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Evaluation of a neutral hydrophilic coated capillary for capillary zone electrophoretic separation of proteins

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Abstract

A neutral, hydrophilic coated capillary with negligible electroosmotic flow was characterized as to migration time reproducibility and separation efficiency for protein separation by capillary zone electrophoresis. Consecutive runs (over 200 runs) of the basic proteins at pH 6.0 yielded excellent migration time reproducibility ($< 2\%$ R.S.D.) and high separation efficiency (ca. $3–5 \cdot 10^5$ plates/m). The acidic proteins were separated at pH 8.0 under the reversed polarity (cathode at the injection end), and excellent migration time reproducibility of less than 3% was achieved. Separation of egg white proteins at pH 3.0 showed migration time reproducibility of less than 0.5% R.S.D. ($n = 36$) for lysozyme, conalbumin and ovalbumin.

1. Introduction

Capillary zone electrophoresis (CZE) can serve as a fast, automated, and easily operated separation tool for analyzing proteins if the adsorption of proteins onto capillary wall can be eliminated for solving the problem of peak tailing and broadening, poor quantitation and irreproducibility of migration time. The adsorption of proteins are known to be incurred by electrostatic, hydrophobic, and, to a lesser extent, hydrogen bonding interactions between proteins and liquid–solid interface [1]. Much research has been devoted to minimize the interactions, and, basically, two major approaches have been employed in the research: the surface modification method [1–12] and the solution/colloids method [13–19]. With the former strategy, the capillary wall is bonded with a coating to deactivate the silanol groups on silica surface, and, for most cases, to increase the surface hydrophilicity; with the latter, buffer additives [13–17], high ionic strength [18,19], or pH extremes [20–23] are employed to prevent the adsorption. Generally, the latter approach is limited by the stability of protein at pH extremes and Joule heating accompanied by high ionic strength. When polymeric additives are used for reducing protein adsorption, the separation time is increased, and, in some cases, the resolution of two species of interest is limited due to the reduction of the difference of electrophoretic mobility with increased viscosity of running buffer. In contrast, with the bonded coating method, the buffer can still remain an adjustable parameter for optimizing the separation.

In this study, we have developed a neutral, hydrophilic coated capillary that allows basic and

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acidic proteins separation at near neutral pH region (pH 6–8). This pH region is especially interesting since proteins often exist in native structures. An extensive evaluation of the coating will be reported, in terms of separation efficiency, migration time reproducibility and long-term stability, as applied to CZE separation of proteins.

2. Experimental

2.1. Materials

All proteins used were purchased as lyophilized powders from Sigma (St. Louis, MO, USA): α-lactalbumin (bovine milk, p*I* 4.8), β-lactoglobulin A (bovine milk, p*I* 5.1), carbonic anhydrase II (human erythrocytes, p*I* 5.4), carbonic anhydrase II (human erythrocytes, p*I* 5.9), myoglobin (horse heart, p*I* 7.2), ribonuclease A (bovine pancrease, p*I* 9.5), cytochrome *c* (bovine heart, p*I* 10.8) and lysozyme (chicken egg white, p*I* 11.0). Benzyl alcohol, hydrochloric acid, sodium hydroxide and the buffers for protein separations and electroosmotic flow (EOF) measurements were also supplied by Sigma. Egg white was taken from fresh chicken egg.

2.2. CZE

A P/ACE 2210 capillary electrophoresis instrument controlled by System Gold software (Beckman, Fullerton, CA, USA) was used in this study. Neutral, hydrophilic coated capillaries of 50 μm I.D. from eCAP neutral capillary methods development kit/proteins (Beckman) were used for protein separation. Protein mixtures were dissolved in deionized water at a concentration of 1 mg/ml for each protein; chicken egg white was diluted 1:10 in water. Unless specified, the separations were carried out at room temperature (25°C), with a field strength of 500 V/cm and detection at 214 nm. The proteins were introduced into the separation capillary by pressured injection for 1 s. Between runs, the column was rinsed with 0.1 *M* HCl for 0.5 min and then with the running buffer for 1.5 min. Running buffer in the vials was changed for every 30 runs.

