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Comparison of the electrophoretic separation of proteins in capillaries with different inner diameter

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Abstract

Two fused-silica capillaries of considerably different inner diameter (75 and 10 μm) were used for the separation of a set of five standard proteins. The separations were run in acid pH (50 m*M* phosphate buffer, pH 2.5). Generally better separations (with minor tailing only) were obtained using a standard capillary [27 cm (20 cm effective length)×75 μm I.D.] in comparison with a narrow bore capillary [27 cm (20 cm effective length)×10 μm I.D.]. The conditions of the electrophoretic separation were the same for both types of capillaries (25 °C; 10 kV; positive polarity at the inlet). The sequence of the proteins was cytochrome *c*, albumin, transferrin followed by a partly resolved peak of catalase and chymotrypsinogen A. In the narrow bore capillary severe tailing was observed – tailing factor ranged from 2.11 to 5.54 or 1.67 to 2.53 depending on the concentration of the analytes injected (2 or 0.2 mg/ml of each test compound injected). The relative [$\Delta(\Delta G^0)$] values of the interaction with the capillary wall in the small bore capillary (with cytochrome *c* taken as initial standard) ranged from −0.74 to −1.04 kJ/mol. The problem of assaying the speed of the endoosmotic flow (EOF) in both capillary types was thoroughly investigated using thiourea and dithiothreitol as EOF markers. It was revealed that if thiourea is used as the EOF marker, the obtained value was dependent on the concentration of the marker injected. Optimum conditions for the EOF determination in acid buffer were specified. The higher speed of the EOF in the narrow bore capillary (10 μm) as compared to the 75 μm I.D. capillary is discussed.

Keywords: Endoosmotic flow; Capillary columns; Cytochrome *c*; Albumin; Transferrin; Catalase; Chymotrypsinogen A; Proteins

1. Introduction

It was demonstrated repeatedly that the inner wall of the fused-silica capillary is a potential site for interactions of the separated analytes [1]. This property has been widely explored in open-tubular electrochromatography in which the capillary wall is modified in such a way that solute–wall interactions contribute or even govern the separation process (for a review, see Ref. [2]). In the particular case of proteins the solute–wall interactions generally spoil the results owing to non-specific attachment of the separated analytes to the capillary wall (sticking) [3]. This results not only in sample loss, but in poor reproducibility and decreased efficiency as well. In order to minimize such effects the separations of protein mixtures are run either at extreme values of pH by which either the dissociation of the silanol

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groups of the capillary wall is minimized (at acid pH) or the dissociation of the free amino groups of the proteinous solutes is decreased (at high pH values) [4]. Another method is based on the application of additives (usually amines and polyamines) that are believed to compete with the free amino groups of proteins for the negatively charged silanol group of the inner capillary wall [5,6]. High salt or zwitterions concentrations in the background electrolyte have been also applied to diminish the ion-exchange interactions of proteins with the capillary wall [7,8]. Dynamic or permanent coating of the capillary represent another possibility how to minimize (abolish) the protein sticking problem [9–14] (for a review, see Ref. [3]).

The adverse effect of protein sticking to the capillary wall was expected to be more pronounced in small bore capillaries in which the proportion of inner capillary surface to the volume of capillary is considerably larger. Therefore comparative experiments were carried out to get the fundamental information to what extent small bore silica capillaries can be used in miniaturized systems.

2. Theory

The widely known interaction of proteins with the capillary wall can be characterized best by considering the process as a kind of capillary electrochromatography. The interactions can be compared on the basis of calculating the change in free binding energy $[\Delta(\Delta G^0)]$. In the past this approach has been used for evaluating interactions of a set of model drugs with an immobilized plug of interacting particles [15,16] in a capillary with minimized endoosmotic flow (EOF), for evaluating of a wide variety of solutes with the liposomes using liposome modified capillary [17] and even for evaluating the interactions between two migrating solutes [18]. There is no reason why a similar approach could not be applied for comparing the interaction, e.g., in two different capillaries. In this case what is needed is to know the migration times, of the test compounds and the endoosmotic flow in the capillaries that are being compared.

