Preparation and evaluation of rigid porous polyacrylamide-based strong cation-exchange monolithic columns for capillary electrochromatography

A CEC monolithic column with strong cation-exchange (SCX) stationary phase based on hydrophilic monomers was prepared by in situ polymerization of acrylamide, methylenebisacrylamide, and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) in a complete organic binary porogenic solvent consisting of DMSO and dodecanol. The sulfonic groups provided by the monomer AMPS on the surface of the stationary phase generate an EOF from anode to cathode, and serve as an SCX stationary phase at the same time. The monolithic stationary phase exhibited normal-phase chromatographic behavior for neutral analytes. For charged analytes, electrostatic interaction/repulsion with the monolith was observed. The strong SCX monolithic column has been successfully employed in the electrochromatographic separation of basic drugs, peptides, and alkaloids extracted from natural products.

Keywords: Basic drugs / Capillary electrochromatography / Hydrophilic monomers / Monolith column / Strong cation-exchange

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1 Introduction

CEC includes features of both HPLC and CE. In CEC, solvent transport is achieved by EOF instead of the hydraulic flow that occurs in HPLC. The advantage of using EOF is the increased column efficiency resulting from the plug-flow profile [1–3]. Traditionally, most CEC separations were performed on silica-based RP stationary phases. Unfortunately, basic analytes will be eluted with serious peak tailing and even cannot be eluted on the stationary phases with negatively charged groups because of electrostatic adsorption. Even though CEC is a widely acceptable analytical technique, it is essential to extend the applications of CEC to charged and hydrophilic analytes, particularly to biomolecules. Smith and Evans [4] suggested to overcome this problem by using strong cation-exchange (SCX) particles instead; as such phases could provide a substantial and stable EOF in a much wider pH range due to the low pKa value of the sulfate group. Capillary electrochromatographic separation of tricyclic antidepressants was carried out by Enlund et al. [5] on a column with strong cation exchangers with different pore sizes. Separation of small peptides was also investigated by Ye et al. [6] by using SCX packing materials as stationary phase. Wu et al. [7] prepared hydrophobic macroporous SCX stationary phases by in situ copolymerization of 2(sulfoxyl) ethyl methacrylate and ethylene dimethacrylate for CEC. A mixed-mode mechanism for the separation of peptides was observed in the monolithic column, comprising hydrophobic and electrostatic interaction as well as electrophoretic migration at a low pH value of mobile phase. A monolithic silica column with SCX stationary phase has been successfully employed in the CEC separation of β-blockers and alkaloids extracted from traditional Chinese medicines by Xie et al. [8].

The utility of acrylamide monoliths for the separation of different analytes was initially reported by Palm and Novotny [9]. Their monolith was prepared using buffered water/formamide/PEG mixtures to dissolve a mixture of acrylamide monomers with an alkyl acrylate as a separation ligand, employing the resulting monolith as a CEC reversed stationary phase with exceptional separation efficiencies. Another approach to create a macroporous acrylamide monolith was studied by Hoegger and Freitag [10–12]. The polymerization is carried out inside the
capillary; an aqueous phase is used as solvent. Monomers based on acrylamides with varying hydrophilicity were used to introduce the interactive moieties together with piperazine diacrylamide as crosslinker and vinylsulfonic acid as provider of the charged, EOF-producing moieties. The separation was found to be governed neither by pure RP nor by pure normal-phase chromatography, even on monoliths, where large amounts of C₆ ligands had been introduced. Svec and coworkers [13, 14] discussed the preparation of porous poly(acrylamide-co-bisacrylamide) monoliths with controlled porous properties using a polymerization process carried out in an organic solvent without water. Molded macroporous monoliths with pore sizes up to 1000 nm have been prepared by changing the composition of porogenic mixture.

The aim of our work is to develop SCX monolithic stationary phase based on hydrophilic monomers for CEC. So monolithic columns with in situ polymerization of acrylamide, methylenebisacrylamide, and charged monomer of 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) in binary organic solvent were prepared, which showed SCX interaction sites for the separation of basic drugs. The EOF property and electrochromatographic performance of the cation-exchange stationary phase were investigated.

2 Experimental

2.1 Instrumentation

All CEC experiments were carried out on a Beckman P/ACE 5510 instrument (Beckman, Fullerton, CA, USA). Fused-silica capillaries (75 µm id, 375 µm od) were obtained from Yongnian Optic Fiber Plant (Hebei, China). A Waters 510 HPLC pump (Waters, Milford, MA, USA) was utilized to flush the columns.

