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Use of liposomes as a dispersed pseudo-stationary phase in capillary electrophoresis of basic proteins

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Abstract

The suitability of liposome capillary electrophoresis for separating basic proteins at different pH values in the acidic domain was evaluated preparing liposomes consisting of large unilamellar vesicles of the phospholipid 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC), whose hydrodynamic diameter and size distribution were evaluated by dynamic light scattering measurements. The study was conducted evaluating different approaches of performing liposome capillary electrophoresis of proteins, using liposomes dispersed in the electrolyte solution of different composition and pH values ranging from pH 6.2 to 4.0. The liposomes were employed as a pseudo-stationary phase dispersed in the electrolyte solution, which was introduced into the capillary as a plug of volume equivalent to that of the capillary tube, whereas liposome-free electrolyte solutions were contained into the electrolyte vessels during electrophoresis. The study was performed with either bare fused-silica capillaries or capillaries previously treated with POPC that was employed as a dynamic coating agent. Such treatment was performed rinsing the capillary tube with a proper volume of electrolyte solution containing POPC and resulted in a significant reduction of the electroosmotic flow, indicative of the adsorptive behavior of liposomes onto the internal surface of bare fused-silica capillaries. The usefulness of using liposome dynamically coated capillaries for separating basic proteins in absence of the dispersed liposome pseudo-stationary phase filled into the capillary prior to injecting the protein sample was evaluated too. The results show that the presence of liposomes reduced drastically the untoward interactions between basic proteins and the capillary wall, also in capillary not subjected to the dynamic coating process with POPC. In addition, the use of liposomes as a pseudo-stationary phase dispersed in the electrolyte solution enhanced the separation of basic proteins also in POPC dynamically coated capillaries.

Keywords: Pseudo-stationary phases; Liposomes; Proteins; Palmitoyloleylglycerophosphocholine

1. Introduction

Liposomes are vesicles formed by phospholipids by a self-assembling process in aqueous solutions, which are composed of one or more lipid bilayer membranes that have entrapped a volume of the surrounding aqueous media during their self-assemblies [1]. On the basis of number of lipid bilayers and size, liposomes are classified into multilamellar vesicles (MLVs), having more than one lipid bilayer and size up to 5.0 μm, and into large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUVs), both having single lipid bilayer and size in the range of 100–400 and 20–50 nm, respectively [2].

The amphiphilic character of the phospholipids and the organization of liposomes in closed structures give to these vesicles the property of encapsulating hydrophobic compounds in the bilayer membrane or hydrophilic molecules in the internal cavity. In addition, depending on phospholipid composition, size and surface characteristics, liposomes can establish a variety of interactions with molecular species and cell surfaces in the surrounding solution [3]. These features enable liposomes to be widely employed as models for biological membranes and as carries for drugs and other agents of therapeutic, diagnostic and cosmetic value [4].

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Liposomes have found a variety of applications in capillary electrophoresis too [5–18]. The first use of liposomes in capillary electrophoresis was reported by Hjertén and coworkers for studying the interactions of liposomes with some model drugs and two octapeptides [5]. Liposomes were employed as a pseudo-stationary phase that was introduced into a polyacrylamide coated electroosmosis-free capillary from the cathodic end to a zone close to the detection windows and the interactions between negatively charged analytes and the liposomes were related to the decrease in the mobility of the analytes toward the anode observed in the presence of the liposomes suspension.

Such approach, termed by Zhang et al. [5] liposome capillary electrophoresis (LCE), has been employed in a number of applications, including the determination of liposome-water partition coefficients for neutral aromatic solutes [6], the study of interaction of a variety of neutral solutes with unilamellar liposomes composed of a mixture of two phospholipids [7], the investigation of the interaction of a set of drugs varying in polarity and chemical structure with liposomes [8], the evaluation of the binding constant for cationic liposome formulations with fluorescein conjugated 2′-*O*-methylphosphorothionate antisense oligonucleotides [9]. Binding studies between drugs and liposomes have also been performed by frontal capillary electrophoresis [10] and by immobilized liposome affinity electrochromatography, using capillaries with immobilized liposomes at the inner wall of a bare fused-silica capillary [11]. Liposomes have also been employed as dynamic coating agents for bare fused-silica capillaries used in CE of proteins [12] and small molecules [13,14].

Moreover, LCE has been applied for separating uncharged solutes by a mechanism based on the distribution of analytes between an aqueous buffer phase and a liposome phase. Except in the work by Zhang et al. [5], such approach has been restricted to low molecular mass analytes such as benzene derivatives [15], steroids [15], riboflavine [16], and phenols [7,15].

