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New physically adsorbed polymer coating for reproducible separations of basic and acidic proteins by capillary electrophoresis

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Abstract

In this work, a new physically adsorbed coating for capillary electrophoresis (CE) is presented. The coating is based on a *N*,*N*-dimethylacrylamide-ethylpyrrolidine methacrylate (DMA-EPyM) copolymer synthesized in our laboratory. The capillary coating is simple and easy to obtain as only requires flushing the capillary with a polymer aqueous solution for 2 min. It is shown that by using these coated capillaries the electrostatic adsorption of a group of basic proteins onto the capillary wall is significantly reduced allowing their analysis by CE. Moreover, the DMA-EPyM coating provides reproducible separations of the basic proteins with RSD values for migration times lower than 0.75% for the same day ($n=5$) and lower than 3.90% for three different days ($n=15$). Interestingly, the electrical charge of the coated capillary wall can be modulated by varying the pH of the running buffer which makes possible the analysis of basic and acidic proteins in the same capillary. The usefulness of this coating is further demonstrated via the reproducible separation of whey (i.e. acidic) proteins from raw milk. The coating protocol should be compatible with both CE in microchips and CE–MS of different types of proteins.

Keywords: Coated capillaries; Proteins

1. Introduction

Although separation of proteins has nowadays become one of the main applications of capillary electrophoresis (CE), practitioners of CE soon realized that capillary electrophoretic separation of proteins using fused-silica capillaries was strongly hampered by solute adsorption onto the capillary wall [1–3].

Adsorption of proteins onto capillary wall seems to be the main reason for observed efficiency loss [4–6], poor reproducibility in migration times and low protein recovery rates [7]. Adsorption is most probably due to electrostatic interactions between positively charged residues of the proteins and negatively charged silanol groups, which are intrinsic to the fused-silica surface of uncoated capillaries [8]. So far, different approaches have been proposed to reduce the noxious interaction between proteins and silica surface.

One of the first solutions proposed was to control the pH or ionic strength of the background elec-

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trolyte [2]. However, this procedure is not very suitable for proteins, because choosing an extreme pH may cause denaturation.

The use of capillaries covalently coated with polymers [3,6–8] or the use of permanently coated capillaries made of multiple polymer layers physically adsorbed [9] have been also proposed. These methods lead in general to high efficiency and reproducible protein separations. However, the preparation of these coatings is labour intensive and frequently they can only be used within a given pH range and for a determined number of injections.

Other possible solution has been the employ of the so-called dynamic coatings that basically consists of using substances (e.g. zwitterions, mono- to oligoamines, surfactants, neutral and charged polymers) within the separation buffer that can quench the undesirable adsorption effect [11]. Some important disadvantages of these dynamic coatings are that the buffer substances can also interact with the proteins and, moreover, the mentioned substances can spoil the MS sensitivity if CE–MS is to be used [12–14].

An interesting strategy to reduce the adsorption phenomena is the use of physically adsorbed polymers [15–17]. The main advantage of this procedure is that coating can be easily prepared just by rinsing the capillary with a solution containing the coating agent that is either a polymer or a low-molecular-mass compound [10,11]. However, because the attachment of the coating to the wall is based on adsorption, a small amount of coating agent is frequently added to the separation medium to keep the coating on the capillary wall surface. This can bring about the same interaction problems with the solutes and CE–MS uncompatibility mentioned above [12–14]. Moreover, as described by Horvath and Dolnik [10], these types of coatings are frequently developed to solve a specific separation problem that reduces considerably their usefulness.

The goal of this work was to develop a simple, fast and reproducible physically adsorbed coating applicable to the CE separation of basic and acidic proteins. In order to make it compatible with a future CE–MS application, the sought coating should not need coating agent into the running buffer and, therefore, its regeneration should be achieved just by flushing the capillary between injections with a solution containing the polymer. Moreover, the new procedure should not require the use of organic solvents, high viscous solutions and elevated temperatures. In this way, its future application to CE separations of proteins in microchips will be much more viable.

