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Capillary electrophoresis of proteins under alkaline conditions

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ABSTRACT

Successful separations of proteins by capillary electrophoresis in uncoated fused-silica capillaries is limited by adsorption and variable rates of electroendosmosis, which can compromise quantitative accuracy and precision. Operation at extremes of pH to minimize these problems is useful in special cases but is not a general strategy for protein separations. Three alternative strategies are described: use of capillaries coated with a linear hydrophilic polymer, the use of acidic solutions to wash the capillary between runs, and the incorporation of additives into the electrophoresis buffer to minimize adsorption during analysis. Applications of these techniques to protein samples is demonstrated.

INTRODUCTION

Migration in capillary electrophoresis is governed primarily by solute charge, so manipulation of the electrophoresis buffer pH is the most direct strategy in optimizing a separation[1]. However, performing free zone separations at elevated pH values can introduce two complications in achieving good resolution and quantitative detector response. First, the increased rate of electroendosmosis can adversely effect resolution and reproducibility. Second, solute adsorption can cause poor peak shape, reduced response, and in worst cases, no elution. Adsorption is a particularly serious problem in the case of proteins because of the multiplicity of polar, charged, and hydrophobic sites on the molecular surface[2]. Acceptable protein separations are typically more difficult to achieve compared to peptides and small molecules. Loss of protein due to adsorption can prevent accurate quantitation, particularly at low sample concentrations. In addition, protein adsorption can change the capillary surface characteristics, affecting results in subsequent runs. When electroendosmosis is used as a transport mechanism during loading and separation, the magnitude of electroendosmosis can drift as the capillary surface is modified by adsorption. Consequently, reproducibility of both migration times and peak areas could be reduced in repetitive analysis of complex mixtures.

A number of strategies have been adopted to minimize problems caused by electroendosmosis and adsorption. First, to achieve good resolution in the presence of

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high rates of osmotic flow, long capillaries are typically used. Unfortunately, the increased surface area of the longer tube exacerbates the problem of adsorption. Second, separations can be performed at pH values where electroendosmosis and adsorption are minimized[3–5]. At pH values below 3 where silanols are fully protonated, both osmotic flow and electrostatic interactions with proteinaceous solutes are reduced. Although some proteins can be separated at low pH, many proteins aggregate and precipitate under acidic conditions, or there may be insufficient difference in their mass-to-charge ratios to achieve good separation. At pH values above 9, it is assumed that both proteins and the silica surface exhibit high negative charge density, and adsorption through electrostatic interaction should be reduced. Unfortunately, some basic amino acid side chains are partially protonated at high pH so some proteins can adsorb, and very high rates of osmotic flow under these conditions may reduce resolution.

Three alternative strategies to minimize adsorption and osmotic flow are the use of coated capillaries, the use of wash solutions to purge the capillary between runs, and the incorporation of additives to the electrophoresis buffer. We wish to report results using these approaches for free zone electrophoresis of proteins.

EXPERIMENTAL

Materials

The following proteins were obtained from Sigma (St. Louis, MO, U.S.A.): albumin (bovine), carbonic anhydrase (bovine erythrocytes), a chymotrypsinogen A (bovine pancreas), cytochrome *c* (horse heart), γ-globulins (human), β-lactoglobulin (bovine milk), lysozyme (chicken egg white), myoglobin (horse heart), ribonuclease A (bovine pancreas). Hemoglobin AFSC electrophoresis standard was obtained from Isolab (Akron, OH, U.S.A.) Methylcellulose (4000 cP at 25°C for 2% solution) was obtained from Sigma. Triton X-100 reduced was obtained from Aldrich (Milwaukee, WI, U.S.A.) Z4A, Z6C, and Z60 are experimental multicomponent mixtures of zwitterionic species.

All capillary electrophoresis was performed using the HPE™-100 high-performance capillary electrophoresis system from Bio-Rad Labs. The capillaries were enclosed in cartridges and, where indicated, were coated on their internal surfaces with a covalently bonded linear polymer. Capillary dimensions were 25 μm I.D. and 20 cm or 50 cm length. Electropherograms were recorded using a Model 804 PC integrator (Bio-Rad Labs.).

