic interaction chromatography (HILIC) that was coined by Alpert in 1990 [5] has been predominantly used in recent years as the nature of the polar stationary phases has recently evolved to encompass a wide range of functional groups on their surfaces. For instance, organic monoliths for use in the HILIC mode either by HPLC or CEC have been designed to bear also some charged groups in their surface ligands in order to induce a mixed mode retention mechanism, e.g., hydrophilic interactions and electrostatic interactions. Also, for CEC, fixed charges were introduced intentionally into monolithic stationary phases in order to support a relatively strong EOF. This led to monoliths of different kinds including neutral (i.e., void of charges) [6, 7], anionic [8–11], cationic [12, 13], zwitterionic [14–18] and amphoteric monoliths [19].

As a continuation to our recent contribution to immuno-monoliths at reduced nonspecific interactions [20], the polar monolith precursors of these immuno-monoliths are further exploited and evaluated here in HI-CEC of polar solutes. Two main polar precursor monoliths are investigated in this research report. One polar precursor monolith (referred to as hydroxyl monolith) is neutral and based on the *in situ* co-polymerization of the functional monomer glyceryl methacrylate (GMM) and the crosslinker pentaerythritol triacrylate (PETA) while the second monolith is a cationic polymer (designated as AP-monolith) that resulted from the *in situ* co-polymerization of the functional monomer *N*-(3-aminopropyl) methacrylamide hydrochloride (NAPM) and the crosslinker ethylene dimethacrylate (EDMA), and proved to be a useful precursor for the oriented immobilization of antibodies [20]. To modulate the retentive properties of the cationic monolith, the NAPM/EDMA monolith (i.e., AP-monolith) was further modified with neutral mono- and oligosaccharides to obtain a series of sugar modified AP-monoliths (SMAP-monoliths). The SMAP-monoliths are viewed stratified phases consisting of a polar amine/amide sub-layer and a sugar top layer (or a polyhydroxy top layer), thus blending the characteristics of hydroxy monolith and AP-monolith.

2 Materials and methods

2.1 Instruments

The HI-CEC experiments were performed on a HP^{3-D} CE system from Hewlett-Packard (Waldbronn, Germany) equipped with a photodiode array detector, and a PC running a HP ChemStation system. The *in situ* polymerization of the monolithic columns was thermally initiated using an Isotemp Model 105 water bath from Fisher Scientific (Fairlawn, NJ, USA). An HPLC pump Model M-45 from Waters Associates (Milford, MA, USA) was used to flush the monolithic columns after polymerization.

2.2 Reagents and materials

Fused-silica capillaries having 100 µm inner diameter (i.d.) and 375 µm outer diameter (o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA). Glyceryl methacrylate (GMM) was from Monomer-polymers & Dajac Labs (Feaster-Ville, PA, USA). 2,2-Azobisisobutyronitrile (AIBN), HPLC grade acetone and acetonitrile (ACN), 1-dodecanol, pentaerythritol triacrylate (PETA), 4-methylphenol, and 4-aminophenol were from Aldrich (Milwaukee, WI, USA). *N*-(3-Aminopropyl) methacrylamide hydrochloride (NAPM) was purchased from Polysciences, Inc. (Warrington, PA, USA). Toluene, phenol, resorcinol and pyrogallol were from Fisher Scientific (Fairlawn, NJ, USA). Cyclohexanol

was from Fluka Chemical Corporation (Milwaukee, WI, USA). Ethylene dimethacrylate (EDMA), 3-(trimethoxysilyl)propylmethacrylate, thiourea, *N*-acetyl glucosamine, cresol, 2-amino-4-cresol, phenol, 4-amino-2-nitrophenol, 4-cyanophenol, resorcinol, catechol, hydroquinone, 4-nitrophenol, Uracil, cytosine, adenine, thymine, adenosine, uridine, inosine and cytidine were from Sigma (St. Louis, MO, USA). Chitotriose and chitopentase were from Seikagaku Kogyo Co. (Chuo-ku, Tokyo, Japan). *N,N*-Dimethylformamide (DMF) and formamide were from EM Science (Gibbstown, NJ, USA).