Recently, we have explored the potentiality of LCE for separating peptides and proteins in either liposome dynamically coated or bare fused-silica capillaies using phosphate buffer at pH 7.4 as the electrolyte solution [19]. This paper reports the results of the extension of our studies for investigating the suitability of LCE at separating basic proteins at different pH values in acidic domain with electrolyte solutions of various composition. The study has been conducted by preparing unilamellar liposomes composed of the zwitterionic phospholipid 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), which have been investigated for their capability at modulating selectivity, resolution and separation performance of basic proteins in acidic pH domain. The study has evaluated different strategies for allowing the liposome to act as a dispersed pseudo-stationary phase. These include the use of either POPC dynamic coated or bare fused-silica capillarised, which are filled with a solution containing the dispersed liposomes whereas protein separation is carried out with liposome-free electrolyte solutions.

2. Experimental

2.1. Chemicals and samples

Reagent-grade phosphoric acid, citric acid, hydrochloric acid, potassium hydroxide, sodium chloride, and chloroform were purchased from Carlo Erba (Milan, Italy). Deionized water was obtained by a Milli-Q water purification system from Millipore (Bedford, MA, USA) and degassed by sonication before use. Cytochrome *c* (from horse heart), lysozyme (from chicken egg white), ribonuclease A (from bovine pancreas), α-chymotrypsinogen A (from bovine pancreas) were obtained from Sigma (Milan, Italy). POPC, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine, was purchased from Avanti Polar Lipids (Alabaster, AL, USA).

2.2. Preparation and characterization of liposomes

Liposomes were prepared as LUVs by adapting extrusion techniques reported in literature [20,21] as described below. The desired amount of POPC was weighted and transferred to a round-bottom flask where it was dissolved in chloroform. The round-bottom flask was placed in a rotary evaporator with the water bath at 30–35 °C to produce a thin layer of phospholipids on the inner surface of the flask. The trace residue of the solvent was removed under vacuum for 24 h. The resulting lipid film was hydrated with 10 mM phosphate buffer at pH 7.4, containing 2.7 mM potassium chloride and 137 mM sodium chloride, under shaking to give a dispersion of multilamellar vesicle (MLV) with a lipid concentration of 40 mM. The MLV dispersion was subjected to five freeze–thaw cycles [22] and processed to LUVs by extrusion through Nucleopore (Whatman, Maidstone, UK) polycarbonate filters of 0.2 and 0.1 μm pore size, using a Lipex Model T 001 extruder (Northern Lipids, Vancouver, Canada). First, the dispersion was passed 10 times through a 0.2 μm pore size polycarbonate filter and then 10 times through a filter of 0.1 μm pore size. The prepared liposome suspension was stored at 4.0 °C in the dark. Average hydrodynamic diameter (126 ± 0.3 nm) and polydispersity (0.087 ± 0.022) of the prepared liposomes were determined by dynamic light scattering (DLS) measurements, which were carried out using a Zetasizer 5000 (Malvern Instruments, Malvern, UK) consisting of a photomultiplier tube, a Malvern 7132 multi-bit digital correlator, and a 5 mW He–Ne laser. All experiments were performed at the scattering angles of 90° (λ = 488 nm) and thermostatically controlled at 25 °C. Hydrodynamic diameter of liposomes was determined from the cumulant analysis of the intensity autocorrelation function as reported elsewhere [23]. The vesicle size was taken as a mean value of three replication of 10 measurements. The particle size distribution (PSD), computed by an inverse Laplace transformation of the data,

were analyzed with the algorithms NNLS (non-negative least squares, smoothing 0.005) and CONTIN built-in procedures of the Malvern Zetasizer 5000.

2.3. Capillary electrophoresis

All experiments were performed using an HP ^{3D}Capillary Electrophoresis system from Agilent (Waldbronn, Germany), consisting of a high voltage power supply, a diode array UV–vis detector and an air-cooling device for temperature control of the cartridge containing the capillary tube. The capillary electrophoresis unit was interfaced with an HP Vectra XM 5 166 MHz personal computer running the HP ^{3D}CE ChemStation software, providing system control in addition to data acquisition and evaluation. Capillary tube of 0.050 mm I.D. and 0.375 mm O.D. was purchased from Quadrex (New Haven, CT, USA). Capillaries having total length of 330 mm and distance from the detection window and capillary end of 85 mm were prepared in house. Prior to use for the first time, the new fused-silica capillary was flushed successively with 0.5 M sodium hydroxide (30 min), water (10 min), and 0.5 M hydrochloric acid (30 min), followed by a second treatment with water (10 min), 0.5 M sodium hydroxide (30 min), water (10 min). Buffers consisting of either a monoprotic or a polyprotic acid and its sodium salt were prepared as previously reported [19] by titrating the proper amount of requested acid to the desired pH value with 1.0 M sodium hydroxide. Buffers of constant ionic strength were prepared in a similar way with the incorporation of the proper amount of sodium chloride calculated as described in literature [24]. The pH was measured with a glass electrode Model 52–02 and Model Basic 20 pH-Meter, both from Crison (Alella, Spain). All solutions were filtered through a type HA 0.22 μm membrane filter (Millipore, Vimodrome, Italy) and degassed by sonication before use. All experiments were carried out at constant applied voltage of 10 kV with the temperature of the capillary cartridge set at 25 °C.

