Journal of Chromatography A, 1218 (2011) 4671–4677

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Novel highly hydrophilic methacrylate-based monolithic column with mixed-mode of hydrophilic and strong cation-exchange interactions for pressurized capillary electrochromatography

Jia Lin[a], Shaofeng Liu[b], Jian Lin[a], Xucong Lin[a,*], Zenghong Xie[a,c,**]

[a] Institute of Food Safety and Environmental Monitoring, Fuzhou University, 350108, China
[b] Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou 450001, China
[c] Xiamen Huaxia Vocational College, Xiamen 361024, China

ARTICLE INFO

Article history:
Received 4 February 2011
Received in revised form 28 April 2011
Accepted 14 May 2011
Available online 23 May 2011

Keywords:
Hydrophilic interaction
Strong cation-exchange
Methacrylate-based monolith
Pressurized capillary electrochromatography

ABSTRACT

A novel highly hydrophilic polymethacrylate-based monolithic stationary phase based on the copolymerization of 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) and pentaerythritol triacrylate (PETA) was designed for pressurized capillary electrochromatography. A typical hydrophilic interaction chromatography mechanism could be observed when the content of acetonitrile (ACN) in the mobile phase exceeded 25%. Slight swelling or shrinking with mobile phases of different polarity was observed in permeability studies. Good retentions and efficient separations of polar analytes, such as neutral amides and phenols, were well achieved in hydrophilic interaction chromatography mode with only about 50% ACN content in the mobile phase. It was remarkably lower than the content of ACN (>90%) used on the hydrophilic polymethacrylate-based monoliths reported previously. Additionally, a mixed mode of hydrophilic interaction (HI) and strong cation-exchange (SCX) could be also obtained in the analysis of charged peptides, and high column efficiency up to 80,000 plates/m was achieved without peak tailing. The prepared hydrophilic stationary phase might provide a potential environmental friendly separation media for polar solutes as it consumes a low volume of organic solvents.

1. Introduction

Hydrophilic interaction chromatography (HILIC), acting as a complementary separation technology to reversed-phase liquid chromatography (RPLC), has been promoted by an increasing demand for the analysis of polar compounds [1,2] by using monolithic columns with obvious predominance such as no frits, simple preparation and wide selection of monomers available with different functional groups [3,4]. Recently, hydrophilic interaction capillary electrochromatography (HI-CEC) based on hydrophilic monolithic column has attracted increasing attention [5–7].

Methacrylate-based monoliths, as one kind of typical polymer-based monolithic columns, are highly favorable and widely adopted attribute to obvious advantages such as chemical stability, flexible modification and good reproducibility [8,9]. Most methacrylate-based monoliths were designed for RP columns [10,11] due to the hydrophobicity of the polymer backbone [7]. In our recent work, we have successfully developed several hydrophilic methacrylate-based monoliths for the separation of polar and charged species in HI-CEC mode [12–16]. However, these monolithic columns showed limited hydrophilicity. The critical compositions of the mobile phases corresponding to the transition from the HILIC to the RP mode were commonly around 70% ACN in water. To achieve sufficient retention and selectivity for polar analytes, mobile phases employing high amount of organic modifier must be used. The separations of polar analytes, such as amides and phenols, were commonly performed with more than 90% ACN content in the mobile phase to achieve enough retention [14–16]. It would be environmentally less friendly as it consumed large volumes of organic solvents [17]. The limited hydrophilicity of the obtained monolithic columns may be due to the limited solubility of strong polar monomers in these reported methacrylate systems [14], which cannot offer enough retention and resolution towards small solutes unless they are very hydrophilic and would restrain the applications of the hydrophilic monolithic column for polar solutes. With more polar stationary phases, the critical composition shifts to a lower content of the organic modifier in the mobile phase, allowing the use of mobile phases with higher water content in the HILIC mode [18]. Additionally, it might be extremely useful for the

* Corresponding author. Fax: +86 591 22866131.
** Corresponding author at: Institute of Food Safety and Environmental Monitoring, Fuzhou University, 350108, China. Fax: +86 591 22866131.
E-mail addresses: linxucong@yahoo.com.cn, xulin@fzu.edu.cn (X. Lin), zhxie@fzu.edu.cn (Z. Xie).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.05.052

effective analysis of some polar solutes of which the solubility was rather poor in the solution with a high content of organic modifier. To promote the development of HI-CEC, further efforts are needed to develop polar stationary phases with higher hydrophilicity for efficient separation of polar compounds.

Monolithic column with mixed-mode of hydrophilic interaction and strong cation-exchange (HI/SCX) is greatly useful for analyzing polar and positively charged species [14]. The utility of an HI/SCX monolithic column, which often contains sulfonate groups, lies in its ability to maintain negative charge even under acidic buffer pH conditions [19]. However, a sulfonate-containing monolith is believed to swell excessively in aqueous buffer [20–22]. If the monolith swells, its throughpores will decrease in size resulting in lower permeability, and further result in the poor reproducibility. Excessive swelling of the sulfonate-containing polymer monolith in aqueous buffer would result in no flow [19]. To fabricate a stable sulfonate-containing methacrylate-based monolithic column with high hydrophilicity and slight swelling or shrinking in mobile phases of different polarity, the challenge lies in fumbling the proper polar monomers, crosslinkers and porogens, and the polymerization mixture should be homogeneous to facilitate handling in methacrylate system. We are not aware of any reports on the investigation of highly hydrophilic methacrylate-based monoliths in HI/SCX mode by CEC.

