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Stabilization of phosphatidylcholine coatings in capillary electrophoresis by increase in membrane rigidity

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Abstract

Divalent cations affect the stability and structure of phospholipid vesicles and also the binding and immobilization of proteins into phospholipid membranes. The effect of calcium, magnesium, and zinc on zwitterionic phosphatidylcholine (PC) coatings in fused silica capillaries for electrophoresis was the primary interest in this work. In addition, the effect of temperature on the coating stability was investigated by coating 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) liposomes at temperatures above and below the gel- to fluid-state transition. All coatings were performed with PC large unilamellar vesicles (LUV) in 40 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) at pH 7.4 as basic solution. HEPES (40 mM) at pH 7.4 was used as background electrolyte (BGE) throughout the study. The stability of the coating was studied by measuring the electroosmotic flow. A molar ratio of 1:3 PC/Ca^{2+} or PC/Mg^{2+} gave the best coating stability owing to the increased rigidity of the phospholipid membrane furnished by the divalent metal ions. Better results were obtained with DPPC in the more rigid gel state than in the fluid state: the electroosmotic flow was much suppressed and the PC coating was stabilized. Coating the fused silica capillary with PC liposome–metal ion buffer solutions resulted in good electrophoretic separation of basic model proteins (p*I*-values 7.8–11.0). The electrophoretic results demonstrate the importance of stabilizing the phospholipid coating on fused silica capillaries, either by the addition of divalent metal ions (Ca^{2+}, Mg^{2+}, or Zn^{2+}) or by working in the gel-state region of the phospholipid.

Keywords: Capillary electrophoresis; Capillary coating; Electroosmotic flow; Divalent metal cations; Phosphatidylcholine; Zwitterionic liposomes; Proteins

1. Introduction

Liposomes, or phospholipid vesicles, are good models for biological membranes [1]. Their amphiphilic nature allows liposomes to be used as drug delivery systems for both hydrophilic and hydrophobic compounds. Phospholipids spontaneously form bilayers in aqueous solutions as they have two fatty acyl chains attached to glycerol along with a phosphate group. Zwitterionic phosphatidylcholine (PC) is considered to form the backbone of cellular membranes. Bilayer-forming lipids exist in gel or fluid state depending on the temperature of their environment.

Divalent cations affect the stability and structure of phospholipid bilayers and also the binding and insertion of proteins [2]. The effect of calcium on negatively charged liposomes has been widely investigated. Calcium is a strong fusogenic agent, promoting the fusion of cells and liposomes [3,4]. The ability of phospholipids to form supported planar bilayers can, therefore, be manipulated through the addition of calcium ions. Previously in our laboratory we showed that calcium stabilizes a 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphatidylcholine (POPC)/phosphatidylserine (PS) coating on fused silica capillaries [5].

Hincha [6] investigated the aggregation of liposomes in fluorometric measurements. He showed that liposomes containing 50% (w/w) of eggPC and 50% of the glycolipid digalactosyldiacylglycerol aggregated at $CaCl_2$ concentrations above 6 mM. The solution contained 5 mg ml^{-1} of the lipids, which is equal to ∼6 mM. Membranes that contained only eggPC did not aggregate in the presence of $CaCl_2$ at concentrations up to 26 mM. Whereas the aggregated liposomes containing glycolipids were stable, aggregation destabilized liposomes containing negatively charged lipids.

Infrared spectroscopic (IR) results have shown that the binding of divalent cations (Mg^{2+}, Ca^{2+}, and Sr^{2+}) to negatively charged 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol (DMPG) induces a crystalline-like gel state with

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highly ordered and rigid all-*trans* acyl chains [7]. The binding of these cations induces a tighter packing of the acyl chains, suggesting a deep penetration of the cations into the polar head group region of DMPG bilayers. The authors suggested that the primary binding sites of these cations are the phosphate group of the phosphodiester moiety, but indirectly also the ester carbonyl groups are affected. In a recent molecular dynamics study [8], however, a sequential binding of calcium ions to the lipid carbonyl oxygens was observed. In the same simulations a significant increase in the fatty chain order was seen. Also, IR results have confirmed the conformational changes in the carbonyl group region of POPC and the stabilization of the gel state induced by divalent cations [9]. Very commonly earth alkali metals such as calcium and magnesium are used to induce membrane fusion, but IR results for the interaction of zinc [10] with phospholipid membranes suggest that zinc, which is a transition metal, is an even more potent divalent cation.

Metal cations have a greater influence on membranes of anionic lipids than on neutral or zwitterionic membranes because of the stronger attractive coulombic forces. It has been demonstrated that cations (e.g. Mg^{2+}, Ca^{2+}, and Ba^{2+}) elute in electrostatic ion chromatography with a *N*-dodecylphosphocholine stationary phase and water as the mobile phase in an order ($Ba^{2+} < Mg^{2+} < Ca^{2+}$) that differs from that of cation exchange [11]. This shows that the zwitterionic phospholipids interact with analyte ions in a unique way, on the basis of coulombic attraction and repulsion and ion-pair formation.