2. Experimental

2.1. Chemicals

2-Ethyl-(2-pyrrolidine) methacrylate (EPyM) was synthesized by reaction of *N*-(2-hydroxyethyl)-2-pyrrolidine with methacryloyl chloride, both from Fluka (Buchs, Switzerland) and purified by a column chromatography, as previously described [18]. *N*,*N*-Dimethylacrylamide (DMA), purchased from Aldrich (Milwaukee, WI, USA), was vacuum distilled. 2,2′-Azobisisobutyronitrile (AIBN) from Fluka was purified by fractional crystallization from ethanol. Tetrahydrofuran (THF) from Fluka was distilled and dried over molecular sieves. Other reagents were used as received.

The proteins, lysozyme from turkey egg white, cytochrome *c* from bovine heart, ribonuclease A and α-chymotrypsinogen from bovine pancreas, α-lactalbumin, β-lactoglobulins A and B, and bovine serum albumin (BSA) from bovine milk, were all obtained from Sigma (St. Louis, MO, USA).

Acetic acid, formic acid, boric acid, orthophosphoric acid, tris(hydroxymethyl)aminomethane (Tris), sodium hydroxide and ethylenediamine tetraacetic acid (EDTA) were from Merck (Darmstad, Germany) and used without further purification in the various running buffers. Distilled water was deionized with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Polymer synthesis and characterization

The monomers EPyM and DMA were copolymerized by free radical polymerization at 50 °C, using AIBN ($[I]=1.5\cdot10^{-2}$ mol l^{-1}) as a radical initiator and THF ($[M]=1.0$ mol l^{-1}) as solvent, a feed molar fraction of 19% EPyM and 24 h of reaction time. Polymerization was carried out in a Pyrex ampoule under an oxygen-free N_2 atmosphere.

The reaction mixture was precipitated in a large excess of a diethyl ether–hexane mixture, purified by reprecipitation, filtered off and vacuum-dried over phosphorus pentoxide.

The copolymeric system was characterized by ^{1}H NMR spectroscopy. Spectra were recorded in $^{2}H_2O$ solutions on a Varian Gemini 200 spectrometer.

2.3. Coating procedure

A very simple coating strategy was used consisting of flushing the capillary, prior to each run, with a diluted polymer solution (0.1 mg ml^{-1} in water) for 2 min and next replacing this solution by flushing the capillary with the separation buffer for 2 min.

2.4. CE conditions

Analyses were carried out on a Beckman P/ACE 2100 System (Fullerton, CA, USA) controlled by Gold Software, with a UV–Vis detector working at 254 nm for electroosmotic flow (EOF) measurements with acetone, and at 214 nm for proteins separation. Bare fused-silica capillaries of 50 and 75 μm I.D. were purchased from Composite Metal Services (Worcester, UK). Sample injections were made at the cathodic end using a N_2 pressure of 0.5 p.s.i. (1 p.s.i. = 6894.76 Pa). Injection times varied from 3 to 20 s.

Before first use, uncoated capillaries were conditioned in the following way: a 20 min rinse with 0.1 *M* NaOH was followed by a water rinse for other 20 min. Between injections, uncoated capillaries were rinsed with water for 2 min, and buffer solutions for another 2 min. In the case of polymer coated capillaries, this routine was changed to 2 min of water, 2 min of diluted polymer solution (0.1 mg ml^{-1}) and 2 min of buffer solution.

2.5. CE buffers

Five different buffer solutions have been used along this work: 0.9 *M* sodium formate/formic acid at pH 2.15; 100 m*M* sodium acetate/acetic acid at pH 5.00; 89 m*M* Tris, 20 m*M* orthophosphoric acid and 2 m*M* EDTA at pH 7.30; 150 m*M* sodium tetraborate/boric acid at pH 8.50; and 100 m*M* sodium tetraborate/boric acid at pH 10.00.

2.6. Electroosmotic flow measurements

Acetone was used as a non-charged marker to determine the EOF of bare silica and polymer coated capillaries. Running buffers were used at different pHs for the EOF measurements.

2.7. Samples

Basic proteins (lysozyme, cytochrome *c*, ribonuclease A and α-chymotrypsinogen) were dissolved in Milli-Q water (0.5 mg ml^{-1} of each protein) and separated in two different capillaries (bare silica and DMA-EPyM coated capillary) both with 47 cm of total length (40 cm of effective length) and 50 μm I.D.

