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Short communication

Performance of a physically adsorbed high-molecular-mass polyethyleneimine layer as coating for the separation of basic proteins and peptides by capillary electrophoresis

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Abstract

A simple method for the preparation of a polyethyleneimine (PEI) coating on the inner surface of fused-silica capillaries for capillary electrophoresis (CE) is reported. The PEI layer can be coated on the silica surface by just flushing the capillary with a solution containing high-molecular-mass PEI. The physically adsorbed layer appears to be very stable and can be used in a pH range of 3–11. In comparison to described methods to fabricate an immobilized PEI layer, the proposed method does not require an immobilization step, is simple and the preparation time of the coating is less than two hours. Good reproducibilities of migration times of basic proteins and peptides were obtained on the same PEI-coated capillary as well as on different PEI-coated capillaries. For basic proteins efficiencies ranging from 300 000–500 000 plates per meter were normally found.

1. Introduction

In CE analysis of proteins, interaction of the biopolymers with the capillary wall seems to be the main reason for the loss in efficiency compared to that predicted by theory. Furthermore, protein adsorption on the internal surface of the capillary can cause poor reproducibility of migration time and low protein recovery. The adsorption is believed to be due to the electrostatic interactions between positively charged residues of the protein and the negatively charged silanol groups which are intrinsic to the fused-silica surface.

Different methods have been developed in CE to diminish interaction between proteins and the silica surface (see Refs. [1–4] for exhaustive and up-to-date reviews). The most successful methods are the ones whereby the silanol groups are shielded by a polymer layer [5–14]. The use of such polymeric coatings has led to high efficiencies and reproducible protein separations.

The PEI coating as developed by Towns and Regnier [8], and adapted by others [10,12,15], seems to be a very interesting solution to this problem. This coating is different from most polymer layers because the surface bears a positive charge. The PEI coating is particularly suited for the separation of basic proteins since at acidic pH the surface and proteins have the same positive charge. Under these conditions the proteins are repelled from the surface in analogy

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to what has been described by Lauer and McManigill [16]. The PEI coating has the additional advantage that the separation of basic compounds is fast, because the basic analytes move to the anode, i.e. in the same direction as the electroosmotic flow.

When testing a high-molecular-mass PEI (M_r , $6 \cdot 10^5$ – $1 \cdot 10^6$) as additive to the buffer in another CE study, we discovered that PEI sticks irreversibly on the silica surface, even under harsh conditions such as flushing the capillary with strong basic and acidic solutions. It is well known that polymers strongly adsorb onto silica surfaces and their adsorption has been applied to mask the silanol groups on the surface of fused-silica capillaries [11,17]. Since the preparation of an immobilized bonded PEI coating is rather laborious and time-consuming (as for most polymer coatings), we found it worthwhile to investigate whether a physically adhered PEI layer can be used as coating as well. The study was focused on the reproducibility, the efficiency and the long-term stability of the coating, using some basic proteins and peptides as test compounds.

2. Materials and methods

2.1. Instrumentation

Separations were carried out using a Prince (Lauer-Labs, Emmen, Netherlands) injection system with temperature controller, connected to a Linear M-200 variable-wavelength UV-Vis detector (Linear Ins. Corp., Reno, NV, USA) operated at 214 nm. Fused-silica capillaries (Polymicro Technologies Inc., Phoenix, AZ, USA) with 75 μm I.D. and 360 μm O.D. were used; the total and effective (from the injection point to the detector) lengths of the capillaries are indicated in the figures. The injection was carried out at the cathodic side using controlled pressure for a fixed time.