The effect of calcium on supported bilayers has been imaged by atomic force microscopy [12]. The organization of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) in supported bilayers of 1,2-dioleyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleyl-*sn*-glycero-3-phospho-L-serine (DOPS) is affected by the presence of calcium. In the absence of calcium, large domains of DPPC were found in mixtures of DPPC/DOPC and DPPC/DOPS, but in the presence of calcium small isolated DPPC domains were found in the DPPC/DOPS mixture.

In this work we examined how divalent cations (Ca^{2+}, Mg^{2+}, and Zn^{2+}) affect the stability of PC coatings in fused silica capillaries and whether the temperature-induced rigidity of the phospholipid bilayer affects the coating stability. The effect of temperature was tested with DPPC (main transition temperature 41 °C) in fluid and gel state. Investigation was also made of the effect on separations of basic model proteins (p*I*-values 7.8–10.5) when divalent cations were added to the liposome solution during coating.

2. Experimental

2.1. Materials

N-(2-Hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), POPC, and the proteins α-chymotrypsinogen A (M_r 25 000; isoelectric point, p*I* = 9.3), lysozyme (M_r 143 000; p*I* = 11.0), ribonuclease A (M_r 13 700; p*I* = 7.8), and trypsin (M_r 23 300; p*I* = 10.5), were purchased from Sigma Chemical Co. (St Louis, MO, USA). DPPC and eggPC were purchased from Avanti Polar Lipids Inc. (Alabaster, USA). The pH solutions (4, 7, and 10) used for calibrating the pH meter and magnesium chloride were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO), sodium hydroxide (1.0 M), and nitric acid (1.0 M) were purchased from Oy FF-Chemicals (Yli Ii, Finland), calcium chloride from Fluka AG (Buchs, Switzerland), zinc chloride from Riedel-de Haën AG (Seelze, Germany), methanol from Mallinckrodt Baker (Deventer, The Netherlands), and chloroform from Rathburn (Walkerburn, UK).

2.2. Equipment

Electrophoretic measurements were made with a Beckman P/ACE System 2000 and 2001 equipped with a wavelength-selectable UV detector (detection at 200 nm and 215 nm) and liquid thermostating of the capillary, or with a Hewlett-Packard ^{3D}CE system (Agilent, Waldbronn, Germany) equipped with a diode array detector (detection at 200 nm and 215 nm) and air thermostating of the capillary. A water bath with a circulating thermostat Lauda RE 104 (Lauda, Lauda-Königshofen, Germany) connected to the Hewlett-Packard ^{3D}CE system was used to control the temperature of the vial carousel.

Uncoated fused silica capillaries used for coating and in CE separations were from Composite Metal Services (Worcestershire, UK). Dimensions were 50 μm i.d. × 375 μm o.d. The length of the capillary to the detector was 30.0 cm with a total length of 37 cm (Beckman) or 38.5 cm (Hewlett-Packard), unless otherwise mentioned.

A nitrogen-evaporating unit (Pierce, Reacti-Therm Heating Module No. 18790, Rockford, IL, USA) and an excicator equipped with a vacuum pump (KNF Neuberger, Freiburg, Germany) were used to evaporate chloroform from the phospholipid mixture. A shaking water bath (SB-16 Techne, Duxford, UK) with a thermostat (HETO, Birkeröd, Denmark) was used to hydrate liposomes into buffer solution, and a vortexer (Heidolph, REAX 2000, Germany) was used to accelerate hydration. Liposomes were extruded to large unilamellar vesicles (LUVs) with a LiposoFast-Basic or a LiposoFast low-pressure homogenizer (Avestin, Ottawa, Canada).

2.3. Methods

2.3.1. Liposome preparation

Appropriate amounts of the lipid stock solutions in chloroform were mixed to obtain the desired compositions. The mixture was evaporated to dryness under a stream of nitrogen, and traces of solvent were removed by evacuation under reduced pressure for 24 h. The lipid residues were hydrated in 40 mM HEPES pH 7.4 at 60 °C to yield multilamellar vesicles (MLV) with a lipid concentration of 2–4 mM, and the vesicles

were maintained at this temperature for 60 min with subsequent vortexing. The resulting dispersion was processed to large unilamellar vesicles by extrusion 19 times through Millipore (Bedford, MA, USA) 0.1 μm pore size polycarbonate filters using a LiposoFast extruder, essentially as described previously [13,14]. DPPC was extruded in fluid state at 60 °C. The other PC solutions were extruded at room temperature. After preparation the liposome solutions were stored in a refrigerator and the stock solutions of phospholipids in chloroform in a freezer.

