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Electrophoresis of proteins in uncoated capillaries with amines and amino sugars as electrolyte additives

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ABSTRACT

The effect of triethylamine and triethanolamine in the running electrolyte on the electroosmotic flow and the migration of four standard basic proteins in bare fused-silica capillaries was examined. At pH 2.5 the direction of the electroosmotic flow was anodal with both additives and at constant ionic strength its magnitude increased with increasing additive concentration. The observations are in qualitative agreement with a theoretical model which is based on the Gouy–Chapman–Stern theory and incorporates a Langmuir-type relationship and also the Von Smoluchowski expression for the electroosmotic mobility, with the approximation to identify the zeta potential with the potential at the Stern plane. From the independence of the electrophoretic mobilities of the additive concentration and type the absence of interactions between the four basic proteins and the two alkylamines is inferred. The utility of glucosamine and galactosamine as additives for the capillary electrophoresis of basic proteins is also demonstrated and their effectiveness is compared with that of the alkylamines.

INTRODUCTION

Protein separations by capillary electrophoresis in untreated fused-silica capillaries are strongly affected by interactions of the proteins with the capillary wall. As a consequence, the efficiency, resolution, reproducibility of migration times and recovery are compromised. Several strategies have been addressed to overcome these problems: chemical or physical coating of the internal surface of the capillary to deactivate the free silanols [1–5]; extremes of electrolyte pH, whether acidic (below pH 2.0), to remove the negative charges to the capillary wall [6], or basic (higher than the isoelectric point), to have both the protein and the capillary wall negatively charged [7]; and the use of electrolytes at high ionic strength, to repress electrostatic interactions between the proteins and the capillary wall [8]. The drawbacks of the above strategies are that coated capillaries often suffer from a gradual loss of surface coverage, particularly at high pH. Extremes of electrolyte pH tend to denature proteins and to compromise recovery by reducing solubility, whereas the current derived from high ionic strength limits the voltage that can be applied.

A further strategy is to use an additive in the running electrolyte. Several compounds have been proposed as electrolyte additives and have been reported to aid in suppressing protein–capillary interactions or to improve separations [9–12]. Few of these additives have been reported to reverse the direction of the electroosmotic flow from cathodic to anodic [13–18]. Reversal of the direction of the electroosmotic flow in fused-silica capillaries occurs when specific adsorption of counter ions at the interfacial region between the capillary wall and the electrolyte takes place. These specifically adsorbed ions are located in the compact part of the double layer, the so-called Stern layer.

The potential changes from ψ_0 (the surface or wall potential) to ψ_δ (the Stern potential), in the Stern layer, and decays from ψ_δ to zero in the diffuse part of the double layer. The potential at

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the surface of shear between the Stern layer (plus that part of the double layer occupied by the solvent associated with the adsorbed ions in the Stern layer) and the diffuse part of the double layer is the zeta potential, ζ, which affects the direction and magnitude of the electroosmotic flow.

Although several papers [19–30] have reported the effect of the electrolyte composition on the electroosmotic flow, an exact relationship between the electroosmotic mobility and the concentration of ions in the running electrolyte which adsorb in the Stern layer has not been reported. The purpose of this study was to gain further insight into the effect of cationic additives on electroosmotic mobility and protein separation performance. We therefore examined the effect of the concentration of two alkylamines, triethylamine and triethanolamine, on the electroosmotic mobility and on the migration behaviour of four model basic proteins. We developed an equation that correlates the charge density at the capillary wall, due to silanol dissociation, and the charge density in the Stern layer, due to counter-ion adsorption, with the electroosmotic mobility. The model is based on the Gouy–Chapman–Stern theory [31] and incorporates a Langmuir-type adsorption equation and also the Von Smoluchowski expression for the electroosmotic mobility, with the approximation to identify the zeta potential with the potential at the Stern plane.