2.3 Capillary column pretreatment and *in situ* polymerization of monoliths

The inner wall of the fused silica capillary was treated with 1.0 M sodium hydroxide for 30 min, rinsed with water for 30 min, flushed with 0.1 M hydrochloric acid for 30 min, and then rinsed with water for 30 min. Thereafter, the capillary was allowed to react with a solution of 50% (v/v) 3-(trimethoxysilyl)propylmethacrylate in acetone for 4 h at room temperature to functionalize the inner wall of the capillary [21]. Finally, the capillary was rinsed with methanol and dried with a stream of nitrogen.

The polymerization solutions used for the preparation of the two main monoliths have each a final weight of 0.2 g of monomers and porogens, and consisted in the case of hydroxy monolith of 7.6-wt% GMM (**I**), 7-wt% PETA (**II**), 59.1-wt% cyclohexanol, 22.9-wt% dodecanol and 3.4-wt% water while in the case of AP-monolith the polymerization solution consisted of 13.8-wt% NAPM (**III**), 10.7-wt% EDMA (**IV**), 48.5-wt% cyclohexanol, 8.7-wt% dodecanol and 18.2-wt% methanol (See structure of monomers below). In both cases, the free radical initiator AIBN was added at 1 wt% with respect to monomers.

Glyceryl methacrylate (GMM) (**I**)

Pentaerythritol triacrylate (PETA) (**II**)

N-(3-Aminopropyl) methacrylamide hydrochloride (NAPM) (**III**)

Ethylene dimethacrylate (EDMA) (**IV**)

Before introducing them into the capillaries, the polymerization solutions were degassed by sonicating for 15 min and purging with nitrogen for 5 min. Prior to sonication NAPM/

EDMA polymerization solution was stirred for 20 min to facilitate the solubility of the monomers. After that, 40 cm pretreated and silanized capillaries were filled with the polymerization solution up to 30 cm, by immersing the inlet of the capillary in the solution vial and applying vacuum to the outlet to prepare a final column of 25 cm. The two ends of the capillaries were sealed with GC septa and NAPM/EDMA monolith (i.e., AP-monolith) was heated in a water bath for 24 h at 50 °C while GMM/PETA monolith (i.e., hydroxy monolith) was thermostated at 60 °C for 15 h. The resulting monolithic columns were rinsed with ACN using an HPLC pump. A detection window was created at 1–2 mm after the end of the polymer bed using a thermal wire stripper. Finally, the column was cut into a total length of 33.5 cm with an effective length of 25 cm.

2.4 Preparation of the sugar modified AP-monolithic columns (SMAP-monoliths)

After completing their *in situ* polymerization, the AP-monolithic capillary columns were rinsed with ACN followed by water. Next, the columns were equilibrated with 0.05 M sodium acetate solution, pH 5.0, for 1 h. After that, 10 % sugar solutions consisting of *N*-acetylglucosamine (GlcNAc) or chitoooligosaccharides prepared in the acetate solution and containing 0.05 M sodium cyanoborohydride were passed through the columns followed by heating at 60 °C in a GC oven overnight. Thereafter, the columns were flushed with water and equilibrated with the running mobile phase consisting of 5% (v/v) of 5 mM ammonium acetate, pH 4.0, and 95% (v/v) ACN.

3 Results and discussion

3.1 GMM/PETA monolith or hydroxy monolith

The functional monomer GMM and the crosslinker PETA have in their structure hydroxyl groups (see structures (I) and (II) shown above), which impart the GMM/PETA monolith with hydrophilic interaction sites, and thus the name hydroxy monolith. After a brief trial and error, and guided by our laboratories years of experience in the design of monoliths, 7.6-wt% of the functional monomer GMM, 7-wt% of the crosslinker PETA, and a ternary porogenic mixture consisting of 59.1-wt% cyclohexanol, 22.9-wt% dodecanol and 3.4-wt% water yielded the hydroxyl monolith that exhibited the best permeability in pressure driven flow and separation efficiency in CEC.