Methods

To study the effect of electrophoresis buffers, wash procedures, and sample type on non-specific adsorption, we have used a flow injection procedure in which sample was introduced into the capillary by vacuum displacement and the amount of protein in the capillary or adsorbed onto the tube wall was estimated by the UV absorbance at 200 nm. Following an initial prewash of the capillary, buffer was drawn into the capillary and the detector was zeroed. Buffer was then displaced by buffer containing protein, and UV absorbance recorded after 1 min. To measure residual adsorption of protein to the capillary, the tube was then washed with 20 volumes of buffer and remaining UV absorption recorded. To measure the effectiveness of wash solutions,

the protein sample was displaced with 20 tube volumes of wash solution, followed by 20 tube volumes of buffer before recording remaining UV absorbance.

Electroendosmosis was measured by filling the capillary with a 0.05 *M* sodium borate buffer (pH 9) and placing 0.2*M* sodium borate buffer (pH 9) in the inlet and outlet reservoirs of the instrument. When voltage is applied using positive-to-negative polarity, electroendosmosis will sweep buffer ions into the tube and monitored current will double at the time when 67% of the tube volume is filled with concentrated buffer; we define this as the osmosis time. This method is simple in that a UV detector is not required, and we use it for assessing coating chemistries and for monitoring capillary lifetime.

RESULTS AND DISCUSSION

Protein adsorption on an uncoated fused-silica capillary is illustrated in Fig. 1. Myoglobin (p*I* 7.0) and serum albumin (p*I* 4.8) were separated under alkaline conditions using an uncoated capillary. At low sample concentration, response for the more basic protein is severely reduced, demonstrating that loss of protein due to

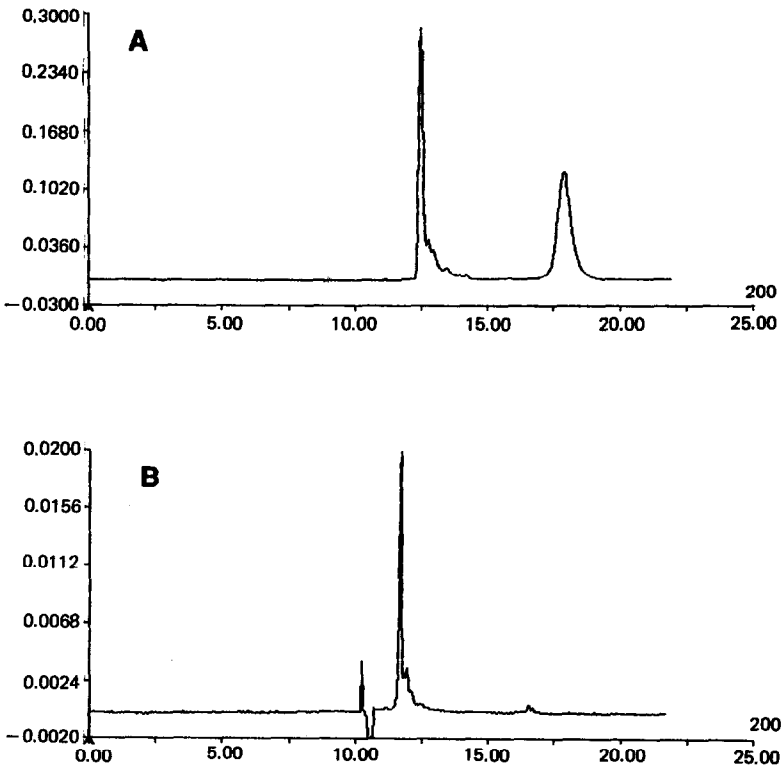

Fig. 1. Protein adsorption on a fused-silica capillary. Electrophoresis was performed in a 50 cm × 50 μm I.D. uncoated capillary at 8 kV using a 0.1 *M* sodium phosphate buffer (pH 8). Bovine serum albumin (first peak) and horse heart myoglobin (second peak) were prepared in 0.01 *M* sodium phosphate (pH 8.0) at (A) 5 mg/ml and (B) 0.5 mg/ml. Ordinate-axis values indicate absorbance at 200 nm. Retention times in min.

