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Phospholipid Bilayer Coatings for the Separation of Proteins in Capillary Electrophoresis

Jennifer M. Cunliffe, Nicole E. Baryla, and Charles A. Lucy*

Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2 Canada

The double-chained, zwitterionic phospholipid 1,2-dilauroyl-*sn*-phosphatidylcholine (DLPC, C_{12}) was investigated for its use as a wall coating for the prevention of protein adsorption in capillary electrophoresis. DLPC forms a semipermanent coating at the capillary wall, which allows excess phospholipid to be removed from the capillary prior to electrophoretic separation. A DLPC-coated capillary allowed for the separation of both cationic and anionic proteins with efficiencies as high as 1.4 million plates/m. Migration time reproducibility was less than 1.3% RSD from run to run and less than 4.0% RSD from day to day. Protein recovery was as high as 93%. Cationic and anionic proteins could be separated over a pH range of 3−10, all yielding good efficiencies (*N* up to 1 million plates/m). The chain length of the phospholipid affected the performance of the wall coating. The C_{10} analogue of DLPC (DDPC) did not form a coating on the capillary wall while the C_{14} analogue of DLPC (DMPC) formed a stable coating that prevented protein adsorption to the same extent as its C_{12} counterpart.

Recently, the Human Genome Project has captured the attention of many scientists.[1] Almost all of the human genes have now been decoded, several years earlier than expected. This premature completion is largely in thanks to the fast, efficient separations that capillary electrophoresis (CE) can provide.[2] Currently, researchers are moving to the next step and are trying to determine which proteins particular genes express. This emerging field is called proteomics. In essence, proteomics is the true goal of understanding the human makeup, since it is the proteins that dictate the biological activity of an organism. Since CE was such an invaluable separation tool for the genome,[3] much research is centered on CE's possible role in proteomics.

CE offers advantages of faster separation times, higher efficiencies, smaller volumes, and automation[4] over more traditional protein separation methods such as chromatography and slab gel electrophoresis. However, the separation of proteins using CE is not always possible. Cationic proteins have a tendency to adsorb to the negatively charged capillary wall. This makes it virtually impossible to separate cationic proteins without altering the chemistry at the silica wall. Fortunately, there has been a tremendous amount of research in this area and many different capillary coatings are available. Popular methods include permanent covalently bonded coatings,[5−8] adsorbed cationic polymers,[9,10] and surfactant-based dynamic coatings.[11−15] A detailed discussion of capillary coatings can be found in Horvath and Dolnik's recently published review article.[16]

Surfactants are becoming increasingly popular for deactivation of the silanols at the capillary wall since they are inexpensive and simple to apply. Surfactants that have an affinity for the capillary wall are added to the electrophoretic buffer. They adsorb to the capillary surface and thus alter the surface charge, effectively shielding the negative silanols from the bulk solution. However, traditional surfactant coatings require that the monomers be present in the buffer to maintain a stable coating.[17] This reduces the possibility of coupling the CE separation with electrospray ionization mass spectrometry (ESI-MS) detection,[18,19] an increasingly popular and powerful method for protein identification.

Melanson et al. recently investigated the double-chained cationic surfactant didodecyldimethylammonium bromide (DDAB) as a dynamic coating in CE.[20] The monomers aggregated to form a flat bilayer structure at the capillary wall, which was confirmed through atomic force microscopy (AFM) imaging.[21] The homogeneous DDAB coating was incredibly stable, such that the excess surfactant could be removed from the buffer prior to CE separation and the electroosmotic flow remained constant for 75 min.[20] DDAB

* To whom correspondence should be addressed. Facsimile: (780) 492-8231. Electronic mail: charles.lucy@ualberta.ca.

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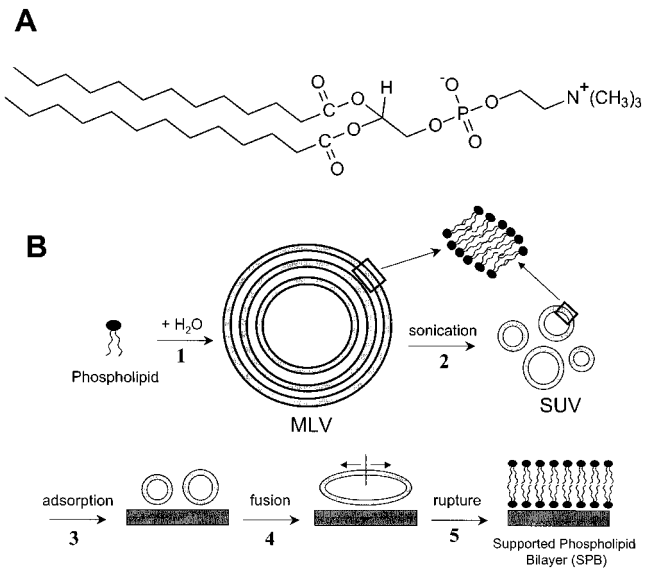

Figure 1. (A) Structure of 1,2-dilauroyl-*sn*-phosphatidylcholine (DLPC). (B) Steps in the formation of a supported phospholipid bilayer (SPB). See text (Background) for details.

was thus termed a semipermanent coating as opposed to a dynamic coating. Although a DDAB-coated capillary can efficiently separate cationic proteins,[20,21] anionic protein adsorption is inevitable due to DDAB's cationic headgroup.

