Effects of pH Gradients on Liposomal Charge States Examined by Capillary Electrophoresis

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Received February 12, 2002. In Final Form: May 26, 2002

Liposomes have been used for biomimetic containers and to study phenomena ranging from photosynthetic systems to membrane fusion and dynamics. An important aspect of many preparations and in biological processes is the presence of a pH gradient across the membrane. Here, experiments were conducted using capillary electrophoresis to investigate the effects of this gradient on liposomes composed of phosphatidic acid, phosphatidylcholine, and cholesterol. pH gradients for the liposomes were created by titration of the exterior buffer; then the electrophoretic properties were analyzed by capillary electrophoresis and the size was measured by laser light scattering. Our results show that the presence of a pH gradient has a significant effect on the electrophoretic migration of liposome samples, induced principally by a change in effective charge. The differences in charge for the liposome samples are evaluated with regard to acid−base equilibria, which is shown to be inadequate to describe the dynamics of the system. A more complex capacitive theory incorporating elements of the Overbeek−Booth theory and the relaxation effect appears to more effectively describe the results and could aid in predicting liposome behavior under various pH gradient conditions.

Introduction

Since Bangham first described liposomes in 1965, liposome studies have expanded to include pharmaceutical, cosmetic, cellular, immune, and membrane research.[1] Liposomes have been utilized in a large variety of settings, from cheese farms to chemotherapy drugs.[2,3] They are an excellent model system for membrane studies and provide a lipid bilayer system that is relatively cheap and easy to prepare. Liposomes have also been used in fundamental studies of phenomena such as membrane fusion, ion gradients, and antigen processing, in addition to novel fluorescence detection schemes, liposome-based separations and immunoassays, MRI contrast agents, and for light-induced proton transfer.[4−11] The small enclosed volume and membrane properties have led to their use as novel biomimetic containers for reactions between DNA, proteins, and labeling reagents.[12,13] Liposomes have also been used to study pH gradients in membrane systems. pH gradients are used for drug loading and modeling photosynthetic systems, and there is a growing body of evidence to support a role for transmembrane pH gradients in drug and neurotransmitter uptake.[11,14]

Capillary electrophoresis (CE) has been used to study liposomal properties such as membrane fluidity and rigidity, size distributions, phospholipid distribution in the membrane, membrane disruption, and surface charge density.[15−21] Several theories which partially explain the behavior of liposomes in CE have been examined and confirmed.[14,19,21,22] These include the Gouy−Chapman−Stern theory concerning pH changes at the membrane−buffer interface, and the Overbeek−Booth theory concerning the electric double layer and its polarization in an electric field. However, there has been little examination of the changes in liposomal properties (such as charge state) for liposomes with a pH gradient. The effects of a pH gradient on liposomes are governed by many factors, only a few of which are clearly understood.[14,23] A technique capable of providing new information about these effects adds a valuable tool to the study of liposomes. It could also potentially shed new light on the processes by which

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10.1021/la025625k CCC: $22.00
Published on Web 07/24/2002

liposomes can be manipulated to enhance drug loading or used to study fundamental biological processes.

This study focuses on the potential of CE to add relevant information about the charge state of liposomes that contain a pH gradient. A pH gradient should cause a measurable difference in the apparent charge of a liposome, which can be examined and quantitated by CE. Based on the capacitive nature of the membrane and work reported in the literature, the addition or removal of protons in the interior aqueous cavity generates a corresponding change in surface charge on the liposome exterior by capacitive effects or protonation due to acid−base equilibria (pK_a), although there has been some minor disagreement about this.[20,23−25] To determine the amount of charge present, liposomes with a transmembrane pH gradient of 1.4 pH units were separated by CE. Liposomes with a more basic interior (pH 8.8 buffer inside, pH 7.4 buffer outside, labeled $C_{8.8\ in/7.4\ out}$ throughout) and a more acidic interior ($D_{7.4\ in/8.8\ out}$) were studied, as well as liposomes with no transmembrane pH gradient at each corresponding pH ($A_{8.8\ in/8.8\ out}$ and $B_{7.4\ in/7.4\ out}$). The electrophoretic mobilities of each sample were calculated, giving a direct measure of the charge state, and the results compared to the capacitive and pK_a models.