2.2 Materials

Acrylamide, methylenebisacrylamide, AMPS, and γ-methacryloyloxypropyltrimethoxysilane (γ-MAPS) were obtained from Sigma (St. Louis, MO). DMSO, dodecanol, and azobisisobutyronitrile (AIBN) were obtained from Shanghai Fourth Reagent Plant (Shanghai, China), and HPLC-grade methanol and ACN were supplied by the Yuwang Chemical Plant (Zibo, Shandong Province, China). A Milli-Q (Millipore, Milford, MA) system was utilized throughout the experiments. All of the mobile phases were prepared by mixing phosphate buffer with ACN, and the pH values of phosphate buffers before mixing were used as the pH values of the mobile phases. The sample solutions except peptides were prepared by dissolving them in ACN with volume ratio at 1:10, and then further diluted to the appropriate concentration ranging from 0.01 to 0.03 µg/mL with the mobile phase before injection. Peptides were directly dissolved in the mobile phase in the concentration range of 0.1–2 µg/µL.

2.3 Preparation of SCX monolithic column

Prior to the polymerization, the capillary was pretreated with the following procedure:

First, the capillary column with a length of 40 cm was rinsed with 0.1 M NaOH for 1 h and then with water until the outflow reached pH 7.0. After subsequent flushing with methanol for about 10 min, it was dried by passage of nitrogen gas. γ-MAPS solution by its dilution with methanol at a volume ratio of 1:1 was injected into the capillary with a syringe, then the capillary was sealed with rubber at both ends and then submerged in a water bath at 50°C for overnight. Finally, the capillary was rinsed with methanol and water to flush out the residual reagent. Thereby, Si–O–Si–C bonds were formed between the capillary wall and the reactive methacryloyl groups, which are available for subsequent attachment of monolith to the wall during the polymerization reaction.

The monolithic column was prepared from a polymerization mixture consisting of the following monomers: acrylamide (40 mg), methylenebisacrylamide (40 mg), AMPS (15 mg), DMSO (190 µL ~ 208 mg), dodecanol (250 µL ~ 208 mg), AIBN (2 mg). The polymerization mixture was sonicated for 20 min to obtain a homogeneous solution and then purged with nitrogen for 10 min. After the pretreated capillary was completely filled with the mixture, it was sealed at both ends with rubber stoppers. The sealed capillary was submerged into a water bath and allowed to react for 4 h at 60°C. The resultant monolithic capillary column was washed with methanol for 2 h using an HPLC pump to remove unreacted monomers and porogens. At the end of this period, the detection window was made by burning off 1–2 mm of both the coated polymer outside and the monolith inside the capillary using flames. The ashes of the organic monolith inside the capillary were flushed out by methanol for about 30 min with the HPLC pump. Capillaries were cut at both ends to a total length of 27 cm and effective length of 20 cm. Finally, the column was equilibrated with mobile phase at 10 kV for 30 min before running.