Standard acidic proteins from bovine milk (BSA, α-lactalbumin and β-lactoglobulin A and β-lactoglobulin B) were dissolved in Milli-Q water (0.25 mg ml^{-1}) and separated in a DMA-EPyM coated capillary of 37 cm (30 cm of effective length) × 75 μm I.D.

Whey proteins directly extracted from bovine milk were obtained by acidification of milk at pH 4.6 with 1 *M* hydrochloric acid, followed by filtration and centrifugation of the supernatant fraction [19]. The supernatant was directly injected into the CE instrument using the same separation conditions as the aforementioned for standard acidic proteins.

3. Results and discussion

3.1. DMA-EPyM polymer characterization

The yield of the radical copolymerization of EPyM and DMA reaction was 87%. Copolymer characterization by ^{1}H NMR spectroscopy is shown in Fig. 1 based on the following signals assignment δ (ppm): 0.87 (α-CH_3, EPyM), 1.70 (–CH_2–, cycle, EPyM), 2.57 (CH_2, DMA), 2.80 (2× N-CH_3, DMA), 2.87 (2× N-CH_2–, cycle, EPyM), 3.00 (N-CH_2, EPyM), 4.07 (O-CH_2, EPyM). The copolymer composition was determined by integration of the corresponding signals being the EPyM content 17 mol%. The copolymer was proved to be totally soluble in water and polar solvents as chloroform,

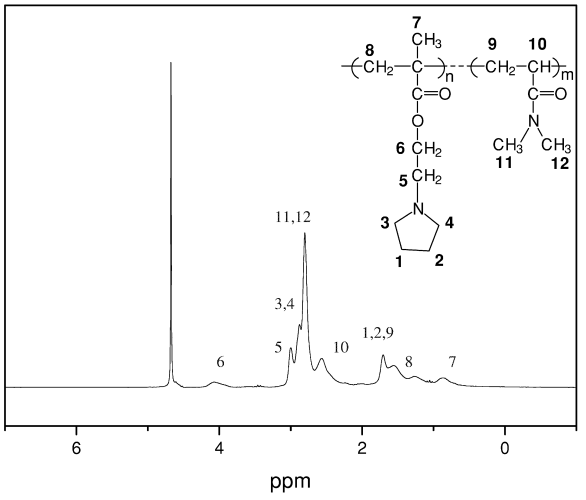

Fig. 1. Chemical structure and ^{1}H NMR spectrum of the copolymer DMA-EPyM (*N*,*N*-dimethylacrylamide-ethylpyrrolidine methacrylate).

ethanol, THF, etc. and showed UV absorbance maximum at 206 nm.

3.2. Electroosmotic flow provided by DMA-EPyM coated capillaries

According to the molecular structure of the DMA-EPyM copolymer (see Fig. 1), the amine group belonging to the ethylpyrrolidine methacrylate monomer is expected to provide some cationic character to this macromolecule. From a CE point of view, an interesting property of this copolymer is that, unlike other high-molecular-mass positively charged polymers already used as CE coatings (e.g. polyethyleneimine [16]), the ethylpyrrolidine methacrylate content in the copolymer is not too high (as it has been demonstrated in the Section 3.1). It could provide a different behavior to the coated capillary

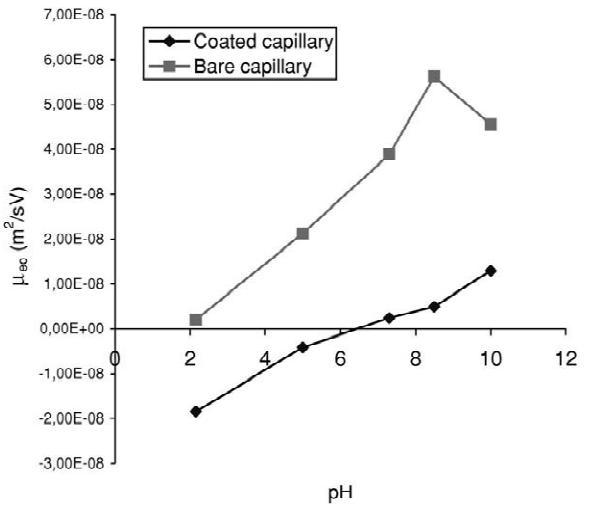

Fig. 2. Electroosmotic flow (EOF) as a function of pH. Comparison between a bare fused-silica capillary and a DMA-EPyM coated capillary.

