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Polymer- and surfactant-coated capillaries for isoelectric focusing

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ABSTRACT

This paper reports a method for deactivation of fused-silica capillaries to be used in capillary isoelectric focusing (cIEF). Deactivation was achieved by adsorbing either a surfactant or hydrophilic polymer to alkylsilane-derivatized capillaries. The surfactant PF-108 and methyl cellulose reduced electro-osmotic flow (EOF) 20 to 30 fold in comparison to underivatized capillaries. Although EOF was reduced sufficiently to allow focusing to permit separations to be completed before proteins were swept through the capillary, there was adequate flow to obviate the need for a separate mobilization step. This reduces the complexity of cIEF and increases reproducibility. Based on resolution of hemoglobin variants, proteins that varied 0.03 pH units in isoelectric point were resolvable. This is equivalent to the highest resolution achieved in conventional slab and tube gel isoelectric focusing.

INTRODUCTION

Fused silica is widely used to fabricate the 10–100 μm I.D. capillaries used in capillary electrophoresis. The principal disadvantage of fused-silica capillaries in the electrophoresis and isoelectric focusing of proteins is that they tenaciously adsorb cationic species and show strong electro-osmotically driven flow [1]. These properties of fused silica arise from silanol ionization above pH 4.0 to produce a negatively charged surface. Whereas solute adsorption diminishes recovery and limits the analytical utility of data obtained from the separation, electro-osmosis compromises separation efficiency by sweeping the ampholyte and sample components out of the system before focusing is complete.

This problem has been addressed in several ways. One has been to adsorb methyl cellulose directly to the surface of the capillary [2,3]. The function of adsorbed methyl cellulose is to (1) physically exclude proteins from contact with the charged capillary and (2) greatly increase the viscosity of the double layer adjacent to the silica surface. Although separations approaching those of conventional acrylamide gel-based systems have been achieved with capillaries coated in this manner, the longevity and shielding properties of this coating have not been established. A second approach is to covalently bond a polymer, such as polyacrylamide, to the capillary wall [4]. The function of the polymer layer and the separations that may be obtained with these capillaries are similar to those of the adsorbed polymer layer. The problem with coatings that contain ester and amide linkages is that they slowly hydrolyze, limiting capillary life [5].

Isoelectric focusing (IEF), as a separation technique in a capillary electrophoretic system may be divided into three steps. The first is creation of the pH gradient. This is achieved by focusing an "ampholyte' solution containing a large number of amphoteric species, each with a different p*I*. When there are a large number of ampholyte species and the difference in their p*I* is small, a continuous pH gradient is formed. The second step is the introduction and focusing of sample components. Because this step is similar to step one. they are often com-

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bined. The time required to focus both ampholyte and sample components depends on the slope of the pH gradient and the applied potential. These initial steps of isoelectric focusing are common to all IEF systems. It is the third step, moiblization, that is unique to capillary isoelectric focusing (cIEF). Mobilization refers to the process of adding an ionic species to the system which triggers the focused ampholyte and proteins to be transported past the detector. It has been the practice in cIEF to carry out focusing and mobilization in discrete steps [6].

This paper reports an alternative approach to surface deactivation that allows (cIEF) to be carried out either in the manner described above or to combine all of the steps into a single operation. The essential elements of this approach are to control both protein adsorption and the magnitude of electro-osmotic flow (EOF) through the adsorption of selected polymers and surfactants to octadecylsilane-derivatized capillaries.

EXPERIMENTAL

Chemicals

IEF markers (hemoglobin C, S, F and A) were a gift from Isolab (Akron, OH, USA). Methyl celluloses (MC), polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), Brij 35, mesityl oxide, toluene and octadecyltrichlorosilane were purchased from Aldrich (Milwaukee, WI, USA). PF108 was a gift from BASF (Parsippany, NJ, USA). Pharmalyte (pH 3-10) was purchased from Pharmacia (Piscataway, NJ, USA).