3. Results and discussion

The suitability of liposome capillary electrophoresis for separating basic proteins at different pH values in the acidic domain was evaluated preparing liposomes consisting of LUVs of the phospholipid POPC. The study was conducted evaluating different approaches of performing liposome capillary electrophoresis of proteins, using the POPC liposomes dispersed in electrolyte solutions of different composition and pH values in the range from pH 6.2 to 4.0. Liposomes were employed both for coating the capillary tube by a dynamic approach and as a pseudo-stationary phase dispersed in the electrolyte solution.

Initial experiments were performed to evaluate the electrophoretic behavior of liposomes at pH 6.2 using 20 mM phosphate buffer, containing 68 mM NaCl, as the background electrolyte solution (BGE). A bare fused-silica capillary was conditioned with BGE until a constant value of the electroosmotic flow (EOF) was measured, using mesityl oxide as the neutral marker. The EOF measured after preconditioning the capillary was 3.99×10^{-8} m^2 V^{-1} s^{-1} as average value of five repeated injections (S.D., 1.77×10^{-10}; R.S.D., 0.44%). The conditioned capillary was employed for subjecting to capillary electrophoresis a solution containing 8 mM POPC in the BGE, which was injected by pressure at 5 kPa for 3.0 s and run with constant applied voltage of 10 kV and cathodic detection at 205 nm, while the temperature of the capillary cartridge was maintained at 25 °C.

Injecting mesityl oxide after having run the POPC sample, that migrated as a broad slow moving peak, revealed that the electroosmotic flow (EOF) was reduced to 2.85×10^{-8} m^2 V^{-1} s^{-1}, indicative of the adsorptive behavior of liposomes onto the internal surface of the bare fused-silica capillary that we [19] and other authors have already reported [11–13,16]. Mesityl oxide was then injected in between sequential runs of POPC samples introduced into the capillary by pressure injection as reported above, corresponding to a sample volume of 7.82 nL. The resulting calculated values of the electroosmotic mobility plotted against the number of sequential injections of the liposome dispersion are reported in Fig. 1, providing evidence of the significant adsorption of liposomes onto the capillary wall with saturation of the adsorption sites after few injections.

On the basis of this observation a bare fused-silica capillary was dynamically coated with liposomes by rinsing the BGE containing 60 μM POPC through the capillary with pressure of 5 kPa (50 mbar) for period times ranging from 248 s, corresponding to the time requested to fulfill the capillary with the above solution (see below), to 30 min. After each rinsing time the electroosmotic flow was measured to evaluate the completeness of the coating. The EOF decreased with increasing the rinsing time down to a minimum value of 1.38×10^{-8} m^2 V^{-1} s^{-1}, which was almost unvaried by rinsing the capillary for times longer than 20 min, indicating

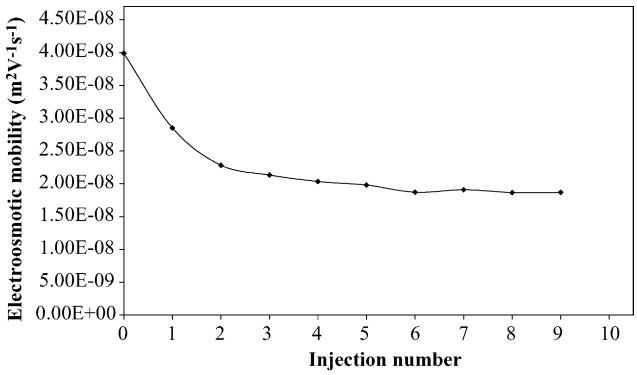

Fig. 1. Electroosmotic flow measured before and between sequential runs of liposome samples consisting of a 8 mM POPC suspended in the BGE. Capillary, bare fused-silica 0.050 mm I.D., 0.375 mm O.D., total length 330 mm (245 mm to the detector); BGE, 20 mM phosphate buffer, pH 6.2, containing 68 mM NaCl; applied voltage 10.0 kV; cathodic detection at 205 nm; temperature of the capillary cartridge, 25 °C.

that an equilibrium state was reached within this period time. Consequently, a rinsing time of 20 min was considered to be appropriate for the dynamic coating of the capillary with POPC.