Holmlin et al. have shown that surfaces terminated in zwitterionic groups are resistant to protein adsorption.[22] Further, zwitterionic surfactants have been successful for the separation of both cationic and anionic proteins in capillary electrophoresis.[14,15,23−25] For example, the single-chained zwitterionic surfactant coco(amidopropyl)hydroxyldimethylsulfobetaine (CAS U) forms a capillary coating that enables protein separations with efficiencies greater than 500 000 plates/m.[14,15] AFM studies have confirmed that the single-chained surfactant CAS U aggregates at the capillary wall as spherical micelles[21] and therefore provides incomplete surface coverage. Also, this coating is unstable in the sense that the surfactant must be present in the separation buffer to keep the coating intact and therefore will not be compatible with MS detection.

The phospholipids termed phosphatidylcholines are essentially double-chained zwitterionic surfactants. They are often used to form supported phospholipid bilayers (SPB) in the study of biological membranes (see Background). Here we present a capillary coating technique for CE using the phospholipid 1,2-dilauroyl-*sn*-phosphatidylcholine (DLPC, Figure 1A). DLPC offers the stability of a semipermanent coating with the ability to separate both cationic and anionic proteins over a wide pH range.

BACKGROUND

Supported Phospholipid Bilayers (SPBs). A phospholipid is defined as a molecule with a polar headgroup (containing a phosphate group) and a double-chained hydrophobic tail. Phospholipids aggregate to form a bilayer where two lipid monolayers combine to form a two-dimensional sheet. Phospholipid bilayers are fundamental to the structure of all biological membranes. Biological membranes define the external boundary of cells and are vital for the survival of living organisms as they regulate the molecular traffic across the boundary. Scientists have used SPBs a lipid bilayer adsorbed on a solid support, to study the physical behavior of biological membranes and of membrane-bound macromolecules.[26,27]

A schematic identifying the steps in SPB formation is shown in Figure 1B. When a phospholipid is suspended in water, a cloudy solution is obtained due to the presence of multilamellar vesicles (MLVs; Figure 1B, step 1). MLVs form when stacks of bilayers become fluid, swell, and finally self-close. Once formed, the size of the particles can be reduced by using sonic energy (sonication). Following sonication, small unilamellar vesicles (SUVs; Figure 1B, step 2) are present in solution.[28] SPB formation is initiated by depositing a SUV solution over a substrate. Vesicle fusion, the formation of supported membranes on substrates from SUVs, is currently the most common and robust way of forming an SPB.[29,30] Several mechanisms have been reported for the formation of SPBs,[30−34] all of which report steps of adsorption, fusion, and rupture (Figure 1B, steps 3−5). For example, Reviakine and Brisson reported a three-step process for formation of SPBs by vesicle fusion.[34] Their model of SPB formation involves an initial step of vesicle adsorption to a surface, followed by fusion, which results in larger vesicles. Finally, the larger vesicles flatten and rupture, leading to the formation of a single bilayer adsorbed onto the surface. SPB formation is dependent on a number of factors including lipid type, buffer, vesicle size, lipid concentration, and the presence or absence of Ca^{2+}.[34,35] Some of these factors have implications in the formation of capillary wall coatings using phospholipids (see Results and Discussion).

Importance of a Uniform Coating in CE. The bilayer structures of double-chained surfactants are attractive for wall coatings in capillary electrophoresis. Flatter and more homogeneous coatings observed with double-chained surfactants translate into greater surface coverage and stability.[20,21] Specifically, prevention of protein adsorption improves[20,21] and a reproducible electroosmotic flow is achieved[20] using the double-chained surfactant didodecyldimethylammonium bromide (DDAB). In comparison, the single-chained surfactant cetyltrimethylammonium bromide (CTAB) does not prevent protein adsorption to the same extent, most likely due to the incomplete surface coverage the coating provides at the capillary wall.[21] Further, the coating was not as stable, as indicated by the electroosmotic flow dropping dramatically following removal of surfactant from the buffer.[20] Since

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phospholipids have proven to be protein resistant[22,36−39] and exhibit great structural integrity, this may translate into a more permanent coating at the capillary wall in CE.

EXPERIMENTAL SECTION

Apparatus. All data were acquired on a P/ACE MDQ capillary electrophoresis system (Beckman Instruments, Fullerton, CA) equipped with a UV absorbance detector. Data acquisition and control were performed using P/ACE Station software (Beckman) for Windows 95 on a 300-MHz IBM personal computer. Efficiencies were calculated with the P/ACE Station software using the peak width at half-height method. All electropherograms were baseline subtracted. The untreated fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) had an inner diameter of 51 μm and an outer diameter of 360 μm.

Chemicals. All solutions were prepared with Nanopure ultrapure water (Barnstead, Duburque, IO). Ultrapure tris(hydroxylmethyl)aminomethane (Tris; Schwarz/Mann Biotech) and 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES, 99%; Sigma) were used to prepare buffers. Tris buffers were adjusted to pH 3 or 7.4 using reagent grade hydrochloric acid (Anachemia). CHES buffers were adjusted to pH 10 using reagent grade sodium hydroxide (BDH). The phospholipids 1,2-dilauroyl-*sn*-phosphatidylcholine (DLPC), 1,2-didecanoyl-*sn*-phosphatidylcholine (DDPC), and 1,2-dimyristoyl-*sn*-phosphatidylcholine (DMPC) (all from Sigma) were used as received. The aggregation process of the phospholipids was accelerated by the addition of calcium chloride dihydrate (Molecular Biology Grade, Sigma). Mesityl oxide (Aldrich) was used as the neutral EOF marker. The proteins lysozyme (chicken egg white), cytochrome *c* (bovine heart), ribonuclease A (bovine pancreas), α-chymotrypsinogen A (bovine pancreas), insulin chain A oxidized (bovine insulin), trypsin inhibitor (soybean), and α-lactalbumin (bovine milk) were used as received from Sigma. Benzyl alcohol (99%, Aldrich) and benzoic acid (reagent grade, BDH) were used as internal standards for the cationic and anionic protein recovery studies, respectively. Fresh chicken egg samples were purchased from a local supermarket.

