Research Article

Hybrid silica monolithic column for capillary electrochromatography with enhanced cathodic electroosmotic flow

A hybrid silica monolithic stationary phase for RP CEC was prepared by in situ co-condensation of (3-mercaptopropyl)-trimethoxysilane (MPTMS), phenyltriethoxysilane (PTES), and tetraethoxysilane (TEOS) via a sol–gel process. The thiol groups on the surface of the stationary phase were oxidized to sulfonic acids by perox trifluoroacetic acid. The introduced sulfonic acid moieties on the monoliths were characterized by a strong and relatively stable EOF in a broad pH range from 2.35 to 7.0 in CEC. Aromatic acids and neutral compounds can be simultaneously separated in this column under cathodic EOF. The CEC column exhibited a typical RP chromatographic mechanism for neutral compounds due to the introduced phenyl groups.

Keywords: Aromatic acid / Capillary electrochromatography / Electroosmotic flow / Silica monolithic column / Sol–gel process DOI 10.1002/elps.200600171

1 Introduction

Recently, CEC has developed rapidly as an attractive alternative separation technique, especially due to the high demand for new miniaturized separation methods having vastly enhanced efficiencies and peak capacities compared with traditional LC techniques [1]. It is well known that the driving force of the mobile phase in CEC is EOF which is generated on the surface of the stationary phases by applying an electrostatic potential across the entire length of a device, such as a capillary or a flat profile cell. In addition to providing sites for the required interactions, CEC stationary phase should also play a role in generating EOF. To attain rapid and reproducible separations, it is important to produce electrochromatographic columns with strong and stable EOF.

Although a variety of approaches for preparation of stationary phases have been described in CEC, the majority of them can be classified into particle packing materials and monolithic materials (continuous bed). Until now, the most commonly used RP stationary phase for packed columns in CEC has usually been standard commercial HPLC-grade ODS [2–5]. However, the rate of EOF in silica packed columns is usually poor with low pH buffer as eluent because the amount of ionized silanol groups on stationary phase is small. Considering the need to maintain a high enough EOF and the hydrophobic surface of the stationary phase, ODS can only work well in the mobile phase with relatively high pH and organic modifier concentration, which creates difficulties in the analysis of acidic and polar compounds. To meet with the demand of stationary phase suitable for CEC, specially designed silica materials, such as mixed-mode stationary phases and mixed-packing materials have been developed [6–10]. Due to the introduced sulfonic groups or quaternary ammonium groups, these materials can attain substantial EOF over a wide pH range. However, these materials face with tedious fabrication procedure or uncontrollable packing uniformity. Recently, monoliths have quickly become a well-established stationary phase format in the field of CEC. The simplicity of their in situ preparation method as well as the good control over their porous properties and surface chemistries make the monolithic separation media an attractive alternative to capillary columns packed with particulate materials [11–15]. Depending on the nature of the monolithic material, two major monolithic columns can

---

Correspondence: Professor Zhike He, College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, P. R. China
E-mail: zhkhe@whu.edu.cn
Fax: +86-27-68754067

Abbreviations: MPTMS, (3-mercaptopropyl)-trimethoxysilane; PTES, phenyltriethoxysilane; SCX, strong cation-exchange; TEOS, tetraethoxysilane

© 2006 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

www.electrophoresis-journal.com
be identified: organic polymer-based and silica-based inorganic monoliths. The organic-based monoliths are often made of a mixture of monomers, crosslinkers and porogens via radical polymerization. The EOF in this type of columns can be easily adjusted by changing the percentage of ionizable monomer or reactive neutral monomer in the polymerization [16, 17]. In contrast to organic-based monolithic columns, silica-based monolithic columns exhibit excellent mechanical stability under the rinsing of various mobile phases and are receiving more and more attentions [5, 14, 18]. Similar to silica particle beads, silica monolithic columns are also often further modified with both alkyl chain as the interaction sites with solutes and ionizable moieties for the manipulation of EOF [19, 20]. Recently, as an alternative of functionally modified silica monolithic column, several kinds of silica-based organic–inorganic hybrid monolithic materials have been introduced in CEC [21–24]. One kind of hybrid silica monoliths is usually synthesized by mixing several types of sol–gel precursors with porogenic solvents [23, 24], which can initially incorporate functional monomers into the silica rod and avoid tedious and restricted on-column further silanization.