When the buffer was changed to a different pH, the column was reconditioned by performing a 10-min rinse with the new buffer. The capillary was then equilibrated with the new buffer for another 10 min prior to performing a run.

3. Results and discussion

3.1. Electroosmotic flow

For the coated capillaries used in this study, the coating inside the capillary wall consists of two polymeric layers covalently bonded to each other [24]. The function of the first layer is to deactivate the silanol groups, and, thus, to reduce the electrostatic interaction between silica surface and proteins; the second layer serves as a hydrophilic shield to suppress the hydrophobic interactions between capillary wall and protein molecules. The coverage of the coating on capillary wall was evaluated by monitoring EOF of the coated capillaries. The plot of EOF vs. pH is given in Fig. 1. As shown in Fig. 1, the EOF of the coated capillary showed values ranging $1 \cdot 10^{-6}$–$5 \cdot 10^{-6}$ $cm^2/V\,s$ at pH values 5–10, which are two orders of magnitude less than the EOF of fused-silica capillaries. The extremely low EOF in the coated capillaries can be attributed to at least two causes. First, the deactivation reaction of silanol groups on silica surface during coating processes reduces the surface charges arising from deprotonated silanol groups. Second, the viscosity of running buffer at the solid–liquid interface also plays an important role in affecting the magnitude of EOF. When hydrophilic polymers are bonded onto the silica surface, the viscosity at the interface is known to increase [25], leading to a slower EOF.

3.2. Basic protein separations

The separations of four basic proteins at pH 6.0 are shown in Fig. 2. Due to the strong

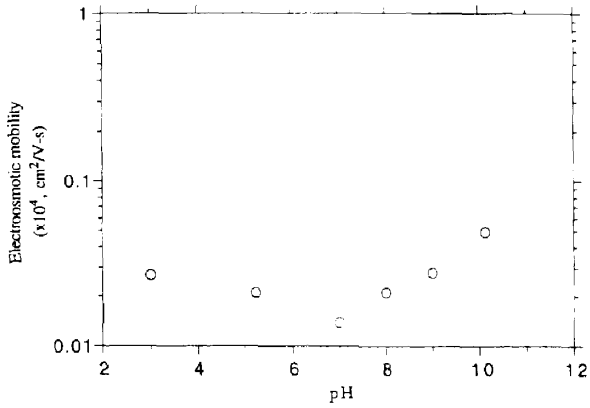

Fig. 1. EOF of the neutral coated capillaries. Conditions: 500 V/cm; 20 cm capillary separation length; benzyl alcohol used as EOF marker. The running buffers were: 20 m*M* citrate at pH 3.0, 50 m*M* 2-(N-morpholino)ethanesulfonic acid (MES) at pH 5.2, 20 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) at pH 7.0, 20 m*M* N-[tris(hydroxymethyl)methyl]glycine (Tricine) at pH 8.0, 20 m*M* 2-(N-cyclohexylamino)ethanesulfonic acid (CHES) at pH 9.0 and 20 m*M* 3-cyclohexylamino-1-propanesulfonic acid (CAPS) at pH 10.1.

electrostatic interactions between positively charged proteins and deprotonated silanol groups, the basic proteins tend to adsorb onto bare silica surface, leading to poor peak shape and irreproducibility of migration time. In this sense, the basic proteins can serve as a sensitive probe of the charging state of the capillary surface. As shown in Fig. 2, all the four basic proteins showed good peak shape under the separation condition. After 216 consecutive runs under a high field strength of 500 V/cm, the migration time of the four proteins showed minimal change of less than 4 s, and the peak shapes of lysozyme, cytochrome *c* and ribonuclease A still remained good. The slightly tailing peak shape of myoglobin was due to the degradation of myoglobin kept in the sample vial for 61 h at 27°C during the 216 consecutive runs. The degradation of myoglobin with time at room temperature can be alleviated by using commercially available sample cooling systems, which use circulating coolant to keep the proteins at 4°C. The separation efficiency and migration time reproducibility of the basic proteins are shown in Table 1. The separation efficiencies of