The repeatability of EOF generated by the dynamically coated capillary was evaluated by performing repeated runs of the neutral marker mesityl oxide. Before each injection the capillary was first rinsed with the BGE containing the liposomes and then with the liposome-free BGE. Both solutions were rinsed through the capillary by pressure of 90 kPa (900 mbar) for 1.0 min. The relative standard deviation of the electroosmotic mobility calculated from the migration times of five repeated injections of mesityl oxide was 2.6%, indicative of a quite stable dynamic coating.

Four standard proteins with isoelectric points (p*I*) ranging from 9.5 (cytochrome *c*) to 11.0 (lysozyme) and molecular masses varying from 12 400 (cytochrome *c*) to 25 000 (α-chymotrypsinogen A) [25] were employed for evaluating the suitability of LCE for separating basic proteins. The study was performed by two different approaches. A first strategy consisted in filling the POPC dynamically coated capillary with 60 μM POPC dispersed into the BGE and running the protein separation with 20 mM phosphate buffer pH 6.2, containing 68 mM NaCl (i.e. with POPC-free BGE). Between runs, the capillary was rinsed with the BGE containing 60 μM POPC for 5.0 min at 5 kPa in order to renewing the POPC dispersion into the capillary.

Alternatively, after the dynamic coating process the capillary was filled with the BGE employed as the running electrolyte solution for separating the protein test mixture. Between runs the capillary was first rinsed with the BGE containing 60 μM POPC for 1.0 min with pressure of 90 kPa (900 mbar) in order to refreshing the coating and then filled again with BGE before injecting a new protein sample.

Fig. 2 shows the separation of the basic protein test mixture carried out with the liposome dynamically coated capillary either in presence or in absence of POPC in the solution filled into the capillary. In both cases the BGE consisted of 20 mM phosphate buffer pH 6.2, containing 68 mM NaCl. It can be seen that both approaches allowed the separation of basic proteins that otherwise were irreversibly adsorbed onto the capillary wall of the bare fused-silica capillary when electrophoresis was carried out with phosphate buffer at pH

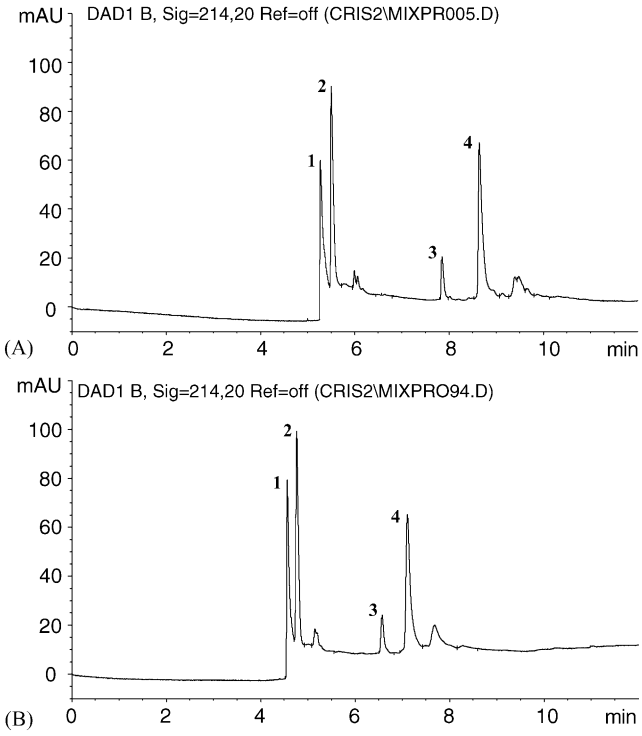

Fig. 2. Separation of basic proteins at pH 6.2 in POPC dynamically coated capillary either filled with 60 μM POPC dispersed in the BGE (A) or with POPC-free BGE (B). BGE, 20 mM phosphate buffer, pH 6.2, containing 68 mM NaCl; capillary and other conditions as in Fig. 1 except detection at 214 nm. Proteins: (1) lysozyme, (2) cytochrome *c*, (3) ribonuclease A, and (4) α-chymotrypsinogen A.