2.2. Samples and chemicals

Lysozyme (chicken egg white), cytochrome c (horse heart), ribonuclease A (bovine pancreas),

trypsinogen and α -chymotrypsinogen (bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and used as received. All the short peptides were from Nutritional Biochemicals Corporation (Cleveland, OH, USA) and the long ones from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Proteins and peptides were dissolved at the concentrations indicated (ranging from 0.2 to 2 mg/ml) in water, previously purified by passage through a PSC filter assembly (Barnstead, Boston, MA, USA). The samples were stored at -20°C and warmed to room temperature before use. Polyethyleneimine (PEI, molecular mass range $6 \cdot 10^5$ – $1 \cdot 10^6$) was from Fluka (Fluka AG, Buchs, Switzerland). Acetic acid and formic acid (E. Merck, Darmstadt, Germany), succinic acid (BDH Laboratory Chemicals Division, Poole, UK), chloro-acetic acid, malonic acid, Tricine (N-tris[hydroxymethyl]methyl-glycine), CAPS (3-cyclohexylamino-1-propanesulfonic acid) and MES (all from Aldrich, Axel, Netherlands) were used in the various running buffers. These buffers were used in a 50 mM concentration and at pH values of 3 (chloro-acetic), 5.5 (acetic, MES, succinic, malonic), 7 and 7.5 (MES), 8 and 8.5 (Tricine), 9–11 (CAPS). The buffers were stored at 4°C and warmed to room temperature before use.

2.3. Coating procedure

Before coating the capillary with PEI the external polyimide coating was burned-off over a length of 5 mm in order to make the detection window. The fused-silica capillary was first etched by flushing the capillary with a solution of 1 M sodium hydroxide for 30 min at 1 bar and with water for 15 min at the same pressure. Then the capillary was flushed with a solution of PEI in water at 1.5 bar for 10 min and the PEI solution left in the capillary for one hour. Next the polymer solution was pressed out of the capillary with air at 1.5 bar. Finally the capillary was rinsed with water for 15 min and with running buffer for 15 min. A washing step of 1 min with buffer was used between injections. This procedure can be carried out by using the

Prince instrument in automatic mode, which allows to coat each capillary unattended overnight.

3. Results and discussion

The PEI molecule has many positive charges and interacts strongly with negatively charged silanol groups on the surface of the fused-silica capillary [8]. This irreversible adsorption creates a PEI layer on the capillary wall and thus masks the underlying silanol groups from unwanted interactions with biopolymers. Moreover, the adsorbed PEI layer has a positive charge over a wide pH range, which results in an electroosmotic flow towards the anode. This anodic electrophoretic flow favours the separation speed of substances with a positive charge such as basic proteins and peptides.

3.1. Effect of the PEI concentration in the coating solution

In order to determine whether the PEI concentration in the coating solution has an effect on the nature of the dynamically generated PEI layer, the electroosmotic flow was measured as function of the pH on capillaries coated with 0.1–10% (w/v) solutions of PEI. Different buffers were used to cover the pH range of 3–10.4. Fig. 1 shows the effect of the pH on the electroosmotic flow. As can be seen the electroosmotic flow is always towards the anode over the investigated pH range. This behaviour indicates that the basic PEI molecules are strongly adsorbed on the silica surface and that the residual amine groups of the PEI create a positively charged surface. The electroosmotic flow is relatively constant over the pH range 3–6 and then gradually decreases at higher pH. This latter effect can be attributed to deprotonation of the amine groups and the higher ionization of silanol groups on the capillary wall [8].

From Fig. 1 it can be seen that capillaries coated with 1, 5 and 10% of PEI exhibit similar behaviour. However, in the capillary coated with 0.1% PEI a significantly larger electroosmotic

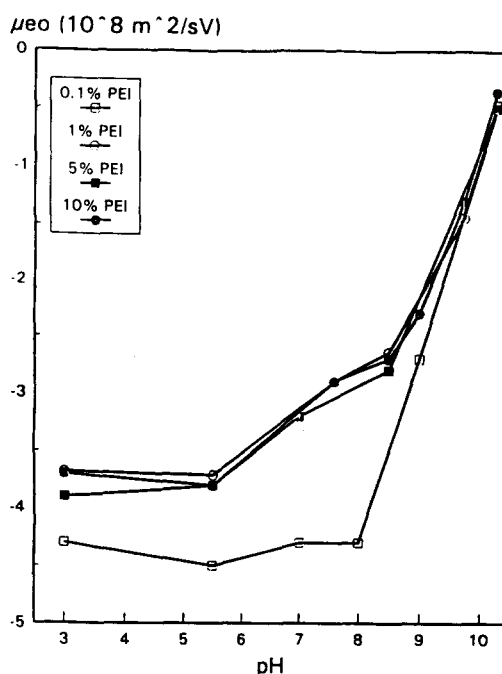


Fig. 1. Plots of electroosmotic flow versus pH on capillaries coated with 0.1, 1, 5 and 10% (w/v) PEI solutions in water. Acetone was employed as neutral marker.

flow (EOF) was found. A similar behaviour has been observed by other authors [8].

