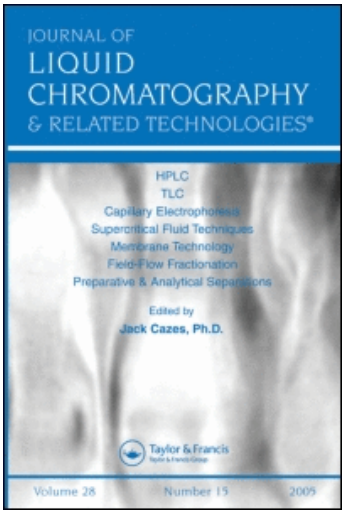

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FREE SOLUTION CAPILLARY ELECTROPHORETIC SEPARATION OF BASIC PROTEINS AND DRUGS USING CATIONIC POLYMER COATED CAPILLARIES

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ABSTRACT

A simple capillary coating which exhibits reversed (anodic) electroosmotic flow (EOF) has been developed for the separation of cationic compounds by free solution capillary electrophoresis. Poly(2-aminoethyl methacrylate hydrochloride) (PAEM) was chemically bound onto the fused-silica capillary inner wall. Capillaries modified by this method exhibit an essentially pH-independent EOF in the pH range from 3 to 5.5, and have been applied to the improved separation of basic proteins and drugs (e.g., beta-adrenergic blocking drugs). High efficiency and precise migration (relative standard deviation was less than 1%) were observed.

INTRODUCTION

Capillary electrophoresis (CE) has been developed into a new powerful analytical separation technique.[1-3] Samples ranging from small inorganic ions to biopolymers, *e.g.*, proteins, have been successfully separated by CE. CE separation is based on the different electrophoretic mobility of charged species. It offers the advantages of fast and efficient separation, high resolution, small sample and running buffer volumes, and simple instrumentation. The small sample size requirement makes it to be the preferred choice for bioanalysis. However, since its debut, practitioners of CE have soon realized that capillary electrophoretic separation of proteins with fused-silica capillaries is hampered by solute adsorption onto the capillary inner wall. This phenomena is primarily the result of electrostatic attraction between positively charged proteins and the negatively charged silanol groups on the capillary inner wall, and the hydrogen bonding between the solute and the capillary inner surface. Other types of interactions, i.e., van der Waals force, hydrophobic interaction, etc., may also contribute to this problem. These solute-wall interactions often lead to band broadening, peak asymmetry, nonreproducible migration, and low recovery of separated species. In extreme cases, positively charged analytes may irreversibly adhere onto the capillary inner wall. Although these interactions are sometimes observed with small molecules, the relevant forces increase with the size of molecule so that large molecules, such as proteins are most severely affected and present unique challenges to the separation method.

Several attempts have been taken to eliminate or minimize these solute-wall interactions. Generally, they can be grouped into three main categories: (1) capillary surface modification; [4-22] (2) manipulation of running buffer pH and ionic strength,[23-27] or use of buffer additives;[28-32] and (3) use of a radial potential field.[33] Capillary modification has demonstrated to be the most flexible for the separation of proteins. Extreme acidic or basic buffers have successfully been employed to minimize the undesired solute-wall interactions. Acidic buffers reduce the ionization of silanol groups on the capillary surface and thus diminish the ionic interactions between solutes and the capillary surface. Basic buffers reverse the negative charges on the proteins to positive charges, thus reducing the undesired ionic interactions.

Limitations of running CE under these extreme pH conditions include: a) a more restricted pH operating range; b) the capillary may be degraded under these pH conditions; c) the resolution may be reduced as all the solutes have the similar ionization status; and d) some proteins may be denatured under extreme pH conditions.

High ionic strength buffers are effective in minimizing solute-wall interactions, but they also increase the electricity current and thus increase the Joule heating which may degrade the efficiency and reproducibility of the separation. The use of a radial potential field could directly control the zeta potential and showed the power to reduce solute-wall interaction in acidic pH range, but it did not work well at alkaline pH conditions and it also led to complicated instrumentation. The use of buffer additives is an effective method to reduce the solute-wall interactions. For example, fluorosurfactant[29, 30] has been successfully used to separate basic proteins.