Materials

All materials were used as received unless otherwise noted. Phosphatidylcholine and phosphatidic acid (Egg) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), and cholesterol and tricine were obtained from Sigma-Aldrich (St. Louis, MO). Potassium sulfate was obtained from EM Science (Gibbstown, NJ), sodium hydroxide was obtained from Mallinckrodt Baker Inc. (Paris, KY), and fused silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ). A 30% (w/v) solution of BRIJ 35 was obtained from Sigma Diagnostics (St. Louis, MO) and was diluted to the appropriate concentrations for use in capillary coating and buffers as described below.

Methods

Liposome Preparation. The liposomes were prepared by reverse phase evaporation using a 10:1 molar ratio of phosphatidylcholine to phosphatidic acid, with 20% (mol/mol) cholesterol as described elsewhere.[26] All liposome samples were used within a week and stored at 4 °C when not used immediately.

pH Gradient Formation. All pH gradient liposome preparations were created from pH 7.4 interior and exterior liposomes or pH 8.8 interior and exterior liposomes by slow titration of the exterior buffer to the new pH. The liposome solution was stirred constantly during titration to prevent membrane disruption from the pH change. Once the pH gradient had been created, the liposomes were used immediately. The pH gradient stability was determined to be approximately 3 h before rapid equilibration of the gradient occurred.

Capillary Coating. Fused silica capillaries were coated with a hydrophilic and uncharged surfactant, BRIJ 35, following the method of Towns and Regnier.[27] Briefly, after sodium hydroxide and water rinses, an octadecylsilane layer was deposited on the surface of the capillary, followed by a rinse with 0.5% (w/v) BRIJ 35 solution, resulting in an adsorbed coating of the surfactant on the walls of the capillary. This was necessary to prevent adsorption of the zwitterionic headgroups of the liposomes to the surface. The capillary was then rinsed with the appropriate running buffer. Successful coatings were determined by electroosmotic flow measurements using a neutral marker (mesityl oxide).

Capillary Electrophoresis. Running buffers consisted of 2 mM tricine and 15 mM potassium sulfate titrated to pH 7.4 or 8.8 with 1 M sodium hydroxide and then brought to final volume. Running buffers also contained 0.001% (w/v) BRIJ 35 to minimize leaching of the adsorbed surfactant from the capillary walls into the bulk buffer.[27] The liposome samples were loaded into a 50 μm inner diameter, 360 μm outer diameter coated capillary by pressure injection (500 mbar for 5 s). Capillaries were 62 cm to detector window, 77 cm total length. A voltage of −25 kV was applied and ultraviolet absorption detection was accomplished with a Spectra 100 UV−vis spectrometer (ThermoSeparation Products, Fremont, CA) at 214 nm using the absorption of the phosphate groups in the lipid headgroups. The adsorbed coating of BRIJ 35 covered most of the surface silanol groups, and a small number of residual silanol groups contributed to a weak electroosmotic flow toward the cathode. However, the liposomes were net negatively charged at the pHs used and migrated against the electroosmotic flow; therefore, the polarity of the voltage applied was reversed (−25 kV). All capillary electrophoresis experiments were performed on a Crystal CE automated capillary electrophoresis instrument (Prince Technologies B. V., Emmen, The Netherlands). Data were collected with the accompanying software from Analytical Technology Inc. (Oaks, PA). All electrophoretic mobility data sets were subjected to a Student *t*-test at a 95% confidence level and were determined to be significantly different.

Membrane Disruption Fluorometric Study. In one set of experiments, a fluorescent, pH-sensitive dye (7 μM 9-aminoacridine) was encapsulated in the liposomes and a transmembrane pH gradient was created. The liposomes were placed in a cuvette and monitored in a fluorimeter for leakage of the dye in the presence of BRIJ 35. No leakage of the dye was seen for 3 h, nor did the interior pH change appreciably.