In order to ascertain that the effects described above were indeed due to changes in the pH and degree of coating, all the experiments described above were repeated using the same buffers but adding different quantities of NaCl in order to keep the current the same (ionic strength is then also approximately constant). Some EOF values changed slightly; however, the same trend as shown in Fig. 1 was observed.

3.2. Effect of type of buffer and pH

The effect of the type of buffer on the electroosmotic flow was investigated on a 10% PEI capillary using acetone as neutral marker. Four different buffers at pH 5.5, i.e. malonic acid, succinic acid, MES and acetic acid, at the same concentration (50 mM) were employed. It appeared that the electroosmotic flow was strongly dependent on the type of anion. The electro-

osmotic mobilities with malonic acid, succinic acid, MES and acetic acid were found to be $-18 \cdot 10^{-9}$, $-18 \cdot 10^{-9}$, $-50 \cdot 10^{-9}$ and $-45 \cdot 10^{-9} \text{ m}^2 \text{ s}^{-1} \text{ V}^{-1}$, respectively. As can be seen, the divalent acids, i.e. malonic and succinic acid, have the same electroosmotic flow, but the value is much smaller than those found with the monovalent acids, i.e. acetic acid and MES. A similar behaviour was observed when NaCl was added to the buffers in order to get similar ionic strength, as done above. A strong electrostatic interaction between the divalent anions and the amine groups of PEI, which decreases the charge on the coating and thus the zeta potential, seems to take place, which may explain this effect.

3.3. Efficiency of the PEI coating for biological compounds

The performance of the PEI-coated capillaries was investigated with proteins and peptides. Fig. 2 shows a typical electrophoretogram of a mixture of basic proteins on a 10% PEI-coated capillary at pH 5.5. The PEI coating appears to be very efficient for basic proteins, and plate numbers ranging from 300 000–500 000 plates/m were normally found. Similar efficiencies were found with the 0.1, 1 and 5% PEI coating solutions. Unfortunately acidic proteins interact strongly with the coating and the performance is very poor.

The PEI coating appears to be also suitable for the separation of other types of samples. As an illustration, Figs. 3A and 3B show a separation of a mixture of di- and tripeptides at pH 8.2 and a separation of longer peptides at pH 9.75, respectively. As can be seen, very efficient and fast separations can be realized.

3.4. Reproducibility and long-term stability of the PEI coating

The reproducibility and the long-term stability of the PEI coating was investigated by measuring the plate numbers and retention times of some basic proteins on three 10% PEI coated capillaries. The run-to-run reproducibilities of the migration times of the proteins on the three

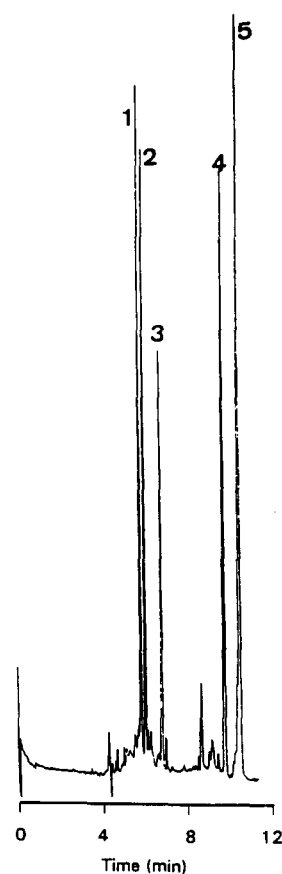


Fig. 2. Separation of five basic proteins. 10% PEI-coated capillary, total length 63.5 cm, effective length 46.5 cm, I.D. 75 μm . Buffer: 50 mM acetate, pH 5.5. Run voltage -28.8 kV . Injection 10 mbar for 6 s. Sample: (1) trypsinogen 0.32 mg/ml, (2) α -chymotrypsinogen 0.32 mg/ml, (3) ribonuclease A 0.72 mg/ml, (4) cytochrome C 0.32 mg/ml and (5) lysozyme 0.32 mg/ml. UV detection at 214 nm.

capillaries were excellent and the relative standard deviations (%R.S.D.) in migration times ranged from 0.5 to 1.5% ($n = 6$). The R.S.D. values for column-to-column reproducibilities of the migration times ranged from 1.9 to 2.8% ($n = 18$) and the R.S.D. in the plate numbers ranged from 10 to 15%.