Capillary modification by both covalent bounding and physical adsorption chemicals to its inner surface has been proven powerful strategy in reducing the solute-wall interactions. In recent years, many chemicals have been used as capillary modification reagents, such as: polyacrylamide,[4] poly(vinyl-pyrrolidinone),[6] polyethyleimine (PEI),[8] vinyl-bound polyacrylamide,[9] poly-ethylene glycol,[7] glycerolglycidoxypropyl,[6] poly(methylglutamate),[12] poly-ether,[13] chitosan,[19] polymers with quaternary ammonium groups,[14,15,18] cryptand,[18] polyvinyl alcohol (PVA),[28] and poly(diallyldimethyl-ammonium chloride) (PDADMAC).[22] Among the listed coatings, PEI, PDADMAC, chitosan, and other positive polymer coatings reverse the charge and zeta potential on the capillary inner wall, and produce a reversed electroosmotic flow (EOF). They are effective in reducing the adsorption of basic proteins onto the capillary inner wall.

In the present paper we report a method to chemically modify fused-silica capillary with Poly(2-aminoethyl methacrylate hydrochloride) (PAEM). Capillaries modified by this method were used to improve the separation of cationic proteins and drugs. Efficient and symmetric peaks were observed using PAEM coated capillaries. High precision of protein migration was also observed. The coating reproducibility was excellent.

EXPERIMENTAL

Instrument

All separations were performed on a laboratory-constructed capillary electrophoresis instrument enclosed in a plexiglas box, which consists of a HPCE high power supply (Spellman, Plainview, NY, U.S.A.), a high power supply local control (Chamnoix Industries, Johnson City, NY, U.S.A.), a

Spectra 100 UV detector (Thermal Separations, Freemont, CA, U.S.A.) and a LCI 100 integrator (The Perkin Elmer, Norwalk, CT, U.S.A.). Injections were accomplished hydrodynamically by elevating the sample reservoir to a height of approximately 5 cm above the other buffer reservoir for approximately 5 seconds.

Reagents and Materials

Fused-silica capillary of 75 μm I.D. was purchased from Polymicro Technology (Phoenix, AZ, U.S.A.). Protein standards, ammonium persulfate, and N,N,N',N'-tetramethylethylene-diamine (TEMED) were purchased from Sigma (St. Louis, MO, U.S.A.). All beta-blockers were gifts from Professor R. Kaliszan (Medical Academy and University of Gdansk, Poland).

Methacryloxypropyltrimethoxysilane was obtained from Hüls American Inc. (Bristol, PA, U.S.A.). 2-aminoethyl methacrylate hydrochloride and all other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Capillary Modification

Fused-silica capillaries were first conditioned with 1 M sodium hydroxide for one hour, then rinsed with deionized water for thirty minutes. A solution of 1% bifunctional compound, methacryloxypropyltrimethoxysilane (adjusted to pH 3.5 by acetic acid), was placed into a capped vial to which one end of the capillaries was immersed, and a syringe was used to apply a pressure to the vial to push the solution into capillaries.

The bifunctional compound was allowed to react with the capillary inner wall for two hours at room temperature. Capillaries were then rinsed with deionized water.

A solution of *ca.* 10% 2-aminoethyl methacrylate hydrochloride (AEM), containing 1 to 3 mg/mL TEMED and *ca.* 1 mg/mL ammonium persulfate was drawn into the capillaries and allowed to react with the bifunctional compound until polymerization was completed. The excess unbound polymer and monomer were removed from the capillaries by rinsing capillaries with deionized water and the capillaries were left to dry at 30 to 40°C overnight.