Dynamic Light Scattering. Data were taken on a Brookhaven Instruments goniometer (Holtsville, NY) and a Protein Solutions DynaPro-LSR (Lakewood, NJ), and analyzed in house. Liposomal pH gradients were created just prior to measurement from each equal pH preparation, and three pH gradient samples were analyzed for each case. The equal pH gradient preparations were measured three times, and the measurements taken were consistent with the sizes measured from other preparations prior to the pH gradient creation. Analysis of multiple samples on multiple instruments revealed a characteristic broad distribution of sizes with this means of preparation as noted in the Results section. The laser light scattering data shown did not have any statistically significant differences in the means of the populations.

Results and Discussion

Capillary electrophoresis involves the electrophoretic migration of molecules or particles according to their size and charge, which results in the separation of different species. This relationship, in its simplest form, is defined by

$$\mu = q/6\pi\eta r \qquad (1)$$

where μ is the electrophoretic mobility, q is the charge of the sample particle or molecule, r is the hydrodynamic radius, and η is the viscosity of the buffer. The only dynamic variables are the charge, q, and radius, r, for the liposome samples. It should be noted that for any given preparation the radius covers a finite range, as noted by Roberts and others, which results in a Gaussian distribution rather than the individual component peaks seen in typical CE (Figure 1).[17,22] Even with these distributions, the dynamics associated with the formation of a pH gradient could be investigated.

Simple pK_a Model of Liposome Charge. The existence of a pH gradient has a marked effect on the electrophoretic migration of liposomes, and hence either the overall charge state or the liposome radius, or both, has been affected (Figures 1 and 2). These liposomes are

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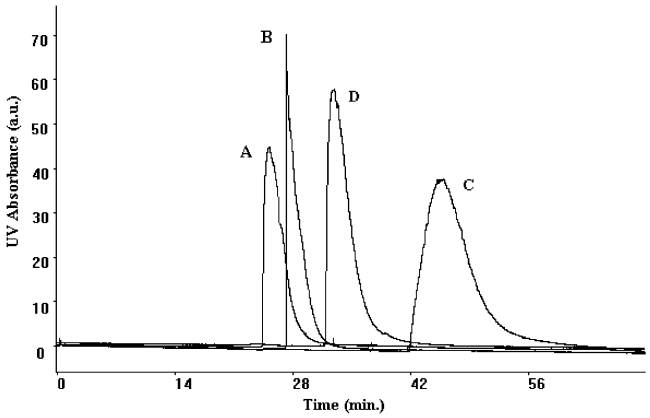

Figure 1. Representative electropherograms of liposomes with different pH interiors and exteriors: $A_{8.8\ in/8.8\ out}$; $B_{7.4\ in/7.4\ out}$; $C_{8.8\ in/7.4\ out}$; $D_{7.4\ in/8.8\ out}$. It should be noted that the liposome peaks resembled a Gaussian distribution, which was expected given the charge/size variations inherent in any liposome preparation.

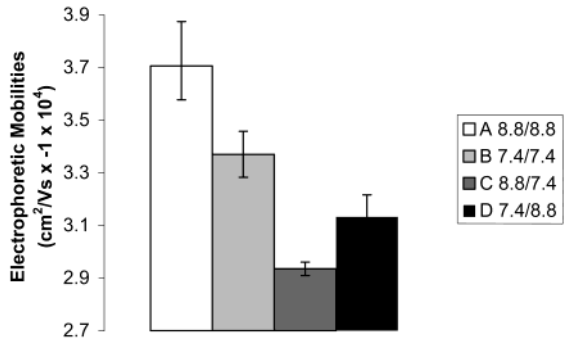

Figure 2. Average electrophoretic mobilities of each liposome population. Error bars represent the standard deviation for a series of runs ($n > 3$ in all cases). Based on the Overbeek–Booth theory for electrokinetic effects on large nonconducting particles and the Ceh–Lasic theory for pH gradient loading of liposomes, the apparent surface charge of the liposomes with a transmembrane pH gradient is affected by both capacitive charge effects and the relaxation effect.