3 Results and discussion

3.1 Column preparation

Monolithic stationary phase has been regarded as a suitable and potential separation material for CEC because of its stability, simple synthesis procedures, and no need for frit fabrication and packing. Moreover, flexibility of surface chemistries of monolithic stationary phases because of a wide variety of monomers enables easy tailoring of the stationary phases with both chromato-
graphic groups and charged functionalities. To synthesize macroporous hydrophilic SCX monolith based on polyacrylamide for CEC, three functional vinyl monomers, namely, acrylamide, methylenebisacrylamide, and AMPS, were used for this study. In here, methylenebisacrylamide was used as crosslinker, and AMPS affords negatively charged functionalities to generate cathodic EOF and provides the cation-exchange interaction sites simultaneously. The selection of the porogenic solvents is crucial for the preparation of the monolithic CEC columns in this system. Peters et al. [15] demonstrated that the composition of the porogenic solvent has great influence on the porous properties of the monolithic materials for CEC. According to Svec and coworkers [13, 14] the binary porogenic system consisting of DMSO/dodecanol was chosen in the preparation of SCX monolith for CEC. It was observed that DMSO/dodecanol binary porogenic system is well suited for the preparation of the monoliths for their compatibility. The effect of porogenic solvent composition on the porosity of the poly(acrylamide-co-methylenebisacrylamide-co-AMPS) monolithic columns was investigated by changing the ratio of DMSO to dodecanol (keeping the monomers/porogens ratio at constant). Translucent gel monoliths with low permeability were observed under microscope by using porogenic mixtures containing less than 47% dodecanol with reaction temperature of 60°C for 4 h. As the content of dodecanol increased to 55%, the permeability of the columns became better and the drops of mobile phase could be seen at the end of the capillary columns when the mobile phase was pumped into the capillary by a syringe pump. It was indicated that a high content of dodecanol in the DMSO/dodecanol binary porogenic system favored production of the columns with good permeability. This is because a higher content of dodecanol resulted in earlier phase separation following more large pores. However, inhomogenous polymerization mixtures were obtained using the porogenic mixtures containing more than 59% dodecanol. Thus, a mixture with DMSO/dodecanol at volumetric ratio of 19:25 was chosen as the binary porogenic solvent. The scanning electron micrographs of the end of the SCX monolithic capillary column are shown in Fig. 1. It can be seen that the monolithic bed with macropores linked to the pretreated capillary wall.

### 3.2 EOF of the SCX column

In contrast to the pH-dependent EOF observed for silica-based stationary phase, a feature of cation exchange material is a substantial and stable EOF that can be maintained over a wide range of buffer pH values. Figure 2 shows the effect of pH on the EOF in SCX column. It can be seen that a much lower degree of dependence of EOF on pH was observed on the SCX column. The EOF increases slightly with pH, probably due to the suppression of ionization of the sulfate group on the surface, as well as the ionization of residual silanol groups on the bare capillary wall, which generate cathode EOF. Even then CEC columns with sulfate groups on the surface of the monolithic bed generated cathode EOF over the whole pH range. The effect of ionic strength on EOF was investigated by keeping 40% ACN in the mobile phase. EOF declines slowly from 1.69 to 1.65 × 10⁻⁸ m²V⁻¹s⁻¹ with increase in ionic strength from 10 to 40 mM, which can be explained by that the thickness of the electrical double layer decreases as the ionic strength increases, i.e., the zeta potential on the surface is reduced, and thus the EOF is lowered. Effect of ACN concentration in the mobile phase on the EOF was also investigated. The EOF almost stays constant at a value of about 1.67 × 10⁻⁸ m²V⁻¹s⁻¹ with increasing ACN concentration from 30 to 60%.

### 3.3 Evaluation of column performance

To test the ability of the column to dissipate excessive Joule heat, the current was measured as a function of the applied voltages by keeping the phosphate concentration at 20 mM. The observed currents increased linearly
from 9.4 to 48.7 μA on increasing the applied voltages from 5 to 25 kV with a linear regression coefficient of 0.9996, which suggested that excessive heat generation does not seem to be a cause of concern. The CEC separation efficiency of the SCX capillary column was evaluated by using the mobile phase containing 40% ACN in 10 mM phosphate buffer at pH 2.8. The Van Deemter plot with neutral unretained toluene as analyte was measured by varying the applied voltages from 5 to 30 kV. The obtained relationship of plate height versus linear velocity is depicted in Fig. 3. It can be seen that the plate height decreases steeply with increasing flow velocity, and then becomes almost constant at velocities exceeding 0.5 mm/s. The curve is relatively flat at high flow velocity, which indicates an efficient mass-transfer process between the mobile phase and the monolithic stationary phase.

The monolithic column was applied for the separation of neutral analytes and pyridine derivatives, and the obtained electrochromatograms are shown in Figs. 4 and 5, respectively. It can be seen that baseline separations for neutral analytes and pyridine derivatives were obtained. Neutral analytes were eluted in the order of toluene < dimethylformamide < formamide < thiourea according to their hydrophilicity on the monolithic columns, which illustrated that the matrix of the stationary
phase possessed relatively strong hydrophilicity. Pyridine derivatives are widely used as intermediates or insecticides in some chemical and agricultural industries. As shown in Fig. 5, no peak tailing was observed for the separation of the pyridine derivatives and column efficiencies of all the peaks were above 200 000 plates/m.