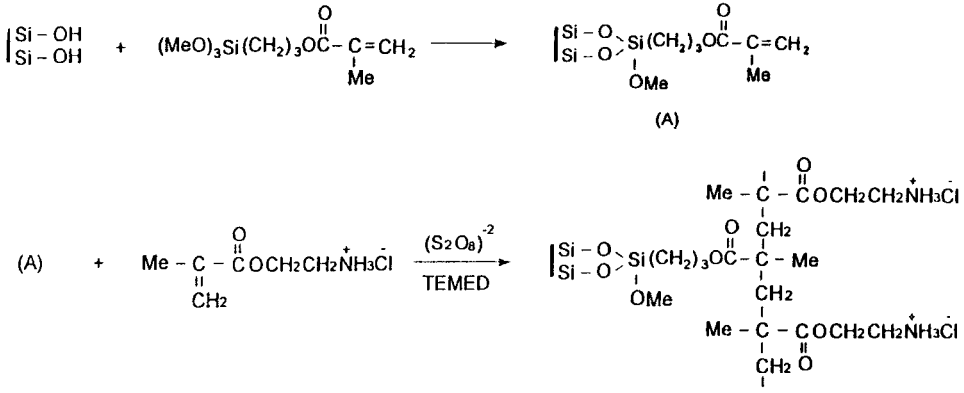

Figure 1. Reaction scheme for the preparation of PAEM chemically coated capillaries.

RESULTS AND DISCUSSION

Capillary Modification

The reaction of bifunctional compound, methacryloxypropyl-trimethoxysilane, with fused-silica capillary inner wall was discussed by Hjerten.[4] The polymerization of 2-aminoethyl methacrylate hydrochloride was previously discussed by Smith and co-workers.[34] Figure 1 shows the chemical reaction scheme for capillary surface modification. When chemically binding PAEM to the capillary inner wall, one must be careful to maintain the monomer solution in acidic condition. It is known that in neutral or basic conditions the monomer may rearrange before polymerization takes place.[34] After polymerization, the polymer chain and the dipole-dipole interaction or hydrogen bonding between the amine and its neighboring ester group prohibit the formation of the cyclic intermediate, therefore no rearrangement takes place after polymerization.[34]

It is interesting to note the potential to further modify the PAEM coated capillary via the active amino groups on PAEM. For example, it should be possible to immobilize proteins or other reagent on PAEM via a carbodiimide linkage to the free amino groups. It may also be possible to tune the EOF by copolymerizing AEM with neutral monomers, such as acrylamide, during the synthesis process.

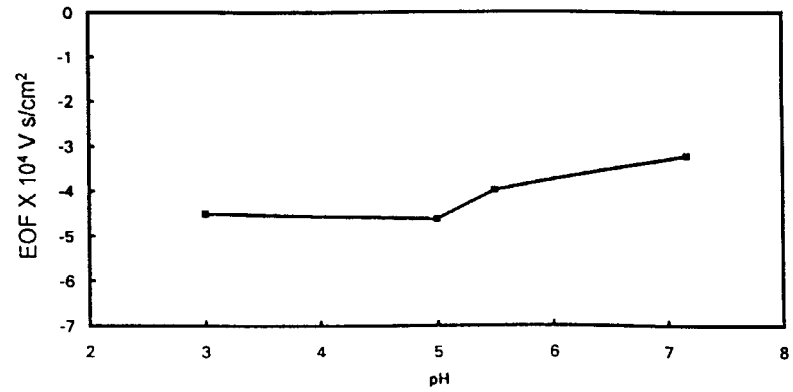

Figure 2. Plot of EOF of PAEM coated capillaries as a function of buffer pH. 75 μm I.D. × 360 μm O.D. PAEM coated capillary, 62.2 cm (45.1 to detector). Hydrodynamic injection at cathode. Acetone was the neutral marker. Detection wavelength was 205 nm. 15 kV applied voltage. Buffers were 10 mM phosphate (pH 3.0 and 7.2) and 17 mM acetate (pH 4.0 and 5.5).