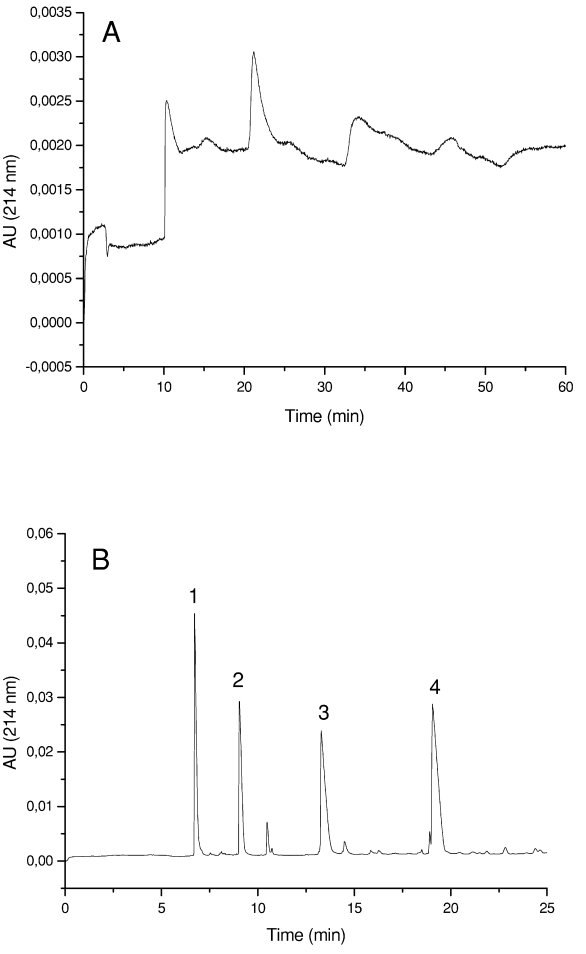

Fig. 3. Electropherograms of basic proteins in a bare capillary (A) and a DMA-EPyM coated capillary (B), both of 47 cm (40 cm effective length)×50 μm I.D. Separation buffer: 100 m*M* sodium acetate/acetic acid at pH 5. Running voltage: 25 kV. Basic proteins: (1) lysozyme; (2) cytochrome *c*; (3) ribonuclease A and (4) α-chymotrypsinogen (0.5 mg ml^{-1} of each protein) injected by pressure.

depending on the pH. In order to study this, the electroosmotic flow of both a bare fused-silica and a DMA-EPyM coated capillary was measured employing running buffers at different pH values. The results obtained from this experiment for both types of capillaries are shown in Fig. 2. As can be seen, the EOF obtained for the bare fused-silica shows a typical dependence on the pH (i.e. EOF close to zero at very low pH, an increase of EOF at pH about 5 and nearly a constant EOF value at pH values higher than 7.5–8). As can be seen in Fig. 2, using the same running buffers with the DMA-EPyM coated capillary the behaviour of the EOF vs. pH is somewhat different from that found in the bare silica capillary. Thus, the DMA-EPyM coated capillary shows an anodal EOF at low pH values, a nearly zero EOF at pHs around 6 and a low cathodal EOF at pHs higher than 8. This behaviour can be explained considering that the global electrical charge onto the capillary wall is due to both the amine groups of the polymer (bearing a positive electrical charge) and the remaining silanol groups onto the silica wall (bearing a negative electrical charge). Thus, under acidic pHs the amine groups are the main charged groups bringing about a global positive charge on the capillary wall and, as a consequence, an anodal EOF. Under very basic pHs the number of positive charges on the polymer decreases and the negative silanol groups become predominant, bringing about a cathodal EOF. It is noteworthy that this EOF is clearly reduced using the DMA-EPyM capillary (for instance, compare in Fig. 2 the EOF values obtained at pH 8.5 using the coated with that from the bare silica capillary) that is a good indication of the shielding effect of the DMA-EPyM coating even at these basic pHs.