composed of phospholipids which contain a charged headgroup. At pH 8.8, the phosphatidylcholine should be zwitterionic and of neutral charge, while the phosphatidic acid will be negatively charged. At pH 7.4, the phosphatidylcholine will remain zwitterionic, but the phosphatidic acids in the membrane will be partially protonated—approximately half of the headgroups will protonate, based on a pK_a for the phosphatidic acid of 7.4. For this simplistic treatment, the $B_{7.4\ in/7.4\ out}$ liposomes would therefore have half the charge of the $A_{8.8\ in/8.8\ out}$ liposomes. Assuming a bilayer thickness of 40 Å, that each headgroup occupies an area of 0.7 nm^2, and a surface area of 8×10^{-10} cm^2 calculated for a sphere of outer radius 80 nm, then approximately 7200 phosphatidic acids will be protonated, which corresponds to approximately 1×10^{-15} C of net negative charge on the liposome. This equates to an electrophoretic mobility of approximately -8×10^{-7} cm^2/(V s), which is orders of magnitude less than the experimental value of -3×10^{-4} cm^2/(V s) (Figure 2). This simplistic model is clearly not adequate to describe the liposome charge states.

pH Gradient Liposome Stability. It is conceivable that these liposomes may become "leaky" under the experimental conditions. The BRIJ 35 surfactant used as an adsorbed coating in the capillary may interact with the liposome membrane in such a way as to allow charge transfer across the membrane. However, a fluorometric study of the liposomes with a pH-sensitive encapsulated dye revealed no significant change in the interior pH over a 3 h period in the presence of identical concentrations of BRIJ 35 (data not shown). Additionally, a literature survey of liposome membrane stability and electroporation effects under similar conditions indicates a stable structure—the voltage field strengths typically used for capillary electrophoresis are well below that required to electroporate a membrane, and the few instances where electroporation is shown at similar voltage field strengths also involve substantial mechanical pressures.[13] It has been assumed therefore that the pH gradient is relatively stable over the time frame of these experiments.

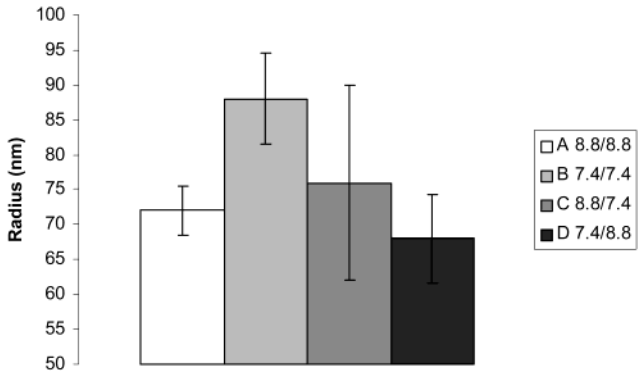

Figure 3. Size distributions for the liposome samples as measured by dynamic laser light scattering. Each column represents three measurements. The error bars reflect the standard deviation associated with the averaged radii from the three data sets. While there has been some evidence in the literature to suggest that the liposome radius can change due to osmotic stress induced by concentration gradient, the large standard deviations do not lend themselves to this argument for the transmembrane pH gradient liposomes.[28,29]

Radius Measurements. Given that both the charge q and the radius r are dynamic variables (eq 1), it was important to assess whether the radius changed in some degree as a response to the pH gradient. There is some evidence for osmotic swelling in the literature in the presence of a high solute concentration gradient.[28,29] With pH gradients existing across the membrane and the relatively high permeability of water across phospholipid membranes, it is possible for the liposomes to swell in an attempt to equalize the pH imbalance existing across the membrane. Laser light scattering indicated some differences between the populations (Figure 3), but there were large distributions associated with each liposome sample, which makes interpretation difficult. In addition, even the presence of high solute gradients will cause only very small changes due to osmotic swelling (~6% of total volume).[28,29] Given the light scattering data and literature evidence, the radius is considered to be relatively constant and the origin of the charge state should be reexamined in more detail. The unique attributes of the lipid bilayer membrane system, in particular its capacitive behavior, should be examined.