In this paper, three sol–gel precursors, (3-mercaptopropyl)-trimethoxysilane (MPTMS), phenyltriethoxysilane (PTES), and tetraethoxysilane (TEOS), were used in one pot for the fabrication of hybrid silica monolithic columns. The introduced thiol groups were oxidized to sulfonic groups under mild conditions. Due to the ionization of sulfonic groups, the column showed strong and relatively stable EOF over a broad range of pH. Aromatic acids could be separated under low pH conditions and some neutral compounds could attain quick separation on this column even with relatively low ACN content.

2 Materials and methods

2.1 Instrumentation

Electrochromatographic experiments were carried out on an HP3D CE instrument from Agilent (Waldbonn, Germany) equipped with a diode array detection (DAD) UV detector. Data collection and instrumental control were performed using the HP ChemStation software.

2.2 Chemicals and materials

TEOS, PTES, and dodecylamine were purchased from ABCR (Karlsruhe, Germany). MPTMS and urea hydrogen peroxide adduct were from Sigma Chemical (St. Louis, MO, USA). HPLC-grade methanol and ACN were supplied by Yuwang Chemical Plant (Zibo, Shandong Prov-ince, China). All other chemicals were obtained from Shanghai Reagent Factory (Shanghai, China). Water used in all of the experiments was doubly distilled and purified by a Milli-Q system (Millipore, Milford, USA). Capillary with 75 μm id × 375 μm od was purchased from Yongnian Optic Fiber Plant (Hebei, China).

2.3 Column preparation

In order to clean the inner surface and expose the maximum number of silanol groups on it, the capillary was first flushed with 1 M NaOH for 0.5 h, then both ends were sealed and placed in a water bath at 60°C for 2 h. After that, it was rinsed with water, 0.1 M HCl, water, and methanol for 0.5 h, respectively. Finally, the capillary was dried at 115°C under nitrogen gas flow for 4 h prior to use. The hybrid monolith was synthesized according to a procedure previously reported by Yan et al. [24] with some modifications. The prepared precursor solution, containing 84 μL of TEOS and 75 μL of PTES, were mixed with the porogen solution, consisting of 200 μL of methanol and 30 μL of 0.2 M HCl solution, in a 1.5 mL vial. After thorough vortexing, the solution was placed in a water bath at 60°C for 3 h. Then 1 μL (for column A) or 5 μL (for column B) of MPTMS was added into the solution. After ultrasonication for 15 min at 0°C, 15 mg dodecylamine was added into the solution. After violent vortexing, the obtained homogeneous mixture was introduced into a 35 cm pretreated capillary for about 10 cm length by a syringe. With both ends sealed by rubber, the capillary was submerged in a water bath at 40°C overnight. The produced column was subsequently rinsed with methanol and ACN for 1 h, respectively. The thiol groups introduced in the monoliths were oxidized by a method similar to the previous reports [25, 26] with some modifications, the ACN solution consisting of urea hydrogen peroxide adduct (UHP; 55 mg/mL) and trifluoroacetic anhydride (TFAA; 60 μL/mL) was used to flush the column at ambient temperature for 3 h. Finally, the monolithic column was flushed with ACN and water for 30 min, respectively, then conditioned with mobile phase prior to use.

2.4 Samples and buffers

The aromatic compounds and neutral solutes were first dissolved in ethanol, and then diluted to the appropriate concentrations with a mixture of ACN/H2O (1:1 v/v) before injection. Phosphate buffer (10 mM) was prepared and adjusted to the desired pH by addition of HCl solution. Mobile phase was prepared by adjusting the buffer to the desired pH, then mixing with the appropriate amount of ACN and water.
2.5 Electrochromatographic experiments

The detection window was created at the end of the monolithic material. The monolithic column with 8.5 cm effective length and 33 cm total length was placed in the CE instrument and equilibrated by applying a low voltage (5 kV) until the current and the baseline signal were stabilized. Ethanol was used as EOF marker. The temperature was kept at 25°C and the detection wavelength was set at 214 nm.

3 Results and discussion

3.1 Column preparation

Sol–gel technology offers a versatile means for the synthesis of both silica-based inorganic porous materials and organic–inorganic hybrid materials based on the polycondensation of siloxane with/without organosiloxane precursors [27]. Usually, silica-based inorganic monolithic columns for HPLC and CEC are synthesized by sol–gel processing with only one type of precursor, tetramethoxysilane, and water under acidic conditions in the presence of porogenic agent. After fabrication of the monolithic support, the surface of the silica monolith is functionalized through conventional silane chemistry to anchor the stationary phase by means of siloxane bond. The most advantage of this method is that batches of bare monolithic columns with excellent mechanical stability could be prepared under optimized conditions while, in addition to the tedious preparation period, one drawback of this approach is that stationary phases usually need to be anchored to the monoliths through restricted silane chemistry reactions.