Instrumentation

cIEF was performed on a component system. A Spellman Model FHR 30P 60/EI (Spellman, Plainview, NY, USA) power supply was used to apply the electric field across the capillary. On-line detection was performed by mounting the surfactant-coated octadecylsilanederivatized capillary (either 50 μm I.D. or 25 μm I.D.) on a 254 nm UV absorbance detector (Bio-analytical System, West Lafayette, IN, USA). The detector signal was recorded with an OmniScribe recorder (Houston Instrument, Austin, TX, USA). High-voltage components of the system were placed in a Lucite cabinet with a safety interlock.

Capillary coating

The coating procedure was applied by a modification of the Town's procedure [7]. Fused-silica capillaries were first treated with 1.0 *M* NaOH for 30 min. They were then washed with deionized water and methanol, respectively, for 30 min each. The capillaries (25 μm I.D. or 50 μm I.D.) were placed in a GC oven at 90°C for 2 h with a nitrogen carrier stream at 400 kPa to evaporate the residual methanol. Octadecyltrichlorosilane with 50% toluene was then pushed through the capillaries by pressure. The capillaries were placed in the oven at 90°C for 6 h with new solution continuously being pushed through the capillaries. After 6 h of silylation, the residual octadecyltrichlosilane and toluene were removed from the capillaries by pressure. The capillaries were then washed with methanol for 20 min and then with deionized water for 30 min. Surfactant solutions were pushed continuously through the capillaries for 6 h to complete the coating process.

cIEF process

The protein sample mixture was prepared by mixing the protein solution and Pharmalyte (pH 3-10) at a final ampholyte concentration of 1 to 2%. In the loading step, the capillary was filled with the sample mixture by positive pressure. The two ends of the capillary were placed into 10 m*M* phosphoric acid anolyte and 20 m*M* sodium hydroxide catholyte, respectively. Focusing was started by applying approximately 500 V/cm to the loaded capillary. The current dropped gradually to a constant "residual" value. This residual current, which is not zero because of EOF in the capillary, indicates the end of focusing.

Cathodic mobilization [6] and EOF mobilization [8] were both used to drive the formed pH gradient with the focused protein zones to the detector window. EOF mobilization utilized EOF in the capillary as the driving force. It combines the focusing and mobilization steps to simplify the operation of cIEF and eliminate possible error. EOF mobilization was employed for the separations reported in this paper. After each run, the capillary was flushed with the surfactant solution for a few minutes.

Capillaries of 25 μm I.D. have been found to have advantages over larger I.D. capillaries. Heat dissipation is more efficient due to the increased sur-

face area-to-volume ratio of smaller capillaries. Therefore higher voltage could be applied to achieve separations more rapidly.

RESULTS AND DISCUSSION

Surface modification

Surface modification to control both adsorption and electro-osmotic flow is an essential element of capillary isolectric focusing. It has been established [7] that surfactants adsorbed to the surface of octadecylsilane-derivatized capillaries effectively control the protein adsorption problem while reducing EOF. These studies also suggested that the degree of reduction in EOF was proportional to the size of the hydrophilic portion of the surfactant. This led to the hypothesis in this work that EOF in a capillary could be adjusted to any desired value through the use of adsorbed surfactants and polymers of various size. It was futher hypothesized that amphiphilic copolymers or oligomers containing both hydrophobic and hydrophilic monomers would adsorb to an octadecylsilane-derivatized surface in such a manner that hydrophobic groups would accumulate at the capillary surface and hydrophilic groups would be turned outward toward the aqueous phase. It was expected that these adsorbed coatings would shield the octadecylsilane surface from contact with proteins as they do in chromatographic applications [9].

These hypotheses were tested using octadecylsilanederivatized capillaries to which one of the following surfactants or polymers had been adsorbed; Brij 35 surfactant, PF-108 surfactant, methyl cellulose, polyvinyl alcohol, or polyvinyl pyrrolidone. Coatings were applied by passing several hundred column volumes of a 0.4% polymer/surfactant solution through the alkysilane-derivatized capillary. Subsequent separations were carried out using buffers containing 0.4% of the surfactant or polymer. It is seen in Fig. 1 that there is approximately a six-fold difference in EOF between the Brij 35 surfactant and methyl cellulose 4000 cP (MC-4000) coated capillaries. The EOF of the MC-4000- and PF- 108-coated capillary was approximately 1/20 to 1/30 that of a native fused-silica capillary, depending on the pH at which the measurements are made. Prior work shows that this reduction in EOF may be attributed to two effects [7]. The first is due to silylation of the capillary wall. Organosilane derivatization reduces the number of surface silanols and reduces the ζ potential which drives EOF. The second is due to the adsorbed polymer increasing the local viscosity at the capillary surface.

