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Journal of Chromatography B, 695 (1997) 163–168

JOURNAL OF CHROMATOGRAPHY B

Short communication

Capillary electrophoresis of peptides and proteins at neutral pH in capillaries covalently coated with polyethyleneimine

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Abstract

Two procedures for the derivatization of the inner wall of fused-silica capillaries for the analysis of peptides and proteins by capillary electrophoresis (CE) at neutral pH are presented. In the first procedure, polyethyleneimine (PEI) is covalently attached to the capillary wall. In the second procedure, PEI is additionally cross-linked. We present analysis of standard peptides and proteins by CE using the coated capillaries. These coatings will have application for the separation of protein complexes at neutral pH, prior to analysis by electrospray mass spectrometry.

Keywords: Peptides; Proteins; Polyethyleneimine

1. Introduction

Capillary electrophoresis (CE) has become a powerful tool for the analysis of proteins and peptides. CE has been widely used in the analysis of proteins and peptides with high resolution, fast separation, and low cost. Peptide and protein adsorption onto the wall of capillaries is a common problem encountered in CE experiment. This adsorption, which is mainly due to the interaction of proteins with negatively charged silanol groups, creates band broadening, thus reducing the resolution of the separation. Different ways to reduce the effects of the negative charges of the capillary wall on the separation of proteins and peptides have been proposed. They include the use of a low pH buffer [1] to reduce the number of ionized silanol groups on the capillary wall and the use of pH above the p*I* of the protein [2] to induce predominantly negative charges on the protein. In both cases proteins can be denatured by the prevalent low and high pH and non-covalent protein–protein interactions, protein–DNA interactions, and other protein–ligand complexes are dissociated. These problems have been avoided by dynamic coating of the capillary wall. This was achieved by coating by hydrostatic interactions between the capillary wall and positively charged molecules added to the buffer [3]. Dynamic coating can be used for separation at physiological pH. However, the significant concentrations of polymer present during electrophoresis can interfere with protein and peptide detection. Charge reversal [4] of the capillary wall, using positively charged polymers attached to the wall by ionic and hydrophobic interactions is another suitable method to prepare capillaries for analysis at physiological pH. In this case, the capillary wall is pretreated with the polymer before analysis and the buffer does not contain any polymer additives. Polymer bleeding from the wall can still interfere with substrate detection, in particular in high sensitivity applications. Covalent binding of molecules to the silanol groups avoids the

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PII S0378-4347(97)00022-4

major problems of non-covalent wall coating. Polyacrylamide [5], hydroxypropylcellulose [6], poly(ethylene glycol) [7], epoxy-diol [8], maltose [8], epoxy polymer [9,10], polyethyleneimine–polyether multilayers [11] are a few examples of surface modifiers that have been successfully covalently attached to the capillary wall.

3-Aminopropylsilane [8,12] is the preferred coating for the separation of proteins and peptides at low pH for CE–electrospray ionization–mass spectrometry (ESIMS). With this coating the wall of the capillary is positively charged below pH 4.8. At basic pH, this coating is not stable. The objective of this paper is to develop new coatings positively charged at neutral pH and compatible with microelectrospray–MS. The requirements are that (a) the flow of buffer towards the mass spectrometer be maintained for use of a sheathless microelectrospray interface, (b) charges on the capillary wall be reversed to make the system compatible with basic proteins, and (c) of coating molecules bleeding to the mass spectrometer during a CE–MS experiment be prevented by covalent coupling. Here we present two procedures to obtain a covalently attached layer of positively charged polymers which is stable at neutral pH. In the first approach, we used 3-glycidoxypropyltrimethoxysilane to attach PEI to the wall of the capillary. In the second approach, PEI was similarly covalently attached to the wall using 3-glycidoxypropyltrimethoxysilane and then cross-linked using ethylene glycol diglycidyl ether (EDGE). These coatings are positively charged at pH 7.7. Their performances were compared to the performance of an uncoated capillary for the separation of peptides and proteins at physiological pH using CE with UV detection. The buffer concentration was similar to the concentration that we will be using to perform CE–MS at a neutral pH.