In contrast to the inorganic monolithic column mentioned above, the recently introduced hybrid organic–inorganic monolithic column represents another attractive direction in CEC. Based also on sol–gel technology, various silane coprecursors containing moieties of interest could be directly incorporated in the reaction mixture with the aid of catalysts and appropriate solvents [23, 24, 28]. In our experiment, three sol–gel precursors, MPTMS, PTES, and TEOS, were used for the preparation of monolithic columns by a two-step catalytic sol–gel process [24], in which the sol was prepared from acid-catalyzed hydrolysis of the three precursors at elevated temperature, then the polycondensation reactions were accelerated by the addition of dodecylamine as the second catalyst. It was thought that the functional organosiloxane groups, such as mercaptopropyl and phenyl, were incorporated into the monolithic materials through the neutral amine assembly pathway [28]. However, similar to that in the preparation of organic polymeric monolithic columns, any changes in the composition of the polycondensation mixture require a corresponding adjustment in the reaction conditions to maintain a porous structure suitable for CEC. It is well known that the ratio of monomer/cross-linker plays an important role in the polycondensation as well as catalysts and porogenic solvents. When a certain amount of MPTMS was introduced, the ratio of PTES/TEOS needed to be optimized. The PTES/TEOS ratio adopted in column A was the optimized result when the ratio of MPTMS in the total volume of precursors was fixed at 0.6% v/v. Compared with the optimized ratio, increasing or decreasing the PTES percentage in the sol–gel solution would lead to the tendency of crack or poor permeability of the monolithic column, respectively. For comparison, column B was prepared under the same contents of PTES and TEOS except MPTMS.

The introduced thiol groups were oxidized to sulfonic acid groups by an ACN solution containing TFA and urea hydrogen peroxide adduct at ambient temperature, in which the in situ generated peroxytrifluoroacetic acid served as oxidant [26]. This method has the advantage of good safety and high reaction efficiency. The reproducibility of column A based on the retention times of DMSO was investigated using the mobile phase containing 20% ACN in 5 mM phosphate buffer at pH 2.35. The values for run-to-run, day-to-day, and column-to-column were less than 2.03% (n = 5), 2.60% (n = 5), and 7.74% (n = 3), respectively.

3.2 Characteristics of EOF

EOF velocity is directly proportional to the zeta potential that, in turn, is directly related to the surface charge. The introduction of reactive monomers offers various possibilities for controlling the exact nature of the surface chemistry. Similar to that in organic polymeric monolithic columns, the ability to control easily the level of the charged functionalities that support the EOF is a vital advantage of the hybrid monolithic columns.

Comparisons among relationships of pH and EOF on these columns are shown in Fig. 1. It could be found that cathodic EOF of columns A and B (after oxidation) remained high and relatively constant in a wide pH range of 2.35–7.0, which indicates that EOFs of these columns were mainly generated under acid conditions from the negatively charged sulfonic groups. In contrast to columns A and B (after oxidation), the EOF of column B (before oxidation) decreased steeply with the decreasing of buffer pH from 7.0 to 3.0 and the magnitude of EOF was very low when buffer pH was lower than 4.5 because the dissociations of silanols were suppressed. In addition, Fig. 1 also shows that the magnitude of EOF of column A...
was usually lower than that of column B (after oxidation) at the same pH, which could be ascribed to the different surface density of charged groups. Considering that column A could provide adequate EOF under low-pH conditions, the separation experiments were mainly performed on column A.

The effect of ACN percentage in mobile phase, while maintaining all other separation parameters constant, was also investigated. As shown in Fig. 2, the EOF of column A barely varied with increasing ACN content from 20 to 50% v/v, which was a little different from that of ODS columns [29]. However, similar phenomenon was observed in both the strong cation-exchange stationary phase (SCX)/ODS mixed-packed stationary phase [9] and the mixed-mode monolithic stationary phase prepared by copolymerization of 2-(sulfooxy)ethyl methacrylate and ethylene dimethacrylate [15]. It is known that EOF is dependent on both the zeta potential and the ratio of dielectric constant and viscosity. Since the ratio of dielectric constant and viscosity is determined by the mobile phase, it can be assumed that the EOF of this column at ACN proportions in the range from 20 to 50% v/v is governed by the zeta potential.