In this work, we attempted to propose a novel methacrylate-based monolithic stationary phase by using a highly hydrophilic functional monomer 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) and the polar cross-linker pentaerythritol triacrylate (PETA), which were specially selected to increase polarity of the stationary phase and decrease unwanted polymer backbone hydrophobicity. The PETA has been used instead of traditional ethylene glycol dimethacrylate (EDMA) as cross-linker to prepare the hydrophilic monolith for CEC with more hydrophilicity due to its hydroxyl sub-layer [13–16]. With the combination of AMPS and PETA, the resulted monolith showed high hydrophilic, which could offer enough retention and resolution towards polar solutes. The composition of the polymerization mixture has been optimized to gain the satisfactory column permeability, efficiency and separation performance. Typical hydrophilic interactions could be observed when the content of ACN in the mobile phase exceeded 25%. The swellability of the highly hydrophilic stationary phases prepared by copolymerization of a high amount of AMPS (up to 50% total monomers) could be well controlled. Good retentions and efficient separations of neutral polar amides and phenols were well achieved with only about 50% ACN content. A mixed mode of HI and SCX could be also obtained in the analysis of charged peptides with high column efficiency up to 80,000 plates/m. The lower content of ACN in the mobile phase was used to achieve efficient separation in HILIC mode, and the prepared column might provide a potential environmental friendly media for the further analysis of polar solutes.

2. Experimental

2.1. Reagents and materials

AMPS, PETA, 2,2′-azobisisobutyronitrile (AIBN), 3-trimethoxysilyl propyl methacrylate (γ-MAPS), were purchased from Aldrich (Milwaukee, WI, USA). Tyr-Gly, Tyr-Gly-Gly, Gly-Gly-Phe-Leu, Gly-Gly-Phe-Met, Tyr-Gly-Gly-Phe-Leu and Tyr-Gly-Gly-Phe-Met were purchased from Sigma (St. Louis, MO, USA). HPLC-grade methanol and ACN were purchased from Chemical Reagent Corporation (Shanghai, China). The water used throughout all experiments was double-distilled water. Other chemicals (Chemical Reagent Plant, Shanghai, China) were of analytical grade. The fused-silica capillaries with a dimension of 100-μm ID (375-μm OD) were purchased from the Yongnian Optic Fiber Plant (Hebei, China).

2.2. Instrumentation

pCEC was performed on a TrisepTM 2010GV CEC system (Unimicro Technologies, Pleasanton, CA, USA) equipped with a UV/Vis detector (190–600 nm), which is comprised of a microvolume pumps, a high-voltage power supply (−30 to +30 kV), a microfluid manipulation module (including a six-port injector), and a data acquisition module. Pressure was applied to the column inlet during the separation. A negative voltage was applied to the outlet of column, and the inlet of column was connected to the split valve and grounded. In this experiment, the isocratic elution system was used. Scanning electron micrographs (SEM) of the monolithic columns were carried out on an XL30 E scanning electron microscope (Philips, The Netherlands).

2.3. Preparation of the monolithic column

Prior to use, the inner wall of the capillary was firstly treated with γ-MAPS [23]. Polymerization solutions weighing 2.0 g each were prepared from monomers (AMPS and PETA) and porogenic solvents in ratios of 20:80 (w/w) monomers/solvents. The mixtures of monomers were weighed and dissolved in ternary porogenic solvents consisting of methanol, ethyl ether and water in various ratios. AIBN (4.0 mg, 1.0 wt% with respect to monomers) was added to the solution. The polymerization mixture was vortexed and then ultrasonicated for 5 min to help dissolve AMPS and eliminate oxygen [19], and then kept in ice until it was injected into the capillary for 35 cm length. The capillary was sealed and submerged into water bath with 60 °C for 20 h. The monolith was washed with methanol and water in sequence to flush out the residual reagents. A detection window was created at 1–2 mm at the end of the polymer bed. Finally, the column was cut to a total length of 50 cm with an effective length of 30 cm. A 2-cm length of the monolith was cut for SEM analysis.

3. Results and discussion

3.1. Column characterization

3.1.1. Optimization of polymerization mixture composition

It is known that the porosity of monolithic column can be varied by minor changes to the composition of the polymerization mixture [25]. The corresponding effects have been evaluated. The permeability, K value [26] ($K = \eta L u/\Delta P$, where u is the linear velocity of the eluent, η is the dynamic viscosity of the mobile phase, L is the column length, and ΔP is the pressure drop across the column), was measured for each column by changing the wt% of AMPS and methanol in the polymerization mixture. Moreover, the optimized column was selected for SEM analysis. The result was presented in Table 1.

With the monomer-to-solvent ratio 30:70 (w/w), the permeability of column H was relatively poor and the measurement could not be made because the column was not applicable. Increasing the concentration of macroporogen (i.e. methanol) to increase the porosity of the monolith did not significantly improve the permeability of the column. Polymerization solutions with high monomer content (30:70 (w/w)) were not considered further [14].

In this study, a ternary porogen solution consisting of methanol, ethyl ether and water was designed to obtain a homogeneous polymerization mixture. Because the solubility of AMPS in organic solvents is low, water was selected as one of the porogens to help dissolve AMPS. Initial trial of a polymerization mixture with more

Table 1
Composition of the polymerization solutions used in the preparation of the different monolithic columns.

Column designation	Monomer-to-solvent ratio	AMPS[a] (% w/w)	Methanol[b] (% w/w)	Water[c] (% w/w)	Permeability[d], K ($\times 10^{-14}$ m^2)
A	20:80	10.0	22.4	6.4	4.14
B	20:80	10.0	24.0	6.4	10.73
C	20:80	10.0	25.6	6.4	17.98
D	20:80	10.0	28.0	6.4	33.12
E	20:80	8.0	24.0	6.4	55.21
F	20:80	9.0	24.0	6.4	19.74
G	20:80	11.0	24.0	6.4	7.72
H	30:70	15.0	21.0	5.6	n/a

n/a: the measurements could not be made because the columns were not applicable.
[a] Percentage of AMPS in the polymerization mixture.
[b] Percentage of methanol in the polymerization mixture.
[c] Percentage of water in the polymerization mixture.
[d] The permeability was measured by using 80/20 ((v/v)) ACN/water. The viscosity of ACN/water (80/20 (v/v)) was obtained from Ref. [24].