Preparation of Solutions Containing Phospholipids. Preparation of the phospholipid solutions involved the addition of 20 mM $CaCl_2$ to the buffer, followed by the addition of the phospholipid. Once all of the reagents were combined, the solution was sonicated (Bransonic 220, Shelton, CT) for 10-min periods. Between each 10-min period there was a 10-min "rest" interval where the solution was stirred at room temperature to cool.[30] This sonicate/stir cycle was repeated about three times or until the solution was clear. Solutions containing phospholipids were stored and used within 6 days. Migration time reproducibility and efficiency of protein separations were compromised after this period of time.

EOF Measurements. New 50-cm (40 cm to the detector) capillaries were used for all coating studies. Each new capillary was pretreated with 0.1 M NaOH for 5 min (20 psi), followed by Nanopure water for 5 min (20 psi). Prior to each injection, a solution of 0.1 mM DLPC in 20 mM Tris-HCl pH 7.4 buffer containing 20 mM $CaCl_2$ was rinsed through the capillary (20 psi) for 2 min. Mesityl oxide was hydrodynamically injected as the neutral EOF marker (0.5 psi for 3 s). A constant voltage of −20 kV (a negative voltage as the EOF was reversed due to the Ca^{2+} in the separation buffer) was applied and detection was at 254 nm. The capillary was thermostated to 25 °C. This procedure was repeated until a stable EOF was achieved (and hence the capillary was completely coated).

Once the coating procedure was complete, the capillary was rinsed with buffer (without calcium and phospholipid) for 1-min intervals to determine the coating stability. The strength of the EOF was measured after each 1-min rinse as an indirect measure of the coating stability upon high-pressure rinsing and applied voltage.

Two methods were used to calculate the electroosmotic mobility (μ_{EOF}). When the μ_{EOF} was greater than 1.0×10^{-4} cm^2/V·s, a single injection method was used as described above, where mesityl oxide was directly injected and a constant voltage was applied. However, when μ_{EOF} is less than 1.0×10^{-4} cm^2/V·s, the above method should not be used because migration times are very long. Williams and Vigh[40] introduced a sequential injection method for the measurement of low EOF. The first mesityl oxide marker was injected using low pressure (0.5 psi) for 3.0 s. This band was pushed through the capillary for 2 min using low pressure (0.5 psi). The second mesityl oxide marker was then introduced by an identical low-pressure injection, and both markers pushed through the capillary for 2 min using 0.5 psi pressure. A constant voltage of 15 kV was applied for 3 min causing the two markers to move within the capillary. A third marker was then injected, and all three bands were pushed to the detector using 0.5 psi pressure. Detection was at 254 nm.

Protein Separations. A new capillary (L_d = 40 cm, L_t = 50 cm) was initially rinsed with 0.1 M NaOH (5 min, 20 psi) and Nanopure water (5 min, 20 psi). It was then coated with 0.1 mM DLPC in 20 mM Tris-HCl pH 7.4 buffer containing 20 mM $CaCl_2$ for 20 min (20 psi). The capillary was rinsed with 20 mM Tris-HCl pH 7.4 buffer (1 min, 20 psi) to flush excess phospholipid and calcium from the capillary before protein samples were introduced. All protein mixtures (0.1 mg/mL in water) were injected for 3 s at 0.5 psi. Separation of proteins occurred in 20 mM Tris-HCl pH 7.4 buffer void of calcium and phospholipid. The applied voltage was 20 kV (+20 kV for cationic protein mixtures, −20 kV for anionic protein mixtures). Protein detection was performed at 214 nm. Between runs the capillary was rinsed with 0.1 mM DLPC in 20 mM Tris-HCl pH 7.4 buffer containing 20 mM $CaCl_2$ (5 min, 20 psi) followed by 20 mM Tris-HCl pH 7.4 buffer (1 min, 20 psi). For the different pH conditions and different phospholipids, the same procedure was used with the appropriate buffer substitutions.

Migration time reproducibility was determined by performing 16 replicate injections on three different days. A variation of the method of Towns and Regnier[41] was used for protein recovery studies.[14] A new 30-cm (20 cm to the detector) capillary was used for the study. Six replicate injections of a protein mixture were performed as previously described (total distance proteins travel to the detector is 20 cm). The 10-cm portion of the capillary was

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then used for the second set of six protein separations. For the cationic proteins, the rinses were adjusted to flow from the outlet vial to the inlet vial, injection was from the outlet vial, and a negative voltage was applied. These settings allowed the proteins to travel 10 cm to the detector (from the outlet to the inlet). An internal standard was used to correct for injection volume variation. The percent recoveries of the proteins were determined by comparing the peak area between the long and short portions of the capillary.

Egg White Protein Analysis. A new capillary (L_d = 40 cm, L_t = 50 cm) was pretreated and coated with DLPC as described in the Protein Separation section above. Egg white was diluted with 20 mM Tris-HCl pH 7.4 buffer in a 1:20 ratio and filtered through a Millex-HA 0.45-μm membrane (Millipore, Bedford, MA) before use. Egg white samples were injected for 3 s at 0.5 psi. Separation was performed in 20 mM Tris-HCl pH 7.4 buffer void of calcium and phospholipid. The applied voltage was 20 kV (+20 kV for lysozyme analysis, −20 kV for ovalbumin analysis). Detection was at 214 nm.