**3.3 Separation of aromatic acids**

On one hand, separation of the acidic solutes in their ionized form in RP CEC with cathodic EOF is relatively difficult because they tend to migrate against EOF, and thereby the migration times are relatively long or cannot even be eluted out; on the other hand, if a column with anionic EOF is used, peak-tailing of acidic compounds is usually another boring problem due to the adsorption by the positively charged surface. So it is very important to prepare a column with strong cathodic EOF at low buffer pH for the effective separation of acidic compounds under ion-suppressed mode. To investigate the column performances, eight aromatic acids, including p-hydroxybenzoic acid (pKₐ 4.58), o-phthalic acid (pKₐ 2.89), 2,5-hydroxybenzoic acid (pKₐ 2.97), benzoic acid (pKₐ 4.20), o-hydroxybenzoic acid (pKₐ 3.0), p-nitrobenzoic acid (pKₐ 3.43), m-nitrobenzoic acid (pKₐ 2.45), and o-bromobenzoic acid (pKₐ 2.85) were selected as the test solutes. The separation of eight aromatic acids under different mobile phase pH is shown in Fig. 3, from which it can be seen that baseline separation can be obtained in 13 min with phosphate buffer of pH 2.35 containing only 20% ACN. However, when the pH is 4.0, all acids, except p-hydroxybenzoic acid and benzoic acid, cannot be eluted and detected within 15 min. Under experimental conditions, the direction of electrophoretic mobilities of acids was opposited to that of EOF. So, the net migrating velocities of some of acids became very small with the increase of pH and could not be eluted out in a short time with mobile phase containing low ACN content (Fig. 3B). The elution order shown in Fig. 3A can be explained by the combined effects of hydrophobicity, electrophoretic mobility, and electrostatic repulsion. For example, o-hydroxybenzoic acid was eluted later than p-hydroxybenzoic acid, and m-nitrobenzoic acid was eluted after p-nitrobenzoic acid, which can be ascribed to the differences in electrophoretic mobilities; o-bromobenzoic acid was eluted after o-hydroxybenzoic acid, which may be ascribed to their hydrophobicity; benzoic acid was eluted after 2,5-hydroxybenzoic acid, which is related with their hydrophobicity and the electrostatic repulsion.
Figure 3. Separation of eight aromatic acids on column A. Experimental conditions: mobile phase, ACN/H₂O/10 mM phosphate buffer 20:30:50 v/v/v; applied voltage, 15 kV; injection, 2 kV x 2 s. Peaks: 1, p-hydroxybenzoic acid; 2, o-phthalic acid; 3, 2,5-hydroxybenzoic acid; 4, benzoic acid; 5, p-nitrobenzoic acid; 6, m-nitrobenzoic acid; 7, o-hydroxybenzoic acid; 8, o-bromo- benzoic acid.

Figure 4. Simultaneous separation of acidic and neutral solutes on column A. Mobile phase: ACN/H₂O/10 mM phosphate buffer (pH 2.35) 30:20:50 v/v/v; other conditions are the same as in Fig. 2. Peaks: 1, p-hydroxybenzoic acid; 2, o-phthalic acid; 3, 2,5-hydroxybenzoic acid; 4, benzoic acid; 5, α-benzene alcohol; 6, o-hydroxybenzoic acid; 7, α-benzene propanol; 8, acetoephone; 9, benzonitrile; 10, benzene; 11, toluene; 12, o-xylene.

between solutes and sulfonic groups. The average separation efficiencies of all eight acids based on five consecutive runs range from 140 000 to 300 000. Recently, separations of acidic compounds on other stationary phases have also been reported. The SCX/ODS mixed-mode stationary phase had been used for the separation of eight acidic drugs in the ion-suppressed mode with a mobile phase (pH 1.5) [30]. The disadvantage of this method is obvious that the void time of column marked by thiourea increased about three-fold from 1.97 to 5.63 min. Ye et al. [31] have compared the ODS-packed column with the strong anion-exchange (SAX)-packed column for the separation of six kinds of aromatic acids in ion-suppressed mode, from which it is found that a mobile phase containing as high as 60% ACN was used in order to reduce the elution times of acids in the ODS-packed column, which, however, lead to the coelution of some acids. On the contrary, the tested acids can be eluted and baseline separation obtained in a short time in our column with the mobile phase containing only 20% ACN. The high column performances exhibited in our columns might be related with the effect of electrostatic-repulsion on the stationary phase due to the introduction of sulfonic groups.