content of water (>6.4% (w/w) in mixture) was not successful. A constant ratio of water in mixture (6.4%) was chosen for the optimization of the monolith, and effect of percentage of methanol and ethyl ether in the porogenic mixture was studied by varied the percentage of methanol in the porogenic mixture. When the wt% of methanol were varied from 22.4% (column A) to 28.0% (column D) with a constant AMPS ratio (10.0 wt%) and a constant monomer-to-solvent ratio (20:80 (w/w)), the column permeability increased from 4.14×10^{-14} m^2 to 33.12×10^{-14} m^2 (Table 1), which indicated that the increase of the wt% of methanol in the porogenic solvent mixture could increase the average pore size. In addition, at constant wt% methanol (10.0%) increasing the wt% of AMPS (columns B and E–G), the column permeability decreased from 55.21×10^{-14} m^2 to 7.72×10^{14} m^2.

To further investigate the relative content of each column in small pores at various wt% of AMPS and methanol, the plate height was measured as a function of mobile phase linear velocity by varying the applied voltage in pCEC. Fig. 1a showed the average plate height for thiourea on the monolithic columns (columns A–D) containing various amounts of methanol in polymeric stationary phases, and Fig. 1b exhibited the same plots for polymerization solutions with different amounts of the AMPS at 24.0 wt% of methanol (columns B and E–G). In general, the monolithic column with a smaller pore size would show a lower H_{min} value. As seen from Fig. 1a and b, when the content of methanol decreased (columns B–D) or the amount of the AMPS increased (columns B, E and F), a higher efficiency was observed and the pore size would be decreased. But when the pore size further decreased, the results showed a reduced mobile-phase velocity and resulted in a lower efficiency in pCEC (columns A and G). The similar decrease in efficiency for monolithic columns with pore size further smaller were reported by Peters et al. [10] and El Rassi and coworkers [27]. It may be explained by double-layer overlap mechanism [28]. When a flow channel was not wide enough in comparison with the thickness of the electrical double layer, the double layers from the opposing walls of the channel would overlap. In the stagnant pore, (almost) no EOF generated and the mass transfer in these regions became diffusion controlled. Therefore, considering peak efficiency and permeability, the monolithic column B seemed to be good in both the efficiency and pressure drop, which was selected for all further experiments. The SEM of column B was also gained in Fig. 2. It showed that this copolymerized monolith composed of spherical units agglomerated into larger clusters interdispersed by large-pore channels, which was the typical characteristic of monolithic structure.

3.1.2. Study of EOF

For the poly(AMPS-co-PETA) monolith, the EOF increases slightly from 0.52 to 0.62 mm/s with the increasing of pH from 3 to 8. It might be due to the suppression of ionization of residual silanol groups on the capillary wall under acidic buffer pH conditions. Effect of ACN content on EOF was also investigated. The EOF increased from 0.51 to 0.72 at pH 4.5 with the increasing of ACN content from 40 to 80%. It was thought to be caused by changes in the viscosity and the zeta potential [16]. With an increase of the organic content of mobile phase, the electroosmotic mobility in proportion to the ratio of dielectric constant to the viscosity was increased, which led to an increase of the EOF. When the concentration of ammonium formate buffer increased from 2 to 30 mmol/L, EOF declined slowly from 0.60 to 0.48 mm/s, which was due to the decreasing of thickness of elec-

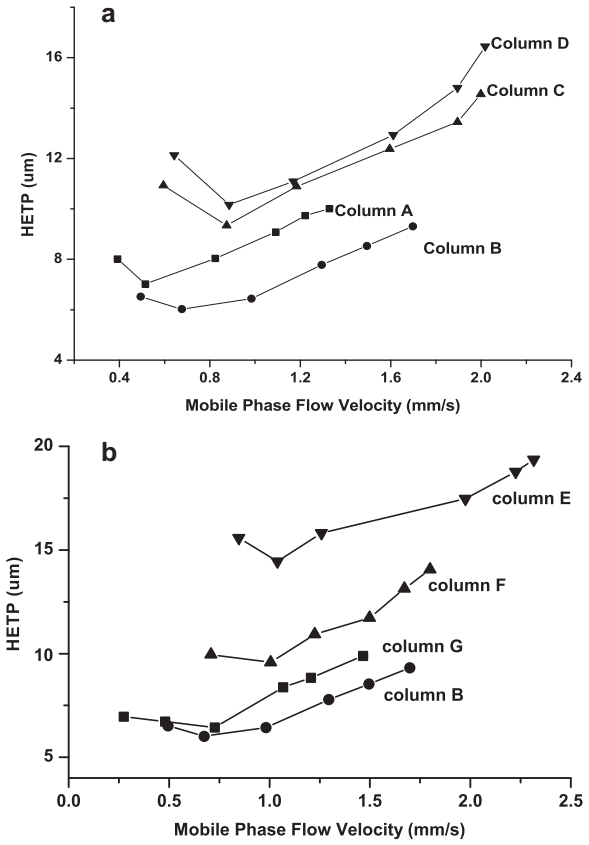

Fig. 1. HETP for various monolithic columns prepared from polymerization solution at different (a) wt% of methanol and (b) wt% of AMPS. The plate height is the average taken for thiourea. Experimental conditions: mobile phase, 5 mmol/L triethylamine phosphate, pH 7.0, at 50% (v/v) ACN; pump flow: 0.1 mL/min; applied pressure 0.8 MPa; running voltage from −5 to −25 kV.