RESULTS AND DISCUSSION

Coating Time and Stability of DLPC. A capillary was coated by rinsing a DLPC solution through the capillary with high pressure. The EOF was used as an indirect measure of the completeness of the coating. The capillary was considered completely coated (refers to maximum coverage and is not necessarily 100%) when the magnitude of the EOF became constant, even after additional rinsing of the capillary with DLPC solution. Figure 2A shows that 0.1 mM DLPC in a pH 7.4 buffer of 20 mM $CaCl_2$ and 20 mM Tris-HCl requires no more than 4 min of high-pressure rinsing to obtain a constant EOF and, hence, to coat the capillary wall. After 4 min of coating, the EOF remains constant at -1.4×10^{-4} cm^2/V·s. To ensure the capillary was always completely coated, new capillaries were coated for at least 20 min prior to use.

The weakly reversed EOF in Figure 2A is consistent with the analogy between electrostatic ion chromatography retention[42] and the EOF generated by zwitterionic coatings reported previously.[15] Hu et al. demonstrated that divalent cations such as Ca^{2+} are retained by electrostatic ion chromatography columns prepared using *N*-dodecylphosphocholine, a single-tailed analogue of DLPC.[42,43] Thus, the weakly reversed EOF in Figure 2A results from the retention of Ca^{2+} by the DLPC coating on the walls of the capillary.

In the absence of Ca^{2+} in the coating buffer (and using a phosphate buffer), the time required to coat the capillary was excessively long (~75 min). The addition of Ca^{2+} to a solution containing phospholipids has been demonstrated to dramatically increase the rate of SPB formation.[33,34,44−46] The calcium ion is a strong fusogenic agent, which promotes the fusion of cells and vesicles.[34,47] Further, it has been suggested that an increase in intracellular Ca^{2+} initiates fusion between biological membranes.[48] Also documented is the effect of buffer on SPB formation. Rapuano and Carmona-Ribeiro examined SPB formation on silica using different buffers.[35] The authors concluded that the use of Tris buffer led to bilayer formation over the largest surface area. They speculated that protonated amino groups on Tris would be attracted to the negatively charged silanols at the silica surface. Subsequently, the phosphate group of the phospholipid could form a hydrogen bridge with the hydroxyl groups of Tris.[35] Thus, Tris buffers containing Ca^{2+} were used for coating the capillaries with DLPC unless otherwise noted.

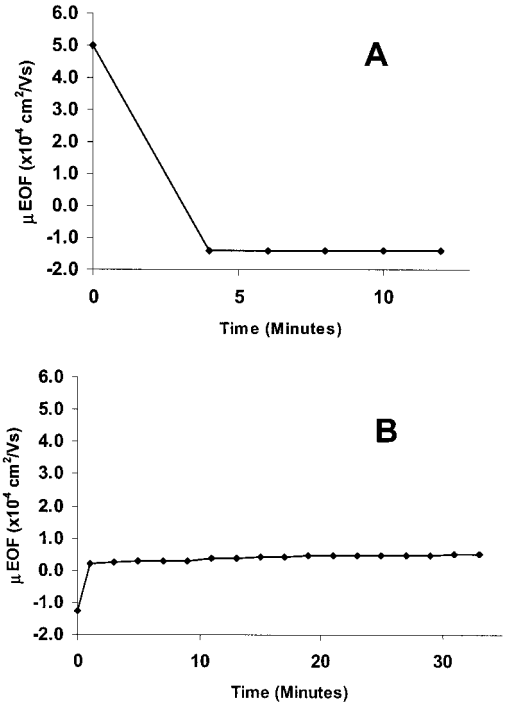

Figure 2. (A) Time required to achieve a stable EOF by rinsing a capillary with 0.1 mM DLPC in 20 mM Tris-HCl pH 7.4 buffer containing 20 mM $CaCl_2$. (B) Coating stability of DLPC as reflected by the EOF as a function of time. Experimental conditions: 50-cm capillary (40 cm to detector); temperature, 25 °C; separation buffer, (A) 0.1 mM DLPC in 20 mM Tris-HCl pH 7.4 containing 20 mM $CaCl_2$, (B) 20 mM Tris-HCl pH 7.4; applied voltage, (A) −20 kV, (B) +15 kV (slow EOF, 3 peak injection method used; see Experimental Section); direct UV detection at 254 nm. (A) EOF was measured after each 2-min rinse until EOF became stable. (B) EOF measured after 1-min rinses with 20 mM Tris-HCl buffer.