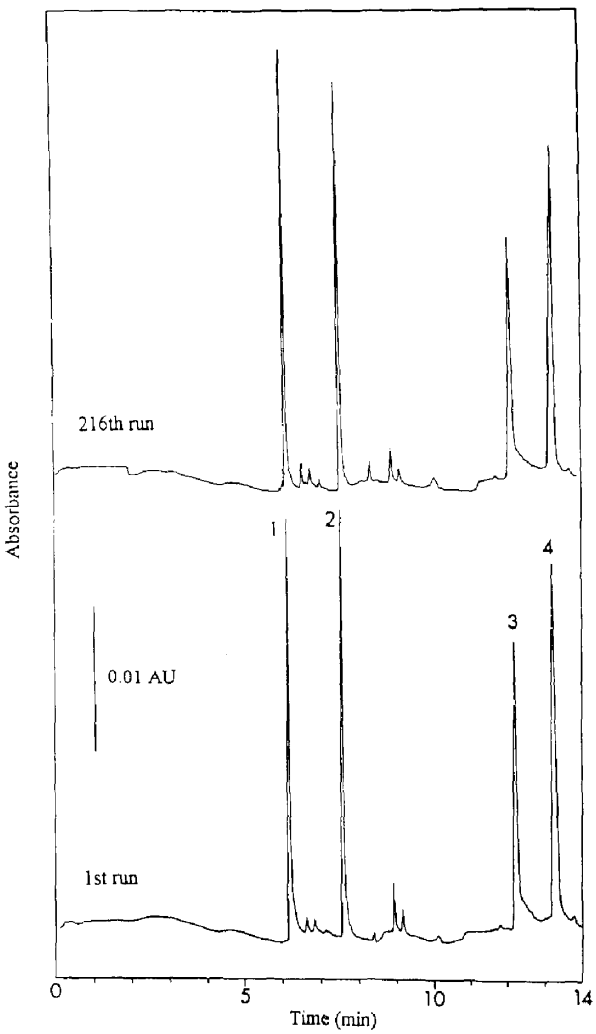

Fig. 2. Capillary electrophoretic separations of the basic proteins as in Table 1. Separation conditions: 20 m*M* citrate and 20 m*M* MES, pH 6.0; 500 V/cm; 20 cm capillary separation length.

the four proteins were all higher than $3 \cdot 10^5$/m for the first run, and, after 215 consecutive runs, the high efficiencies still remained, indicating the excellent stability of the coating. The relative standard deviations (R.S.D.s) of the basic proteins for overall 216 runs were all less than 2%. In this study, the running buffer in the vials was changed for every 30 runs. As will be discussed below, the migration time reproducibility could be further improved if the running buffer was changed more often.

Table 1
Column efficiencies and migration time reproducibility for consecutive runs at pH 6.0

Peak No.	Protein	Efficiency/m ($\times 10^3$)		Migration time[a]	
		1st run	216th run	Average (min)	R.S.D. (%)
1	Lysozyme	389	587	6.1	0.5
2	Cytochrome *c*	525	483	7.5	0.5
3	Myoglobin	587	465	12.1	1.0
4	Ribonuclease A	318	297	13.1	1.0

[a] $n = 216$ (1st–216th runs).

A detailed study of the migration time pattern of three basic proteins (lysozyme, cytochrome *c* and ribonuclease A) was carried out with another coated capillary, and the results were shown in Fig. 3. It is of interest to note that, for every 30 runs with a same vial of running buffer, the migration time of each protein tended to increase slightly with the number of run until the running buffer was replaced. When the running buffer was just changed, the migration time of each protein discontinuously shifted to a lower value. This phenomenon was more significant for the proteins with longer migration time, especially ribonuclease A. There are at least two possible causes for this effect. First, the evaporation of the buffer solution during the consecutive runs resulted in a more concentrated buffer with increased ionic strength, and thus reduced the electrophoretic mobility of proteins and the residual EOF. Second, the polarization of electrodes by an excess of counter-ions (or depletion of co-ions) leaded to a lower effective separation voltage. Thus, the migration times of proteins were increased due to a lower separation field strength. Nevertheless, this study suggests that if a running buffer vial can be replaced more frequently than 30 runs, the elution time of the last run will deviate to a lesser extent from the first run, and better migration time reproducibility can be achieved.

A detailed study of the separation efficiency vs. run number for 210 consecutive runs was also carried out, and no significant trend was observed for the four basic proteins (lysozyme, cytochrome *c*, myoglobin and ribonuclease A).