2.3.2. Sample and buffer preparation

The concentration of the HEPES solution was 40 mM, with pH adjusted to 7.40 with 1.0 M sodium hydroxide (ionic strength 18 mM). Before use the background electrolyte (BGE) was filtered through 0.45 μm Millipore filters (Bedford, MA, USA) using a Millipore vacuum system. The BGE solution did not contain any liposomes.

The liposome solutions used for coating the capillary contained 0.1–3 mM (usually 0.75 mM) of POPC, eggPC, or DPPC in 40 mM HEPES with different concentrations (0–60 mM) of $CaCl_2$, $MgCl_2$, or $ZnCl_2$.

Protein stock solutions were prepared in water (2 mg ml^{-1}). The protein samples were diluted in water from stock solutions. The concentrations were as follows: 200 μg ml^{-1} of ribonuclease A, 250 μg ml^{-1} of lysozyme and trypsin, and 500 μg ml^{-1} of α-chymotrypsinogen A.

The migration time of DMSO (0.05%, v/v) was used as a marker for the electroosmotic flow (EOF). All solutions were stored in a refrigerator, except the stock solutions of proteins in water, which were stored in a freezer.

2.3.3. Capillary coating

The fresh capillary was rinsed at a pressure of 930–940 mbar for 10 min with 0.5 M nitric acid, then for 20 min with water. Phospholipid coating was applied to the capillary inner surface as follows: after preconditioning, the capillary was rinsed for 10 min with liposome solution at 930–940 mbar; then it was left to stand filled with the liposome solution for 15 min.

2.3.4. Capillary electrophoretic separations

CE separation conditions were as follows: voltage 20 kV, temperature of the capillary cassette 25 °C or 45 °C, injection of DMSO for 2 s at 50 mbar and of proteins for 5 s at 50 mbar. Before runs and before each injection the capillary was rinsed for 2 min with the BGE solution or for 1 min with liposome solution and 1 min with BGE solution. During a long series of runs, the quality of the BGE solution was ensured by change of the buffer vials after every fifth or sixth run.

3. Results and discussion

In previous work we demonstrated the coating of capillaries with liposomes composed of zwitterionic PC, anionic PS, and neutral cholesterol in the presence of HEPES or Ca^{2+} or both [5,14]. The capillary coated with PC alone was not stable unless it was rinsed with liposomes before each run [14]. The addition of Ca^{2+} to zwitterionic 1,2-dilauroyl-*sn*-phosphatidylcholine (DLPC) [15] and anionic POPC/PS [5] liposomes improved the coating stability and simplified the optimization of the coating conditions. In this work we investigated the stabilization of a PC coating by the addition of divalent cations Ca^{2+}, Mg^{2+}, and Zn^{2+} and by working in the gel-state region of the phospholipid.

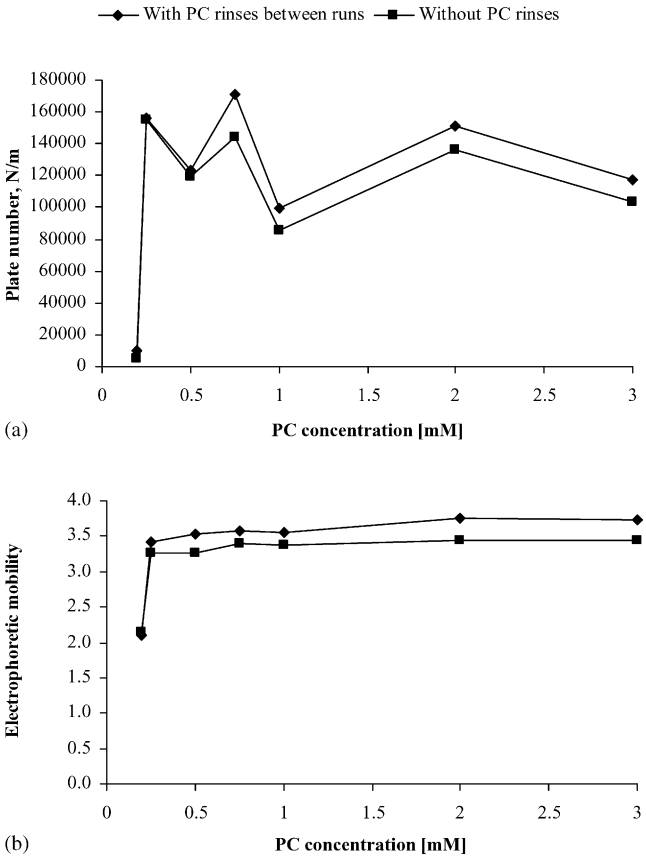

Fig. 1. Effect of PC concentration (0.1–3 mM) on the coating stability. The PC/Ca^{2+} ratio was held constant at 1:20. Capillary 38.5 cm (effective length 30 cm) × 50 μm i.d. × 375 μm, o.d. Injection of 250 μg ml^{-1} lysozyme in water for 5 s at 50 mbar. (a) The electrophoretic mobilities and (b) the plate number for lysozyme. Separation conditions 20 kV, 25 °C, 215 nm.