The migration time (t_{obs}) of a given species is determined by the distance it travels from the injection point (i.e., effective capillary length) and its net velocity:

$$t_{obs} = l/v_{obs} \tag{1}$$

where l is the effective length of the capillary and v_{obs} is the net velocity.

Net velocity represents a sum of electromigration velocity (v_E) and the velocity of the endoosmotic flow (v_{EOF}):

$$t_{obs} = l/(v_E + v_{EOF}) \tag{2}$$

If a particular solute interacts with the capillary wall, the electromigrating velocity is changed by a factor R which specifies the fraction of time the analyte spends by interacting with the capillary wall:

$$v_E^{10} = Rv_E^{75} \tag{3}$$

where 75 and 10 in the exponent refer to velocities observed with the capillaries of the respective I.D.

Using electrophoretic migration times, Eq. (3) can be written as:

$$1/t_E^{10} = R/t_E^{75} \rightarrow t_E^{75}/t_E^{10} = R \tag{4}$$

As demonstrated in Ref. [18] the magnitude R is related to the capacity factor k' by:

$$R = 1/(1 + k') \rightarrow k' = 1/R - 1 \tag{5}$$

Further development of these calculations involves factor Φ which represents the phase ratio that is difficult to asses; however it is related to the capacity factor by the following equation:

$$k' = K\Phi \tag{6}$$

where K is the proportionality constant.

It is therefore reasonable to calculate the free energy change $[\Delta(\Delta G^0)]$ relatively to an arbitrarily chosen standard:

$$\Delta(\Delta G^0) = -R_g T \ln (k'_x/k'_r) \tag{7}$$

where R_g is the gas constant, T is the absolute temperature (taken as 295 in all calculations) and k'_x and k'_r are the capacity factors for the investigated compound (subscript x) and reference standard (subscript r), respectively. The way of calculating the free energy change $[\Delta(\Delta G^0)]$ values for the protein–

wall interactions is a slight modification of the approach published by Barker et al. [18].

The tailing factor (γ) was calculated according to the formula publishing Huang et al. [19]:

$$\gamma = B_{0.1}/A_{0.1} \quad (8)$$

where $B_{0.1}$ is peak width at 10% peak height (to the right with the perpendicular); $A_{0.1}$ is the peak width at 10% peak height (to the left with respect to the perpendicular).

3. Materials and methods

3.1. Apparatus and operating conditions

A Beckman P/ACE 5000 capillary electrophoresis (CE) system (Fullerton, CA, USA) was used throughout this study.

Separations were run at 10 kV and 25 °C in: (1) a bare fused standard capillary: 27 cm (20 cm effective length)×75 μm I.D. and (2) bare fused narrow-bore capillary: 27 cm (20 cm effective length)×10 μm I.D. (both types of the capillaries were purchased from Composite Metal Services, Hallow, UK).

The injection was done hydrodynamically by overpressure (3.45 kPa, 2 s) and the concentrations of the proteins were 0.2 or 2 mg/ml for the 75 μm I.D. capillary and 2 mg/ml for the 10 μm I.D. capillary. The high concentration used with the narrow capillary was necessary to get an adequate detector response. The separations were run with positive polarity at the inlet, detection was done at 214 nm. Phosphate buffer (50 m*M* NaH_2PO_4, pH 2.5) was used as background electrolyte (pH was adjusted by HCl). The postwash and prewash sequences used are summarized in Table 1.

3.2. Chemicals

Standard proteins/peptides: cytochrome *c* (relative molecular mass, M_r 12 500), chymotrypsinogen A (M_r 25 000), bovine serum albumin (M_r 68 000), catalase (M_r 240 000) were purchased from Boehringer (Mannheim, Germany); transferrin (M_r 76 000–81 000), dithiothreitol (DTT) and methanol were from Sigma (St. Louis, MO, USA). Buffer components and thiourea were obtained from Lachema (Brno, Czech Republic). Arginine and histidine were obtained from Millipore (Bedford, MA, USA). All chemicals used were of the analytical-reagent grade or highest purity available. All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA) and before analysis were filtered using Millex-HV filter (Millipore), 0.45 μm.