The capillary-to-capillary study of the migration time reproducibility (R.S.D.) was evaluated using separations of the basic proteins

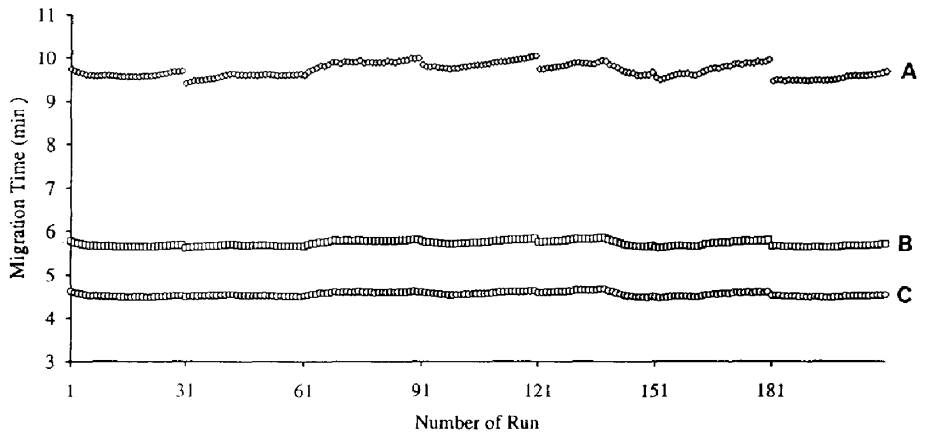

Fig. 3. Migration times of 210 consecutive runs for capillary electrophoretic separations of basic proteins. Separation conditions as in Fig. 2. A = Ribonuclease A; B = cytochrome *c*; C = lysozyme.

(lysozyme, cytochrome *c* and ribonuclease A) at pH 6.0. Six capillaries, with designations of Cap 1–6, were evaluated with 240 consecutive runs. The migration time reproducibility (R.S.D.) based on the first and last 20 runs ($n = 40$) was calculated and shown in Table 2. The Cap 1–3 were randomly chosen from the same lot, and Cap 1, 4, 5 and 6 were from four different lots. By our coating procedure, 140 coated capillaries of 45 cm long were prepared in one lot. As shown in Table 2, the R.S.D.s of migration times were all less than 2%, and the variation of the migration time reproducibility for the capillaries from different lots was not larger than that from the same lot, indicating the consistency of the coating techniques for obtaining coating capillaries with excellent migration time reproducibility.

3.3. *Acidic protein separations*

As mentioned above, the EOF of the coated capillaries is extremely low, and can be regarded as zero for normal CZE separation. For separation of the acidic proteins at the pH values near neutral, the separation has to be carried out under reversed-polarity mode (cathode is at the injection end), to drive the negatively charged proteins toward detection window by their own electrophoretic mobility. The separations of four acidic proteins at pH 8.0 under the reversed polarity are shown in Fig. 4. As shown in Fig. 4, all the four acidic proteins showed good peak shapes under the separation conditions. After 120 consecutive runs of separation under a high field strength of 500 V/cm, the migration times of the four proteins showed minimal change, and the peak shapes of α-lactalbumin, carbonic anhydrase II (p*I* 5.4) and carbonic anhydrase II (p*I* 5.9) still remained good. The broadening of the β-lactoglobulin A peak was found to be caused by degradation of β-lactoglobulin A molecules,

Table 2
Capillary-to-capillary variation of migration time reproducibility at pH 6.0

	Migration time reproducibility (R.S.D., %)[a]		
	Lysozyme	Cytochrome *c*	Ribonuclease A
Cap 1[b]	0.7	0.6	0.7
Cap 2	1.7	1.6	1.9
Cap 3	0.7	0.6	0.6
Cap 4	0.7	0.7	1.0
Cap 5	1.9	1.9	1.5
Cap 6	0.5	0.4	0.8

[a] Based on 1st–20th runs and 221st–240th runs ($n = 40$) for each capillary.
[b] Cap 1–3 were randomly chosen from a lot of 140 capillaries. Cap 1, 4, 5 and 6 were each randomly chosen from different lots; 140 capillaries were prepared in one lot.