Further, we examined the use of two amino sugars, glucosamine and galactosamine, as additivies for the capillary electrophoresis of basic proteins and their effectiveness was compared with that of triethylamine and triethanolamine.

THEORY

According to the Gouy–Chapman–Stern (GCS) model of the double layer, as outlined by Shaw [31], a Langmuir-type adsorption model, modified by the incorporation of a Boltzmann factor, may be used to describe the specific adsorption of ions in the Stern layer from the electrolyte solution.

Considering only the adsorption of counter ions, the surface charge density σ_S of the Stern layer is related to the ion concentration C (mol/l) in the bulk solution by the following equation [32]:

$$\sigma_S = \frac{zen_0 \cdot \dfrac{C}{1000/M} \cdot \exp\left(\dfrac{ze\psi_\delta + \Phi}{kT}\right)}{1 + \dfrac{C}{1000/M} \cdot \exp\left(\dfrac{ze\psi_\delta + \Phi}{kT}\right)} \quad (1)$$

where e is the elementary charge, z is the valence of the ion, k is the Boltzmann constant, T is the absolute temperature, n_0 is the number of accessible sites, M is the molecular mass of the solvent (water in this instance), ψ_δ is the potential at the Stern plane and Φ allows for any specific adsorption potential. The surface charge density of the diffuse part of the double layer is given by the Gouy–Chapman equation [32]:

$$\sigma_G = -\left(\frac{2\kappa\varepsilon kT}{ze}\right) \sinh\left(\frac{ze\psi_\delta}{2kT}\right) \quad (2)$$

which at low potentials reduces to

$$\sigma_G = -\frac{\varepsilon\kappa}{4\pi}\psi_\delta \quad (3)$$

where κ is the reciprocal Debye length, defined as

$$\kappa = \left(\frac{4e^2 I}{\varepsilon kT}\right)^{1/2} \quad (4)$$

in which I is the ionic strength and ε is the permittivity of the aqueous solution.

The ionized surface silanol groups of the fused-silica capillary generate a surface charge density, σ_0, which is related to electrolyte pH by the following relationship [33]:

$$\sigma_0 = \frac{\gamma}{1 + \dfrac{[H^+]}{K_a}} \quad (5)$$

where γ is the sum of the ionized and protonated surface silanol group concentration, $[H^+]$ is the bulk electrolyte hydrogen ion concentration and K_a is the dissociation constant.

Hence the surface charge density, σ_0, of the capillary wall should be considered constant at a given pH and of opposite sign to σ_S if reversal of charge takes place within the Stern layer due to specific adsorption of counter ions.

For overall electric neutrality throughout the whole of the double layer,

$$\sigma_G = \sigma_s - \sigma_0 \quad (6)$$

The electroosmotic mobility, μ_{eo}, can be obtained by using the Von Smoluchowski equation [32]:

$$\mu_{eo} = -\frac{\varepsilon}{\eta} \cdot \zeta \quad (7)$$

where ε and η are the permittivity and the viscosity, respectively, of the running electrolyte and ζ is the zeta potential. For a number of simple systems [32,34,35], the zeta potential can be identified with ψ_δ. If this approximation is made, substituting the value of ψ_δ from eqn. 3, then eqn. 7 can be rewritten as

$$\mu_{eo} = \frac{4\pi}{\kappa\eta} \cdot \sigma_G \quad (8)$$

Thus, the combination of eqns. 1, 5 and 8 yields the following expression for the electroosmotic mobility:

$$\mu_{eo} = \frac{4\pi}{\kappa\eta}\left\{\frac{zen_0 \cdot \frac{C}{55.6} \cdot \exp\left(\frac{ze\psi_\delta + \Phi}{kT}\right)}{1 + \frac{C}{55.6} \cdot \exp\left(\frac{ze\psi_\delta + \Phi}{kT}\right)} - \left(\frac{\gamma}{1 + \frac{[H^+]}{K_a}}\right)\right\} \quad (9)$$