2. Experimental

2.1. Equipment

The CE instrument used in the experiments was a Water Quanta 4000 (Millipore, Bedford, MA, USA). The 50 mm I.D.×185 mm O.D. fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). All the experiments were done using Tris-HCl (8.6 m*M*, pH 7.7) as the running buffer. The capillaries were equilibrated with the running buffer at −30 kV for at least 30 min. Analytes were detected on-column by their UV absorbance at 214 nm.

2.2. Chemicals

3-Glycidoxypropyltrimethoxysilane (96%), PEI (M_r 25 000), and EDGE (tech.) were purchased from Aldrich (Milwaukee, WI, USA). Dimethylformamide, sodium hydroxide and hydrochloric acid were from J.T. Baker (Phillipsburg, NJ, USA). Tris-(hydroxymethyl)aminomethane (Tris) was provided by Boehringer Mannheim (Indianpolis, IN, USA). The peptides and proteins were purchased from Sigma (St. Louis, MO, USA). All the solutions were prepared using water purified with a Milli Q RG Water system (Millipore).

2.3. Capillary coatings

We derived two procedures for covalent coating of fused-silica capillaries from Ref. [13] which described covalent attachment of PEI to silica beads that were used for chromatography experiments. For both coating procedures, the capillaries were prepared by sequential rinses with 1 *M* NaOH for 20 min at room temperature, 3 *M* HCl for 20 min, and water for 30 min.

2.3.1. Procedure 1

A solution of 3-glycidoxypropyltrimethoxysilane (5–8%, w/v) in water at pH 5.5–5.8 was passed through the capillary at 90°C for 2 h. The solution was then flushed out, and a solution of PEI (5%, w/v) in dimethylformamide was passed through the capillary at room temperature for at least 2 h (Fig. 1).

2.3.2. Procedure 2

Procedure 2 adds one more step to procedure 1, which consists of passing EDGE saturated with triethyleneamine through the capillaries for 1 h. Finally, the capillaries were rinsed with the CE buffer before being used (Fig. 1).