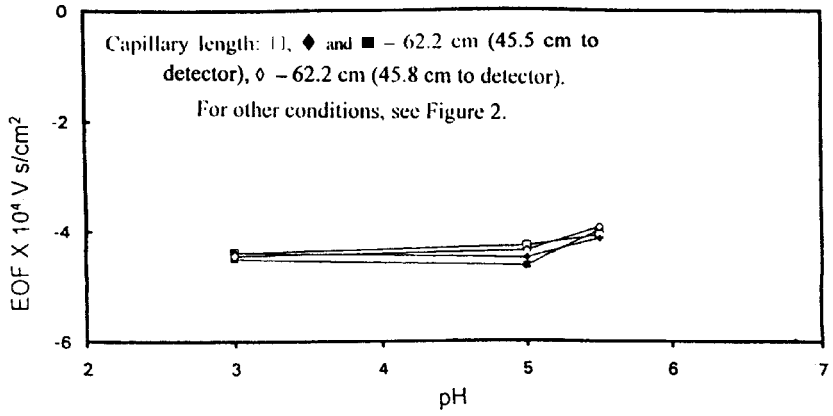

Figure 3. Reproducibility of PAEM coating. EOF of four independently prepared capillaries at selected buffer pH. Capillary length:. □, ◆ and ■ - 62.2 cm (45.5 cm to detector), ◇ - 62.2 cm (45.8 cm to detector). For other conditions, see Figure 2.

Electroosmotic Flow of Capillaries Modified with PAEM

The positive charges on the polymer, poly(2-aminoethyl methacrylate hydrochloride) (PAEM), reverse the charges on the capillary inner wall and the zeta potential of the capillary inner surface in acidic pH range, thus reversing the electroosmotic flow (EOF). Figure 2 shows the EOF as a function of running buffer pH. Acetone was used as the neutral marker when measuring the EOF. As shown in Figure 2, at pH values below 5.5, the EOF remained quite constant, because the amino groups on the polymer were almost fully protonized at this pH range. At pH values higher than 5.5, EOF changed with pH as the amino groups become partially ionized. There may be some active silanol groups remaining on the capillary surface, thus at higher pH their dissociation also affects the EOF.

Reproducibility and Stability of PAEM Coating

To test the reproducibility of PAEM coating, EOF of four independently prepared capillaries was measured at various pH values. The results are shown in Figure 3. As observed from Figure 3, the reproducibility was excellent. The relative standard deviation of EOF ranged from 1% to 3%.

To determine the stability of PAEM coated capillaries, a PAEM coated capillary was used for one week. During this period of time, the capillary was subjected to an electric field of *ca.* 250 V/cm under different pH conditions (in the range from 3 to 7.2) for a total of *ca.* 15 hours. Afterward, the capillary was subject to a continued electric field of 250 V/cm at pH 4.5 (25 mM acetate buffer) for 30 hours. No significant change in the EOF was observed (from $\mu_{eo} = 4.68 \times 10^{-4}$ $cm^2V^{-1}s^{-1}$ initially to $\mu_{eo} = 4.66 \times 10^{-4}$ $cm^2V^{-1}s^{-1}$). When an electric field of 250 V/cm was applied to this capillary for additional 18 hours, the EOF changed significantly (from 4.68×10^{-4} to *ca.* 2.6×10^{-4} $cm^2V^{-1}s^{-1}$). Thus the total lifetime for this capillary was more than 45 hours but less than 63 hours, under continuous high field conditions.

Separation of Basic Proteins and Drugs

It is usually difficult to separate basic proteins using bare fused-silica capillaries due to the solute-wall interactions. When PAEM coated capillaries were used, the separation of basic proteins was dramatically improved. As

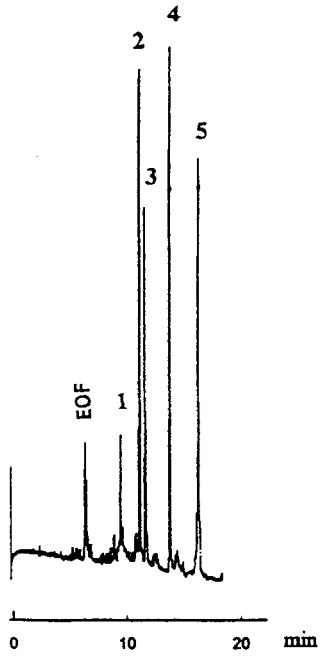

Figure 4. Separation of 5 proteins using PAEM coated capillary. 62.1 cm (44.6 cm to detector). 25 mM acetate pH 4.5 buffer. 15 kV applied voltage. Hydrodynamic injection at cathode. Analytes: 1- myoglobin, 2- ribonuclease A, 3- trypsin, 4- cytochrome C, 5- lysozyme. All analyte concentrations were *ca.* 1 mg/mL. Detection wavelength was 210 nm.