EXPERIMENTAL

Instrumentation

Capillary electorphoresis was performed on a P/ACE 2100 instrument, operated under System Gold control, data acquisition and analysis software (Beckman, Fullerton, CA, USA). A fused-silica capillary (Quadrex, New Haven, CT, USA), 0.075 mm I.D., 0.375 mm O.D., with a total length of 37 cm (30 cm to the detector), was mounted in the cartridge. The capillary tube temperature was maintained at 25 ± 1°C by means of a fluorocarbon liquid continuously circulating through the cartridge. A deuterium light source with either a 214- or 280-nm bandpass filter was used. The samples were injected by applying 0.5 p.s.i. (1 p.s.i. = 6894.76 Pa) pressure for 1 s, and the approximate sample volume of 9 nl was calculated according to ref. 36.

Chemicals

Triethylamine was obtained from Pierce (Rockford, IL, USA), triethanolamine from Fluka (Buchs, Switzerland) and D-glucosamine hydrochloride, D-galactosamine hydrochloride and 5-(hydroxymethyl)-2-furaldehyde from Aldrich (Milwaukee, WI, USA). Cytochrome *c* (from horse heart), lysozyme (from chicken egg white), ribonuclease A (from bovine pancreas) and α-chymotrypsinogen A (from bovine pancreas) were supplied by Sigma (St. Louis, MO, USA). Analytical-reagent-grade phosphoric acid, hydrochloric acid, sodium hydroxide, sodium chloride and HPLC-grade water were obtained from Carlo Erba (Milan, Italy) and were used as received.

Procedures

All experiments were carried out with uncoated fused-silica capillaries. Prior to use, the untreated capillary was flushed successively with 0.5 *M* sodium hydroxide solution (30 min), water (10 min) and 0.1 *M* hydrochloric acid (30 min), followed by a second treatment with 0.5 *M* sodium hydroxide solution (30 min) and water (10 min), and then rinsed with the running electrolyte.

The running electrolyte was renewed after five or six runs, and before each run the capillary was rinsed with the running electrolyte for 3 min. For storage the capillary was rinsed with water for 10 min and then dried by flushing nitrogen for 10 min.

The capillary was flushed with 0.5 *M* sodium hydroxide solution (3 min) and water (3 min) each time a running electrolyte of new composition was used. All experiments were carried out applying a constant voltage of 10 kV.

The electroosmotic mobility was determined by measuring the migration time of 5-(hydroxymethyl)-2-furaldehyde, used as an inert tracer, detected at 280 nm at the anodic end of the capillary, upon reversing the polarity. Protein solutions of 1.0–3.0 mg/ml were prepared in water. Running electrolytes at various additive

concentrations and constant ionic strength were prepared by adding the appropriate amount of sodium chloride to the electrolyte solution, containing 50 m*M* phosphate buffer (pH 2.5), for compensation of the changes in the additive concentration. All solutions were filtered through a 0.22-μm Type HA membrane filter (Millipore, Bedford, MA, USA) and degassed by sonication before use.

RESULTS AND DISCUSSION

All experiments were performed with running electrolytes containing 50 m*M* phosphate buffer (pH 2.5). Under such conditions basic proteins are fully protonated and the internal wall of the fused-silica capillaries still exhibits negative charges, as a consequence of incomplete silanol protonation [37]. However, in the presence of positively charged additives that adsorb at the interfacial region between the capillary wall and the electrolyte, the zeta potential becomes positive and the concomitant electroosmotic flow is directed towards the anode.

We first investigated the effect of the concentration of triethylamine (TEA) and triethanolamine (TEOHA) on the electroosmotic mobility and the migration behavior of proteins. Examination of eqn. 9 suggests that at a given pH and constant ionic strength, the electroosmotic mobility would depend mainly on the surface density of adsorbed counter ions in the Stern layer, which should follow a Langmuirian-type adsorption model. Hence, in order to verify this prediction, the effect of varying the additive concentration in the running electrolyte was examined for both additives at the same constant ionic strength and pH. The above requirements were obtained by adding the appropriate amounts of sodium chloride to the running electrolyte for compensation of the changes in the amine concentration.