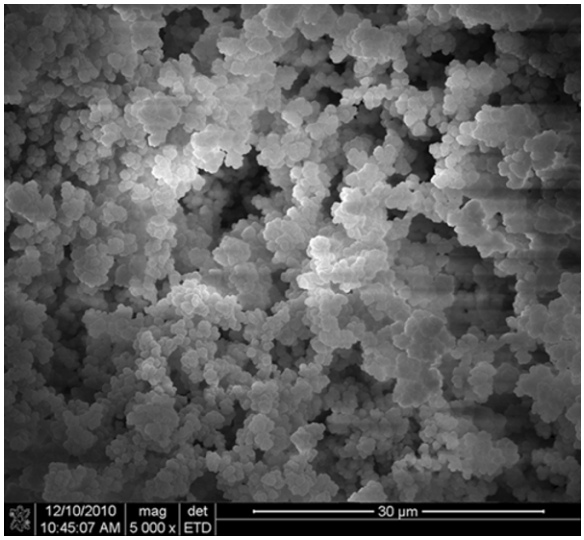

Fig. 2. Scanning electron microphotograph of monolithic column B.

Table 2
Reproducibility of EOF, retention time, and column efficiency on column B.[a]

	EOF (RSD%)	Retention time (RSD%)	Column efficiency (RSD%)
Batch-to-batch[b] ($n=4$)	3.6	3.8	4.8
Day-to-day[c] ($n=5$)	2.3	2.5	3.4

[a] Capillary column, 30 cm effective length, 50 cm total length_100 mm ID; experimental conditions: mobile phase, 5 mmol/L triethylamine phosphate buffer, pH 5.0, in ACN/H_2O (50/50 (v/v)); applied voltage: −20 kV; supplement pressure: 0.3 MPa; pump flow rate: 0.1 mL/min; detection wavelength: 214 nm.
[b] The meaning of batch-to-batch ($n=4$) was that the monoliths were prepared from four batches and every batch contained three columns.
[c] The RSD% of day-to-day was calculated by the same column.

trical double layer and zeta potential by increasing ionic strength [16].

3.1.3. Monolithic column reproducibility and stability

The reproducibility of column production was assessed through the percent RSD (RSD%) using thiourea as model solutes. Twelve monolithic columns were prepared from different batches of polymerization mixtures with the same composition. As seen from Table 2, the reproducibility data were favorable and in close agreement to those reported in the literatures [10,26,29]. Due to the one-step in situ synthesis protocol, the rate of success in preparing such monolithic capillary columns was satisfying.

Permeability is a good index to reflect swelling or shrinking of the monolith. If a monolith swells, its throughpores will decrease in size, resulting in lower permeability, and vice versa. As seen from Table 3, with the use of organic solvents, the permeability decreased with an increase in solvent relative polarity. It indicated that the monolith swelled in polar solvents and shrank in less polar solvents. However, the decreasing of the permeability (only 48%) for the monolithic column was smaller than the sulfonate-containing monolithic column Gu reported (94%) when the flushing solution was changed from acetonitrile to water. Although the poly(AMPS-co-PETA) monolith swelled in aqueous buffer and shrank in organic solvents, no detachment of the monolith from the capillary wall was observed under any conditions, likely due to covalent attachment to the capillary wall. Furthermore, the column flow rate reached a constant value after equilibration with a new solvent. This indicated reversible shrinking or swelling of the monolith existed under a variety of solvent conditions. To investigate the influence of swelling on the reproducibility, the retention time and column efficiency of three test compounds toluene, acrylamide and thiourea were measured in a mobile phase consisting of ACN/water (95:5 (v/v)) firstly. Then, the column was kept at a mobile phase consisting of ACN/water (5:95 (v/v)) for 60 min and preequilibrated with a mobile phase consisting of ACN/water (95:5 (v/v)), the retention time and column efficiency were measured again in a mobile phase consisting of ACN/water (95:5 (v/v)). The whole processes were repeated four times. The RSD value ($n=5$) of the retention time and column efficiency for three test compounds ranged in 2.3–2.9% and 3.2–3.8%, respectively. It indicated that the performance of this monolithic column did not appear to deteriorate after slightly swelling or shrinking under different solvent conditions. The polymer monolith could be used continuously over 90 days, by continued flushing with the buffer solution for at least 4 h every day under a pressure above 12 MPa and no obvious decline of column efficiency was observed. Excessive swelling of the sulfonate-containing polymer monolith in aqueous buffer, which would result in no flow [19], was not observed for the poly(AMPS-co-PETA) monolith reported in this study.

3.1.4. HILIC retention mechanism

In order to investigate the HILIC properties of poly(AMPS-co-PETA) monolith, toluene, acrylamide and thiourea were used as test compounds. The content of ACN was changed from 95% to 25% while the ammonium formate concentration was kept constant at 5 mmol/L. The influence of ACN content on the retention factors (k) of three test compounds was shown in Fig. 3. The nonpolar toluene was almost not retained on this polar monolith due to its hydrophobicity. Therefore, the k of toluene kept almost constant with the ACN content decreasing from 95% to 25%. Thiourea, commonly used as the void time marker in RPLC, had the strongest retention on the polar monolith due to its highest hydrophilicity in the three test compounds. The k of thiourea increased with the increasing content of ACN. Acrylamide behaved similar to thiourea but with less retention. When the ACN content in the mobile phase decreased to 25%, the retention factors of toluene and acrylamide were overlap. These results demonstrated a typical HILIC retention mechanism when the content of ACN in the mobile phase exceeded 25%. The content of ACN in the mobile phase for the transitions from HILIC to RP mode in hydrophilic polymethacrylate-based monoliths by CEC reported previously, which is a convenient measure of the degree of polarity of the HILIC stationary phase, were above 70% [12–14]. The critical value gained in this poly(AMPS-co-PETA) stationary phase was remarkable lower. This result indicated that this monolith

Table 3
Permeability of the optimized Poly(AMPS-co-PETA) monolith.