3.1. Phosphatidylcholine concentration

As a first step we optimized the PC concentration in the coating step. The phospholipid–metal ion molar ratio was held constant at 1:20. Earlier we found solutions containing 3 mM PC to result in a stable coating [5,14]. Phospholipids are of relatively high cost, however, and it was desirable to find the lowest concentration that would give a good coating in the capillary. The estimation of coating stability was based on the run-to-run electrophoretic mobility and the plate number of the lysozyme used as model basic protein. Fig. 1 shows that the liposomes did not coat the capillary at PC concentra-

tions below 0.2 mM. The best results (highest plate numbers) were obtained with 0.75 mM PC, and this concentration was chosen for further experiments. The electrophoretic mobility was fairly stable at all PC concentrations from 0.25 mM PC to 3 mM PC (Fig. 1), showing that the net charge of the surface was almost constant and the silanols on the fused silica capillary were shielded by the PC coating. Fluctuations in the plate number of lysozyme were probably due to lysozyme interacting in different ways with the PC coating. The thickness of the coating may have varied with the PC concentration, or lysozyme may have interacted with the calcium added to the PC solution, or with PC lipids leaking out from the capillary. All these factors can affect the plate numbers.

3.2. Metal concentration

The effect of adding calcium or magnesium to the liposome solution during the coating step was then investigated. The PC concentration was held constant at 0.75 mM and metal ions were added up to a molar ratio of 1:20 for PC/metal ion. The electroosmotic flow as a function of number of injections is shown in Fig. 2. In the first 10 runs, flushing was done for 1 min with the liposome solution and 1 min with the BGE before each injection. In the following 10 runs the capillary was only flushed with BGE, for 2 min before each injection. The dotted line (Fig. 2) indicates the change in flushing conditions.

From Fig. 2 we can see that both calcium and magnesium affected the phospholipid coating. For PC/Ca^{2+}, the electroosmotic flow was most strongly suppressed at a molar ratio of 1:3 and the stability of the coating (smallest R.S.D. for the electroosmotic mobilities) was then the best. In the case of PC/Mg^{2+}, molar ratios of 1:3 and 1:10 gave the best and equally good results. Increasing the metal concentrations to a molar ratio of 1:20 did not improve the coating stability: evidently the PC membrane was saturated with metal ions.

Calcium and magnesium form complexes with the phosphate groups and dehydrate them [9] or they are immobilized into the membrane and cause conformational changes in the carbonyl region of PC [8]. A combined effect is also possible. The atomic weights of calcium and magnesium are 40.1 and 24.1 and the ionic radii are 99 pm and 72 pm, respectively, and these different ion sizes could explain why calcium and magnesium affect the PC coating in different ways. In the case of immobilization in the phospholipid membrane, the size of the metal ion might determine how well it can be immobilized. Since magnesium is smaller it can more easily penetrate into the membrane, and a higher concentration of magnesium than of calcium can stabilize the coating.

As seen from the electroosmotic mobilities in Fig. 2, neither metal ion was able to stabilize the coating well enough without a liposome solution flush between the runs. This was as expected because divalent cations do not interact strongly with zwitterionic PC liposomes [10]. Ca^{2+} interacts fairly strongly with anionic liposomes [5] through electrostatic interactions. However, the electrostatic interactions with zwitterionic liposomes such as PC are weak.