Although void of fixed charges on its surface, the hydroxy monolith under investigation generated a significant cathodal EOF velocity at pH 7.0. As can be seen in Fig. 1, the EOF velocity increased from 1.49 mm/s to 1.9 mm/s when the %ACN (v/v) in the mobile phase was increased from 92% to 98%. This represents a 27% increase in the EOF velocity for 6% increase in the ACN content of the mobile phase. On the other hand, at 95% (v/v) ACN in the mobile phase, the EOF velocity was nearly unaffected by the pH, and increased marginally from 1.8 mm/s to 1.9 mm/s when the pH was increased from 5.0 to 6.0, and stayed the same thereafter at 1.9 mm/s at pH 8.0 and 9.0. It is believed that the EOF was due to the adsorption of mobile phase ions onto the monolithic surface, which imparted the monolith with the zeta potential necessary to generate the EOF. This phenomenon resembles that observed with other neutral monoliths introduced by El Rassi and co-workers [7, 22–29]. The polar hydroxyl and ester groups present in both the monomer and the crosslinker are believed to be responsible for the binding of ions from the background electrolyte to the monolithic surface. Since the EOF is cathodal, the zeta potential is therefore negative, and can be attributed essentially to the adsorption of the negatively charged acetate ions. The observed EOF velocity was largely unaffected by pH changes in the range 6.0 to 9.0 may be due to the fact that the acetate ions are fully negatively charged at pH > 6.0.

On the other hand, and as shown in Fig. 1, the effect of the ACN content of the mobile phase on the magnitude of the EOF is noticeable, and shows that this magnitude increased with the %ACN at an almost constant rate in the range studied. This may indicate that the adsorption of ions to the monolith is based on polar interactions. In other words, the higher the CAN content of the mobile phase the greater the amount of adsorbed acetate ions to the hydroxy monolith, and in turn the higher the EOF velocity. In addition, increasing the EOF velocity with increasing the ACN content of the mobile phase is due to the fact that at elevated ACN content the viscosity of the mobile phase decreases, a fact that favors a stronger EOF since the EOF velocity is inversely proportional to the viscosity of the mobile phase [30]. The viscosity of ACN-water mixture goes through a slight maximum value at ~20% (v/v) ACN, and this viscosity is about 4 fold higher in pure water than in pure ACN [31].

In order to investigate the HILIC retention properties of the hydroxy monolith under investigation, some phenols and small polar molecules were electrochromatographed on the hydroxy monolithic column. The mobile phase content in ACN was changed from 92 % to 98 % (v/v) while the ammonium acetate concentration was kept constant at 5mM. The results for six test compounds are shown in Fig. 2 in terms of $\log k'$ vs. the %ACN (v/v) in the mobile phase. As expected, $\log k'$ increased quasi linearly with increasing % ACN (v/v), and the k' values of the phenols increased with the number of hydroxyl groups in the phenol molecule. A typical electrochromatogram of the 3 phenols is shown in Fig. 3A. Other phenol derivatives, namely the 4-methylphenol, 4-nitrophenol, 4-cyanophenol and 4-aminophenol, were retained in the order of increasing polarity (see Fig. 3B) with the methyl substituted phenol eluting first, the nitro substituted second, the cyano substituted third and amino substituted last. Also, Fig. 3C shows the typical HILIC retention mechanism as portrayed by the elution order of the three test solutes, which is *N,N*-dimethylformamide < formamide < thiourea. Thiourea, a solute that is usually used as the EOF marker in RP-CEC eluted last on the hydroxy monolith. The average separation efficiencies per meter of column are around 146 000, 120 000 and 92 000 for Fig. 3A, 3B and 3C, respectively.

Finally, the retention behaviors of some nucleic acid bases, nucleosides and nucleotides on the hydroxy monolith were examined. These solutes are important probes for evaluating the polarity of the stationary phase under investigation due to the multiplicity of their polar groups, and in turn, the influence of functional groups on HILIC retention. Good separations of some nucleic acid bases and nucleosides were obtained with 95% ACN (v/v), containing saturated ammonium phosphate (~3.5 mM, pH 7.0) as the mobile phase (see Fig. 4A and B). The nucleic acid bases under investigation, namely uracil, thymine, cytosine and adenine (Fig. 4A), eluted more or less in the order of increasing number of amine functions, particularly primary and secondary amines. The conjugation of the nucleic acid bases with ribose as in their corresponding nucleosides increased the interaction of the polar conjugates with the hydroxy monolith as it is the case of uridine with respect to uracil, but this interaction stayed almost the same for cytidine when compared to cytosine (compare Fig. 4A and B). To demonstrate the selectivity of the novel hydroxy monolith, two nucleotides isomers differing in the position of the phosphate groups, namely 3' AMP and 5' AMP were separated with a good selectivity as shown in Fig. 4C.

3.2 NAPM/EDMA monolith or AP-monolith

To address the need for a monolith that would exhibit different HILIC retention properties than the above hydroxy monolith, an attempt was made to evaluate a hydrophilic monolith containing charged moieties in its structure. In this regard, an amine/amide bearing compound, namely *N*-(3-aminopropyl) methacrylamide hydrochloride was used here as the functional monomer in the making of the AP-monolith under investigation. This should in

principle provide a monolith with more polar functionalities than the above hydroxy monolith since amine/amide groups are known to be more polar than the hydroxyl groups.