The long-time reproducibility of the migration times of proteins was followed on one capillary for one month. During this period 70 injections of the test proteins were performed. The migration times changed about 5% and no noticeable loss in efficiency was observed.

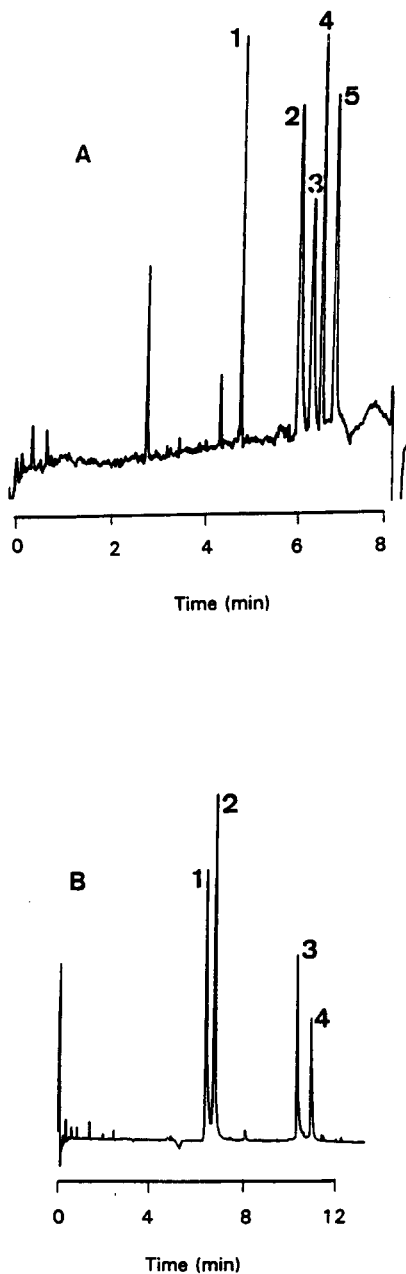


Fig. 3. Separation of short peptides (A) and long peptides (B) on a 5% PEI-coated capillary. Total length 85 cm, effective length 50 cm, I.D. 75 μm . Run voltage -24 kV . Injection 10 mbar for 6 s. Sample concentration ranging from 0.2 to 0.6 mg/ml. UV detection at 214 nm. (A) Buffer: 50 mM Tricine, pH 8.2; peptides: (1) GE, (2) GGG, (3) LGF, (4) AA, (5) SGG. (B) Buffer: 50 mM CAPS, pH 9.75; peptides (1) WAGGDASGE, (2) ELAGAPPEPA, (3) SYSMEHPRWG, (4) LQAAPALDKL.

In order to test the stability of the coating at very basic pHs we left a 50 mM CAPS buffer at pH 11 in a 5% PEI-coated capillary for 60 h. Analysis times were measured before and after this experiment using the five basic proteins as analytes at pH 5.5. After 60 h the migration times of all proteins were slightly increased, i.e. about 3%, while the efficiency was not altered. This result indicates that the dynamically generated PEI layer is more or less irreversibly attached to the silica surface.

4. Conclusions

A stable high-molecular-mass PEI layer can be dynamically generated on the inside surface of fused-silica capillaries by just filling the capillary with a solution of a high-molecular-mass PEI (M_r $6 \cdot 10^5$ – $1 \cdot 10^6$) and flushing the capillary after a certain time. The preparation of the coating is very simple, reproducible and takes less than two hours. The coating is very stable in the pH range 3–11. For basic proteins efficiencies of 300 000–500 000 plates/m were obtained. The coating appears to be suitable for the separation of biological compounds such as basic proteins and peptides but cannot be recommended for acidic proteins.

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