4. Results and discussion

4.1. Comparison of the separation in the standard (75 μm) bore capillary at different concentrations of the protein mixture

With a capillary (75 μm I.D.) and 50 m*M* phosphate (pH 2.5) nearly complete separation of the five-membered model set of proteins was obtained at the concentration 2 mg/ml (chymotrypsinogen A and catalase were only partly separated). If the concentration of proteins in the sample was decreased 10 times (0.2 mg/ml) the result was better (Fig. 1, Table 2) (the separation of chymotrypsinogen A and catalase was better indicated). As expected the tailing factors for all separated solutes were higher in the relatively overloaded run (2 mg/ml of each protein injected).

Table 1
Postwash and preconditioning systems used

Diameter of the capillary (μm I.D.)	Postwash and reconditioning sequence
75	1 min buffer postwash; preconditioning: 1 min water, 1 min buffer
10	1 min buffer postwash; preconditioning: 1 min water, 5 min buffer

Note: For the estimation of the electroosmotic flow with thiourea/dithiothreitol (75 and 10 μm I.D. capillary) the following scheme was applied: 1 min buffer postwash; preconditioning: 1 min water, 10 min 1 mol/l NaOH, 1 min water, 10 min 3 mol/l hydrochloric acid, 1 min water, 10 min buffer. This procedure was necessary to remove sorbed thiourea/dithiothreitol from the capillary.

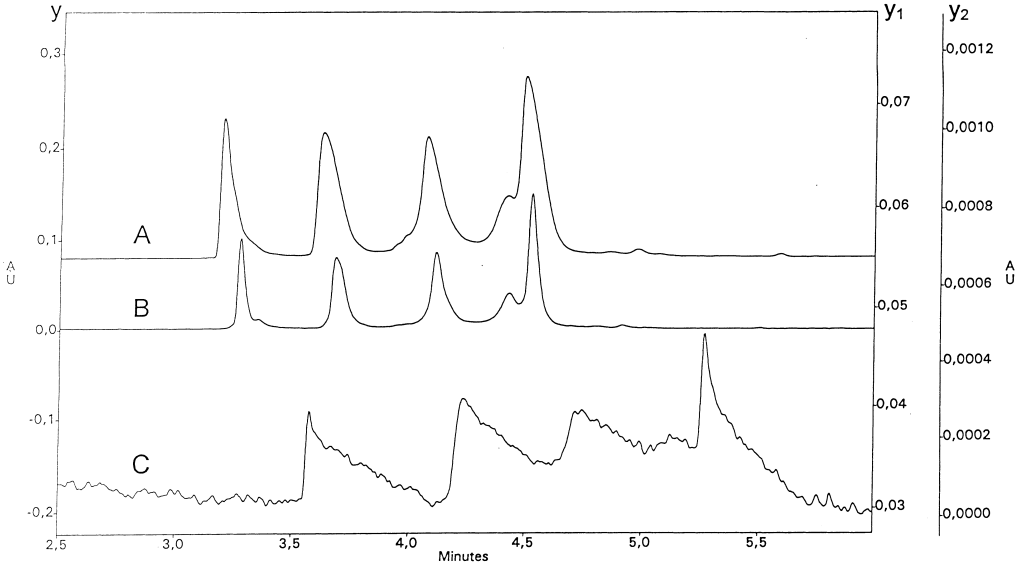

Fig. 1. Comparison of the capillary electrophoretic separation of the protein mixture in the 75 μm I.D. (2 and 0.2 mg/ml of each component injected) and 10 μm I.D. capillary (2 mg/ml of each component injected). The absorbance at 214 nm. *y*-Axis on the left: 75 μm I.D. capillary – injection 2 mg/ml; y_1-axis on the right: 75 μm I.D. capillary – injection 0.2 mg/ml; y_2-axis on the right: 10 μm I.D. capillary – injection 2 mg/ml. (A) 75 μm I.D. capillary, 2 mg/ml of each component injected; (B) as for A, 0.2 mg/ml injected; (C) 10 μm I.D. capillary, 2 mg/ml of each component injected.