The dependence of the electroosmotic mobility on the concentration of the two additives in the running electrolyte, as measured with 5-(hydroxymethyl)-2-furaldehyde as a neutral marker, is reported in Fig. 1. Experimental trends in the data are in good agreement with the theoretical prediction of eqn. 9. The curves, the slopes of which decrease, are Langmuirian or quasi-Langmuirian in shape with an initial linear region which is typical of adsorption behaviour at low concentration [38,39].

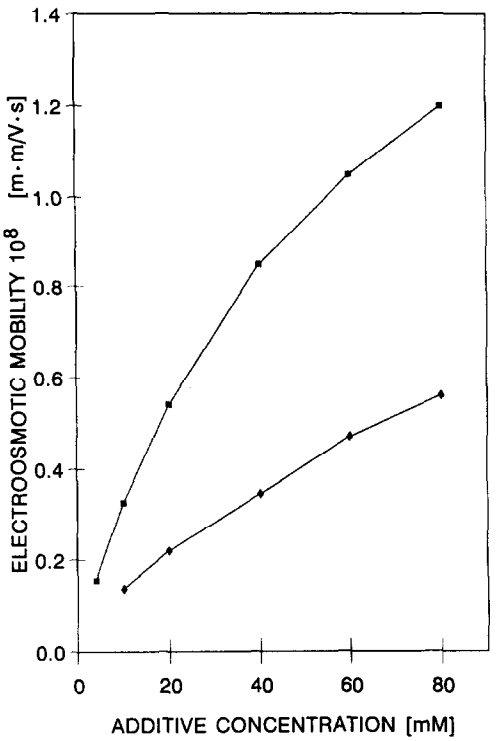

Fig. 1. Dependence of electroosmotic mobility on the concentration of additive in the running electrolyte at constant ionic strength (83 m*M*) and pH (2.5). Capillary, fused-silica, 0.075 mm I.D. × 370 mm total length (300 mm to the detector); applied voltage, 10 kV; temperature, 25°C; neutral marker, 5-(hydroxymethyl)-2-furaldehyde; detection wavelength, 280 nm at the anodic end. ■ = Triethylamine; ◆ = triethanolamine.

The differences in the slopes of the two curves in Fig. 1 can be accounted for by differences in the specific adsorption energy of triethylamine and triethanolamine. Specific adsorption of TEA is likely to involve hydrogen bonding between the silanols and the amino group and may be stabilized by hydrophobic interactions between the alkyl chains and the siloxane groups, which are known to exhibit hydrophobic character [37,38]. Triethanolamine is also likely to have specific interactions via the amine group, but it is expected to establish much lower hydrophobic

interactions than TEA, which does not have polar groups besides the amino function.

The effect of varying the concentration of TEA or TEOHA in the running electrolyte on the electrophoretic mobility, μ_e, of four standard basic proteins was investigated at pH 2.5 over the same concentration range, constant ionic strength and experimental conditions employed to study the influence of these additives on the electroosmotic mobility. The values of the electrophoretic mobility of proteins vary within 4.4% (R.S.D. of the mean value) when measured with either additive over the whole investigated concentration range, and within 2.4% and 2.6% when measured over the same concentration range with TEOHA or TEA, respectively. This is an indication that the electrophoretic mobilities are virtually independent of the nature and concentration of the two additives, revealing the absence of interactions between proteins and additives. On the other hand, the apparent mobility, μ_{app}, which is the resultant of the oppositely directed vectors of the electrophoretic and electroosmotic mobility, vary from TEOHA to TEA and decreases with increasing additive concentration. For each additive a plot of the apparent mobility versus the additive concentration in the running electrolyte yields a set of curves that parallel each other and differ in shape and slope from those obtained with the other additive (see Fig. 2). Thus, for either TEA or TEOHA, an increase in the additive concentration leads to an increase in the protein migration times without any practical improvement in separation.