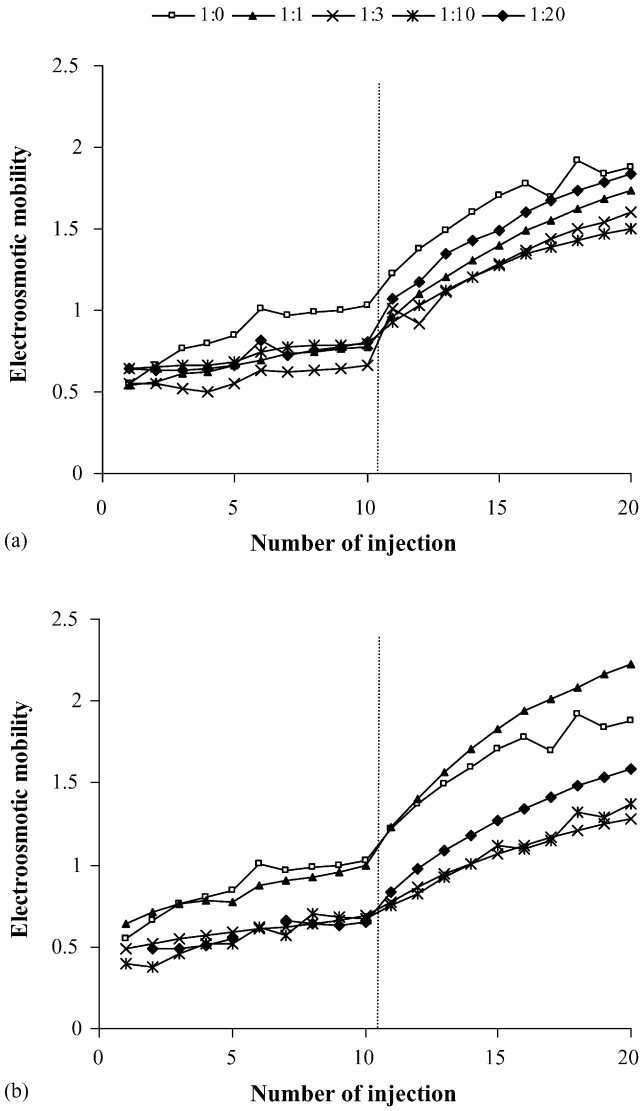

Fig. 2. Effect of (a) Ca^{2+} and (b) Mg^{2+} on the coating stability. The concentration of PC was 0.75 mM and the PC/metal ion molar ratio varied between 1:0 and 1:20. Capillary as in Fig. 1. Injection of 0.05% DMSO in BGE for 2 s at 50 mbar. Separation conditions 20 kV, 25 °C, 215 nm. The dotted line indicates the change in flushing conditions from flushing 1 min with liposomes solution plus 1 min with BGE to flushing 2 min with BGE.

As noted by Garidel et al. [7], the addition of calcium or magnesium to lipid bilayers makes the bilayer more gel-like. Evidently, interaction with multivalent cations affects the packing of phospholipids in the bilayer through rearrangements of the zwitterionic head group [16]. In previous work [14] we demonstrated that adding cholesterol to the liposome solution resulted in a stable coating. Cholesterol minimizes the movement of the acyl chains in the bilayer and makes the bilayer more rigid and gel-like [17]. On the basis of these findings, we infer that making the liposomes gel-like and rigid is essential for achieving a stable coating on a fused silica capillary.

3.3. Gel-fluid states of the liposomes

Puu and Gustafson [2] found DPPC to exhibit much better fusion than POPC when proteoliposomes containing 50% of DPPC or POPC were transferred onto platinum or silicon supports. They suggested that nonperfect liposomes, prepared under their transition temperature, may more easily form planar lipid membranes than liposomes prepared at higher temperature.

Depending on the temperature of the environment, phospholipids exist in gel or fluid state. The phase transition behavior of liposomes has been reviewed by Taylor and Morris [18]. In the gel state, the hydrocarbon chains are fully extended and all in *trans* conformation and the polar head groups are relatively immobile. When the system is heated the phospholipids undergo transition to the fluid state where the polar head groups are more mobile and the hydrocarbon chains are disordered due to changes from all *trans* to *trans* and *gauche* conformations of the carbon bonds. The transition temperature depends on the nature of the polar head group and on the length and degree of saturation of the hydrocarbon chains [18]. The transition temperature increases with the degree of saturation and length of the hydrocarbon chain. Thus, DPPC, which is a fully hydrated long chain PC, has a higher transition temperature (41 °C) than POPC (−2 °C) or eggPC (−5 °C to −15 °C) [17].

We investigated the effect of the temperature-induced gel-to fluid-state transition on the coating stability by studying DPPC above and below its transition temperature (41 °C). The capillary was coated as described in the experimental section. After coating, the liposomes were flushed out of the capillary for 2 min with BGE. Before the runs were started a 20 min period was allowed for heating (from 25 °C to 45 °C) or cooling (from 25 °C to 45 °C) the capillary. Four different systems were investigated: working at (1) 25 °C or (2) 45 °C during both coating and runs, (3) coating of the capillary at 25 °C with runs made at 45 °C, and (4) coating at 45 °C with runs made at 25 °C. Between the runs the capillary was flushed with BGE for 2 min. The stabilities of these four systems during runs are presented in Fig. 3.

As seen from Fig. 3, the coating is most stable and the eof is most strongly suppressed when both coating and runs are done at 25 °C. The eof is much less suppressed with coating and runs at 45 °C and it increases with each injection evidently owing to the slow leak of lipids out of the capillary. Coating at 25 °C with runs at 45 °C shows an initial suppression of the eof to almost the same level as when both coating and runs were made at 25 °C. With continued heating at 45 °C, however, there is a similar trend as for coating and heating at 45 °C as the liposomes are transferred to their fluid state and leak out of the capillary. With the fourth system (coating at 45 °C, runs at 25 °C) the eof is less suppressed, but after cooling of the capillary to 25 °C the eof is stable and the liposomes do not leak out of the capillary because they are in the gel state.