These EOF values seem to indicate that the capillary is indeed coated by this copolymer. However, the applicability in CE of this coating was further studied by trying to separate a group of basic proteins. Moreover, the same group of basic proteins was used to determine the reproducibility of our coating procedure.

3.3. Separation of basic proteins using bare silica and DMA-EPyM coated capillaries

Fig. 3A shows the electropherogram of the separation of four basic proteins (i.e. lysozyme, cytochrome *c*, ribonuclease A and α-chymotrypsinogen) obtained using a bare silica capillary together with a running buffer at pH 5. As can be seen, under these separation conditions the adsorption phenomenon between the proteins (positively charged) and the capillary wall (negatively charged) is so strong that makes impossible the analysis of these proteins by CE. The use of a DMA-EPyM coated capillary under identical separation conditions brings about an adequate separation of these four basic proteins as shown in Fig. 3B with efficiencies slightly higher than 50 000 theoretical plates per meter of column for the most basic proteins (i.e. lysozyme and cytochrome *c*). This result demonstrates the capability of this coating to reduce the harmful adsorption phenomenon.

The main advantage of our coating is that it is easily implemented by flushing the capillary for 2 min with a dilute solution of the DMA-EPyM polymer in water (0.1 mg ml^{-1}) and next the capillary is rinsed with the separation buffer (for other 2 min) prior to each run. Therefore, no coating agent is added into the separation buffer avoiding in this

Table 1
Intra-day and inter-day reproducibility of migration times of basic proteins using a DMA-EPyM coated capillary

Protein	Same day ($n=5$)		Three days ($n=15$)	
	Average migration time (min)	RSD (%)	Average migration time (min)	RSD (%)
Lysozyme	6.68	0.45	6.75	1.22
Cytochrome *c*	9.00	0.60	9.17	1.89
Ribonuclease A	13.28	0.69	13.65	2.83
α-Chymotrypsinogen	19.12	0.75	19.84	3.90

Conditions: 47 cm total length×50 μm I.D. DMA-EPyM coated capillary; buffer 100 m*M* sodium acetate/acetic acid at pH 5.00; separation voltage 25 kV; detection wavelength 214 nm.

way any interaction with the solutes. Considering that for physically adsorbed coatings and under typical separation conditions, coating agent is added to the running buffer in order to keep the coating on the capillary wall surface [10] a study about the reproducibility of our procedure is mandatory.

In order to carry out the coating reproducibility study we have preferred to use as sample the set of basic proteins instead of the more usual EOF marker. The choice of the four basic proteins as sample allows us to study the reproducibility of the coating protocol together with its capability to shield the detrimental adsorption under more demanding conditions. Moreover, we have used a long capillary (40 cm detection length) in order to amplify any adsorption effect. The migration time reproducibility obtained for the same day and 3 different days is given in Table 1. As can be seen, under these conditions the RSD values obtained were lower than 0.75% for the same day and 3.90 for 3 different days for these proteins, which seems to indicate an acceptable reproducibility of our coating protocol. Moreover, the shape and efficiency of the peaks kept stable along the 3 days experiment, that is a good indication of the shielding properties and stability of the DMA-EPyM coating.

3.4. Separation of acidic proteins from whey using DMA-EPyM coated capillaries

It has been frequently observed that one of the main limitations of this type of physically adsorbed coatings for CE is that the developed coating is habitually used to solve a specific separation problem [10], mainly the CE separation of basic proteins that, therefore, reduces considerably its applicability.

The modulation of the EOF of the DMA-EPyM coating with the pH can be favorably used to separate both basic and acidic proteins. To do this, a standard group of acidic proteins (i.e. four bovine milk proteins, namely, BSA, β-lactoglobulin A, β-lactoglobulin B and α-lactalbumin) was selected and their separation was tried using a DMA-EPyM coated capillary at pH 8.5. Fig. 4A shows the electropherogram of the separation of these four proteins with efficiencies up to 59 000 theoretical plates m^{-1} for the last peak (i.e. α-lactalbumin). As can be seen, a good separation of these four proteins was obtained in less than 12 min, demonstrating that the DMA-EPyM coated capillaries can be used to separate both basic and acidic proteins. This makes broader the usefulness of this coating. Besides, as expected, the migration order of these proteins is the inverse than that obtained using a bare fused-silica capillary at similar pH [20].