Capacitive Effects. It has been argued that membrane capacitance has an effect on the surface charge density only when a transmembrane potential and ion gradient exist.[24] Logically, the liposome surface in a more acidic environment would exhibit a higher degree of protonation, whereas a liposome surface in a more basic environment would be expected to have a lesser degree of protonation. Surface charge is therefore defined by both the pK_a's of

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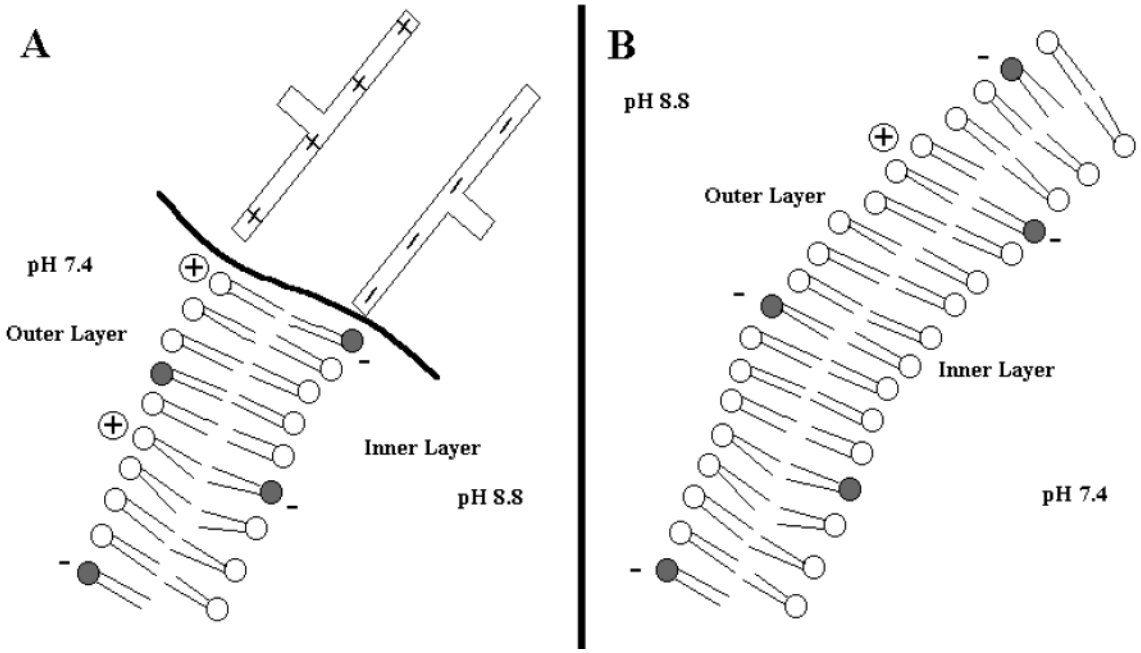

Figure 4. Schematic of capacitive effect theory. Phosphatidic acids are indicated by a shaded headgroup. (A) For the $C_{8.8\ in/7.4\ out}$ liposomes, a large amount of positive capacitive charge (indicated as a circled positive charge) would be present at the exterior surface. This positive capacitive charge would mask a large amount of the negative charge present on the exterior surface at pH 7.4, resulting in a greatly slowed migration in the electric field as compared to $A_{8.8\ in/8.8\ out}$. A decrease of 7.7×10^{-5} cm^2/(V s) in electrophoretic mobility was observed. This is diagrammed more simply in the upper half of (A) as a plate capacitor, where the charge on the interior surface capacitively induces an equal and opposite charge on the opposite side of the membrane. (B) In the case of the $D_{7.4\ in/8.8\ out}$ liposomes, less of the interior layer's phosphatidic acids will be negatively charged, resulting in less capacitively induced charge on the liposome exterior and therefore less of a reduction in electrophoretic mobility as compared to the $B_{7.4\ in/7.4\ out}$ liposomes. A decrease of only 2.2×10^{-5} cm^2/(V s) in electrophoretic mobility was observed in this case.