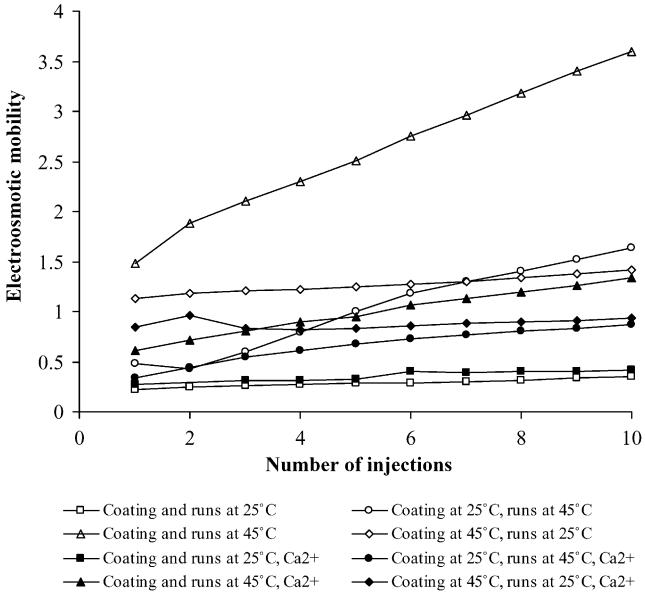

Fig. 3. Effect of temperature on stability of DPPC coating with and without added calcium. Coating and running temperatures were as follows: Symbols on left, coating and runs at 25 °C or 45 °C, symbols on right, coating at 25 °C and runs at 45 °C, coating at 45 °C and runs at 25 °C.

In addition, the effect of calcium on the DPPC coating was studied. The same four systems were used but with the addition of Ca^{2+} to the DPPC solution (molar ratio 1:3 for DPPC/Ca^{2+}) (Fig. 3). Adding calcium generally improved the coating by lowering and stabilizing the eof. For the system where both coating and runs were made at 25 °C, however, calcium did not affect the coating, suggesting that working in the gel state does not require the addition of calcium. In other words, working in the gel state improves the coating stability more than adding calcium to DPPC.

Working with DPPC in the gel state suppressed the eof more than when working with PC and adding calcium or magnesium to the system (cf. Figs. 2 and 3). The mean electroosmotic mobility of 10 runs with DPPC was 0.288 × 10^{-8} m^2 s^{-1} V^{-1}, whereas for PC/Ca^{2+} (1:3) it was 0.588 × 10^{-8} m^2 s^{-1} V^{-1} and for PC/Mg^{2+} (1:3) it was 0.596 × 10^{-8} m^2 s^{-1} V^{-1}. The coating is clearly better when the liposomes are in the gel state, because the liposomes, being more rigid, more easily form a planar bilayer.

3.4. Evaluation of phosphatidylcholine coating with proteins

Basic proteins cannot be studied by electromigration techniques at neutral pH on an uncoated fused silica capillary because of the strong interactions between the negative silanol groups and the positively charged proteins. We examined the shielding of silanols by coating the capillary with PC/Ca^{2+} and using proteins as model analytes.

The PC/Ca^{2+} capillaries were coated as described in the experimental section. A sample containing lysozyme, trypsin, ribonuclease A, and α-chymotrypsinogen A was

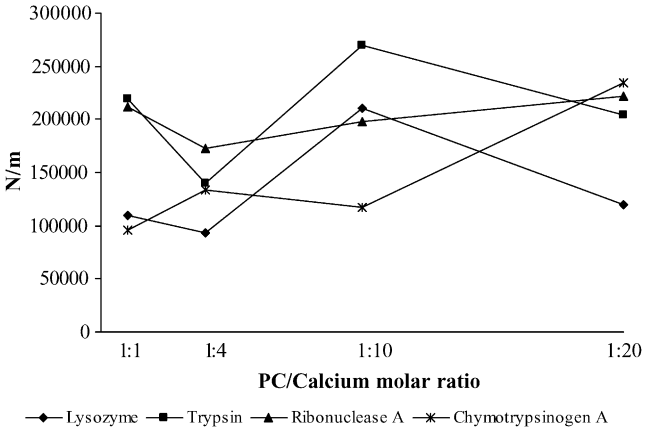

Fig. 4. Plate numbers for lysozyme, trypsin, ribonuclease A, and α-chymotrypsinogen A as a function of PC/Ca^{2+} ratio. The capillary was rinsed for 1 min with liposome solution and for 1 min with BGE before each injection of proteins for 5 s at 50 mbar. The plate numbers are an average of six injections. BGE: 40 mM HEPES at pH 7.4. Separation conditions: capillary 37 cm (effective length 30 cm) × 50 μm i.d. × 375 μm o.d.), 20 kV, 25 °C, 215 nm.