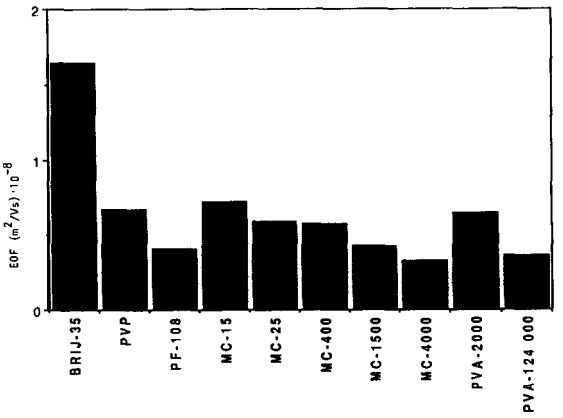

Fig. 1. Electra-osmotic flow of various polymer- and surfactant-coated octadecylsilane (C_{18}) derivatized capillaries. Experimental conditions of CZE: 50 pm I.D. 120 cm separation length and 30 cm total length capillaries were employed to measure the retention time of the neutral marker (mesityl oxide) at pH 6 under 9 kV.

Adsorption of polymers at surfaces is generally thought to be by a loop-and-train mechanism [10]. As the molecular weight of the polymer becomes larger, the adsorbed coating becomes thicker and more viscous. Coating thickness is also proportional to the concentration of the coating solution [10]. This should cause the EOF of a coated capillary to decrease inversely with the molecular weight and concentration of polymer in the coating solution.

TABLE I

THE EFFECT OF POLYMER CONCENTRATIONS ON EOF

Experimental conditions: a PVA-2000-coated octadecylsilane-derivatized capillary, 75 μm I.D., 30/20 cm in length, pH 6 phosphate buffer, under 9 kV.

PVA in buffer (%)	PVA in capillary (%)	Retention of mesityl oxide (min)
0	0	9
0.4	0	14
0.4	0.4	18
0.4	4	24