The stability of the wall coating in the absence of DLPC can be determined by monitoring the EOF.[10] The capillary was first coated with DLPC as described in the Experimental Section. The excess DPLC and Ca^{2+} were then rinsed from the capillary with a series of 1-min high-pressure rinses of Tris-HCl pH 7.4 buffer. Figure 2B shows the EOF observed after each rinse. Prior to the rinses with buffer void of DLPC and Ca^{2+}, the EOF was weakly reversed, as described above. The EOF rapidly changes from weakly reversed in the presence of Ca^{2+} to weakly forward ($+0.22 \times 10^{-4}$ cm^2/V·s). This is equivalent to the EOF observed after coating a capillary with 0.1 mM DLPC without Ca^{2+} for 75 min (unpublished results). After the first rinse, the EOF was stable

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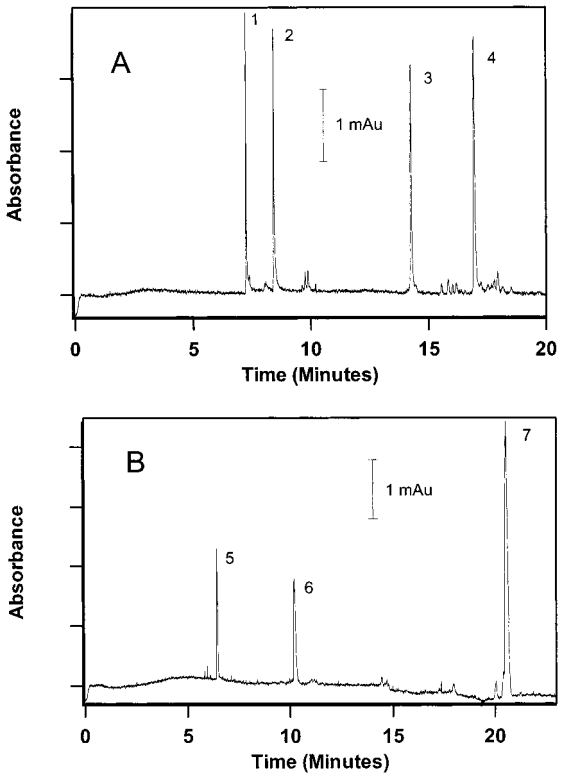

Figure 3. (A) Separation of cationic proteins at pH 7.4. Peaks: (1) lysozyme, (2) cytochrome *c*, (3) ribonuclease A, and (4) α-chymotrypsinogen A. (B) Separation of anionic proteins at pH 7.4. Peaks: (5) insulin chain A, (6) trypsin inhibitor, and (7) α-lactalbumin. Experimental conditions: 50-cm capillary (40 cm to detector); temperature, 25 °C; separation buffer, 20 mM Tris-HCl at pH 7.4; sample, 0.1 mg/mL protein mixture in water; applied voltage, (A) +20 kV, (B) −20 kV; direct UV detection at 214 nm. Coating procedure: 20-min rinse with 0.1 mM DLPC in 20 mM Tris-HCl pH 7.4 buffer containing 20 mM $CaCl_2$ followed by a 1-min rinse with separation buffer to remove excess phospholipid; between runs, phospholipid rinse time was shortened to 5 min.

over 35 runs (35 min of rinsing and 105 min of high voltage) as seen in Figure 2B. The EOF only slightly increased from +0.22 × 10^{-4} (first high-pressure rinse) to +0.5 × 10^{-4} $cm^2/V\cdot s$ (35th high-pressure rinse). These results demonstrate that DLPC generates a semipermanent coating, similar to the double-chained surfactant DDAB.[20] DLPC can therefore be removed from the electrophoretic buffer prior to performing a separation. Thus, unwanted buffer additive−analyte interactions that can deteriorate the separation are eliminated and detection schemes that are compromised in the presence of surfactants may be applied. For example, with DLPC in the separation buffer, numerous spikes in the electropherogram are present that interfere with analyte peaks.

Protein Separations Using a Semipermanent DLPC Wall Coating. When DLPC and calcium are removed from the buffer, the EOF generated in the capillary at pH 7.4 is suppressed (∼+0.3 × 10^{-4} $cm^2/V\cdot s$). Thus, cationic analytes can be separated using a positive polarity and anionic analytes can be separated using a negative polarity. Both cationic and anionic proteins are efficiently separated using DLPC as a capillary wall coating at pH 7.4. Figure 3A shows the separation of four cationic proteins: lysozyme, cytochrome *c*, ribonuclease A, and α-chymotrypsinogen A in under 20 min. In theory, CE is capable of achieving protein efficiencies of 1−2 million plates/m. Efficiencies of the protein peaks obtained using a DLPC-coated capillary were between 780 000 and 1.4 million plates/m. These values exceed what has previously been achieved using a permanently coated capillary and are comparable to efficiencies obtained using dynamic, surfactant-based coatings.[14,15,20] Commonly used permanent coatings such as polyacrylamide give efficiencies of 50 000−600 000 plates/m for cationic proteins[49,50] while dynamic coatings have yielded efficiencies nearing 1 million plates/m.

Table 1. Efficiency, Migration Time Reproducibility, and Recovery of Cationic and Anionc Proteins Separated Using a DLPC-Coated Capillary at pH 7.4

protein	efficiency (plates/m)	migration time (% RSD) run to run	migration time (% RSD) day to day	recovery (%)
lysozyme	1 400 000	0.3	4.0	64 ± 5
cytochrome *c*	1 000 000	0.3	4.0	76 ± 6
ribonuclease A	780 000	0.4	4.0	83 ± 7
α-chymotrypsinogen A	880 000	0.4	3.8	75 ± 7
insulin chain A	170 000	0.2	3.4	93 ± 7
trypsin inhibitor	150 000	0.4	3.3	78 ± 6
α-lactalbumin	310 000	1.3	2.8	92 ± 8

The separation of three anionic proteins, insulin chain A, trypsin inhibitor, and α-lactalbumin, at pH 7.4 using a DLPC-coated capillary is shown in Figure 3B. Efficiencies of the anionic protein peaks ranged between 150 000 and 310 000 plates/m. Although lower than the theoretical 1−2 million plates/m, these efficiencies are consistent, if not superior to the efficiencies obtained with a common method used for anionic protein analysis, namely, use of an uncoated capillary.[51]

Migration time should be reproducible from run to run and from day to day if proteins do not adsorb onto the capillary. For the cationic and anionic proteins, the RSD of the migration times were as low as 0.2% (run to run, $n = 16$) and 2.8% (day to day, $n = 3$). Table 1 summarizes the peak efficiencies and migration time reproducibilities achieved for the above proteins at pH 7.4.