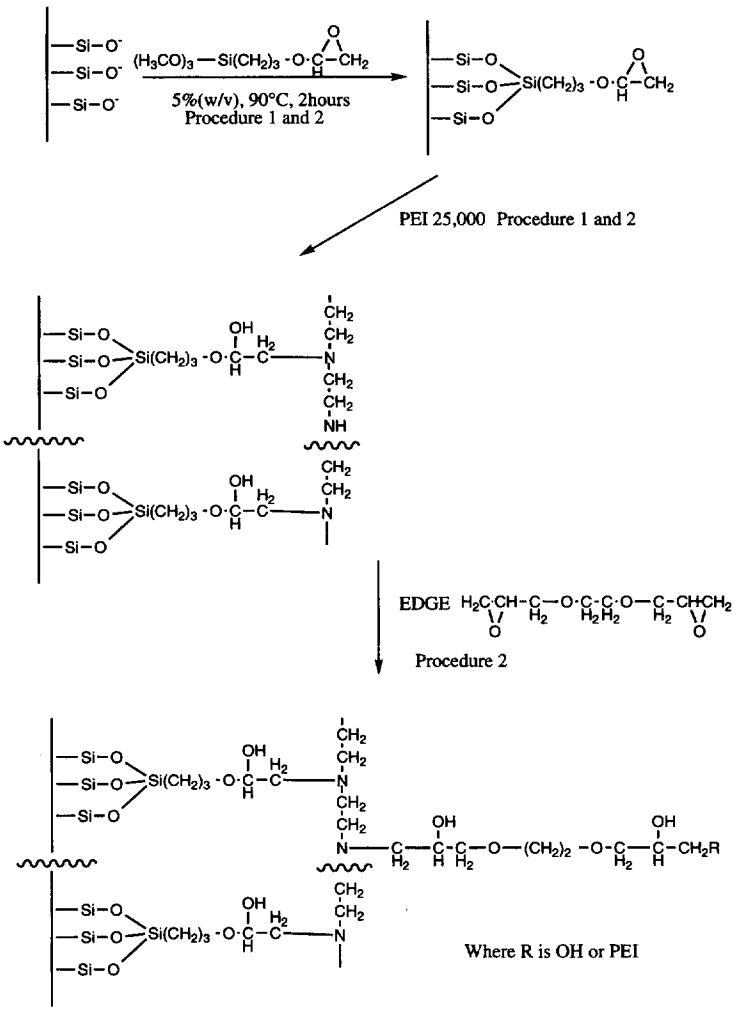

Fig. 1. Schematic representation of the chemistry to coat fused-silica capillaries using procedures 1 and 2.

3. Results and discussion

The study of protein interactions by CE–microelectrospray–mass spectrometry requires that the separation be performed near neutral pH. At that pH some proteins are still positively charged and interact with silanol groups on the capillary wall. This effect can be reduced by coating of the capillary. A suitable coating has to generate electroosmosis in the capillary toward the mass spectrometer to sustain a sheathless microelectrosprayer. Using a neutral coating, such as polyacrylamide, would not be suitable because the electroosmosis would be reduced so much that a microelectrospray would not be sustainable. Also, the coating should not bleed off the wall and into the mass spectrometer. PEI is positively charged in a wide range of pH and does not interact with proteins.

PEI has been previously used by Towns and Regnier [14] to non-covalently coat capillaries for CE. In the present study, we used a chemical linker to covalently attach PEI to the capillary wall. In the first procedure PEI was covalently attached to the wall of the capillary. In the second procedure, the attached PEI molecules are further cross-linked to increase the stability of the coating.

We first tested whether the electroosmotic flow was reversed by the coating, by injecting a neutral marker (formamide 0.2%). The electroosmotic mobility was $-5.8\pm0.2\times10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$ for a capillary coated with procedure 1, compared to a value of $7.77\pm0.02\times10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$ for an uncoated capillary. The electroosmotic flow was therefore reversed by our coating procedure.

We then tested the performance of the coated capillaries by separating a peptide standard composed of bradykinin, bradykinin fragment 1–5, substance P, [arg^8]-vasopressin, luteinizing hormone releasing hormone (LHRH), bombesin, leucine enkephalin, methionine enkephalin, and oxytocin. Fig. 2 shows the separation of this standard sample at neutral pH performed on a capillary coated using procedure 1. Each peptide was also injected individually to determine the respective migration time. All the peptides were separated except for leucine enkephalin and methionine enkephalin which co-migrated in the first peak. No band broadening due to peptide adsorption was noticed. The same peptides, without oxytocin, were separated under the same conditions on an uncoated capillary (Fig. 3). In this experiment, LHRH and bradykinin 1–5 were not resolved, bombesin consisted of two peaks one which was isolated and the other one was located under the peak of bradykinin 1–5. Substance P peak was very broad, leucine enkephalin and methionine enkephalin comigrated and bradykinin also eluted as a broad peak, in part comigrating with leucine enkephalin and methionine enkephalin. A comparison between Figs. 2 and 3 clearly showed the advantage of using a coated capillary for CE at neutral pH. If there were no interactions between the peptides and the capillary wall, one would expect a reversed order of migration of the analytes. On the