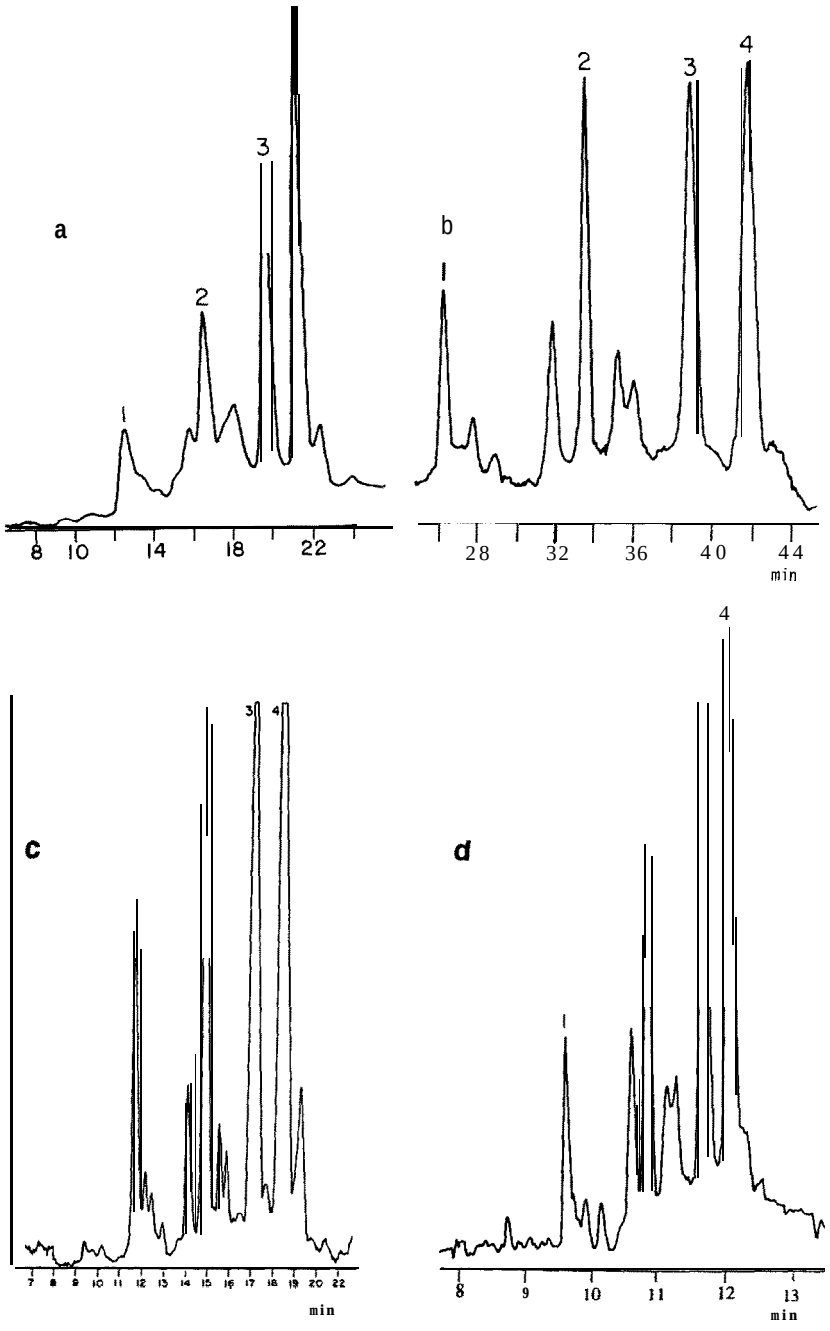

Fig. 2. Electropherograms of Hb variants separated by (a) a methyl cellulose (15 cP)-coaled octadecylsilane-derivatized (MC-I 5+C_{18}) capillary (25 μm I.D. with 16 cm separation Iength and 20 cm total length); (b) a methyl cellulose (25 cP)-coated octadecylsilane-derivatized (MC-25 + C,,) capillary (25 μm I.D. with 16 cm separation length and 20 cm total length); (c) a methyl cellulose (4000 cP)-coated octadecylsilane-derivatized capillary (25 μm I.D. with 12 cm separation length and 16 cm total length); (d) a polyvinyl alcohol) 124 000-coated octadecylsilane-derivatized (PVA-I24 000 + C_{18}) capillary (25 μm I.D. with 1 I cm separation length and 16 cm total length). Sample solutions: 1mg/ml Hb in 1–2% ampholyte with a 0.2% polymer additive. Voltage: 500 V/cm. EOF mobilizations were employed without interrupting the experiments. The major peaks 1, 2, 3 and 4 are HbC, HbS, HbF and HbA, respectively.

As expected, EOF was inversely related to molecular weight of the coating polymer in both the case of methyl cellulose- and polyvinyl alcohol-coated capillaries (Fig. 1). However, less than a two-fold change in EOF was achieved by a large variation in polymer molecular weight. To achieve large differences in EOF, it is seen that different polymers must be used.

The impact of polymer concentration on EOF is seen in Table I. Columns initially coated with a 0.4% solution of PVA were further treated with 10 column volumes of either 0, 0.4 or 4% PVA. These capillaries were then operated with a buffer solution containing either 0 or 0.4% PVA. A capillary treated and operated with 0.4% PVA served as the control. The mesityl oxide transport time in the control system was 18 min. It is seen that pretreatment with 4% PVA increased the transport time to 24 min. The clear implication is that a thicker coating resulted from exposing the column to a higher concentration of polymer. In contrast, it is seen that pretreatment and operation with buffers that contain no polymer substantially reduced the transport time of the neutral marker. This behavior implies that PVA adsorption on an octadecylsilane-derivatized surface is very dynamic, readjusting rapidly with variations of polymer concentrations in the buffer.

EOF is perceived as a negative phenomenon in cIEF that causes protein mixtures to be swept past the detector before they are fully separated. Systems with no EOF are also limited in that a post-focusing mobilizer must be added to transport solutes to the detector [6]. An alternative to these two extremes would be to use a polymer-coated capillary in which EOF was adjusted to allow both complete focusing and transport simultaneously.