Flushing fluid	Relative polarity[a]	Viscosity, η (cP)[b]	Column back pressure, ΔP (MPa)	Linear velocity, u (mm/s)[c]	Permeability, k ($\times 10^{-14}$ m^2)
Acetonitrile	0.460	0.369	4.9	24.2	18.2
Methanol	0.762	0.544	4.9	13.1	14.5
Water	1.000	0.890	6.9	7.3	9.4
Water/acetonitrile 50/50 (v/v)	/	0.820	6.9	8.4	10.1

[a] Relative polarity data were from http://virtual.yosemite.cc.ca.us/smurov/orgsoltab.htm.
[b] Viscosity data were from online CRC Handbook of Chemistry and Physics, 85th ed., CRC, Boca Raton, 2004–2005.

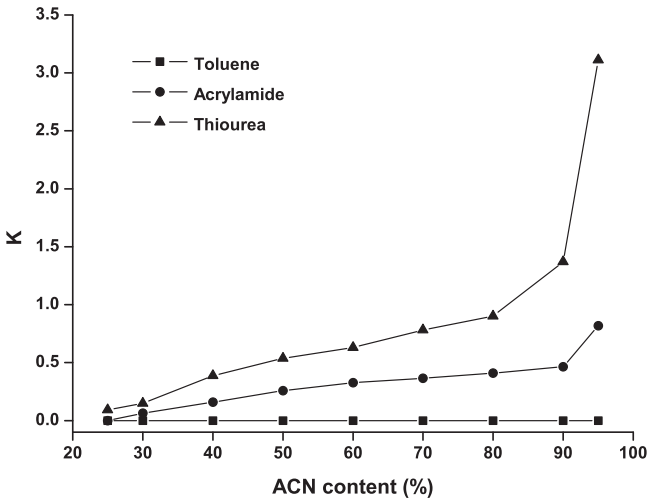

Fig. 3. Relationship between retention factor and acetonitrile concentration on poly(AMPS-co-PETA) monolithic column. Conditions: capillary column B, 30 cm effective length, 50 cm total length 100 MM ID; mobile phase, 5 mmol/L triethylamine phosphate buffer (TEAP), pH 7.0, in ACN/H_2O; applied pressure 0.8 MPa; detection wavelength: 214 nm; applied voltage: −10 kV; pump flow: 0.1 mL/min.

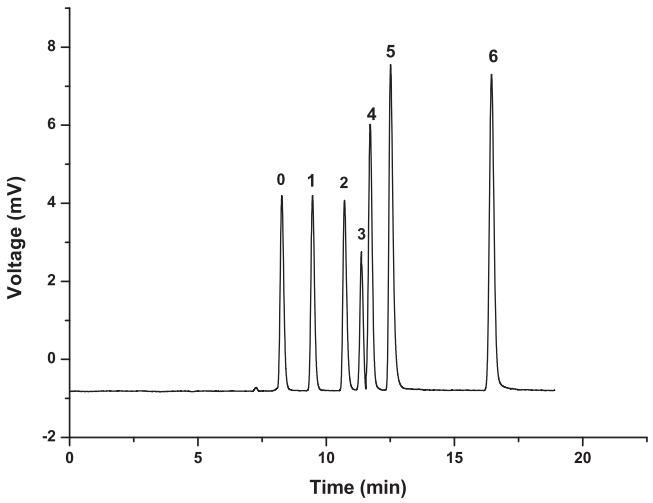

Fig. 4. Electrochromatogram of phenols. Experimental conditions: mobile phase, 5 mmol/L TEAP, pH 7.0, in ACN/H_2O (50/50 (v/v)); applied voltage: −15 kV; supplement pressure: 0.8 MPa; other conditions were as in Fig. 3. Solutes: 0. Toluene, 1. Phenol, 2. Hydroquinone, 3. Resorcinol, 4. Catechol, 5. Pyrogallol, 6. Phloroglucinol.

exhibited high hydrophilicity with a low critical composition of ACN in the mobile phase (about 25% ACN in mobile phase) corresponding to the transition from the HILIC to the RP mode. The increased hydrophilicity of this monolith should be due to the incorporation of hydrophilic monomer AMPS and cross-linker PETA. The AMPS molecule contains the hydrohphilic sulfonate group and the hydrophilic acrylamido group, which connect to the short alky chain. The high percentage (50%) of AMPS copolymerized into the monolith backbone increased the hydrophilicity effectively. The PETA was used instead of traditional EDMA as cross-linker to prepare the monolith with more hydrophilicity due to its hydroxyl sub-layer. It was believed that the critical concentration shifted to a lower content of ACN, and would allow the use of mobile phases with higher water content in HILIC mode.

3.2. Applications

3.2.1. Neutral compounds

3.2.1.1. Separation of phenols. The polar poly(AMPS-co-PETA) surface can provide a hydrophilic environment. To further characterize the polymethacrylate-based monolith under investigation, a series of phenols were selected as model analytes in pCEC. As shown in Fig. 4, the retention increased with the rise in hydroxyl groups within the phenol molecule. The elution order of the phenols was listed as follows: less polar phenol with one hydroxyl group first, and then followed by more polar hydroquinone, resorcinol and catechol with two phenolic hydroxyl groups, and finally the most polar pyrogallol and phloroglucinol with three phenolic hydroxyl groups. All positional isomers of phenols could be well separated. The result showed that the extent of polar interaction depended on the position and the number of the hydroxyl groups. Six phenols were well separated with 50% ACN content in the mobile phase while more than 90% content of ACN had to be used in the reported hydrophilic methacrylate-based monoliths [14–16].

3.2.1.2. Separation of amides. Another mixture of amides including formamide, acrylamide, acetamide, DMF and N, N-dimethylenebisacrylamide was used to investigate the separation of structurally related neutral compounds that was difficult to achieve in RPLC. As can be seen from Fig. 5, good separation was obtained with 50% ACN content. However, in the previous work, the separation of these amides were gained in hydrophilic methacrylate-based monoliths by using a high content (more than 90%) of ACN in the mobile phases [14,15]. Additionally, the order of elution was listed as DMF < N,N-dimethylenebisacrylamide < acrylamide < acetamide < formamide. It was well according to the polarity of the solutes, which indicated a typical HI separation mechanism.