First, a polymerization mixture was prepared using the recipe given in a recent contribution from our laboratories by Jmeian and El Rassi [32] and replacing the glycidyl methacrylate by NAPM while the percentages of crosslinker and porogens were kept constant. This composition consisted of 18-wt% NAPM, 12-wt% EDMA, 59.5-wt% cyclohexanol and 10.5-wt% dodecanol. The free radical initiator AIBN was added to the polymerization mixture (1-wt% with respect to monomers). Being a hydrophilic monomer the solubility of NAPM, which is a solid compound at room temperature, was limited in the binary porogenic mixture consisting of cyclohexanol and dodecanol (mentioned above) and a clear solution could not be obtained. Based upon this observation, a sufficient volume of methanol was added to the polymerization mixture in order to solubilize NAPM and obtain a clear solution. The choice of methanol was successful, and a monolithic column with a good permeability was achieved by using 18.2-wt% methanol in the polymerization mixture and adjusting the content of NAPM, EDMA, cyclohexanol and dodecanol to 13.8-wt%, 10.7-wt%, 48.5-wt% and 8.7-wt%, respectively, in the polymerization mixture.

A set of 3 AP-monoliths was prepared by varying the wt% of NAPM while increasing or decreasing the wt% of the crosslinker EDMA accordingly, see Table 1. The content of the other components (i.e., 48.5-wt% cyclohexanol, 8.7-wt% dodecanol and 18.2-wt% methanol) in the polymerization mixture was kept constant. The retention factor k' and the selectivity factor α of a test mixture containing toluene, DMF, formamide and thiourea were compared (Table 1). As shown in this table a subtle change in the monomer composition in the polymerization mixture changed the chromatographic properties of the monolith significantly. When the percentage of NAPM was increased, the k' decreased. This could be due to the fact that when the percentage of the crosslinker EDMA was decreased it might have affected the structure and the porosity of the AP-monolith thus decreasing its performance. The column at 13.8-wt% NAPM provided a relatively good selectivity factor for the solute pair DMF/formamide and for the solute pair formamide/thiourea and higher k' values, and was used for the rest of the study.

The best AP-monolith at 13.8-wt% NAPM yielded k' values of 0.135, 0.916 and 2.75 for DMF, formamide and thiourea, respectively, as opposed to k' values for the same solutes of 0.045, 0.167 and 0.36, respectively, obtained on the hydroxy monolith. These results confirm that indeed the AP-monolith possesses a higher polarity than the hydroxy monolith as manifested by the greater k' values obtained on the former than on the later monolith for the same solutes and under the same mobile phase composition.

The direction of EOF depends on the sign of zeta potential of the monolith, and as a result an anodal EOF was observed with the AP-monolith in the pH ranging from 4 to 8, and the EOF velocity as a function of pH is shown in Fig. 5. This figure shows that the EOF velocity increased with increasing the pH of the mobile phase up to pH 6 and thereafter the EOF velocity remained unchanged. This behavior may be explained by the ionization of the surface amino groups and the interplay of the ionization and shielding effects of the adsorbed mobile phase ions to the surface of the monolithic stationary phase. In the pH range studied, the ionization of the amino groups decreased, which will lower the electrostatic attractions and in turn the neutralization/shielding effect exercised by the negatively charged acetate ions. This can be seen in the apparent jump of the EOF velocity in the pH range 4 to 6. In this pH range, the ionization of the acetate ions increases and then become fully charged at pH > 6.5. On the other hand, in the pH range 6 – 8, the surface amino groups will be more and more de-protonated, which will favor the adsorption of the ammonium ions to the monolithic surface as the monolithic surface becomes less repulsive

towards the ammonium ions, a fact that would counterbalance the loss of the fixed positive charges, thus manifesting itself in the constancy of the EOF velocity. Similar behavior were reported earlier by Bedair and El Rassi [33] with a cationic stearyl acrylate monolith that was introduced for reversed phase CEC in the sense that the direction and magnitude of the EOF on such cationic stearyl monolith were largely affected by the adsorption of mobile phase ions.