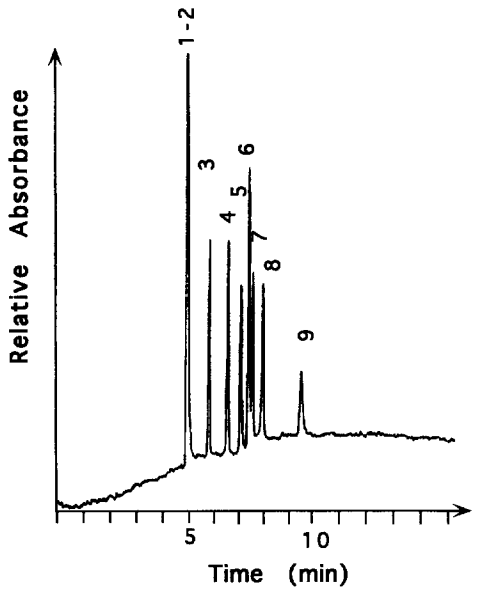

Fig. 2. Separation of a mixture of peptides on a 76.5 cm×50 μm I.D×185 μm O.D. fused-silica capillary in Tris-HCl 8.6 m*M*, pH 7.7 at −30 kV. The capillary was coated according to procedure 1. The injection was 10 s at −10 kV of a total peptide concentration of 5 μg μl^{-1}. Peaks: 1 and 2=leucine enkephalin and methionine enkephalin, 3=oxytocin, 4=bradykinin 1–5, 5=bombesin, 6=LHRH, 7=[arg^8]-vasopressin, 8=bradykinin, and 9=substance P.

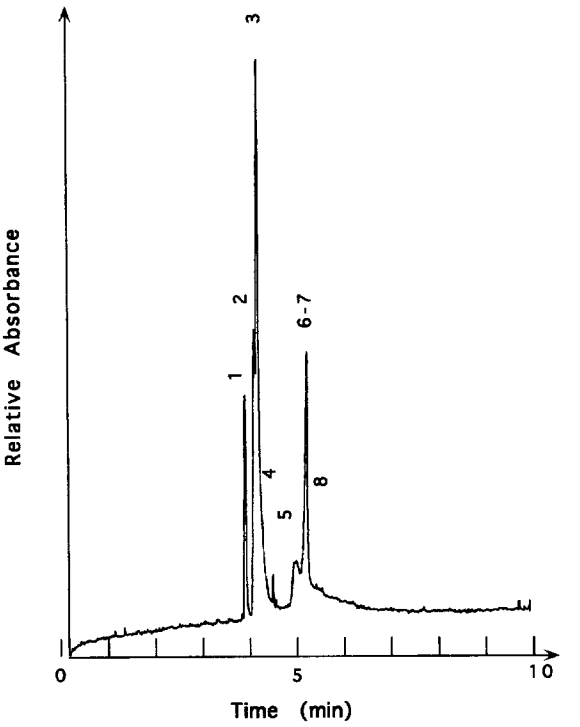

Fig. 3. Separation of a mixture of peptides on a 76.5 cm×50 μm I.D×185 μm O.D. uncoated fused-silica capillary. The injection was 10 s at −10 kV. Peaks: 1=[arg^8]-vasopressin 0.09 μg μl^{-1}, 2=LHRH 0.15 μg/μl, 3=bradykinin 1–5 0.12 μg μl^{-1}, 4=bombessin 0.09 μg μl^{-1}, 5=substance P 0.09 μg μl^{-1}, 6 and 7=leucine enkephalin 0.09 μg μl^{-1} and methionine enkephalin 0.09 μg μl^{-1}, 8=bradykinin 0.24 μg μl^{-1}. The buffer and electrophoresis conditions were as described in Fig. 2.

uncoated capillary bombesin, substance P and bradykinin migrated slower than expected and their peaks were broader and tailing more than other peaks in the electropherogram. This clearly indicates that bombesin, substance P, and bradykinin interacted with the uncoated capillary. On the coated capillary, the resolution was good and no band broadening resulting from adsorption to the wall was observed.