The effectiveness of TEA and TEOHA with decreasing protein–capillary wall interactions at pH 2.5 was compared with that of the amino sugars glucosamine (GluA) and galactosamine (GalA). In all experiments the running electrolyte consisted of 40 m*M* additive in 50 m*M* phosphoric acid, adjusted to pH 2.5 with 0.1 *M* NaOH, and containing 40 m*M* chloride ions added either as hydrochloric acid with the additive (GalA and GluA) or as NaCl. Hence all running electrolytes had the same additive concentration, the same ionic strength ($I = 83$ m*M*) and concentrations of phosphate and chloride ions in the same ratio.

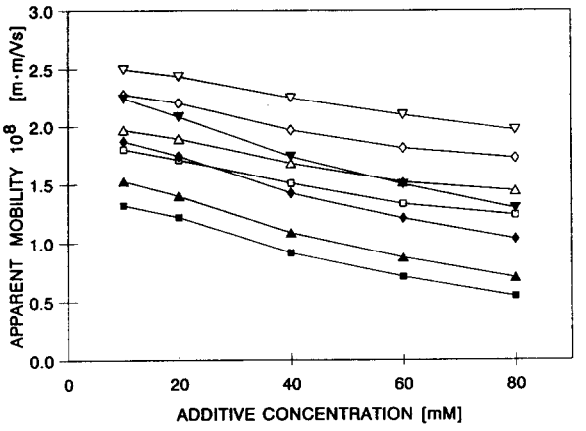

Fig. 2. Apparent mobility of standard basic proteins as a function of additive concentration in the running electrolyte. Open symbols, triethylamine; closed symbols, triethanolamine. ▽, ▼ = cytochrome *c*; ◇, ◆ = lysozyme; △, ▲ = ribonuclease A; □, ■ = α-chymotrypsinogen A. Detection at 214 nm at the cathodic end. Other conditions as in Fig. 1.

Typical electropherograms of the four model basic proteins performed with TEA, TEOHA, GluA and GalA at an applied voltage of 10 kV are depicted in Fig. 3. The values of the measured current were 104, 96, 82 and 89 μA with TEA, TEOHA, GluA and GalA, as the additive, respectively.

The electropherograms show significant dissimilarity in the migration times, which reflects the differences in the electroosmotic mobility produced by the four additives. The model basic proteins are positively charged at pH 2.5 and therefore move towards the cathode against the electroosmotic flow, which takes place in the opposite direction in the presence of the additives.

The average theoretical plate number per metre, N_{AV}, is reported on each electropherogram. These efficiency values may indicate differences in the effectiveness of the additives in preventing protein–capillary wall interactions, which contribute to peak broadening [40,41]. Another factor that has been reported to play a significant role in peak broadening is the longitudinal diffusion that takes place during the migration time between injection and detection [40,41]. Further, it should be considered that the