As in the case of the hydroxy monolith, the AP-monolith retained the solutes DMF, formamide and thiourea in the order of increasing solute polarity (Fig. 6A). That is the elution order agreed well with the hydrophilic interaction mode where the highest polar species thiourea eluted last while the nonpolar toluene eluted first at the dead time of the column t_0 . To further evaluate the HILIC properties of the monolith under investigation, three phenols namely phenol, resorcinol and pyrogallol were also separated using the AP-monolith (Fig. 6B). The observed elution order parallels the number of hydroxyl groups present in the solute molecule. The more polar the molecule (highest number of hydroxyl groups) the more it was retained on the column. As was observed with DMF, formamide and thiourea, the retention of the phenols was much higher on the AP-monolith than on the hydroxy monolith confirming once more the stronger polar character of the former monolith than the later monolith. In fact, the k' values obtained on the AP-monolith for phenol, resorcinol and pyrogallol were 0.12, 0.54 and 1.44, respectively, as opposed to k' values of 0.04, 0.096 and 0.19, respectively, obtained on the hydroxy monolith under otherwise the same elution conditions. The average separation efficiencies per meter of the AP-monolithic column are around 69 000 and 36 000 in Fig. 6A and 6B, respectively.

As shown in Fig. 7, ten phenols and substituted phenols were also separated according to their hydrophilicity. Cresol which has a methyl group (least polar) eluted first followed by 2-amino-4-cresol and phenol, which are the next compounds in the order of increasing polarity. Pyrogallol with three hydroxyl groups is the most polar compound in the mixture, and hence most retained on the column. The AP-monolith was also able to separate positional isomers namely catechol, resorcinol and hydroquinone as well. These ten phenols and substituted phenols were not as well separated on the hydroxy monolith as on the AP-monolith. On the other hand, and due to its charged amino groups, the AP-monolith did not allow the elution and separation of oppositely charged nucleotides.

To further characterize the NAPM/EDMA monolith under investigation, three other slightly polar molecules, namely 2-aminobenzamide (2-AB), aniline and caffeine were also separated using the AP-monolithic column. The k' values of caffeine, aniline and 2-AB were 0.053, 0.092 and 0.31, respectively. The least polar caffeine did not show a considerable retention on the monolith while the most polar 2-AB exhibited the highest retention.

3.3 Sugar modified AP-monoliths - Stratified hydrophilic monoliths

Although the above AP-monolith is quite hydrophilic and proved useful in HI-CEC, its surface-active primary amines may react with some solutes or impurities in the samples (e.g., aldehydes and ketones), a fact that may lead over an extended period of use to a monolithic column of unstable retention properties. In order to deactivate the surface of the AP-monolith, mono- and oligosaccharides were covalently attached to its surface yielding the so-called sugar modified AP-monolith (SMAP-monolith) or stratified hydrophilic monoliths. In this regards, *N*-acetylglucosamine (GlcNAc), chitotriose, and chitopentaose were covalently attached to the amine bearing monolithic surface *via* reductive amination, which converts the primary amines to the much less reactive secondary amines. The SMAP-monoliths can be viewed as stratified hydrophilic monoliths covered with a sub-layer of amine/amide groups, and a top sugar layer or polyhydroxy layer. While the sub-layer reflects in some way the characteristics of the AP-monolith, the top polyhydroxy layer may

exhibit to a certain extent the HILIC retention properties of the hydroxy monolith. Thus, the SMAP-monoliths can be viewed as a blend of both monoliths; that is a combination of hydroxy monolith and AP-monolith.

As expected, the SMAP-monoliths yielded an anodal EOF, due to the fact that the reductive amination does not remove the amine groups; it rather transforms the primary amines to secondary amines, and therefore positive charges are still present on the surface of the monoliths to give the positive zeta potential required to maintain the anodal EOF. For a monosaccharide such as GlcNAc, the EOF velocity on the SMAP-monoliths was similar to that of the bare AP-monolith. However, for a trisaccharide such as chitotriose, the EOF velocity on the SMAP-monolith was 58% of that of the bare AP-monolith, namely 0.9 mm/s on the former versus 1.55 mm/s on the later under otherwise the same running conditions which consisted of a hydro-organic mobile phase made of 5 mM NH_4Ac at 95% ACN (v/v) and at -20 kV applied voltage. The reduction in the EOF velocity may be attributed to the higher shielding effect of the trisaccharide for the binding of the mobile phase counter ions to the amine groups on the surface of the SMAP-monolith and perhaps to the viscous effect of the trisaccharide top layer.