Evaluation of coated capillaries

The human hemoglobin (Hb) variants (C, S, F and A) have been widely used in the evaluation of isoelectric focusing systems. Literature values [11] for the isoelectric points of these proteins are as follows; HbC = 7.42, HbS = 7.20, HbF = 7.05, and HbA = 6.98. [It should be noted that the p*I* of these proteins in a separation system could be slightly different than these values.] These variants were readily separated in all of the methyl cellulose and polyvinyl alcohol-coated capillaries (Fig. 2). Based on the resolution of the hemoglobin F and A pair, proteins that vary as little as 0.03 pH units in isoelectric point can be baseline resolved. Partial resolution can probably be achieved between proteins that vary 0.01 p*I* units.

The quality of these isoelectric focusing separations was evaluated in several ways. Visual inspection of the electropherograms shows that the best resolution was achieved with the MC-25-coated column. One is left with the clear impression that the coatings play a role in resolution, as will be shown below. The type of coating also contributes to analysis time because mobilization is achieved by EOF. By selecting from the series of polymers and surfactants in Fig. 1, it is possible to control both resolution and analysis time.

Other measures of the quality and resolution of an isoelectric focusing system are the linearity and slope of the pH gradient, *i.e.* d(pH)/d(length). The more linear the pH gradient and the smaller the slope, the better the resolution of the system. Unfortunately it is not possible to measure pH directly in a capillary. Instead, it is necessary to determine the gradient from the elution time of standards. A plot of pH *versus* time is seen for a series of columns in Fig. 3. Slopes for these curves (m_t), linearity (R), and the standard deviation of data points is seen in Table II. The linearity is quite good, ranging from 0.995 to 1.000.

The most widely used method of specifying resolution in isoelectric focusing is in terms of the difference in p*I* of adjacent peaks, *i.e.* their resolution is defined as being equal to 1. If we assume that (i) the pH gradient is linear, (ii) these adjacent bands are of equal width, and (iii) the solutes are similar in diffusion coefficient, then

$$R_s = (t_2 - t_1)/0.5(\Delta t_2 + At,) \quad (1)$$

where t_2 and t_1 are the elution times and At, and Δt_1 are the peak widths of proteins 2 and 1, respectively. Based on the assumptions that the peaks are of equal width and $R_s = 1$, this equation reduces to

$$At, = t_2 - t_1 \quad (2)$$

Since peak width may also be defined in terms of standard deviation where $At = 4\sigma$, then

$$4\ \sigma_2 = t_2 - t_1 \quad (3)$$

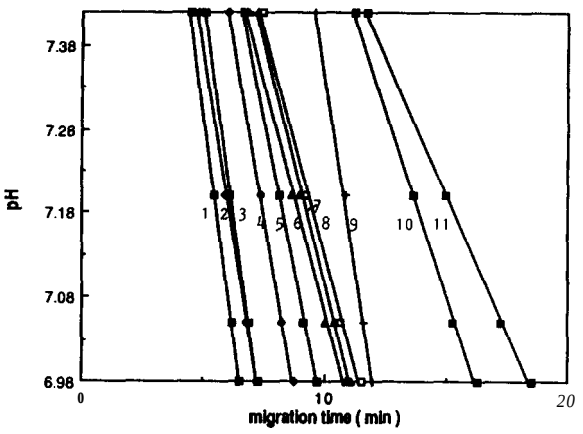

Fig. 3. The calibration curves of the separated Hb variant zones in a linear pH gradient by the coated capillaries. Experimental conditions of cIEF: 25 μm I.D. coated capillaries with 10–15 cm separation length and 14-20 cm total length. Sample solutions: 1 mg/ml Hb in 1–2% ampholyte with a 0.2% polymer additive. Voltage: 500 V/cm. EOF mobilizations were employed without interrupting the experiments. 1-5 = MC-15; 6–8 = MC-25: 10, 11 = MC-4000 and 9 = PVA 124 000.