functional groups and capacitive charge. Therefore, liposomes with pH gradients would not have interior and exterior surfaces of the same charge state as liposomes with no pH gradient, and would not be expected to have similar electrophoretic mobilities.

The liposomes have a net negative charge at pH 8.8, defined by the pK_a of the phosphatidic acid headgroups that constitute approximately 10% of the membrane. At pH 7.4, approximately half of these phosphatidic acid headgroups are protonated, which would decrease the charge by half. Therefore, the $B_{7.4\ in/7.4\ out}$ liposomes should migrate more slowly in the electric field than the $A_{8.8\ in/8.8\ out}$ liposomes. This was observed by a decrease of 3.6×10^{-5} cm^2/(V s) (an approximately 10% change) in electrophoretic mobility between $A_{8.8\ in/8.8\ out}$ and $B_{7.4\ in/7.4\ out}$. However, if the electrophoretic mobility differences observed are a result solely of changes in apparent charge state, then a difference corresponding to roughly 50% would be expected from the pK_a effects, but was not observed. Some additional mechanisms must be present in addition to the simple equilibrium pK_a effects.

Coexisting Mechanisms for Apparent Charge States. It is intuitive that, in addition to simple pK_a effects, additional mechanisms whereby charge transfer can occur will exist at bilayer membrane interfaces when an ion gradient is present. One well-known source of transmembrane charge effects is the ability of the phospholipid membrane to behave as a capacitor. Therefore, in addition to modeling the surface charge of a liposome as a simple function of acid−base equilibria, the capacitive nature of the membrane must be taken into account for the pH gradient liposomes.

If the capacitive nature of the membrane can effect changes in the apparent surface charge, then it follows that, in the case of $C_{8.8\ in/7.4\ out}$, a large amount of capacitively induced charge would be present at the exterior surface in addition to the pK_a induced charge from the deprotonated phosphatidic acid groups. From the membrane's behavior as a capacitor, a large amount of positive capacitive charge will be present at the exterior surface for $C_{8.8\ in/7.4\ out}$ from the pK_a induced negatively charged interior surface at a pH of 8.8. This will result in the masking of some of the remaining negative charge on the exterior surface, which is at pH 7.4 (Figure 4A). This in turn would result in a reduced migration in the electric field. In agreement with this model, a corresponding reduction in the migration rate is observed (decrease of 7.7×10^{-5} cm^2/(V s)).

In the case of $D_{7.4\ in/8.8\ out}$, the exterior surface will have a high negative surface charge from the pK_a induced deprotonation of the phosphatidic acid groups at pH 8.8. Additionally, it would be expected that the capacitive nature of the membrane would not contribute as strongly due to the partially protonated interior surface, which is at pH 7.4. Therefore, it is surprising that this liposome population is not faster than the $B_{7.4\ in/7.4\ out}$ liposomes. To explain this result, a more careful study of the factors which contribute to electrophoretic mobility must be made.