3.4 Separation of basic drugs with SCX monolithic column

The prepared monolithic column was applied for the separation of basic drugs using 30 mM phosphate buffer containing 40% ACN at pH 2.8 as mobile phase and the typical electrochromatogram is shown in Fig. 6. Four basic drugs were baseline separated in 8 min with excellent peak symmetry and column efficiencies above 220 000 plates/m, and the RSD values of the migration times of these analytes were less than 1% for five consecutive runs, which showed the good stability of such monolithic stationary phase. In order to gain a further insight into the separation mechanism of the basic drugs on the monolithic column, the effect of the phosphate concentration on the retention of the basic drugs was investigated. To describe the elution of charged solutes in CEC, here we defined a nominal retention factor ($k^*$) based on the chromatographic formalism as following:

$$ k^* = \frac{t_R - t_0}{t_0} $$

(1)

where $t_R$ and $t_0$ denote the migration time of the analyte and that of an inert, neutral tracer, respectively. Obviously, the $k^*$ value reflects the concurrence of both chromatographic and electrophoretic processes. Under the low pH value of 2.8, four basic drugs should be positively charged. Except caffeine the other three drugs all eluted after the void time at various ionic strengths. It can be seen in Fig. 7 that the retention factors of the three most retained drugs increased as the ionic strength of eluent decreased, which was a typical behavior for separation of analytes in ion-exchange chromatography. But the retention factor of caffeine had no obvious relation with the ionic strength of the eluent and this may attribute to the combination of the least retention of caffeine on the SCX monolithic column and the fast electrophoretic mobility of caffeine. In conclusion, the separation of basic drugs on the SCX monolithic column the mainly based on cation-exchange interaction and the electrophoretic mechanism contributed less.

Natural products and their active components as sources for new drug discovery have attracted attention in recent years. The root of *Coptis chinensis* Franch is a...
monly used traditional Chinese medicine which contains protoberberine alkaloids such as berberine, palmatine, coptisine, epiberberine, jatrorrhizine, and columbamine. Now the prepared monolithic column with SCX stationary phase was also used for the separation of the extracted quaternary alkaloids from the root of *C. chinensis* Franch, and a typical electrochromatogram obtained with 30 mM phosphate buffer at pH 2.8 as mobile phase is shown in Fig. 8. As seen in the electrochromatogram, all the alkaloids were baseline separated in 20 min.

### 3.5 Separation of peptides with SCX monolithic column

In contrast to the CEC separation of small neutral molecules the separation of charged analytes such as peptides and proteins is difficult. Amphoteric peptides will exhibit inverse electrophoretic migrations by changing the conditions of mobile phase in the presence of the electric field. Separations of five peptides were successfully carried out on the SCX monolithic columns which were shown in Fig. 9. The effect of the ionic strength of the mobile phase on the retention of peptides in SCX monolithic column is summarized in Fig. 10. The retention factors of the five peptides increased as the ionic strength of eluent decreased, which is also a typical behavior of the separation of solutes in ion-exchange chromatography. This can be explained by the fact that under the low pH condition, the peptides are protonated and undergo electrostatic interactions with the negatively charged monolithic bed.

![Figure 9](image_url)

**Figure 9.** Electrochromatograms for separation of peptides by SCX monolithic column. Experimental conditions: mobile phase, 40% v/v ACN in 20, 25, 30, and 35 mM phosphate buffers (pH 2.0); injection 10 kV for 2 s; separation voltage 12 kV. Solutes: (1) ProMet, (2) Gly-Gly-Phe, (3) Met-Phe, (4) Phe-Gly, (5) Phe-Ala.

![Figure 10](image_url)

**Figure 10.** Influence of the phosphate concentration on the retention factor $k^*$ of peptides.
4 Concluding remarks

A CEC monolithic column with SCX stationary phase based on hydrophilic matrix was prepared by in situ polymerization of acrylamide, methylenebisacrylamide, and AMPS in a binary organic porogenic solvent consisting of DMSO and dodecanol. The stationary phase can provide a substantial and stable EOF over a wide range of buffer pH values in CEC. Efficient separations of basic drugs, peptides, and alkaloids extracted from a natural product were obtained. High efficient separations based on SCX mechanism primarily profit from the use of the biocompatible hydrophilic monomers. Good separation of the alkaloids extracted from the root of *C. chinensis* Franch and the hydrophilicity of monolithic matrix showed that this monolithic column had great potential in the analysis of complex systems such as the lysate of cells.

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5 References