4.2. Comparison of the narrow (10 μm) and standard (75 μm) bore capillary

Using a narrow bore capillary (10 μm) and a higher concentration (2 mg/ml) of the protein sample (the higher amount injected was necessary to obtain an adequate detector response), the result shown in Fig. 1 was obtained.

The resulting profile (peak shapes) obtained with the standard protein mixture depends on the inner diameter of the capillary used: in the small bore capillary all proteins tested were considerably tailing (for comparison of the tailing factor in narrow and standard bore capillary compare Table 2 and Table 3). It is feasible to assume that this tailing results from the interaction with the capillary wall: the respective $[\Delta(\Delta G^0)]$ values (relative to cytochrome *c*) are summarized in Table 4.

There is one considerable problem with calculating the $\Delta(\Delta G^0)$ values that concerns the estimation

Table 2
Comparison of the resolutions (R_s) and tailing factors (γ) for individual standard proteins at two different sample loads in the standard bore (75 μm I.D.) fused-silica capillary

Model protein	Resolution		Tailing factor	
	$c=2$ mg/ml	$c=0.2$ mg/ml	$c=2$ mg/ml	$c=0.2$ mg/ml
Cytochrome *c*	–	–	5.54	2.53
Albumin	2.61	4.49	3.98	2.39
Transferrin	2.22	3.74	2.11	1.67
Catalase	1.50	2.15	NE	NE
Chymotrypsinogen A	0.32	0.64	3.1	1.87

Resolution calculated according to the formula $R_s=2(t_1-t_2)/(w_1+w_2)$ where t_1 and t_2 are migration times of more slowly and faster moving two subsequent peaks, and w_1 and w_2 are the respective peak widths at the baseline.
NE—Not estimated.

Table 3
Tailing factors (γ) of the compounds investigated in the narrow bore (10 μm I.D.) fused-silica capillary

Model protein	Resolution	Tailing factor
Cytochrome *c*	–	21.38
Albumin	1.51	7.32
Transferrin	0.73	6.04
Chymotrypsinogen A + catalase	1.06	14.10

of the endoosmotic flow. We have realized rather soon that the t_{EOF} value estimated by thiourea depends not only on the size of the capillary and other electrophoretic conditions but also on the amount of the endoosmotic marker injected: with a thiourea sample containing 0.02 mg/l the t_{EOF} value was 33.4 min in the 75 μm capillary; if the thiourea concentration was increased to 1 mg/ml the t_{EOF} value approached 200 min. All thiourea samples were dissolved in the background electrolyte and the lower (0.02 mg/ml) concentration was used for t_{EOF} estimation. If dithiothreitol is used as an EOF marker an average value (under otherwise identical conditions) was 33.6 min (average from six runs) which differs from the thiourea estimate by about 0.6%. The estimate of t_{EOF} by dithiothreitol in the narrow bore capillary was 22.8 min which is quite close to the 22.6 min obtained with thiourea. It is noticeable that the sticking problems with thiourea were not seen if dithiothreitol was used as an EOF marker.

The time for the endoosmotic flow in the narrow bore capillary (estimated by using both EOF markers, i.e., thiourea and dithiothreitol in the background electrolyte buffer) was considerably shorter (22.6 and 22.8 min, respectively); this corresponds to $\mu_{EOF} = 2.69–2.72 \cdot 10^{-9}$ m^2 s^{-1} V^{-1} for the wide bore capillary and $3.94–3.98 \cdot 10^{-9}$ m^2 s^{-1} V^{-1} for the capillary with the smaller diameter.

All attempts to use other compounds as EOF markers (typically methanol or piperidine) failed completely.