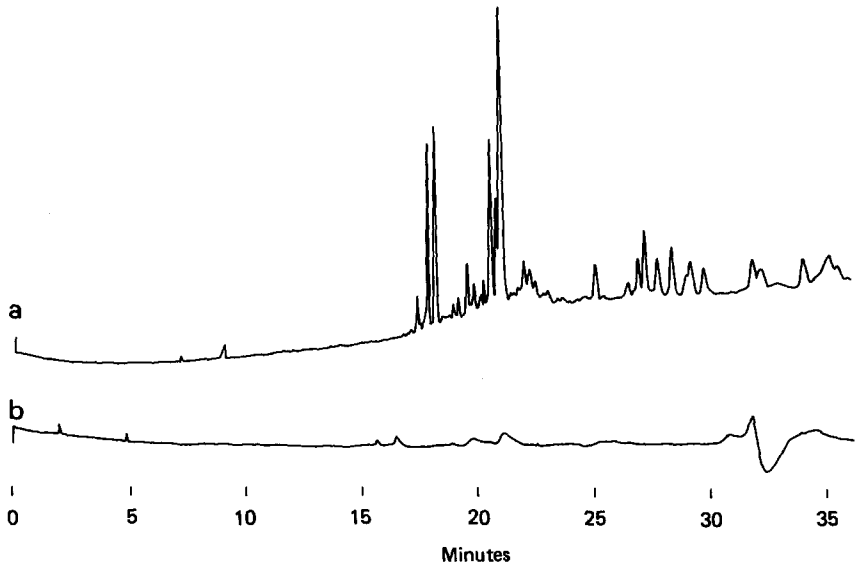

Fig. 2. Separation of a tryptic digest of bovine serum albumin on (a) coated and (b) uncoated 50 cm × 50 μm I.D. capillaries. Albumin (1 mg/ml) was digested with trypsin for 4 h at 37°C in 10 m*M* Tris chloride (pH 8). Electrophoresis was performed in 0.1 *M* potassium borate (pH 8.5)–0.2% methylcellulose–Z60 at 8 kV. Detection was at 200 nm. (a) Negative to positive polarity; (b) positive to negative polarity.

adsorption can prevent accurate quantitation. Protein adsorption will also reduce the rate of electroendosmosis from 10.83 cm/min for a fresh 50 cm × 50 μm I.D. uncoated capillary to 2.76 cm/min for a capillary washed with 10 mg/ml cytochrome *c* in 0.1 *M* phosphate buffer (pH 8), suggesting that quantitative precision can be compromised by a drift in the electroendosmotic flow during analysis of protein-containing samples. The problems of adsorption and variable electroendosmosis should be minimized using coated capillaries.

The use of polymer-coated tubes for capillary electrophoresis was first described by Hjertén[6], who demonstrated that a linear hydrophilic polymer attached to the

TABLE I

NON-SPECIFIC ADSORPTION OF HEMOGLOBIN TO AN UNCOATED CAPILLARY

50 cm × 50 μm I.D. uncoated capillary; column B, capillary prewashed before introduction of 10 mg/ml hemoglobin with 20 m*M* sodium hydroxide; column A, capillary prewashed with 0.1 *M* phosphate (pH 2.5).

Testing buffer	*Hemoglobin absorbance (mAU, 200 nm)*	*Remaining absorbance (mAU, 200 nm)*	
		B	*A*
0.1 *M* boric (pH 9.0)	668	3.5	2.5
0.1 *M* phosphate (pH 7.0)	654	9.5	9.5
0.1 *M* acetate (pH 4.8)	658	30.5	7.5
0.1 *M* phosphate (pH 2.5)	666	0	0

capillary surface reduced electroendosmosis. His patented procedure employs linear polyacrylamide as the coating; we have modified this procedure to produce a coating which exhibits enhanced stability at alkaline pH. As seen in Fig. 2, a capillary coated with this technique exhibits good performance in electrophoresis of a tryptic digest of bovine serum albumin using tris-borate buffer at pH 8.3. Under the same conditions most of the peptide components are strongly adsorbed to the capillary surface when an uncoated tube is used. In addition to reducing solute adsorption, it can be shown that coating the capillary greatly diminishes electroendosmosis. This permits separations to be performed in short capillaries with low total voltages. Coated capillaries typically exhibit a 40-fold reduction in electroendosomosis, *viz.* 0.25 cm/min for a coated capillary and 10.0 cm/min for an uncoated capillary at the following conditions: 20 × 25 μm I.D. capillary; 0.1 *M* borate buffer (pH 8.5); 400 V/cm.