By modifying the AP-monolith with *N*-acetylglucosamine, chitotriose and chitopentaose different HILIC retention properties were observed. Figure 8A shows a typical electrochromatogram for phenol, resorcinol and pyrogallol obtained on the chitotriose modified AP-monolith and Fig. 8B shows the bar charts for the comparison of the k' values of these three phenols obtained on the AP-monolith and the various SMAP-monoliths. Figure 8A shows at a glance the gain realized upon modifying the AP-monolith with chitotriose as far as the strength of HILIC retention is concerned (compare to Fig. 6B). Also, Fig. 8B shows a considerable effect on the retention of phenols upon the immobilization of sugars on the AP-monolith. For instance, and for the most polar/most retained pyrogallol, its k' values obtained on the SMAP-monoliths were 2.31, 2.92 and 3.54 fold higher on *N*-acetylglucosamine-, chitotriose- and chitopentaose-modified AP-monoliths, respectively, than that obtained on the bare AP-monolith for the same %ACN in the mobile phase. Overall, the k' values of phenol, resorcinol and pyrogallol increased with increasing the number of repeating sugar unit in the immobilized saccharides. This observation is in agreement with the fact that increasing the overall polarity of the stationary phase increases the retention of polar compounds. The full potentials of stratified hydrophilic monoliths are still under investigation and the results will be the topic of an upcoming research report.

Returning to the electrochromatogram shown in Fig. 8A, it shows clearly peak broadening and tailing for the most polar species pyrogallol on the SMAP-monolith. This is because the retention of pyrogallol is quite high leading to slow sorption kinetics and increased longitudinal molecular diffusion. A very retentive stationary phase in CEC does not favor the realization of fast and high resolution separations for components widely differing in their k' values simply because CEC is practiced nowadays using isocratic elution due to the unavailability of CEC instrumentation for gradient elution.

4 Reproducibility of column fabrication

Briefly, some data on run-to-run, day-to-day and column-to-column reproducibilities of some of the optimal monolithic columns are reported here in terms of t_0 , EOF velocity and the retention factor (k') of some solutes. The percent relative standard deviation (%RSD) for k' of DMF were 1.7 ($n = 3$) and 12.43 ($n = 2$) for run-to-run and day-to-day, respectively, on the AP-monolith (under running conditions of Fig. 6A), while the %RSD for k' of phenol were 1.82 ($n = 3$) and 2.24 ($n = 2$) for run-to-run and day-to-day, respectively, on the hydroxy monolith (under running conditions of Fig. 3A). The %RSD for t_0 as measured

from the retention time of toluene, were 2.15 ($n = 4$) and 11.66 ($n = 2$) for run-to-run and day-to-day, respectively, on the AP-monolith under conditions of Fig. 6A while the %RSD for t_0 were 1.42 ($n = 3$) and 0.22 ($n = 2$) for run-to-run and day-to day, respectively, on the hydroxyl monolith under conditions of Fig. 3. Finally, the %RSD of the EOF velocity for column-to-column was 7.44 ($n = 2$) on the hydroxy monolith under conditions of Fig. 3. All of the above %RSD values are similar to those previously reported [28] for another kind of monolith.

5 Conclusions

The various hydrophilic monoliths described in this research report provided HILIC properties covering a wide spectrum of retention levels for polar solutes. While the neutral hydroxy monolith allowed isocratic HI-CEC for a number of polar species including charged nucleotides, the more polar AP-monolith exhibited very unique selectivity for neutral, and polar positional isomers but was not suitable for the separation of negatively charged species such as nucleotides. The relatively strongly hydrophilic SMAP-monoliths offered high retention and required gradient elution to exploit its full potentials in the separation of many polar compounds. The SMAP-monoliths are being investigated in nano-LC and analytical HPLC columns since sophisticated HPLC systems are currently available for performing gradient elution analysis. Despite its neutrality, the hydroxy monolith exhibited a moderate EOF velocity that can be conveniently varied by the mobile phase composition, e.g., pH and to a larger extent % ACN. Besides that, the AP-monolith provided a stable and relatively strong anodal EOF. It allowed the achievement of broad selectivity for the various polar solutes investigated. The modification of the surface of the AP-monoliths with GlcNAc, chitotriose and chitopentaose yielded highly polar surfaces exhibiting strong hydrophilic interactions with polar compounds and requiring gradient elution for species covering a wide range of k' values.