According to Fig. 3, it is seen that

$$\mathrm{pH} = \mathrm{m},(\mathrm{t}) + b \tag{4}$$

Substituting eqn. 4 in eqn. 3, it may be shown that

$$4\ \sigma_2 = \mathrm{d(pH)}/m_t \tag{5}$$

where d(pH) is the difference in the pH between the peak maxima of the two solutes. But this difference in pH is the difference in the isoelectric point of the two proteins, therefore,

$$'4\ \sigma_2 = \mathrm{d(p}I)/m_t \tag{6}$$

A more exact description of resolution is given by the equation

$$\Delta \mathrm{p}I = 3[D(m_t)/E(-\mathrm{d}u/\mathrm{pH})]^{1/2} \tag{7}$$

where D is the diffusion coefficient of the analyte, E is the applied field strength in V/cm, m_t is the slope of the pH vs. time calibration curve, and $-\mathrm{d}u/\mathrm{pH}$ is the change in mobility with pH [12]. The problem with eqn. 7 is that it is difficult to obtain D and $-\mathrm{d}u/\mathrm{pH}$ for many solutes. Eqn. 6 is more tractable experimentally. Although the assumptions made in deriving resolution eqn. 6 may not be valid in many cases, it is probably suitable for evaluating coating quality where the same analytes are used in all cases under nearly identical conditions. The impact of coating chemistry on resolution in terms of $\Delta \mathrm{p}I$ is shown in Table II. The MC-25-coated capillary had more than twice the resolution of any other capillary. However, it also took the longest to achieve a separation. Resolution appears to be compromised in very rapid separations.

The effect of ampholyte concentration on resolution

Increasing the ampholyte concentration from 0.8 to 2% increased resolution ($\Delta \mathrm{p}I$ values according to eqn. 6 are 0.054 an 0.038, respectively) (Fig. 4). It is thought that this was due to an increase in the buffer capacity and viscosity of the more concentrated ampholyte. Data to support this viscosity hypothesis will be provided below.

The impact of viscosity on resolution

According to eqn. 7, the diffusion coefficient *(D)* of a protein is directly proportional to $\Delta \mathrm{p}I$. This suggests that increasing the viscosity of the ampholyte to reduce D would enhance resolution. This was found to'be true when 2% glycerol was used to increase viscosity (Fig. 5). However, it should be noted that elution time is substantially longer in the case of the separation with 2% glycerol. This means that the rate of electro-osmotic pumping was reduced with the higher viscosity ampholyte. Because

TABLE II

ANALYSIS OF THE RESOLUTION AND LINEARITY OF THE VARIOUS COATED CAPILLARIES

Coatings	n	mean of R^2	S.D.	m.r.$\Delta \mathrm{p}I^a$	slope $m_t(\Delta \mathrm{pH}/\Delta t)$
C_{18} + MC-15	5	0.997	0.0010	0.050	-0.1001
C_{18} + MC-25	3	0.995	0.0015	0.029	-0.0287
C_{18} + MC-4000	2	0.998	0.0007	0.039	− 0.0648
C_{18} + PVA-124000	I	1.000	—	0.046	−0.1815

[a] m.r.$\Delta \mathrm{p}I$ stands for the minimum resolvable $\Delta \mathrm{p}I$.