6.2 without dynamically coating or filling the capillary with POPC. In addition, it clearly appears that the presence of the POPC suspended in the BGE filled into the capillary causes an improvement of resolution, selectivity and peak shape of the two completely separated proteins (i.e. ribonuclease A and α-chymotrypsinogen A) at the expenses of increased migration time (see Tables 1 and 2). Repeated runs of the protein test mixture were carried out to evaluate the repeatability of migration times of the separations performed by the two different approaches. The relative standard deviation (R.S.D.) of protein migration times ranged from 2.20 to 3.60% in absence of POPC in the BGE filled into

Table 1
Effect of POPC dispersed in the BGE filled into the capillary on efficiency using either bare-fused-silica or POPC dynamically coated capillaries

pH	Capillary	[POPC] (μM)	Efficiency (plates/m)			
			Lys	Cyt	RNase	Chy
6.2	POPC-coated	60	14987	168710	498130	238538
6.2	POPC-coated	0[a]	125616	124714	256942	138534
5.2	POPC-coated	60	13048	194628	267783	212742
5.2	POPC-coated	0[a]	76000	76881	45338	37073
6.0	Bare-fused-silica	120	214008	77277	180477	150000
5.0	Bare-fused-silica	120	273538	254755	288591	204363
4.0	Bare-fused-silica	120	226040	304567	378640	200946

Lys: Lysozyme; Cyt: cytochrome *c*; RNase: ribonuclease A; Chy: α-chymotrypinogen A.
[a] To indicate capillary filled with POPC-free BGE.

Table 2
Effect of POPC dispersed in the BGE filled into the capillary on resolution using either bare-fused-silica or POPC dynamically coated capillaries

pH	Capillary	[POPC] (μM)	Resolution	
			Lys/Cyt	RNase/Chy
6.2	POPC-coated	60	1.70	11.54
6.2	POPC-coated	0[a]	3.16	7.04
5.2	POPC-coated	60	1.71	6.71
5.2	POPC-coated	0[a]	1.91	2.73
6.0	Bare-fused-silica	120	3.70	1.03
5.0	Bare-fused-silica	120	1.51	3.30
4.0	Bare-fused-silica	120	6.33	9.88

Lys: Lysozyme; Cyt: cytochrome *c*; RNase: ribonuclease A; Chy: α-chymotrypinogen A.
[a] To indicate capillary filled with POPC-free BGE.

the capillary and from 2.90 to 4.40% in presence of the liposomes.

Due to the poor buffering capacity of phosphate at pH values ranging from 6.0 to 4.0, which is outside the range taken as one pH unit below pK_{a2} (7.20) and one pH unit above pK_{a1} (2.15) of phosphoric acid, defining lower and upper limit of the buffering range around pK_{a2} and pK_{a1}, respectively, the experiments within this pH range were conducted using citrate buffer, having effective buffering capacity at pH ranges 3.12 ± 1, 4.76 ± 1, 6.40 ± 1, corresponding to one pH unit above and below the three pK_a values of citric acid. Therefore, experiments at pH 5.2 were performed with 20 mM citrate buffer, containing 68 mM NaCl using the two strategies described above. First, a bare fused-silica capillary was rinsed with citrate buffer of above composition and pH for the period time necessary to reach a constant value of the electroosmotic flow, which resulted to be 2.93 × 10^{-8} m^2 V^{-1} s^{-1} (S.D. 7.99 × 10^{-10} m^2 V^{-1} s^{-1}; R.S.D., 2.73%). Then, the capillary was dynamically coated by rinsing a 20 mM citrate pH 5.2 solution, containing 68 mM NaCl and 60 μM POPC, through the capillary with pressure of 5 kPa (50 mbar) for 20 min. After the dynamic coating the EOF generated in the capillary was 7.97 × 10^{-9} m^2 V^{-1} s^{-1} (S.D. 1.62 × 10^{-10} m^2 V^{-1} s^{-1}; R.S.D., 2.04%), which is about one fourth of the EOF measured before rinsing the capillary with the solution containing the dispersed liposomes. The separations of basic proteins carried out with the POPC dynamically coated capillary in presence and in absence of liposomes in the BGE filled into the capillary are reported in Fig. 3A and B, respectively. The electropherograms and data reported in Tables 1 and 2 show that resolution and selectivity of the completely separated proteins were improved by the presence of POPC in the capillary, similarly to the behavior already observed with phosphate buffer at pH 6.2. From this it can be inferred that the action of POPC in suppressing the adsorption of proteins onto the capillary wall may also result from the competitive interactions of proteins with the liposomes dispersed in the solution, rather than simply from the masking effect of POPC for the silanol groups on the capillary wall, which is the mechanism generally accepted for the