3.4 Simultaneous separation of acidic and neutral solutes

Theoretically, both the ionic and neutral compounds can be separated by CEC. However, most of the reported applications of CEC focused on the analysis of neutral compounds. As mentioned in Section 3.3, separation of acidic compounds in RP CEC is recommended with ion-suppressed mode using a low-pH mobile phase. Difficulties in the simultaneous separation of acidic and neutral hydrophobic compounds on RP stationary phase under low-pH conditions mainly originate from the weak cathodic EOF. It is well known that the migration of charged species can be driven by both electrophoretic mobility and EOF, while neutral species is only driven by EOF. Therefore, the weak EOF would lead to tedious analysis times for neutral compounds. Considering that the strong cathodic EOF can be generated in our columns under low pH mobile phase, the separation of a mixture of 12 solutes, including five aromatic acids and seven neutral solutes, was investigated on the hybrid monolithic column. As is shown in Fig. 4, simultaneous separation of acids and neutral solutes can be obtained. It is also found that o-hydroxybenzoic acid could be eluted later than
\( \alpha \)-benzene alcohol, which clearly shows that electrophoretic mechanism contributes to the separation of acids. The elution order of neutral solutes coincides with their hydrophobicity. The RSD values for the retention times of all solutes were less than 2.75% for five consecutive runs and less than 3.29% for day-to-day runs \((n = 5)\). The column-to-column reproducibility \((n = 3)\) evaluated in terms of RSD values for the retention times of all solutes were less than 9.47%.

### 3.5 Retention mechanism of hybrid monolithic stationary phase

The effect of ACN concentration in the mobile phase on the retention of neutral and acidic solutes in CEC with hybrid column was also studied. Similar to that in LC, the \( k^* \) in CEC was calculated according to the following equation:

\[
k^* = (t_r - t_0)/t_0
\]

where \( t_r \) is the migration time of a solute and \( t_0 \) is the migration time of a neutral and chromatographically unretained solute. In the experiment, ethanol was selected as \( t_0 \) marker. The electrophoretic retention factor \( k^* \) has been used to describe the migration process of both neutral and charged solutes. The effects of ACN content on \( \log k^* \) of neutral and acidic solutes are plotted in Fig. 5.

As shown in Fig. 5A, the good linear relationship between the logarithm value of \( k^* \) and volume fraction of organic modifier in the mobile phase demonstrated that the retention mechanism of neutral solutes on hybrid stationary phase was similar to that on ODS columns. The RP mechanism in the hybrid columns was related with the introduced phenyl groups in the sol–gel process. However, it is observed from Fig. 5B that the influence of ACN on the retention of acidic solutes was a little different from that of neutral solutes. A linear relationship was obtained with ACN content being lower than 40% v/v while a deviation from linear relationship was observed with ACN content being more than 40% v/v. It was obvious that electrophoretic mechanism participated in the separation in addition to chromatographic mechanism. Theoretically, the electrophoretic retention factor \( k^* \) can also be expressed as follows [32, 33]

\[
k^* = (k' - \mu_{ep}/\mu_{eo})(1 + \mu_{ep}/\mu_{eo})
\]

where \( k' \) is the retention factor caused by chromatography alone, \( \mu_{eo} \) and \( \mu_{ep} \) are the electrophoretic mobility and electroosmotic mobility of solute, respectively. For neutral solutes, \( \mu_{eo} = 0 \) and \( k^* \) becomes \( k' \), reflecting a purely chromatographic process. Therefore, a linear relationship can be observed from Fig. 5A for neutral solutes. For weak acids in ion-suppressed mode, their electrophoretic mobilities were small and EOF in this column was very strong; consequently, the \( \mu_{ep}/\mu_{eo} \) ratios of acids were also very small. It is known that the magnitude of \( k' \) is notably influenced by the ACN content in mobile phase. When a mobile phase containing low ACN content was used, compared with \( k' \), the \( \mu_{ep}/\mu_{eo} \) ratios of acids could be neglected. Thus \( k' \) could substitute for \( k^* \) approximately and the RP mechanism played the main role in the elution of acids under ion-suppressed mode. When ACN content was higher than 40%, the ratio of \( \mu_{ep}/\mu_{eo} \) could not be neglected and \( k^* \) could not be substituted by \( k' \). This led to the deviation from linear relationship when high content of ACN was used.

### 4 Concluding remarks

Due to the introduced sulfonic acid groups, the hybrid silica monolithic column with enhanced cathodic EOF is suitable for the simultaneous separation of aromatic acids and hydrophobic neutral compounds in CEC. The simplicity of the preparation of hybrid monolithic stationary phase provides us versatility to integrate the advanta-
geous properties of organic and inorganic materials and thereby the characteristics of the created hybrid organic–inorganic stationary phases could be fine-tuned for the separations in CEC.

Financial support from the National Nature Science Foundation of China to H. Z. (No. 20475054) is gratefully acknowledged.

5 References