Contributions to Electrophoretic Mobility: The Relaxation Effect. To better understand the parameters which govern electrophoretic mobility, it is necessary to consider in more depth the factors that contribute to the apparent charge which is measured by electrophoretic mobility. In particular, attention must be paid to the zeta (ζ) potential, which can be simply defined as the electrokinetic potential drop across the electric double layer surrounding a charged sphere in an electric field. In paying attention to the ζ potential, eq 1 becomes the Smoluchowski equation

$$\mu = \zeta\epsilon/\eta \qquad (2)$$

where μ is the electrophoretic mobility, ζ is the zeta

potential of the particle, ϵ is the dielectric permittivity of the buffer, and η is the viscosity of the buffer. The magnitude of the ζ potential is proportional to the ionic strength of the buffer and the radius of the particle. Both these factors contribute to the size of the electric double layer, which in turn contributes to the ζ potential, and are described by the term κR. It is expected that, as κR increases, the ζ potential will increase. The ζ potential is also dependent on the surface charge density of the particle. As already discussed for the liposome systems in question, the surface charge density differs drastically among the populations. It is therefore hardly surprising that the electrophoretic mobilities are significantly different, given the difference in ζ potentials that will result for each population from the pK_a and capacitively induced charges.

In the case of $D_{7.4\ in/8.8\ out}$, a large amount of negative charge would be expected from both the pK_a effects at the exterior pH 8.8 surface and from decreased positive capacitive effects from the inner pH 7.4 surface. This should result in a large κR and subsequently a large ζ potential, and it was expected that these liposomes would have the fastest mobility. So why was this not observed experimentally? It has been shown in the literature that in certain regimes increases in surface charge can decrease the electrophoretic mobility due to relaxation effects dominating the system.[34,35] The relaxation effect is caused by the distortion of the electric double layer and the particle's ion cloud by the electric field, and results in a pull in the opposite direction of the particle's electrophoretic mobility. It is likely given the relatively high ionic strength of the buffer and the large amounts of surface charge that in this case the κR is in a region whereby relaxation effects dominate the electrophoretic mobility despite the increased ζ potential.

It should be noted that the transbilayer movement of the phosphatidic acid from the outer to inner monolayer could be a factor in the changes in electrophoretic migration.[30−32] According to work by Eastman et al., for liposomes with a molar ratio of 10:1 phosphatidylcholine to phosphatidic acid, up to 60% of the phosphatidic acid present in the outer layer of the liposomal membrane will migrate to the inner layer in the presence of a pH gradient of 5 pH units with a more basic interior.[31] Since a greater difference in electrophoretic mobility was observed for the $C_{8.8\ in/7.4\ out}$ liposomes, which have a more basic interior, and the liposomal composition is nearly identical, it is possible that some of the phosphatidic acid lipids could have migrated to the inner layer, further contributing to the positive capacitive charge that would build up on the exterior surface. However, no cholesterol was present in the liposomes formulated by Eastman et al., but cholesterol is present in the pH gradient liposomes used here. Cholesterol is a well-characterized membrane stabilizer and contributes to the plasticity of the membrane.[33] Since cholesterol interacts with the hydrophobic carbon chains of the lipids, it is likely that its presence would negatively affect the ability of the phosphatidic acid to move from one monolayer to the other. Additionally, the activation energy required for the transbilayer movement of phosphatidic acid is relatively high (28 kcal/mol), and therefore this mechanism would not be expected to contribute overwhelmingly to the changes in apparent charge state observed.[31]

Conclusions

We have demonstrated the usefulness of CE as a tool capable of probing the changes in apparent charge of liposomal preparations which contain a transmembrane pH gradient. This work is applicable to a wide variety of studies, including but not limited to analysis of drug preparations prepared by pH gradient loading and research of membrane-dependent processes such as endocytosis and hormone secretion that could rely on pH gradients for selective partitioning of species of biological importance. Future studies will focus on biologically important pH gradients, such as those involved in neurotransmitter accumulation, using liposomes as a biomimetic membrane system.

Acknowledgment. This work was supported by ACS PRF (No. 99-0286) and Arizona State University. The authors gratefully acknowledge the assistance of David O'Brien and Sanchao Liu of the University of Arizona for the use of their Brookhaven Instruments goniometer for the dynamic laser light scattering experiments. The authors also acknowledge insightful discussions of liposome behavior with Laurie Locascio of the National Institute for Standards and Technology and with Scott Saavedra of the University of Arizona.

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