Run-to-run reproducibility of migration time and peak area should be improved by washing the capillary between analyses to remove adsorbed protein. We have used the flow injection technique to evaluate between-run wash procedures. In Table I, non-specific adsorption of hemoglobin to an uncoated capillary is compared for acidic or basic prewash solutions and for hemoglobin solutions prepared in different test buffers (hemogobin was purged from the capillary with 20 tube volumes of test buffer prior to reading residual absorbance). Non-specific adsorption was observed for hemoglobin prepared in pH 4.8, pH 7 and pH 9 buffers, but not for hemoglobin prepared in pH 2.5 phosphate buffer. Also, prewashing the capillary with pH 2.5 phosphate buffer reduced non-specific adsorption in two cases (hemoglobin in pH 4.8 and pH 9 test buffers). Table II summarizes the results for a similar experiment using a coated capillary. In all cases using coated capillaries, non-specific adsorption was reduced. Note that prewashing the tube with pH 2.5 phosphate was more effective than base (as observed for the uncoated tube) for hemoglobin in pH 4.8 and pH 9 buffers but the opposite was seen for hemoglobin in pH 7 buffer. These results indicate that prewashing the tube with acidic phosphate is generally more effective than base prewashes in preventing adsorption of hemoglobin to the capillary wall.

The use of additives to the electrophoresis buffer has been suggested for reducing protein adsorption[7,8]. The effect of different test buffer additives on non-specific adsorption is shown in Table III. For this study, hemoglobin was prepared in pH 4.8

TABLE II

NON-SPECIFIC ADSORPTION OF HEMOGLOBIN TO A COATED CAPILLARY

50 cm × 50 μm I.D. coated capillary; column B, capillary prewashed before introduction of 10 mg/ml hemoglobin with 20 m*M* sodium hydroxide; column A, capillary prewashed with 0.1 *M* phosphate (pH 2.5).

Testing buffer	*Hemoglobin absorbance (mAU, 200 nm)*	*Remaining absorbance (mAU, 200 nm)*	
		B	*A*
0.1 *M* boric (pH 9.0)	598	1.5	0.1
0.1 *M* phosphate (pH 7.0)	597	3.5	6.5
0.1 *M* acetate (pH 4.8)	562	26.5	6
0.1 *M* phosphate (pH 2.5)	608	0	0

TABLE III

EFFECT OF BUFFER ADDITIVES ON NON-SPECIFIC ADSORPTION OF HEMOGLOBIN

50 cm × 50 μm I.D. capillary; 0.1 *M* acetate (pH 4.8) buffer supplemented with additives as shown; capillary prewashed with 20 m*M* sodium hydroxide before introduction of 10 mg/ml hemoglobin.

Additive	*Remaining absorbance (mAU at 200 nm)*
0.05% Methylcellulose	30.5
0.25% Z60 zwitterion solution	6.0
0.25% Z4A zwitterion solution	29.5
1% Ethylene glycol	27.0
0.05% Triton X-100 R	27.0
None	30.5

acetate buffer supplemented with the different additives; an uncoated tube prewashed with 20 m*M* sodium hydroxide was used. Only the addition of the Z60 zwitterionic solution significantly reduced non-specific adsortion. This and the observation that detergents or methylcellulose had no effect suggests that non-specific adsorption is due primarily to electrostatic interactions.