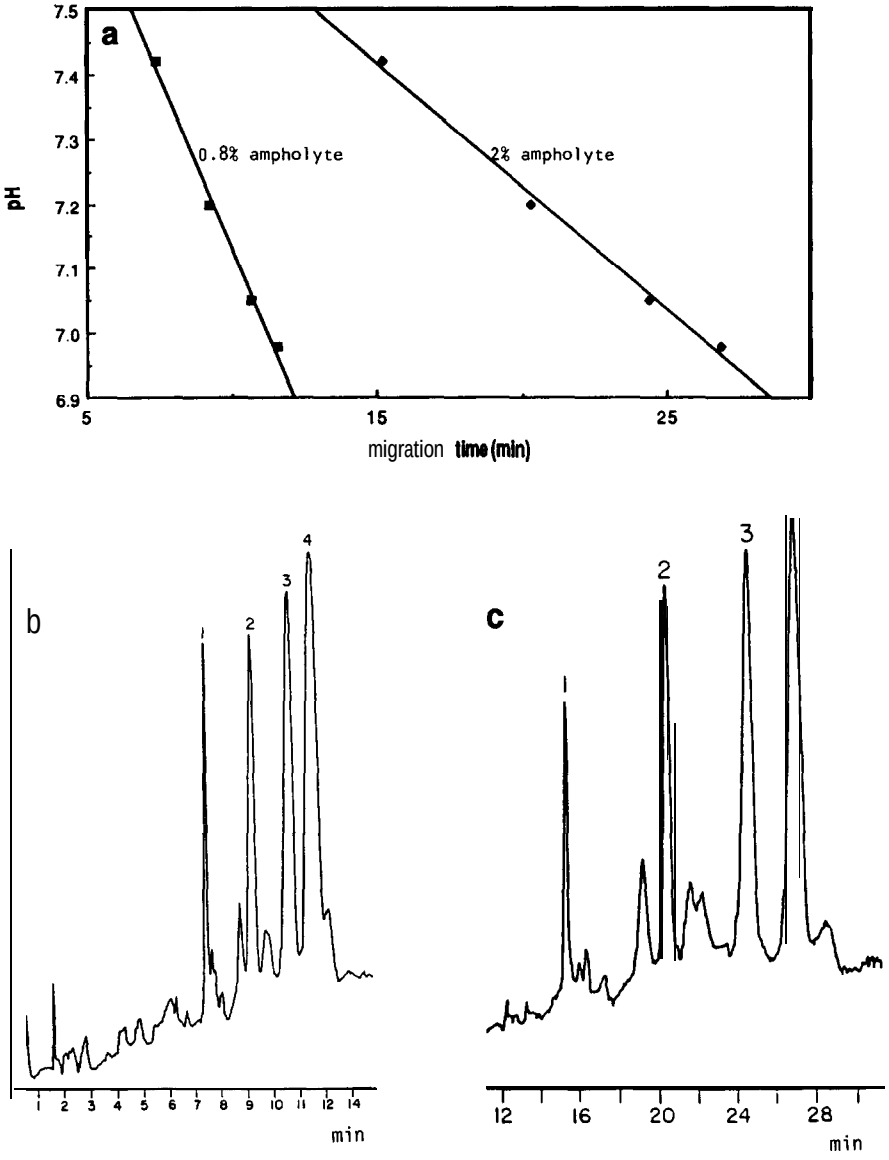

Fig. 4. The effect of the ampholyte concentration on resolution. (a) The effect on the calibration curves of the separated Hb variant zones. (b,c) Electropherogram comparison. (b) 0.8% ampholyte; (c) 2% ampholyte. cIEF conditions: A MC-25-coated capillary, 25 μm I.D. with 14 cm separation length and 18 cm total length. Sample solutions: 1mg/ml Hb in (a) 0.8 and(b) 2.0% ampholyte with a 0.2% polymer additive. Voltage: 500 V/cm. EOF mobilizations were employed without interrupting the experiments. The major peaks 1, 2, 3 and 4 are HbC, HbS, HbF and HbA, respectively.

focusing and elution were achieved in 5-8 min, it could be that more complete focusing was achieved in the column with the lower electro-osmotic flow, *i.e.* the column with the 2% glycerol additive. Focusing at still higher viscosity proved negative (data not shown). This was attributed to the fact that focusing was achieved at 500 V/cm and too high viscosity causes overheating.

Reproducibility

Reproducibility between runs and batches was examined in the separation of hemoglobin variants.