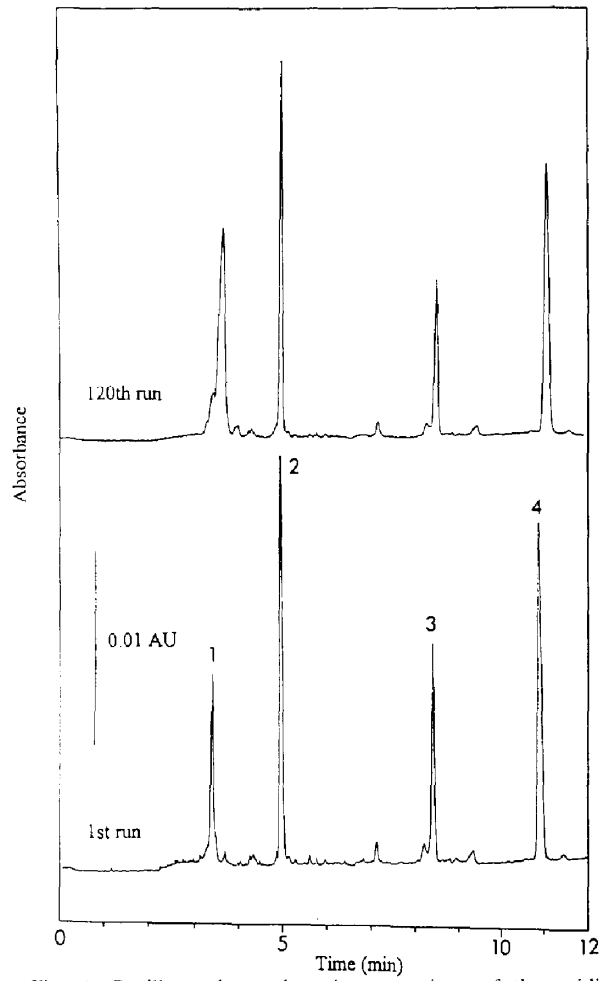

Fig. 4. Capillary electrophoretic separations of the acidic proteins as in Table 3. Separation conditions: 20 m*M* Tricine, pH 8.0; 500 V/cm; 20 cm capillary separation length.

as confirmed by the fact that a symmetrical and sharp peak shape was restored after replacing with a fresh sample vial. The separation efficiency and migration time reproducibility of the acidic proteins are shown in Table 3. As indicated in Table 3, after 120 consecutive runs, the efficiencies were still high (the low efficiency of β-lactoglobulin A is owing to degradation). Regarding the migration time reproducibility, except for β-lactoglobulin A, the R.S.D.s of the acidic proteins for 216 consecutive runs were less than 2%. Due to the degradation of protein molecules, β-lactoglobulin A showed a higher value of R.S.D. of 3%.

3.4. Egg white protein separations

The three major components of egg white proteins are lysozyme (p*I* 11.0, M_r 14 000), conalbumin (p*I* 6.6, M_r 77 000) and ovalbumin (p*I* 4.7, M_r 43 500) [26]. To elute these three components with a wide p*I* range in one run, 20 m*M* citrate buffer at pH 3.0 was used to separate the three proteins. As shown in Fig. 5, these three components eluted according to their charging states; lysozyme, the most positively charged protein, eluted first followed by conalbumin, the protein with the middle p*I*. The identification of each peak was confirmed by individual standard protein. Both lysozyme and conalbumin appeared with good peak shapes, while the ovalbumin peak appeared with a shoulder that has been observed and attributed to impurity [26,27].

Reproducibility of migration time of the egg white proteins was studied for over 36 consecutive runs, and the results were shown in Table 4. As seen in Table 4, the relative standard deviation (R.S.D.) for each component was all less than 0.5%. Data of reproducibility of the peak area and peak height for over 9 runs are listed in Table 5. The R.S.D.s of peak area and peak height for these three proteins were around 3%, suggesting little, if any, loss of protein molecules due to adsorption and the possibility of using CZE to quantify the egg white proteins.