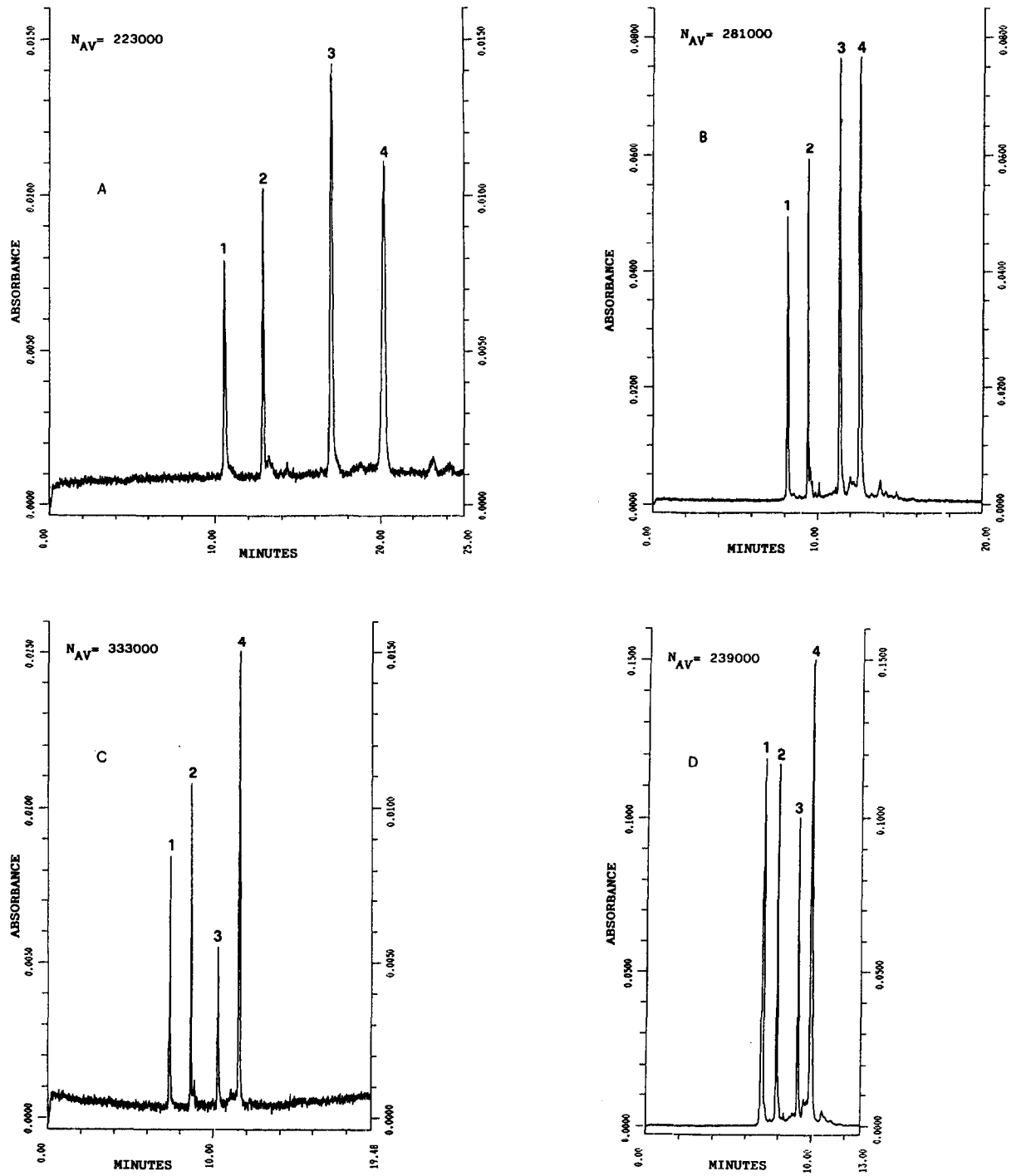

Fig. 3. Electropherograms of standard basic proteins obtained with different additives at the same concentration (40 m*M*), ionic strength (83 m*M*) and pH (2.5). (A) Triethylamine; (B) triethanolamine; (C) galactosamine; (D) glucosamine. Proteins: 1 = cytochrome *c*; 2 = lysozyme; 3 = ribonuclease A; 4 = α-chymotrypsinogen A. Conditions as in Fig. 2, except detection at 280 nm with galactosamine.

TABLE I

REPRODUCIBILITY OF MIGRATION TIMES WITH VARIOUS ADDITIVES

Electrophoretic conditions as in Fig. 3.