Fig. 4B shows a real sample of whey from raw milk injected under the same conditions of Fig. 4A.

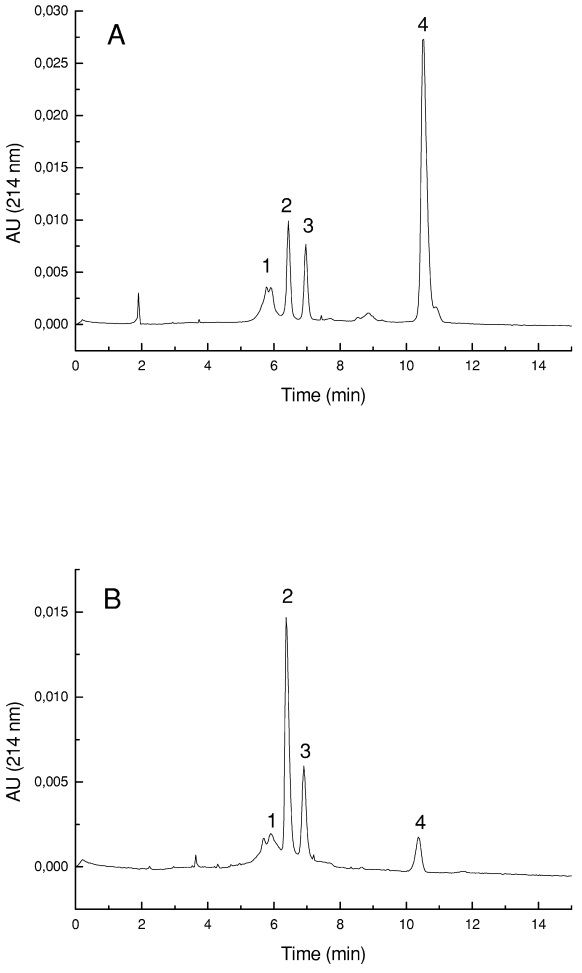

Fig. 4. Electropherograms of a mixture of standard proteins (A) and proteins from whey obtained as described in Section 2 (B). Separations were carried out using a DMA-EPyM coated capillary of 37 cm (effective length 30 cm)×75 μm I.D. Separation buffer: 150 m*M* sodium tetraborate at pH 8.5. Separation voltage: −17 kV. A) Standard proteins (0.25 mg ml^{-1} of each): (1) bovine serum albumin (BSA); (2) β-lactoglobulin A; (3) β-lactoglobulin B and (4) α-lactalbumin. (B) Proteins found in whey.

Table 2
Reproducibility of migration times of acidic proteins in a standard sample and in a whey from raw milk ($n=5$)

Sample	Standard proteins		Whey proteins	
	Average migration time (min)	RSD (%)	Average migration time (min)	RSD (%)
BSA	5.87	0.86	5.95	1.07
β-Lactoglobulin A	6.37	0.97	6.44	0.61
β-Lactoglobulin B	6.90	1.07	6.95	0.66
α-Lactalbumin	10.37	1.43	10.59	1.39

Conditions: 37 cm total length×75 μm I.D. DMA-EPyM coated capillary; running buffer: 150 m*M* sodium tetraborate/boric acid at pH 8.50; separation voltage: −17 kV; detection wavelength: 214 nm.

As can be seen the four proteins could be easily detected in whey under these conditions. Moreover, the reproducibility of the separation of the four acidic proteins in both standard and real sample was also studied and the results are given in Table 2. As can be seen, good migration time reproducibility is again obtained for these proteins (with RSD values lower than 1.43%, see Table 2) without any noticeable change in the separation efficiency which corroborates the usefulness of our approach.

4. Conclusions

A simple, fast and reproducible polymer coating for CE of basic and acidic proteins has been developed. The coating is physically adsorbed onto the capillary wall and it is regenerated just by flushing the capillary between injections with a dilute solution containing the polymer. In this CE procedure, separation of proteins is achieved with no polymer added to the running buffer which make it compatible with CE–MS. This coating does not require the use of organic solvents, high viscous solutions or elevated temperatures, therefore, its future application to CE separations of proteins in microchips can be anticipated.

Acknowledgements

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