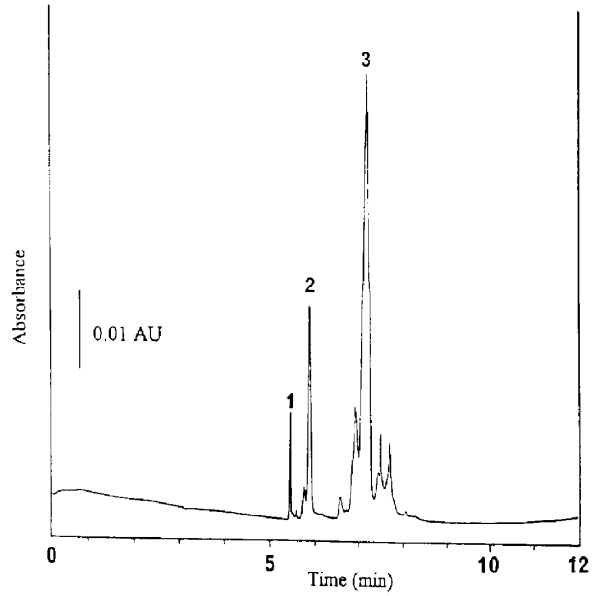

Fig. 5. Capillary electrophoretic separations of egg white proteins as in Table 4. Separation conditions: 20 m*M* citrate, pH 3.0; 324 V/cm; 30 cm capillary separation length.

Table 3
Column efficiencies and migration time reproducibility for consecutive runs at pH 8.0

Peak No.	Protein	Efficiency/m ($\times 10^3$)		Migration time[a]	
		1st run	120th run	Average (min)	R.S.D. (%)
1	β-Lactoglobulin A	73	35	3.5	3.0
2	α-Lactalbumin	117	142	5.0	0.4
3	Carbonic anhydrase II (p*I* 5.4)	548	263	8.4	1.0
4	Carbonic anhydrase II (p*I* 5.9)	334	249	10.9	1.3

[a] $n = 120$ (1st–120th runs).

Table 4
Reproducibility of migration time for egg white proteins at pH 3.0

Peak No.	Protein	p*I*	Migration time[a]	
			Average (min)	R.S.D. (%)
1	Lysozyme	11.0	5.3	0.4
2	Conalbumin	6.6	5.8	0.3
3	Ovalbumin	4.7	7.1	0.4

[a] $n = 36$.

4. Conclusions

A neutral, hydrophilic coated capillary with negligible EOF was evaluated to show high separation efficiency, excellent migration time reproducibility, and long term stability as applied to CZE for protein separations. From the results shown above, the coated capillary combined with three buffers at pH 3.0, 6.0 and 8.0 can serve as a convenient analyzing tool for separations of proteins with a wide p*I* range. The pH 6.0 buffer is used for separations of neutral and basic proteins (p*I* > 6.7) under normal separation polarity (anode at the injection end), and the pH 8.0 buffer can be used for acidic proteins (p*I* < 6.6) under reversed separation polarity (cathode at the injection end). With a field strength of 500 V/cm and a capillary separation length of 20 cm, model proteins with p*I* values ranging 3.5–6.6 (or 6.7–11.0) were observed to elute in 20 min with high separation efficiency by using the pH 6.0 (or the pH 8.0 buffer); with the pH 3.0 buffer, proteins with p*I* values ≥4 would elute in 10 min under a field strength of 500 V/cm and capillary separation length of 20 cm. In this sense, an unknown sample can be quickly scanned by carrying out the separation with pH 3.0 buffer, followed by the separations with pH 6.0 and/or pH 8.0 buffers to achieve better separation efficiency and resolution.

For CZE separation, development of a method for protein separation is by no mean an easy task, which involves the optimization of buffer type, ionic strength, pH, field strength and capillary separation length. The conjunction of the neutral coated capillary with three buffers of appropriate pH values (pH 3.0, 6.0 and 8.0) is then expected to make easier the development of methodology for protein separations by CZE.

Table 5
Reproducibility of peak area and peak height for egg white proteins at pH 3.0

Peak No.	Protein	R.S.D. (%)[a]	
		Peak area	Peak height
1	Lysozyme	3.0	2.5
2	Conalbumin	3.5	3.7
3	Ovalbumin	3.6	2.9

[a] $n = 9$.

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