3.2.2. Separation of peptides

A major limitation of RPLC for peptides separation is the lack of adequate retention of small hydrophilic peptides. HI mode has proved to be a very useful separation mode for small hydrophilic peptides [30,31]. To investigate the selectivity of the poly(AMPS-co-PETA) monolith for small hydrophilic peptides, six peptides (Tyr-Gly, Tyr-Gly-Gly, Gly-Gly-Phe-Leu, Gly-Gly-Phe-Met, Tyr-Gly-Gly-Phe-Leu and Tyr-Gly-Gly-Phe-Met) were selected and some parameters were optimized.

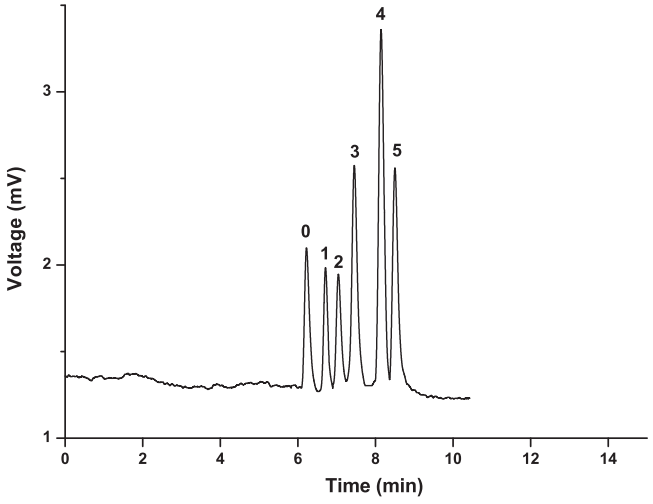

Fig. 5. Electrochromatogram of amides. Experimental conditions: mobile phase, 5 mmol/L ammonium formate, pH 7.0, in ACN/H_2O (50/50 (v/v)); supplement pressure: 1.2 MPa; applied voltage: −10 kV; other conditions were as in Fig. 4. Solutes: 0. Toluene, 1. DMF, 2. N,N-dimethylenebisacrylamide, 3. acrylamide, 4. Acetamide, 5. Formamide.

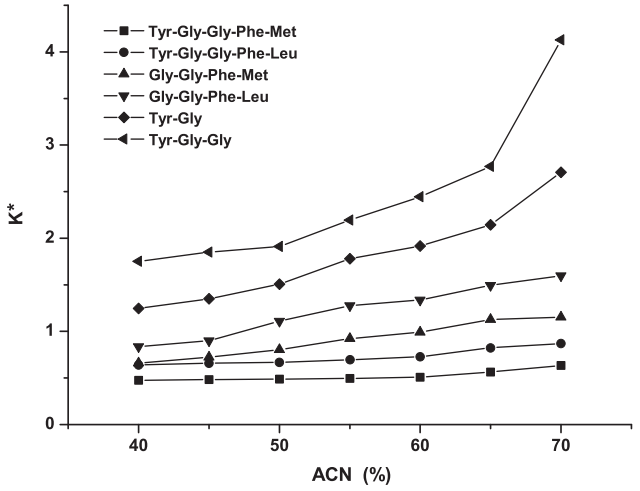

Fig. 6. Effect of acetonitrile concentration on the k^* value. Experimental conditions: mobile phase, 80 mmol/L ammonium formate pH 4.5 in ACN/H_2O; applied voltage: −10 kV; supplement pressure: 3.4 MPa; other conditions as in Fig. 3.

3.2.2.1. Effect of the organic modifier concentration. To describe the elution of charged solutes in pCEC, the retention factor (k^*) was defined by the following equation:

$$k* = \frac{(t_r - t_0)}{t_0} \quad (1)$$

where k^* is the retention factor, t_r is the migration time of the analyte and t_0 is the migration time of the EOF marker in the pCEC.

The effect of the ACN concentration ranging from 40% to 70% (v/v) on the retention of the peptides was investigated and shown in Fig. 6. With increasing the content of ACN, both the retention time and the resolution of the solutes increased. It might attribute to the result of enhanced hydrophilic interactions between the solutes and the hydrophilic stationary phase. All six tested peptides could be baseline separated when the concentration of ACN was above 50% (v/v).

3.2.2.2. Effect of pH value. The influence of pH on the retention of peptides was studied at pH value of 4, 4.5, 5, 6 and 7. The results were shown in Fig. 7. It illustrated the dependence of k^* of some peptides on pH values of the mobile phase. The retention of the

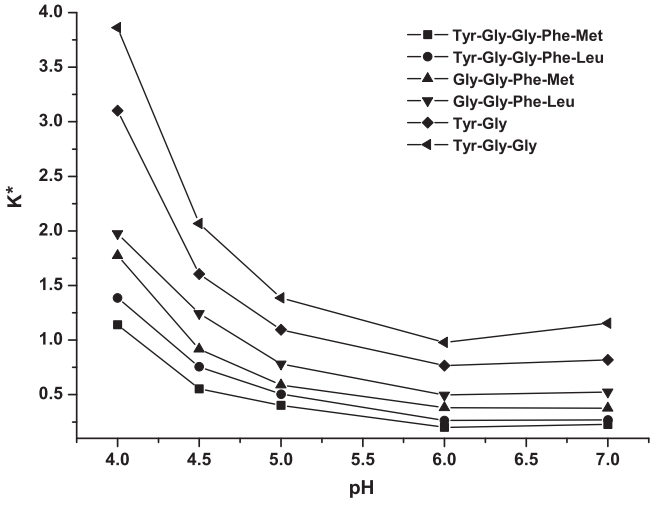

Fig. 7. Effect of pH on the k^* value. Experimental conditions: mobile phase, 80 mmol/L ammonium formate with pH 4, 4.5, 5, 6 and 7 in ACN/H_2O (50/50 (v/v)); applied voltage: −10 kV; supplement pressure: 3.4 MPa; other conditions as in Fig. 3.