Additive	Protein							
	Cyt		Lys		RNase		Chy	
	Mean (min)	R.S.D. (%)	Mean (min)	R.S.D. (%)	Mean (min)	R.S.D. (%)	Mean (min)	R.S.D. (%)
TEA	11.04	0.52	13.16	0.48	17.62	0.61	20.08	0.85
TEOH	8.17	0.33	9.41	0.37	11.31	0.62	12.54	0.43
GluA	7.08	0.52	7.95	1.06	9.24	1.62	10.02	1.91
GalA	7.43	0.54	8.69	0.66	10.32	0.79	11.56	0.86

separated zones do not pass the detector at the same velocity. This causes unequal peak widths arising solely from differences in zone velocity [42], which induce differences in N_{AV} not related to protein–capillary wall interactions.

The run-to-run reproducibility of the protein migration times was determined by replicate injections ($n = 6$) on the same capillary. No washing of the capillary was performed between runs in order to detect irreversible protein adsorption that would affect the electroosmotic mobility and consequently the migration times. The high reproducibility of the migration times reported in Table I indicate that irreversible protein adsorption does not take place, as also evidenced in the electropherograms by the absence of peak tailing.

Peak capacity, which is another measure of the separation efficiency, was calculated using the equation $n = 1 + 0.25\sqrt{N}\ln(t_\omega/t_\alpha)$ [43]. where t_ω and t_α are the migration times of the last and the first peak in the electorpherogram, respectively. The calculation values of peak capacity were 41, 32, 35 and 24 with triethylamine, triethanolamine, galactosamine and glucosamine as the additive, respectively. The high value of the peak capacity obtained with triethylamine may be primarily related to the large separation space, which is taken in this case as the interval between cytochrome *c* and α-chymotrypsinogen A, obtained with this additive, owing to the high counter-electroosmotic flow. On the other hand, a high value of the peak capacity is also obtained with galactosamine, which displays a shorter separation space and a higher separation efficiency. The effect of the different additives on the selectivity is apparent from examination of the four electropherograms. The migration order of the four basic proteins is not affected by changing the additive and with all running electrolytes secondary peaks, due to impurities present in the authentic protein standards, are detected.

From the results reported in Table II, it is observed that the high resolution obtained with TEA and TEOHA, which generate a counter-electroosmotic flow, is in agreement with the expression for resolution proposed by Giddings [44] and Jorgenson and Lukacs [45], which predicts that the best resolution in capillary electrophoresis is obtained when electroosmotic

TABLE II

RESOLUTION OF PROTEINS WITH DIFFERENT ADDITIVES

Electrophoretic conditions as in Fig. 3.

Additive	Cyt–Lys	Lys–RNase	RNase–Chy
TEA	13.02	19.07	10.02
TEOHA	9.07	12.08	5.02
GluA	7.09	12.01	5.04
GalA	11.09	14.05	8.05

and electrophoretic mobilities are of the same order of magnitude and opposite in direction. However, it should be noted that galactosamine, which almost suppresses the electroosmotic flow, displays similar resolution, owing to the higher separation efficiency.

CONCLUSIONS

We have shown that at a given pH and constant ionic strength, increasing the concentration of a cationic species that adsorbs at the silica–electrolyte interface leads to an increase in the electroosmotic mobility. The effect depends on the nature and concentration of the cationic additive and has been qualitatively described by a model based on the Gouy–Chapman–Stern theory. Eqn. 9 correlates a complete expression for the Stern model of the double layer with the electroosmotic mobility. Although it inevitably entails oversimplifications, this model provides a framework for the study of the effect on the electroosmotic mobility of additives that are useful to control electroosmotic flow and solute–capillary wall interactions. Extrapolation of our theoretical model to the analysis of the double-layer parameters from capillary electrophoretic data obtained under a variety of conditions is under investigation.

Triethylamine and triethanolamine were selected as model cationic additives owing to their known effectiveness at masking silanophilic activity in RP-HPLC [46]. It is seen that the two alkylamines are also effective at preventing protein–capillary wall interactions, although the conditions selected may not be the most appropriate for protein analysis. Similar results are also displayed by the two amino sugars galactosamine and glucosamine.

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