The reason for shorter t_{EOF} values observed consistently with narrow bore capillary is difficult to evaluate. It was proposed that phosphate creates in acidic background electrolytes an adsorbed phosphate layer that avoids protein/peptide adsorption and reduces adsorption of separated analytes on the inner capillary wall [20]. This assumption would, however, decrease the surface charge of the capillary resulting in decreased EOF. In addition, the smaller diameter of the narrow bore capillaries is expected to result in a higher hydrodynamic resistance which also would result in a slow-down of the endoosmotic flow. Consequently it is proposed that neither of these effects plays the decisive role in the speed of the endoosmotic flow in narrow bore capillaries. Apparently the effect of the 7.5-times higher surface to volume ratio in the narrow bore capillary is not only capable to compensate for the above adverse effects but is capable of supplying enough charge density to keep (or even increase) the endoosmotic flow.

This conclusion is further supported by an observation published by Altria [21] which says that levels of EOF also decrease significantly with in-

Table 4
Comparison of free binding energy changes [$\Delta(\Delta G^0)$] in the 10 μm I.D. capillary relatively to cytochrome *c* (in kJ/mol)

Model protein	t^{75}_{obs}	t^{75}_{E}		t^{10}_{obs}	t^{10}_{E}		$R = t^{75}_{E}/t^{10}_{E}$		$k' = 1/R - 1$		$[\Delta(\Delta G^0)]$	
		TU	DTT		TU	DTT	TU	DTT	TU	DTT	TU	DTT
Cytochrome *c*	3.21	3.55	3.54	3.49	4.13	4.12	0.85	0.85	0.17	0.17	refer.	refer.
Albumin	3.65	4.10	4.09	4.13	5.07	5.04	0.80	0.79	0.25	0.26	−0.94	−1.04
Transferrin	4.11	4.69	4.68	4.58	5.76	5.72	0.81	0.81	0.23	0.23	−0.74	−0.74
Chymotrypsinogen A + catalase	4.55	5.28	5.26	5.10	6.61	6.56	0.79	0.80	0.26	0.25	−1.04	−0.94
Arginine	2.47	2.66		3.18	3.70		0.71		0.40		−2.09	
Histidine	2.77	3.02		3.11	3.60		0.83		0.20		−0.39	

The electrophoretic migration times were calculated using thiourea (TU) and dithiothreitol (DTT) as EOF markers

Note: Minimum interaction was assumed in the 75 μm I.D. capillary; refer stands for the reference protein. t_{obs} – Observed migration time in min, t_E – electrophoretic migration time in min, TU and DTT, respectively, stand for thiourea and dithiothreitol as EOF markers, $t^{75}_{EOF} = 33.4$ min (estimated by TU), 33.6 min (estimated by DTT), $t^{10}_{EOF} = 22.6$ min (estimated by TU), 22.8 min (estimated by DTT), data for arginine and histidine refer to TU as EOF marker.

creased capillary bore. The rational background for this statement may be seen in the slight technological differences during the preparation of wide and narrow bore capillaries which may result in different properties of the inner capillary surface ([22], see also Ref. [23]).

The relative free energy of binding changes [$\Delta(\Delta G^0)$] values were calculated relatively to the values obtained for cytochrome *c*. The reason was that the actual value of k' for this protein was the lowest; attempts were made to relate the changes in free energy of binding to an amino acid that would give a good detector response: arginine and histidine were selected for this purpose. As it emerges from Table 4 the k' values for both these amino acids were higher than that of cytochrome *c*. It is evident that regarding the size of the molecule also low molecular species are prone to bind to the capillary surface (see also the problems with EOF estimation by thiourea).

5. Conclusions

The following conclusions can be drawn from our experiments:

1. The peaks of separated proteins exhibit considerably distinct tailing in the narrow bore capillary.
2. The free energy changes [$\Delta(\Delta G°)$] of binding of the solutes in the narrow bore capillary relatively to the peak of cytochrome c were evaluated. Only small differences between the three proteins that could be evaluated were observed. The two amino acids investigated were also bound to the capillary surface.
3. In order to carry out the [$\Delta(\Delta G°)$] calculations it was necessary to determine the t_{EOF}. Two EOF markers were used, namely dithiothreitol and thiourea. No successful measurements could be obtained with either methanol or piperidine. With thiourea the concentration of the marker should be about 0.02 mg/ml, as increased concentration of the marker leads to unrealistically increased values for t_{EOF}, caused apparently by sticking of this marker to the inner capillary surface.

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