Non-specific adsorption of acidic (β-lactoglobulin, p*I* 5.1) and basic proteins (bovine pancreatic α-chymotrypsinogen A, p*I* 9, and horse heart cytochrome *c*, p*I* 9) was investigated using uncoated and coated capillaries (Tables IV and V). Proteins prepared at 10 mg/ml in phosphate buffers ranging in pH from 2 to 10 were introduced into a base-prewashed capillary, then the tube was purged with phosphate buffer of the same pH. This study demonstrates that adsorption was higher for basic proteins as compared to acidic proteins, that adsorption was reduced using a coated capillary, and that adsorption increased for all proteins above pH 4. Capillary wash procedures were also compared for a variety of acidic and basic proteins (Table VI). The data in Table VI indicate that both sodium hydroxide and pH 2.5 phosphate buffer washes are effective in removing adsorbed proteins. However, we have observed that adsorption in subsequent protein injections is higher in base-washed tubes, suggesting that the state of the silica surface following protein removal affects nonspecific adsorption.

The results of these studies demonstrate that most proteins exhibit some degree of non-specific adsorption to capillary walls, and that the level of adsorption is dependent upon the pH of the buffer, the nature of the protein, and the previous history of the tube. Coating the internal surface of the capillary with a linear hydrophilic polymer reduces adsorption. Washing the capillary following a separation is necessary to remove adsorbed protein prior to a subsequent analysis, and acidic phosphate buffers were shown to be most effective in prewash and between-run wash steps. The observations are consistent with the report by McCormick[5] that phosphate forms complexes with silanols on the silica surface, reducing electroendosmosis and protein–silica interaction. In cases where proteins adsorb to coated capillaries and can not be effectively removed by post-run washes, addition of zwitterionic species to the electrophoresis buffer can reduce adsorption during separation. Using the techniques reported here, we were able to obtain improved results with proteins separated under alkaline conditions (Fig. 3). We observed good reproducibility of peak height, peak

TABLE IV

ADSORPTION OF PROTEINS TO AN UNCOATED CAPILLARY AT DIFFERENT pH VALUES

50 cm × 50 μm uncoated capillary; proteins prepared in 0.1 *M* phosphate buffer at the designated pH; following protein introduction, capillary washed with phosphate buffer of the designated pH.

Protein	*Remaining absorbance (mAU at 200 nm)*									
	pH 2.0	*pH 2.5*	*pH 3.0*	*pH 4.0*	*pH 5.0*	*pH 6.0*	*pH 7.0*	*pH 8.0*	*pH 9.0*	*pH 10.0*
Chymotrypsinogen	0	0	0	5	11	18	14	11	8	1
Cytochrome *c*	0	0	0	3	11	24	20	20	15	5
β-Lactoglobulin	0	0	[a]	[a]	9	8	6	5	3	4

[a] Precipitation.

TABLE V

ADSORPTION OF PROTEINS TO A COATED CAPILLARY AT DIFFERENT pH VALUES

50 cm × 50 μm coated capillary; proteins prepared in 0.1 *M* phosphate buffer at the designated pH; following protein introduction, capillary washed with phosphate buffer of the designated pH.

Protein	*Remaining absorbance (mAU at 200 nm)*									
	pH 2.0	*pH 2.5*	*pH 3.0*	*pH 4.0*	*pH 5.0*	*pH 6.0*	*pH 7.0*	*pH 8.0*	*pH 9.0*	*pH 10.0*
Chymotrypsinogen	0	0	1	0	1	1	1	2	2	4
Cytochrome *c*	0	0	0	1	2	6	6	6	9	
β-Lactoglobulin	0	0	0	0	2	2	2	3	2	2

TABLE VI

NON-SPECIFIC ADSORPTION UNDER ALKALINE CONDITIONS

50 cm × 50 μm uncoated capillary; proteins prepared at 1.0 mg/ml concentrations in 0.1 *M* phosphate buffer pH 8; following protein introduction, capillary washed with the designated rinse. Data in mAU.

Proteins	*pI*	*Remaining absorbance (mAU at 200 nm)*		
		Washing solution		
		Phosphate pH 8.0	*Phosphate pH 2.5*	*Sodium hydroxide 20 mM*
BSA	5.07	0	0	0
β-Lactoglobulin	5.14	7	0	0
Hemoglobin AFSC	6.9–7.41	7	0	0
Horse myoglobin	7.33	14	0	0
Chymotrypsinogen	8.8	11	0	0
Ribonuclease A	8.88	12	0	0
Cytochrome *c*	9.28	20	0	0
Lysozyme	10–11	10	0	0
Human γ-globulins	[a]	21	0	0

[a] Wide range.

shape, and migration time for a human red blood cell lysate in 49 consecutive separations using pH 9 borate as the electrophoresis buffer (Fig. 4). We conclude that the combined strategies of using coated capillaries, purging the capillary between runs, and adding zwitterionic species to the electrophoresis buffer can improve performance in capillary electrophoresis of proteinaceous samples.