Protein recovery studies (see Experimental Section) were conducted for lysozyme, cytochrome *c*, ribonuclease A, α-chymotrypsinogen A, insulin chain A, trypsin inhibitor, and α-lactalbumin at pH 7.4. For the cationic proteins (lysozyme, cytochrome *c*, ribonuclease A, α-chymotrypsinogen A), the recoveries were 64 ± 5%, 76 ± 6%, 83 ± 7%, and 75 ± 7%, respectively. Since the recoveries are not quantitative, it is evident that protein adsorption is occurring at the capillary wall. Indeed, the cationic peaks exhibit some tailing (Figure 3A), which is indicative of protein adsorption. Since the EOF is forward (+0.3 × 10^{-4} $cm^2/V\cdot s$), the wall has a slight negative charge, and therefore, electrostatic attraction could exist between any uncoated portions of the wall and the cationic proteins. Another possibility is that proteins are interacting with the zwitterionic headgroup. Holmlin et al. demonstrated that zwitterionic self-assembled monolayers (terminating in the phosphatidylcholine headgroup) prevented protein adsorption sub-

(49) Schmalzing, D.; Piggee, C. A.; Foret, F.; Carrilho, E.; Karger, B. L. *J. Chromatogr., A* **1993**, *652*, 149−159.
(50) St'astna, M.; Radko, S. P.; Chrambach, A. *Electrophoresis* **2000**, *21*, 985−992.
(51) Legaz, M. E.; Pedrosa, M. M. *J. Chromatogr., A* **1993**, *655*, 21−29.

stantially more than monolayers terminated in a single charge.[22] They tested lysozyme (cationic) and fibrinogen (anionic) at pH 7.4 for adsorption. However, adsorption of proteins was not completely eliminated at a zwitterionic phosphatidylcholine-terminated surface,[22] which is consistent with our protein recovery data.

The anionic proteins also partially adsorb to the capillary wall (again consistent with the data of Holmlin et al.[22]) but to a lesser degree than do the cationic proteins. For insulin chain A, trypsin inhibitor, and α-lactalbumin, the recoveries were 93 ± 7%, 78 ± 6%, and 92 ± 8%, respectively. The results of the protein recovery studies are summarized in Table 1.

Effect of pH on the DLPC Capillary Coating. To judge the versatility of the coating, DLPC was tested for its ability to maintain a stable coating and minimize protein adsorption over a wide pH range. The phosphate group of the phosphatidylcholine headgroup has a pK_a of ∼1. Thus, to ensure DLPC will act as a true zwitterion, it is best to work at a pH greater than 3. At pH 3, the capillary wall still carries a slight negative charge, as indicated by the positive EOF (∼+0.3 × 10^{-4} $cm^2/V\cdot s$) generated in a bare capillary at this pH. Consequently, cationic proteins adsorb to a bare capillary wall at pH 3.[21] This indicates that a coating is necessary even when proteins are separated at low pH.

EOF is normally quite variable at low pH in a bare capillary.[52] Therefore, a coating would be attractive if it produces a stable EOF at low pH. It required only 5 min of high-pressure rinsing of 0.1 mM DLPC in a 20 mM Tris-HCl pH 3 buffer (containing 20 mM $CaCl_2$) to reach a stable EOF (−1.6 × 10^{-4} $cm^2/V\cdot s$) and hence coat the capillary wall. The EOF upon rinsing the capillary (1 min) with 20 mM Tris-HCl pH 3 buffer (removing DLPC and calcium) is −1.4 × 10^{-4} $cm^2/V\cdot s$. The EOF remained constant at this value after 10 min of identical rinses (RSD = 3%, n = 10), indicating that DLPC forms a stable coating on the capillary wall at low pH.

A mixture of cationic proteins (lysozyme, cytochrome *c*, ribonuclease A, α-chymotrypsinogen A) was separated at pH 3 (20 mM Tris-HCl) using a DLPC-coated capillary. Figure 4A shows that all four proteins were separated in less than 20 min with efficiencies ranging from 560 000 to 1 000 000 plates/m. The migration time RSD for the proteins was less than 1.8% (n = 16). Table 2A summarizes the efficiency and migration time reproducibility obtained for each of these proteins at pH 3. Separating the anionic proteins insulin chain A, trypsin inhibitor, and α-lactalbumin using a negative voltage would not be practical because they would not be observed in a reasonable time. These proteins have a pI ∼ 4 and would migrate very slowly, against the already suppressed EOF.

DLPC was used to coat a capillary at pH 10. A solution of 0.1 mM DLPC in 20 mM CHES pH 10 buffer (containing 20 mM $CaCl_2$) required 25 min of high-pressure rinsing to achieve a constant EOF of −1.3 × 10^{-4} $cm^2/V\cdot s$ and hence coat the wall. The coating time is much slower than was observed at pH 3 and 7.4, most likely due to the fact that CHES rather than Tris buffer was used (CHES has a better buffering capacity at pH 10). As discussed above, phosphoplipid dissolved in Tris buffer leads to improved bilayer formation over a silica surface.[35] Upon rinsing with CHES pH 10.0 buffer for 1 min, the EOF changes to +1.1 × 10^{-4} $cm^2/V\cdot s$. Upon 15 min of additional rinsing, the EOF remains constant, verifying the coating stablity.