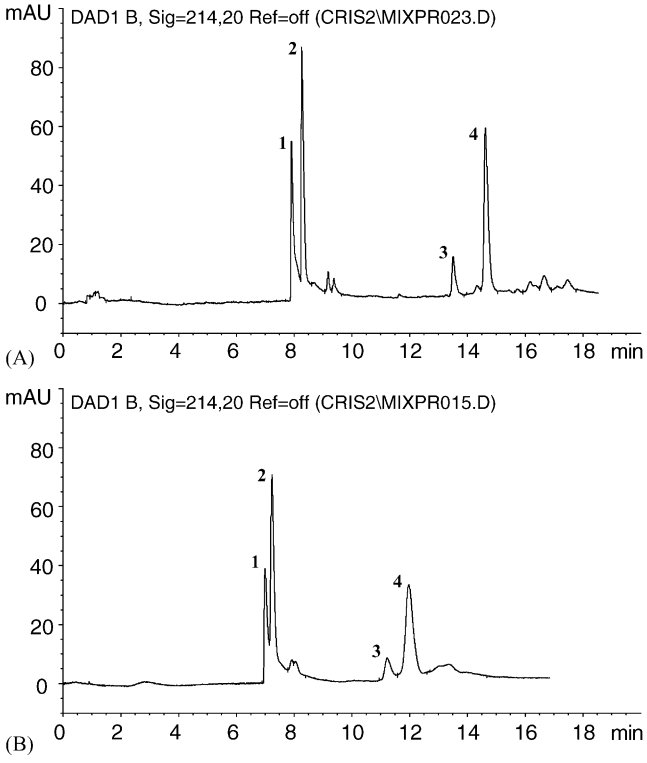

Fig. 3. Separation of basic proteins at pH 5.2 in POPC dynamically coated capillary either filled with 60 μM POPC suspended in the BGE (A) or with POPC-free BGE (B). BGE, 20 mM citrate buffer pH 5.2, containing 68 mM NaCl; capillary, experimental conditions and proteins as in Fig. 2.

additives adsorbed at the interface between the capillary wall and the electrolyte solution [26,27].

Subsequent experiments were performed to evaluate the influence of POPC dispersed in the BGE on the electrophoretic behavior of basic proteins using bare fused-silica capillaries, which were not preventively treated for the dynamic coating with POPC. The investigation was carried out using a bare fused-silica capillary that was equilibrated with the POPC-free BGE and then filled with the BGE containing POPC at various concentrations ranging from 32 to 120 μM by injecting into the capillary a plug of each solution equivalent to the volume of the capillary tube. Volumes of both the capillary tube and plug were just about calculated by the CE Expert Version 1.0 software from Beckman (Fullerton, CA, USA) on the basis of the injection time and applied pressure with the approximation of having considered the viscosity of the solution containing the liposomes equal to that of distilled water, while capillary dimensions and temperature of the capillary cartridge requested by the software were those reported in the Experimental section. Accordingly, the volume of plug, equivalent to the capillary volume, was 648 nL, corresponding to the hydrodynamic injection of the POPC-containing solution at 5 kPa for 248 s. The experiments were carried out by injecting the protein mixture after having filled the capillary with 648 nL of the BGE containing the dispersed liposomes. Between runs the capillary was flushed with the liposome-free BGE for

1.0 min with pressure of 90.0 kPa (900 mbar) to remove the plug introduced before the previous sample injection.

The study was performed using citrate buffer at three different pH values ranging from 6.0 to 4.0 and constant ionic strength of 0.111 M, adjusted by incorporating the proper amount of sodium chloride to the electrolyte solution consisting of 20 mM citric acid titrated to the desired pH value with 1.0 M sodium hydroxide. Ionic strength was maintained at a constant value in order to level off the contribution of this parameter to the generation of the electric double layer at the interface between the capillary wall and the electrolyte solution and between the surface of liposomes and the electrolyte solution with changing pH [28]. Ionic strength is known to influence both the size of ion atmosphere around ions and colloidal particles and the thickness of the electric double layer at a solid-liquid interface [29]. In addition, ionic strength has proven to alter the size dependent electrophoretic behavior of liposomes [30].

Separations of the protein test mixture carried out at pH 6.0, 5.0, and 4.0 in presence of a column volume of POPC suspension introduced into the capillary just before injecting the protein sample are displayed in Fig. 4, whereas data on efficiency and resolution are reported in Tables 1 and 2. The concentration of POPC in the BGE filled into the capillary was 120 μM. This concentration was selected on the basis of the results obtained at pH 6.0 using solutions containing POPC at concentration lower than 120 μM, which were more unsatisfactory than those displayed in panel A of Fig. 4 due to the poor resolution of ribonuclease A from α-chymotrypsinogen A observed at this pH value with citrate buffer. With lower concentrations of POPC all proteins exhibited higher mobility and lower resolution (see below).