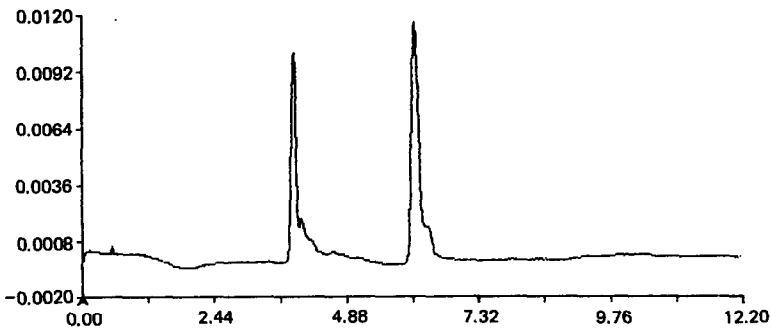

Fig. 3. Separation of cytochrome *c* (p*I* 9.3, first peak) and lysozyme (p*I* 10, second peak). Proteins were prepared at 0.1 mg/ml in a 1:10 dilution of the electrophoresis buffer. Electrophoresis was performed in a 12 cm × 25 μm I.D. coated capillary at 6 kV using a 0.05 *M* phosphate buffer (pH 8.0)–Z6C. Detection was at 200 nm. Retention times in minutes.

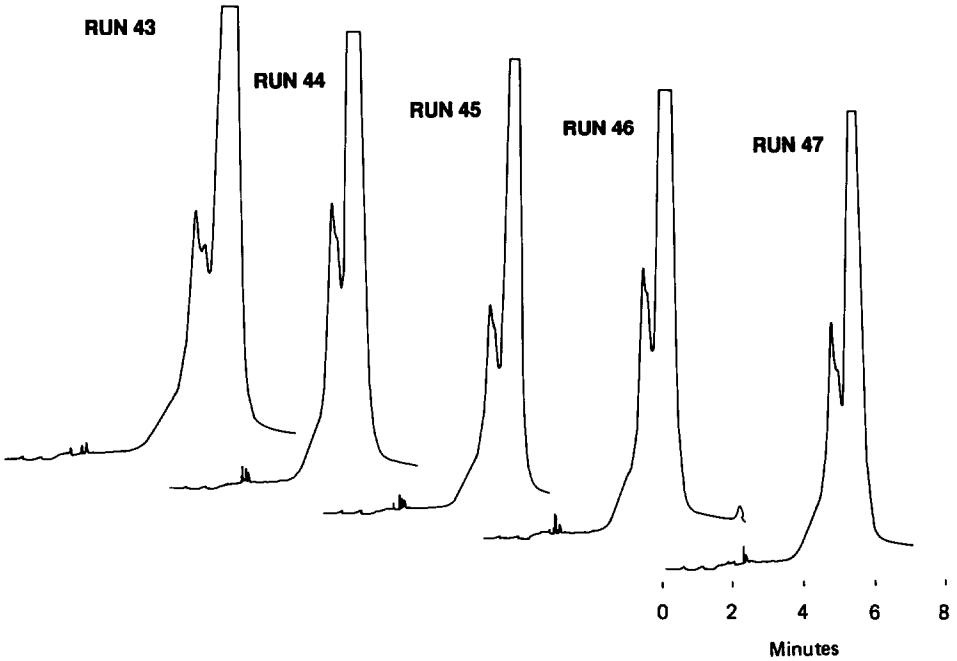

Fig. 4. Repetitive analyses of a human red blood cell lysate. Electrophoresis was performed in a 20 cm × 25 μm I.D. coated capillary at 8 kV using a 0.1 *M* phosphate buffer (pH 9.0)–Z60. Detection was at 200 nm.

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