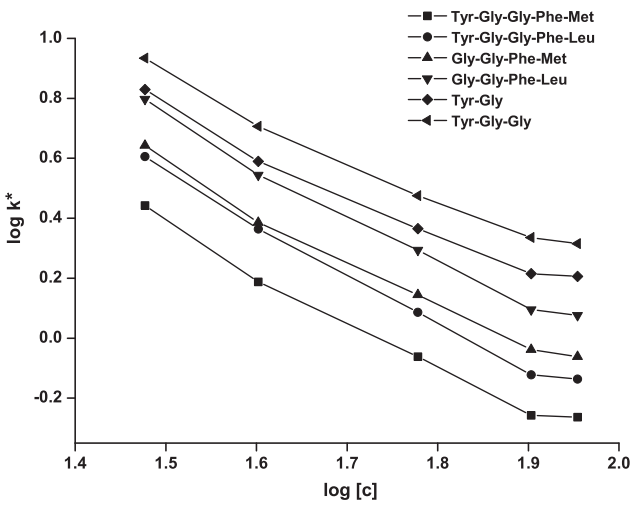

Fig. 8. Effect of salt concentration on the k^* value. Experimental conditions: mobile phase: 50% (v/v) acetonitrile in ammonium formate with varied concentration from 30 to 90 mM (pH 4.5); applied voltage: −10 kV; supplement pressure: 3.4 MPa; other conditions as in Fig. 3.

peptides was remarkably dependent on the pH of the mobile phase owing to their amphoteric property. Since the p*I* values of the selected peptides are near 6.1, the peptides were positively charged in the mobile phase with a low pH value, and both hydrophilic and electrostatic interaction could contribute to the retention of the peptides, while electrophoresis accelerated the solutes towards the cathode. The combination of these mechanisms resulted in the moderate retention of peptides at low pH. The retention of peptides decreased with an increasing pH value of the mobile phase from 4.0 to 6.0. It could be attributed to the fact that the electrostatic interaction reduced because peptides were deprotonated and changed neutral gradually. When the pH value increased further, peptides gradually would become negatively charged and cause electrostatic repulsion from the stationary phase. However, the negatively charged peptides tend to migrate against the EOF, and the migration time of peptides almost stayed the same. Therefore, considering the elution time and the resolution, pH 4.5 was selected for the following optimization.

3.2.2.3. Effect of ionic strength. Effects of salt concentration on the retention of peptides were also investigated by changing the concentration of ammonium formate from 30 to 90 mmol/L in the mobile phase. At pH 4.5, the stationary phase surface was negatively charged due to the ionization of sulfonate groups from AMPS (pK_a of 1.2) and residual silanol groups [19]. Therefore, electrostatic interactions between positively peptides and negatively charged stationary phase surface would happen. As can be seen in Fig. 8, the retention of peptides decreased with increasing ionic strength of the mobile phase. For the analytes and the stationary phase with different charge, a higher salt concentration would lead to a lower retention factor. The tendency was similar to that in the case of ion-exchange CEC [32,33]. Considering the effect of the joule heat caused by the high ionic strength, the elution time and the resolution, 80 mmol/L was selected as best salt concentration for the separation of the peptides. The result indicated that ion-exchange mechanism participated in the separation. A mixed mode of HI and SCX could be also obtained in the analysis of these charged peptides.

3.2.2.4. Effect of applied voltage and supplementary pressure. The influence of the applied voltage (−4 to −16 kv) on the migration behavior of peptides was investigated. The retention factor of all peptides decreases with increasing applied voltage, and the elution order remains the same. This may be caused that at pH 4.5,

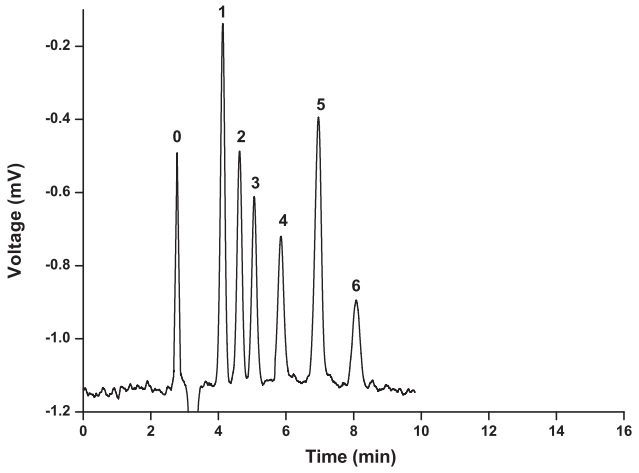

Fig. 9. Electrochromatogram of peptides. Experimental conditions: mobile phase, 80 mmol/L ammonium formate pH 4.5 in ACN/H_2O (50/50 (v/v)); applied voltage: −10 kV; supplement pressure: 3.4 MPa; other conditions as in Fig. 3. Solutes: 0. Toluene, 1. Tyr-Gly-Gly-Phe-Met, 2. Tyr-Gly-Gly-Phe-Leu, 3. Gly-Gly-Phe-Met, 4. Gly-Gly-Phe-Leu, 5. Tyr-Gly, 6. Tyr-Gly-Gly.

the peptides bear positive charge, and their electrophoretic velocity increased with the increasing of the applied voltage. The applied voltage could improve the peak shape, but too high voltage would increase baseline noise. A voltage of −10 kV was applied for a relatively rapid and baseline separation.