injected into a coated capillary (see experimental section for molecular masses and p*I*-values). Fig. 4 shows the plate numbers for the four proteins as a function of PC/Ca^{2+} molar ratio for separations with liposome flushes before each injection. The plate numbers are the average of six protein injections. As can be seen from Fig. 4, the plate numbers for ribonuclease A and α-chymotrypsinogen A improve as the amount of calcium increases. This might be because the coating becomes more positively charged at higher PC/Ca^{2+} molar ratios and repels the positively charged proteins. For lysozyme and trypsin the plate numbers reach a maximum at a molar ratio of 1:10. Lysozyme and trypsin interacted to some extent with the phospholipids or calcium, or with both. Most proteins contain some histidine or tryptophan on their surface and have an affinity for heavy metals, forming complexes with them [19]. In immobilized metal ion affinity chromatography (IMAC), proteins are eluted from the gel in an order depending on the histidine or tryptophan content on the surface of the proteins. Any metal ion with an excess of d-electrons is suitable for IMAC. The most popular metals are Ni^{2+}, Cu^{2+}, Zn^{2+}, and Co^{2+}, but Ca^{2+}, which is an earth alkali metal, has been used as well. Since proteins have an affinity to calcium, the decrease in plate numbers for lysozyme and trypsin at PC/Ca^{2+} molar ratio 1:20 might be due to the excess calcium in the coating. The relative standard deviations (%R.S.D.) for the migration times of the proteins in PC/Ca^{2+}-coated capillaries were less than 4.5% for the runs with liposome flush before each injection, and as much as 9% when the capillary was only flushed with BGE before each injection.

For further study of the phospholipid coating, comparison was made of capillaries coated with PC/Ca^{2+} (1:20) and PC/Mg^{2+} (1:20) (Table 1). For both calcium and magnesium the runs with liposome flush before each injection gave better R.S.D. of the migration times. As can be seen, in general the R.S.D. for magnesium are twice as large as those for calcium.

Fig. 5 shows the separations of proteins on PC-coated capillaries. The liposome solution contained PC and either Ca^{2+}or Mg^{2+} at a molar ratio of 1:20. Lysozyme is tailing, probably due to the above-noted interactions with the coating. Proteins adsorb onto phospholipid surfaces [20], but to a lesser extent on phospholipids like PC with no net charge. Holmlin et al. [21] showed that zwitterionic self-assembly monolayers terminating in a phosphatidylcholine headgroup decrease the adsorption of proteins from aqueous buffer but cannot completely eliminate it. α-Chymotrypsinogen A decomposes slightly under the influence of trypsin, which works as a proteolytic enzyme. The other proteins in the sample may also be digested by trypsin in some degree. However, this did not cause any major problems since the sample was only used at 25 °C for a few hours and a fresh sample was prepared each time a new coating was tested.

In study of a series of mono- and divalent metal cations, including calcium and magnesium, Binder and Zschörnig [9] found zinc to possess the strongest effect on the lipid phase behavior, followed by calcium. Zinc forms bridges between neighboring phospholipid molecules, dehydrating the headgroups and increasing the fusion of the lipid membranes [10]. In view of that result, we examined how zinc, which also is a divalent ion but a transition metal, interacts with the PC coating. Zinc precipitates at pH 7.4 as $Zn(OH)_2$ and the experiments were therefore carried out at pH 6.5. Fig. 6 shows a comparison of Zn^{2+} with Ca^{2+} and Mg^{2+} where a fused silica capillary was coated with PC/metal ion in molar ratio 1:20 and flushed between runs with the liposome solution. A protein sample was injected and the average plate numbers for the proteins were calculated. The plate numbers for the

Table 1
Relative standard deviations (%R.S.D.) of the migration times for proteins separated on capillaries [37 cm (effective length 30 cm) × 50 μm i.d. × 375 μm o.d.)] coated with PC/Ca^{2+} or PC/Mg^{2+}, molar ratio 1:20

	PC/Ca^{2+}, 1:20		PC/Mg^{2+}, 1:20	
	Liposome flush (%)	BGE flush (%)	Liposome flush (%)	BGE flush (%)
Lysozyme	0.13	1.08	0.27	2.07
Trypsin	0.43	1.77	0.3	2.59
Ribonuclease A	0.28	2.48	0.49	3.84
α-Chymotrypsinogen A	0.59	1.99	1.28	4.02

Six runs were made with liposome flushes between the runs and six runs with BGE flush between the runs. BGE: 40 mM HEPES at pH 7.4. Separation conditions: 20 kV, 25 °C, detection 215 nm.