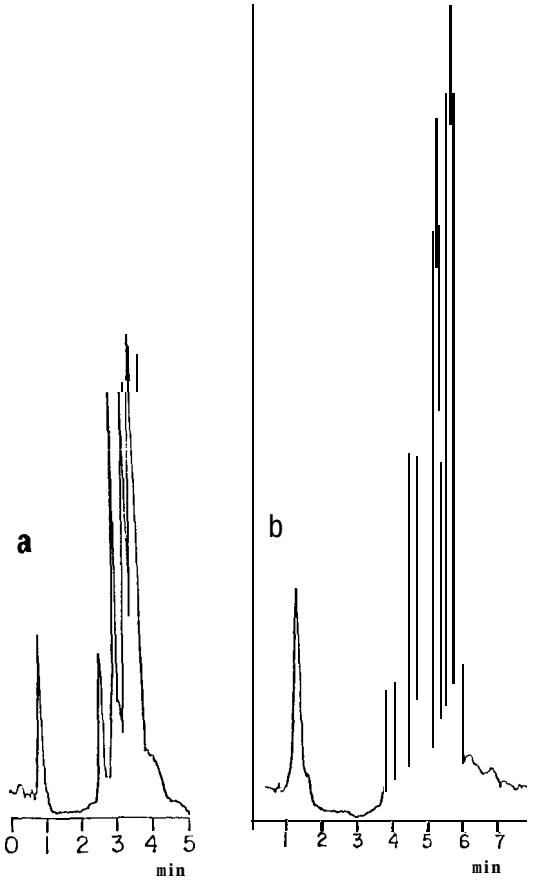

Fig. 5. Viscosity effects: (a) without glycerol, (b) with 2% glycerol. cIEF conditions: A MC-25-coated capillary, 25 μm I.D. with 10 cm separation length and 14 cm total length. Sample solutions: 1 mg/ml Hb in 0.2% ampholyte with a 0.2% polymer additive. Voltage: 500 V/cm. EOF mobilizations were employed without interrupting the experiments, The major peaks I, 2, 3 and 4 are HbC, HbS, HbF and HbA, respectively.

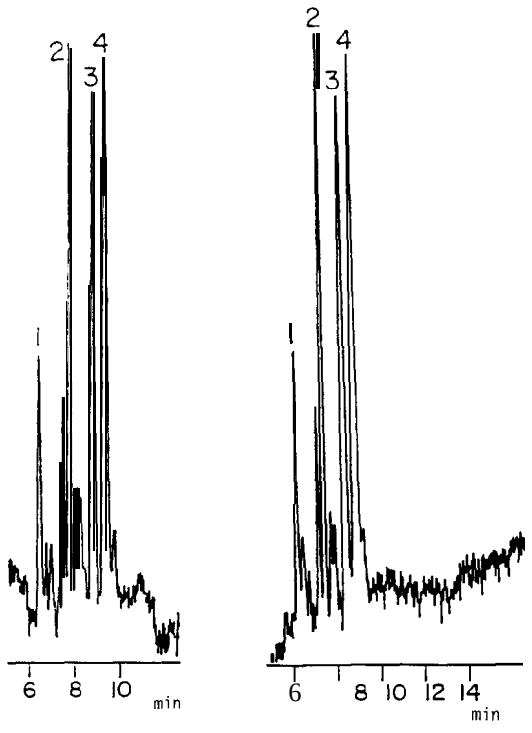

Fig. 6. Electropherograms of the two consecutive cIEF experiments CIEF conditions: A MC- 15-coated capillary, 25 μm I.D. with 10 cm separation length and 14 cm total length. Sample solutions: 1mg/ml Hb in 1–2% ampholyte with a 0.2% polymer additive. Voltage: 500 V/cm. EOF mobilizations were employed without interrupting the experiments. The major peaks 1. 2, 3 and 4 are HbC, HbS, HbF and HbA, respectively.

A run to run comparison in a fast separation is shown in Fig. 6. Batch to batch comparisons were made with two different preparations of MC-25-coated capillaries. The coefficients of linearity for the two plots were 0.988 and 1.000 with an R.S.D. of 0.007. Resolution of the individual preparations computed from the peak width of hemoglobin F was 0.029 and 0.039 ΔpI.

CONCLUSIONS

It may be concluded that both surfactants and hydrophilic polymers adsorbed to octadecylsilane-derivatized capillaries can effectively control electro-osmotic flow and the adsorption of proteins at the internal surface of fused-silica columns. Higher-molecular-weight poymers were more effective than low-molecular-weight surfactants in controlling electro-osmotic flow. Through control of electro-osmotic flow it was possible to combine the focusing and mobilization steps. Under ideal conditions the resolution of this capillary isoelectric focusing system approached that of conventional slab and tube gel systems, Moreover, separations were achieved in less than an hour in all cases.

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