The significant different migration behavior of the protein pair α-chymotrypsinogen A and ribonuclease A observed with pH 6.2 phosphate and with pH 6.0 citrate buffers (see Figs. 3 and 4) can be related to the selective ion-pair formation of these basic proteins with phosphate and citrate anions. Such effect is expected to varying the effective charge-to-hydrodynamic radius of proteins and hence their electrophoretic mobility, as it has already described to elucidate the influence of buffer anions on the electrophoretic mobility of proteins [31].

However, it should be recall that in absence of POPC all basic proteins were irreversibly adsorbed on the capillary wall of the bare fused-silica capillary. Therefore, it can be inferred that POPC influences significantly the electrophoretic behavior of basic proteins, also in absence of the preventive dynamic coating of the capillary wall with the liposomes.

The dependence of the electrophoretic behavior of basic proteins from the concentration of POPC in the BGE filled into the capillary before injecting the protein sample investigated at pH 4.0 with the concentration of POPC in the BGE filled into the capillary ranging from 32 to 120 μM in 20 mM citrate buffer at pH 4.0 and constant ionic strength (0.111 M) is depicted in Fig. 5 as plots of the migration times against the concentration of dispersed POPC. It is noted that the migration times of all four proteins increases with increasing the concentration of POPC in the BGE introduced into the capillary before injecting the protein sample, indicating significant interactions between proteins and POPC. This is further evidenced in Fig. 6, where the intrinsic selectivity of the system, defined as the ratio of the mobility of faster moving analyte to that of the slower one [32], is plotted as a function of the concentration of POPC for the two pairs proteins cytochrome *c*–lysozyme and α-chymotrypsinogen A–ribonuclease A. For both protein pairs the selectivity increases almost linearly, but with different slope, with the concentration of POPC present in the capillary. The above findings suggest that the variations in selectivity should be the results of differentiated

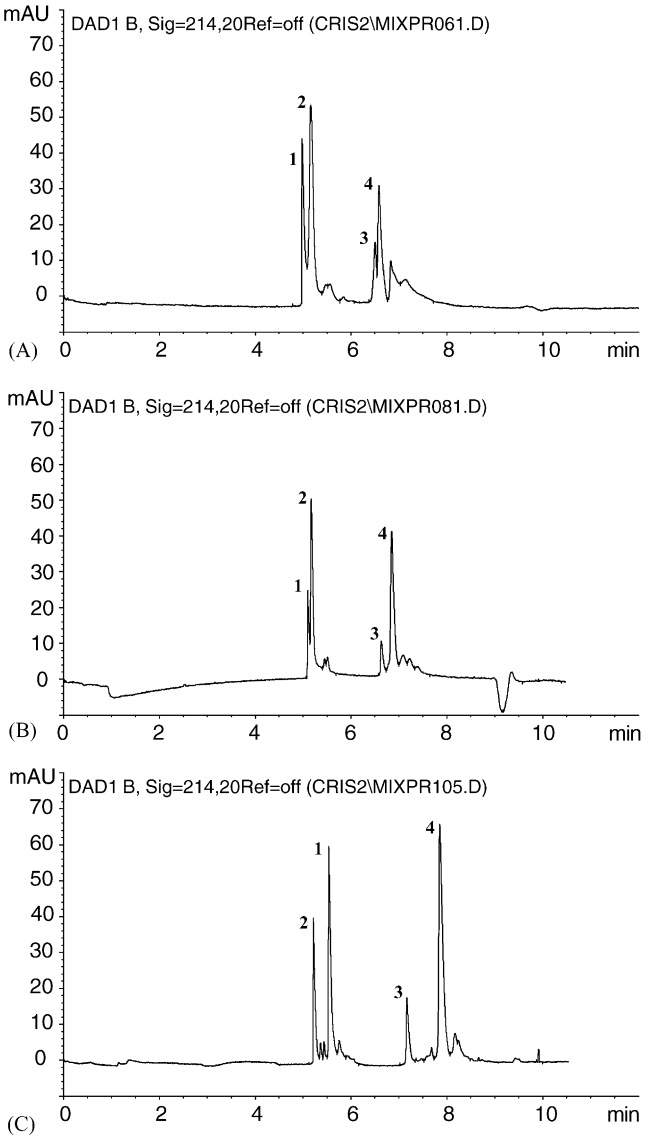

Fig. 4. Separation of basic proteins in bare fused-silica capillary filled with one column volume of 120 μM POPC suspended in 20 mM citrate buffer of constant value of ionic strength (0.111 M) and different pH values. (A): pH 6.0; (B): pH 5.0; (C): pH 4.0. BGE, 20 mM citrate buffer of constant value of ionic strength (0.111 M) at pH 6.0 (A), pH 5.0 (B), and pH 4.0 (C); capillary, experimental conditions and proteins as in Fig. 2.