mentioned above there are many interactions that contribute to the problem of solute adsorption onto the capillary inner surface. Hydrogen bonding, Van der Waals force, and hydrophobic interactions may play an important role in the interaction of fused-silica surface with the solute. For the PAEM modified capillary, the authors believe that ionic interactions is the primary contribution to the reduction of solute adsorption. The positive charges on PAEM reversed the charge and the zeta potential of capillary inner wall, and eliminated the electrostatic attraction between capillary inner wall and the basic proteins. Furthermore, basic proteins were repelled by the capillary inner wall by means of electrostatic repulsion. Therefore, the peak shape and efficiency were improved. Figure 4 shows the separation of 5 proteins using a PAEM coated capillary.

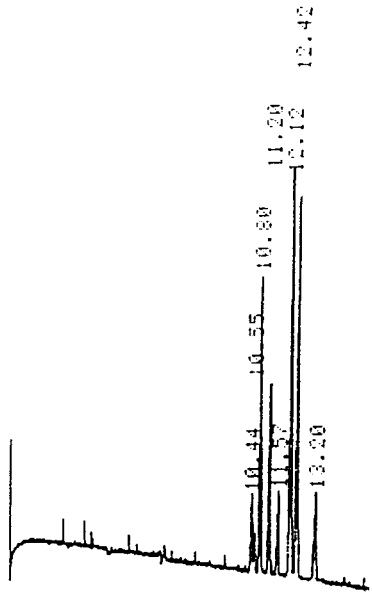

Figure 5. Separation of 8 beta-blockers using a PAEM coated capillary. 62.1 cm (44.6 cm to detector). 25 mM acetate pH 4.7 buffer. 15 kV applied voltage. Hydrodynamic injection at cathode. Numbers above the peaks are migration times in minutes. Peak identification: 10.44 min —acebutolol, 10.55 min —betaxolol, 10.80 min —dilivalol, 11.20 min —carteolol, 11.57 min —atenolol, 12.12 min — propanolol, 12.42 min — pindolol, 13.20 min —nifenalol.

The sample was injected at the cathode. Because PAEM coated capillary has a reversed EOF which was faster than the electrophoretic flow of all analytes, all proteins migrated from the cathode to the anode. As observed from Figure 4, highly efficient (N=400,000 plates per meter) and symmetric peaks were obtained. The relative standard deviation (%RSD) of migration time of proteins was less than 1% with five measurements. No concentrated buffer solution was required for the separation of basic proteins when PAEM coated capillaries were used.

The separation of small cationic compounds, for example, beta-blockers, by bare capillary could also encounter the solute-wall interaction problem.[35] The peaks could be tailing and wide. Using PAEM coated capillaries, the separation of beta-blockers was also improved. Figure 5 shows the separation

Table 1

Reproducibility of Migration Times (in Minutes) of 8-Beta-blockers using a PAEM Coated Capillary*

Run	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8
1	10.47	10.59	10.84	11.24	11.61	12.15	12.45	13.24
2	10.43	10.55	10.80	11.18	11.54	12.18	12.36	13.14
3.	10.44	10.55	10.80	11.20	11.57	12.12	12.42	13.20
%RSD	0.20	0.22	0.21	0.27	0.30	0.25	0.37	0.38

* For peak identification and separation conditions, see Figure 5 and the text.

of 8 beta-blockers at pH 4.7, 25 mM acetic buffer. Numbers above the peaks are migration times in minutes. As observed from Figure 5, highly efficient and symmetric peaks were obtained. Table 1 shows the reproducibility of migration times in minutes of these beta-blockers. As shown in Table 1, highly reproducible migration was obtained.

The pH-independent EOF coating in the pH range from 3 to 5.5 should also expand the applications of micellar electrokinetic chromatography (MEKC). It is well known that bare capillaries exhibit long and poor reproducible MEKC migration in this pH range. The fast anodal EOF should shorten the migration in MEKC. The pH-independent EOF should produce more reproducible migration in MEKC.

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