Acknowledgments

The financial support of this research in part by a Grant No. 1R15GM096286-01 from the National Institutes of Health is greatly appreciated.

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Highlights

- Various monoliths were successfully introduced and evaluated in HI-CEC
- A hydroxy monolith based on diol functions yielded cathodal EOF
- A cationic monolith based on primary amine functions (AP-monolith)
- 3 sugar modified monoliths (SMAP-monoliths) called stratified monoliths blending the amine and hydroxy monoliths
- The polarity of the various monoliths followed the order: hydroxy monolith < AP-monolith < SMAP-monoliths

Figure 1.

Plot of EOF velocity (mm/s) vs. %ACN (v/v) in the mobile phase. Conditions: hydroxy monolithic capillary column, 33.5 cm (effective length 25 cm) $\times$ 100 μ m i.d.; running voltage, 20 kV; column temperature, 20 $^{\circ}$ C; hydro-organic mobile phase, 5 mM NH_4Ac (pH 7.0) at various %ACN; EOF tracer, toluene; sample injection, pressure at 5 bar for 10 s.

Figure 2.

Plots of $\log k'$ of three phenols (A) and of three other compounds (B) vs. % ACN (v/v) in the mobile phase. Solutes were injected by pressure at 5 bar for 10 s. Other conditions as in Fig. 1.

Figure 3. Separation of three phenols (A), four phenol derivatives (B) and three other compounds (C) obtained on the hydroxy monolith. Conditions: hydro-organic mobile phase, 5 mM NH_4Ac (pH 8.0) at 95 % ACN (v/v). Other conditions as in Fig. 1.

Figure 4. Separation of four nucleic acid bases (A), three nucleosides (B) and (C) two nucleotide isomers on the hydroxy monolith. Conditions: hydro-organic mobile phase, saturated with ammonium phosphate (~3.5 mM), pH 7, 95% ACN (v/v) in (A) and (B); hydro-organic mobile phase, 5 mM NH₄Ac, pH 7, 80 % ACN (v/v) in (C). Solutes are as indicated in (A), (B) and (C).

Figure 5.

Plot of EOF velocity (mm/s) vs. pH of the mobile phase. Conditions: AP-monolithic capillary column, 33.5 cm (effective length 25 cm) $\times$ 100 μ m i.d.; running voltage, -20 kV; column temperature, 20 $^{\circ}$ C; hydro-organic mobile phase, 5 mM NH_4Ac at 95% ACN (v/v); EOF tracer, toluene.

Figure 6.

Separation of small polar compounds (A) and three phenols (B) obtained on the AP-monolith. Conditions are the same as in the Fig. 5, except that pH = 4. Solutes are in (A): 1, toluene; 2, DMF; 3, formamide; 4, thiourea and in (B): 1, toluene; 2, phenol; 3, resorcinol; 4, pyrogallol.

Figure 7.

Separation of 10 polar compounds obtained on the AP-monolith. Conditions are the same as in the Fig. 5, except that pH = 4 and %ACN = 97. Solutes: 1, cresol; 2, 2-amino-4-cresol; I, impurity from mixture, 3, phenol; 4, 4-amino-2-nitrophenol; 5, 4-cyanophenol; 6, resorcinol; 7, catechol; 8, hydroquinone; 9, 4-nitrophenol; 10, pyrogallol.

Figure 8.

Separation of three phenols on the chitotriose modified AP-monolith (A) and bar charts for the k' values of these three phenols obtained on the AP-monolith and the three SMAP-monoliths (B). Conditions are the same as in Fig. 5, except that pH = 4. Solutes in (B): 1, toluene; 2, phenol; 3, resorcinol; 4, pyrogallol.

Table 1

Effect of the monomer NAPM percentage on chromatographic properties. Conditions: AP-monolithic capillary column, 33.5 cm (effective length 25 cm) $\times$ 100 μ m i.d.; running voltage, -20 kV; column temperature, 20 $^{\circ}$ C; hydro-organic mobile phase, 5 mM NH₄Ac, pH 4.0 at 95% ACN (v/v); EOF tracer, toluene.

Wt% of NAPM/EDMA	k'			
	DMF (D)	Formamide (F)	Thiourea (T)	k' _F /k' _D
13.8/10.7	0.135	0.916	2.75	3.00
14.8/9.7	0.114	0.787	1.75	2.22
15.8/8.7	0.116	0.787	1.69	2.15