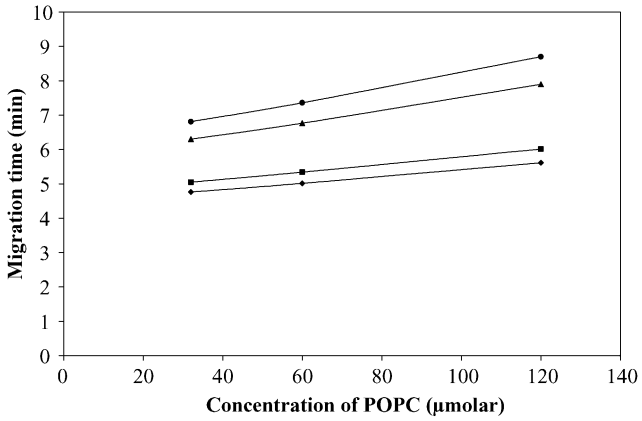

Fig. 5. Plots of migration times as a function of POPC concentration in 20 mM citrate buffer pH 4.0 for the four basic proteins: (♦) cytochrome *c*, (■) lysozyme, (▲) ribonuclease A, (●) α-chymotrypsinogen A. Capillary and experimental conditions as in Fig. 2.

interactions of proteins with POPC rather than the effect of the variations in parameters that are expected to contribute indifferently to the mobility of samples, such as the electroosmotic flow or the viscosity of the liposome suspension.

On the other hand, it should be considered that filling a bare fused-silica capillary with POPC dispersed in the BGE has the effect of suppressing the interaction of basic proteins with the silanol groups on the capillary wall. However, this effect could only explain the effectiveness of POPC at preventing protein–capillary wall interaction without justifying the differences in selectivity observed for the two protein pairs with increasing POPC concentration.

It is worth mentioning that at pH values higher than 4.0 the migration order of the protein pair lysozyme–cytochrome *c* is reversed with respect to that observed at pH 4.0. This is the results of the increase of the electrophoretic mobility with decreasing pH, which below pH 5.0 is greater for cytochrome *c* than for lysozyme. The effect has been evidenced with a variety of electrolyte solutions by plotting the electrophoretic mobility of the two proteins as a function of pH

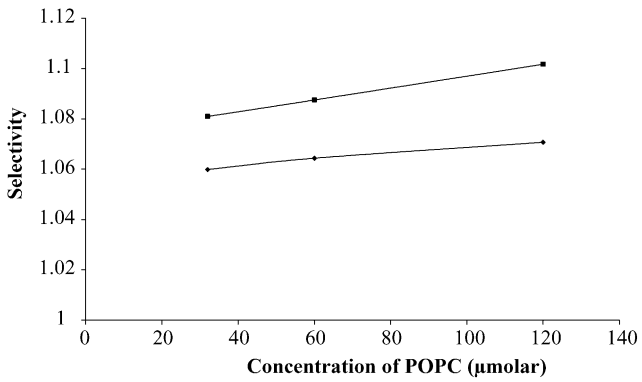

Fig. 6. Plots of the selectivity for the two protein pairs: (♦) cytochrome *c* and lysozyme; (■) ribonuclease A and α-chymotrypsinogen A as a function of POPC concentration in 20 mM citrate buffer pH 4.0. Capillary and experimental conditions as in Fig. 2.

[31,33]. As expected, such plots show for both basic proteins the increasing of electrophoretic mobility with decreasing pH (in accordance with the increasing degree of protonation of amino groups) and the inversion of migration order of cytochrome *c* and lysozyme arising from the different slopes of the curves describing the dependence of the electrophoretic mobility from pH for these proteins, which cross each other between pH 4.0 and 5.0.

4. Conclusions

The results of the present study strongly suggest that the separation of basic proteins can be performed with liposomes consisting of the phospholipid 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine used as a pseudo-stationary phase dispersed in the electrolyte solution. The liposomes formed by this phospholipid show strong affinity for the capillary wall of bare fused-silica capillary, resulting in the variation of the electroosmotic flow and in the repression of the interactions between basic proteins and the capillary.

The enhancement of protein separation and the variations in the intrinsic selectivity of the system observed in the presence of POPC in the capillary are indicative of selective interactions between the basic proteins and liposomes. This observation evidences the potentiality of protein liposome capillary electrophoresis, which can be significantly enlarged if we consider the possibility of using liposomes tailored for a given separation problem by preparing vesicles with phospholipids of properly engineered composition.

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