We then tested the effect of covalent wall coating on the separation of proteins at neutral pH. A mixture of formamide (neutral marker), myoglobin, and ribonuclease A was separated on a coated capillary prepared according to procedure 1. The separation was performed in 5 min. Results are shown in Fig. 4. Ribonuclease A migrated in 2 peaks; the smaller one probably being an impurity or degradation product. This coating allows the separation of peptides and proteins without getting any significant band broadening due to protein adsorption. The stability of this coating was evaluated by performing subsequent separation of peptides and proteins on the same capillaries. Similar separations were obtained for an average of 5 days using the same capillaries. In contrast, if aliquots of the same sample were separated on an uncoated capillary under similar condition, no protein peaks were observed.

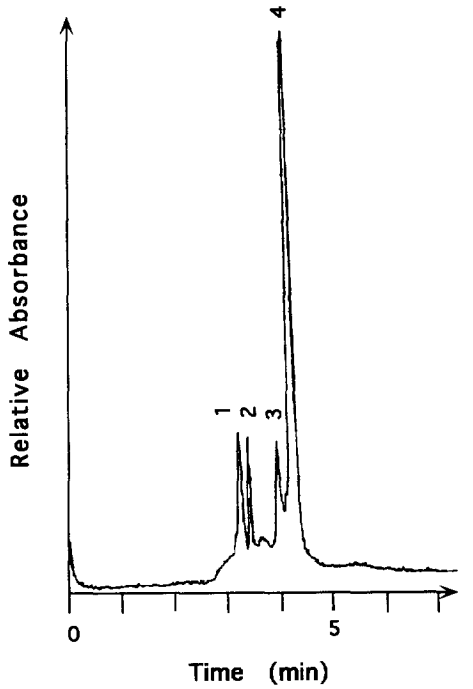

Fig. 4. Separation of a mixture of proteins on a 47 cm×50 μm I.D×185 μm O.D. fused-silica capillary at −20 kV. The capillary was coated according to procedure 1. The injection was 3 s at −3 kV. Peaks: 1=formamide 0.1% (v/v), 2=myoglobin (bovine) 0.06 μg μl^{-1}, 3 and 4=ribonuclease A (bovine) 9.4 μg μl^{-1}. Electrophoresis buffer was as in Fig. 2.

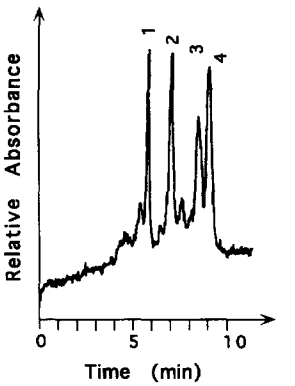

Fig. 5. Separation of a mixture of proteins on a 47 cm×50 μm I.D×185 μm O.D. fused silica capillary at −20 kV. The capillary was coated according to procedure 2. The injection was 3 s at −3 kV. Peaks: 1=myoglobin (bovine) 0.4 μg μl^{-1}, 2=α-chymotrypsin (bovine) 3.5 μg μl^{-1}, 3=ribonuclease A (bovine) 2.8 μg μl^{-1}, and 4=trypsin (porcine) 4.5 μg μl^{-1}. Electrophoresis buffer was as in Fig. 2.

To further improve the stability of the coating, we cross-linked the attached PEI (procedure 2). We tested this coating using a protein standard made of myoglobin, α-chymotrypsin, ribonuclease A, and trypsin (Fig. 5). All four components were separated within 10 min on a 57-cm long capillary. This coating remained stable at a neutral pH for at least a month. The resolution obtained for the separation of proteins was as good as the resolution obtained in capillaries coated with procedure 1.

4. Conclusion

These results indicate that either procedure 1 or 2 can be use to covalently attach PEI to the inside capillary wall and that the stability of the coating is improved by cross-linking PEI using EDGE. These coatings will be used for studies of protein complexes by CE–MS.

Acknowledgments

This work was supported by the National Science Foundation Science and Technology Center for Molecular Biotechnology. D.F. acknowledges a post-doctoral fellowship from NSERC (Canada).

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