Figure 4B shows the separation of a mixture of three anionic proteins (insulin chain A, trypsin inhibitor, α-lactalbumin). The proteins were separated in under 20 min with efficiencies up to 360 000 plates/m. The migration times for insulin chain A, trypsin inhibitor, and α-lactalbumin also proved to be quite reproducible over 16 runs, yielding RSD values less than 2.3%. Efficiencies and

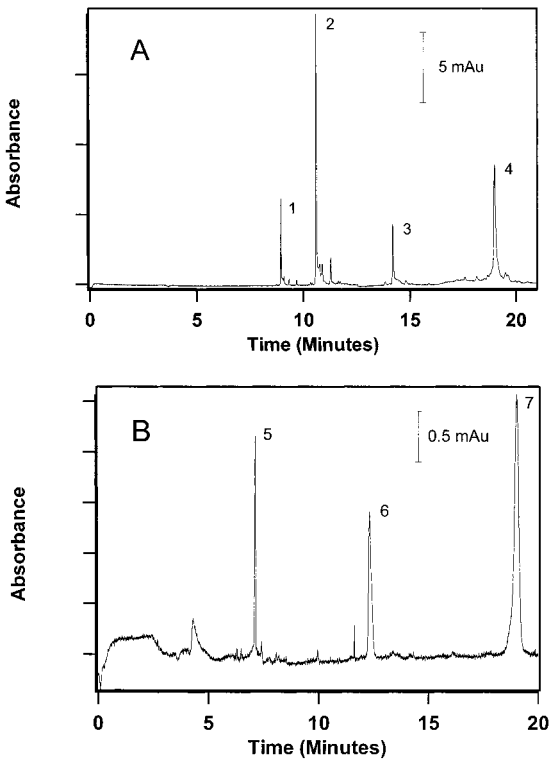

Figure 4. (A) Separation of cationic proteins at pH 3. Peaks: (1) cytochrome *c*, (2) lysozyme, (3) ribonuclease A, and (4) α-chymotrypsinogen A. (B) Separation of anionic proteins at pH 10. Peaks: (5) insulin chain A, (6) trypsin inhibitor, and (7) α-lactalbumin. Experimental conditions: 50-cm capillary (40 cm to detector); temperature, 25 °C; separation buffer, (A) 20 mM Tris-HCl pH 3, (B) 20 mM CHES pH 10; sample, 0.1 mg/mL protein mixture in water; applied voltage, (A) +20 kV, (B) −20 kV; direct UV detection at 214 nm. Coating procedure: 20-min rinse with 0.1 mM DLPC in (A) 20 mM Tris-HCl pH 3 buffer containing 20 mM $CaCl_2$ or (B) 20 mM CHES pH 10 buffer containing 20 mM $CaCl_2$, followed by a 1-min rinse with separation buffer to remove excess phospholipid; between runs, phospholipid rinse time was shortened to 5 min.

Table 2. Efficiency and Migration Time Reproducibility of Proteins

protein	efficiency (plates/m)	migration time run to run (% RSD)
(A) Separated Using a DLPC-Coated Capillary at pH 3		
lysozyme	1 000 000	1.0
cytochrome *c*	1 000 000	0.7
ribonuclease A	790 000	1.3
α-chymotrypsinogen A	560 000	1.8
(B) Separated Using a DLPC-Coated Capillary at pH 10		
insulin chain A	360 000	0.9
trypsin inhibitor	96 000	1.1
α-lactalbumin	100 000	2.3

(52) Ciccone, B. *Am. Lab.* **2001**, *33*, 30−33.

migration time RSD of the proteins using DLPC at pH 10 are summarized in Table 2B. As with the anionic proteins at pH 3, the cationic proteins lysozyme, cytochrome *c*, ribonuclease A, and α-chymotrypsinogen A were not separated at pH 10. This would not be practical since they all have a p*I* ∼ 9 and would approximately migrate with the suppressed EOF, making the separation time extremely long.

Analysis of Egg White Proteins. The major protein components of egg white are lysozyme (p*I* ∼ 11) and ovalbumin (p*I* ∼ 5). At biological pH (7.4), lysozyme is positively charged and ovalbumin is negatively charged. Thus, separation of both proteins on a bare capillary is impossible. CE methods used for the analysis of egg white proteins involve working with high-salt buffers (e.g., >300 mM phosphate) at neutral[53] or high pH[54] or with low-pH buffer conditions using poly(ethylene glycol) additives.[55] Efficiencies were never reported for the protein peaks in these previously reported methods. However, on the basis of their electropherograms, it is estimated that the efficiencies were less than 10 000 plates/m. Using a DLPC-coated capillary at pH 7.4, lysozyme and ovalbumin were analyzed in an egg white sample. Figure 5 shows the electropherograms for analysis of (A) ovalbumin (negative voltage applied) and (B) lysozyme (positive voltage applied). Identities of the proteins were confirmed by injecting standards. The efficiency of the ovalbumin peak was 15 000 plates/m, and the efficiency of the lysozyme peak was 750 000 plates/m. After three runs, the lysozyme peak degraded, indicating adsorption was occurring to the wall. This is consistent with the protein recovery data obtained for lysozyme at pH 7.4 (64% recovery).