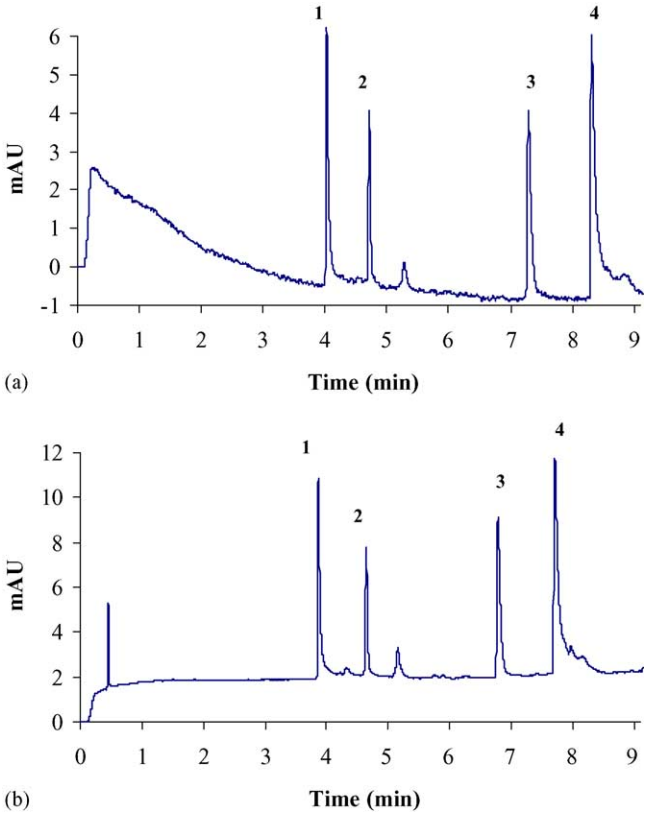

Fig. 5. Protein separations on a fused silica capillary (as in Fig. 4) coated with (a) PC/Ca^{2+} (1:20) and (b) PC/Mg^{2+} (1:20) in 40 mM HEPES at pH 7.4. Injection of (1) lysozyme, (2) trypsin, (3) ribonuclease A, and (4) α-chymotrypsinogen A for 5 s at 50 mbar. Separation conditions: 20 kV, 25 °C, 215 nm.

proteins are lower at pH 6.5 than at pH 7.4 (cf. Fig. 4). The plate numbers at pH 6.5 are similar in the presence of calcium and magnesium and considerably lower in the presence of zinc. This is probably due to interactions between zinc and the proteins. Zinc is one of the most widely used metal ions in IMAC since it has an affinity for proteins and peptides

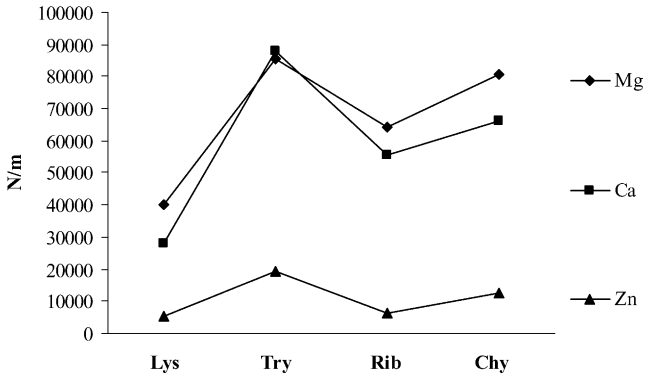

Fig. 6. Plate numbers for proteins separated on a fused silica capillary (as in Fig. 4) coated with POPC and Zn^{2+}, Ca^{2+}, or Mg^{2+}. PC/metal ion molar ratio of 1:20, in 40 mM HEPES at pH 6.5. The protein sample was injected for 5 s at 50 mbar and the separation was made in 25 °C at 20 kV and detection at 215 nm.

and forms complexes with them [19]. The migration times of the proteins were similar for zinc, calcium, and magnesium (data not shown). The R.S.D. for the migration times were less than 1% when coating was done with PC and Zn^{2+}, Ca^{2+}, or Mg^{2+}. Even though the plate numbers were worse when zinc was added, the stability of the coating was as good for zinc as for the other metal ions.

Protein separations were also carried out on DPPC-coated capillaries (results not shown). A sample of lysozyme, ribonuclease A, and α-chymotrypsinogen A was injected to a capillary coated with DPPC in 40 mM HEPES at pH 7.4. The R.S.D. for the migration time of the proteins on the DPPC-coated capillary were less than 1%, but the plate numbers were poor (maximum 100 000 m^{-1}).

4. Conclusions

The effect of divalent metal ions calcium, magnesium, and zinc on phospholipid membrane was studied by coating a fused silica capillary with a liposome solution containing metal ion. All metal ions improved the stability of the PC-coated capillary. Increased rigidity, induced by metal ions immobilized into the membrane, is suggested to be the major reason for the improvement. Of the three metal ions, calcium appeared to improve the stability of the coating most. The effect of the temperature-induced gel- to fluid-state transition on the coating stability was investigated by coating a fused silica capillary with DPPC above and below the transition temperature of the liposome. Working in the gel state significantly increased the stability of the coating, confirming that rigidity of the membrane is essential in improving the coating stability. Injection of basic proteins to the PC-coated capillaries showed that silanols in the capillary were almost completely shielded by the PC coating. Some tailing of the protein peaks was observed, most probably due to interactions between the proteins and the PC lipids or the immobilized metal ions.

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