The influence of the pressure on the migration behavior of peptides was also studied. With the introducing of supplementary pressure in pCEC, the mobile phase was propelled by an EOF and a pressurized flow with a little expense in column efficiency. Along with the pressure increased from 1.1 to 7.0 MPa, a decrease in retention time of all analytes were observed and the k^* almost kept constant. Finally, a supplementary pressure of 3.4 MPa was chosen to achieve the compromise between analysis time and resolution.

Under the optimum conditions, all six peptides were separated with baseline resolution within 9 min without peak tailing (Fig. 9). The column efficiency up to 80,000 plates/m for Gly-Gly-Phe-Leu could be achieved on this monolith.

4. Conclusion

A novel hydrophilic polymethacrylate-based monolithic column based on the copolymerization of AMPS and PETA in the present of porogens was prepared and successfully used as a polar stationary phase in HI mode by pCEC. The favorable features of poly(AMPS-co-PETA) monolith are highly hydrophilic and satisfying stability. Due to the highly hydrophilic, effective separations for neutral polar amides, phenols, and charged peptides could be obtained with the content of ACN only about 50%. It was remarkably lower than the content of ACN (>90%) used on the hydrophilic polymethacrylate-based monoliths reported previously, making it ideal for providing a potential environmental friendly separation media for polar solutes. Due to the improvement of the stability for monolith containing high amount of solfonate groups, well chromatography performance and easy of preparation, the poly(AMPS-co-PETA) monolith is expected to find many applications.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgements

This work was supported by National Natural Science Foundation (20907009, 40976071, and 20905079), Foundation of the Ministry of Science and Technology (2009GJC40009), Key Science & Technology Project of Fujian Province (2008Y2004).

References

[1] A.J. Alpert, J. Chromatogr. 499 (1990) 177.
[2] Z. Jiang, N.W. Smith, P.D. Ferguson, M.R. Taylor, Anal. Chem. 79 (2007) 1243.
[3] U. Pyell, J. Chromatogr. A 892 (2000) 257.
[4] N. Ishizuka, H. Minakuchi, K. Nakanishi, N. Soga, H. Nagayama, K. Hosoya, N. Tanaka, Anal. Chem. 72 (2000) 1275.
[5] T. Ikegami, K. Tomomatsu, H. Takubo, K. Horie, N. Tanaka, J. Chromatogr. A 1184 (2008) 474.
[6] J. Ou, J. Dong, X. Dong, Z. Yu, M. Ye, H. Zou, Electrophoresis 28 (2007) 148.
[7] X. Dong, R. Wu, J. Dong, M. Wu, Y. Zhu, H. Zou, Electrophoresis 30 (2009) 141.
[8] F. Svec, E.C. Peters, D. Sykora, J.M.J. Fréchet, J. Chromatogr. A 887 (2000) 3.
[9] M.M. Dittmann, K. Masuch, G. Rozing, J. Chromatogr. A 887 (2000) 209.
[10] E.C. Peters, M. Petro, F. Svec, J.M.J. Fréchet, Anal. Chem. 70 (1998) 2288.
[11] E.G. Vlakh, T.B. Tennikova, J. Chromatogr. A 1216 (2009) 2637.
[12] J. Wang, H. Lu¨, X. Lin, Z. Xie, Electrophoresis 29 (2008) 928.
[13] X. Wang, H. Lu¨, X. Lin, Z. Xie, J. Chromatogr. A 1190 (2008) 365.
[14] J. Lin, G. Huang, X. Lin, Z. Xie, Electrophoresis 29 (2008) 4055.
[15] X. Wang, X. Lin, Z. Xie, Electrophoresis 30 (2009) 2702.
[16] X. Wang, X. Lin, Z. Xie, J.P. Giesy, J. Chromatogr. A 1216 (2009) 4611.
[17] V. David, M. Calley, J. Chromatogr. A 1217 (2010) 858.
[18] Z. Jiang, N.W. Smith, P.D. Ferguson, M.R. Taylor, J. Sep. Sci. 32 (2009) 2545.
[19] B. Gu, Z. Chen, C.D. Thulin, M.L. Lee, Anal. Chem. 78 (2006) 3509.
[20] Y. Ueki, T. Umemura, J. Li, T. Odake, K. Tsunoda, Anal. Chem. 76 (2004) 7007.
[21] E.C. Peters, M. Petro, F. Svec, J.M.J. Fréchet, Anal. Chem. 69 (1997) 3646.
[22] E.F. Hilder, F. Svec, J.M.J. Fréchet, J. Chromatogr. A 1053 (2004) 101.
[23] B. Xiong, L. Zhang, Y. Zhang, H. Zou, J. High Resolut. Chromatogr. 23 (2000) 67.
[24] H. Wode, W. Seidel, Ber. Bunsen Phys. Chem. 98 (2004) 927.
[25] Y. Ueki, T. Umemura, Y. Iwashita, T. Odake, H. Haraguchi, K. Tsunoda, J. Chromatogr. A 1106 (2006) 106.
[26] P.A. Bristow, J.H. Knox, Chromatographia 10 (1977) 279.
[27] M. Bedair, Z. El Rassi, Electrophoresis 23 (2002) 2938.
[28] S. Eeltink, J. Herrero-Martinez, G.P. Rozing, P.J. Schoenmakers, W.T. Kok, Anal. Chem. 77 (2005) 7342.
[29] A. Palm, M.V. Novotny, Anal. Chem. 69 (1997) 4499.
[30] T. Yoshida, J. Biochem. Biophys. Methods 60 (2004) 265.
[31] A.J. Alpert, Anal. Chem. 80 (2008) 62.
[32] G. Choudhary, C. Horváth, J. Chromatogr. A 781 (1997) 161.
[33] M. Ye, H. Zou, Z. Liu, J. Ni, J. Chromatogr. A 869 (2000) 385.