DLPC Analogues for Use as Capillary Coatings. The C_{10} analogue of DLPC, DDPC, required 5 min to achieve a stable EOF in a pH 7.4 Tris-HCl buffer containing calcium. However, upon rinsing the capillary with 20 mM Tris-HCl pH 7.4 buffer in 1-min intervals, the EOF rapidly increased. This demonstrates that the coating desorbs from the wall after each rinse. Thus, DDPC acts more like a dynamic coating (single-chained surfactants, e.g., CTAB[20]) than a semipermanent coating (double-chained surfactants, e.g., DDAB[20] and DLPC, this work) as a stable EOF is achieved only when phospholipid is present in the separation buffer. It is possible that the hydrophobic attraction between the tails is not sufficiently strong to overcome the electrostatic repulsion between the zwitterionic headgroups for bilayer aggregation to dominate. The poor coating that DDPC provides at the capillary wall was confirmed by attempting a separation of cationic proteins at pH 7.4. The cationic proteins lysozyme, a-chymotrypsinogen A, cytochrome *c*, and ribonuclease A completely adsorbed to the DDPC-coated wall such that no peaks were detected.

The C_{14} analogue of DLPC, DMPC, performs similarly to its C_{12} counterpart with one difference. DMPC appears to aggregate in solution more rapidly than DLPC based on the time it takes for a solution to become clear upon sonication. Aggregation is expected to be more favorable for DMPC as the longer hydrocarbon chain length will promote increased hydrophobic attraction. DLPC can require up to 15 min to coat the capillary at pH 7.4 while DMPC never required more than 5 min under the same conditions (0.1 mM phospholipid in 20 mM Tris-HCl pH 7.4 buffer containing 20 mM $CaCl_2$). Upon a 1-min high-pressure rinse with 20 mM Tris-HCl pH 7.4 buffer, the EOF was $+0.2 \times 10^{-4}$ cm^2/V·s. Rinsing the capillary for an additional 10 min only changed the EOF to $+0.27 \times 10^{-4}$ cm^2/V·s, indicating the ability to remove excess surfactant from the buffer and still achieve a stable EOF. A DMPC-coated capillary was capable of separating lysozyme, cytochrome *c*, ribonuclease A, and α-chymotrypsinogen A with efficiencies as high as 1.3 million plates/m and migration time RSDs less than 1.0% ($n = 16$). Anionic proteins (insulin chain A, trypsin inhibitor, α-lactalbumin) were separated with efficiencies as high as 300 000 plates/m and migration time RSD values lower than 1.1% ($n = 16$). Since DMPC displays similar behavior and performance to DLPC at pH 7.4, it is assumed that it will also be an effective and stable coating for enabling the separation of highly adsorbing proteins over a wide range of pH values.

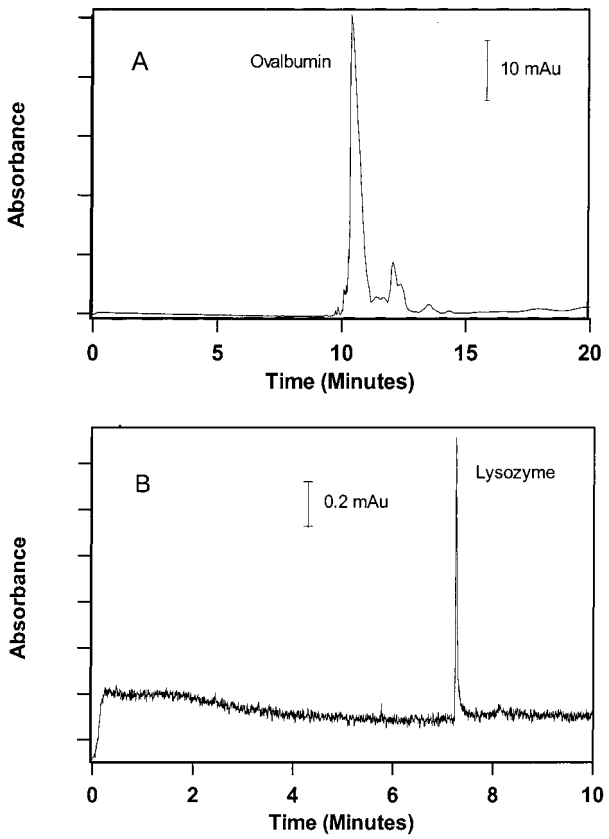

Figure 5. Analysis of (A) ovalbumin and (B) lysozyme in an egg white sample. Experimental conditions: 50-cm capillary (40 cm to detector); temperature, 25 °C; separation buffer, 20 mM Tris-HCl at pH 7.4; sample, 1:20 diluted egg white in buffer; applied voltage, (A) −20 kV, (B) +20 kV; direct UV detection at 214 nm. Coating procedure: 20-min rinse with 0.1 mM DLPC in 20 mM Tris-HCl pH 7.4 buffer containing 20 mM $CaCl_2$ followed by a 1-min rinse with separation buffer to remove excess phospholipid; between runs, phospholipid rinse time was shortened to 5 min.

CONCLUSIONS

For capillary electrophoresis to be useful in the emerging field of proteomics, simple, robust, and effective wall coatings are necessary for the prevention of protein adsorption. The phospholipid DLPC (essentially a double-chained, zwitterionic surfactant) forms a stable coating at the capillary wall that effectively

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(55) Besler, M.; Steinhart, H.; Paschke, A. *Food Agric. Immunol.* **1998**, *10*, 157−160.

suppresses the EOF and prevents the adsorption of both cationic and anionic proteins over the pH range of 3−10. The coating is semipermanent in nature. Thus, phospholipid can be removed from the buffer prior to separation allowing the possibility of MS detection. This is a subject of future investigations.

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