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Size-Dependent Electrophoretic Migration and Separation of Liposomes by Capillary Zone Electrophoresis in Electrolyte Solutions of Various Ionic Strengths

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The size-dependent electrophoretic migration and separation of liposomes was demonstrated and studied in capillary zone electrophoresis (CZE). The liposomes were extruded and nonextruded preparations consisting of phosphatidylcholine/phosphatidylglycerol/cholesterol in various ratios and ranging from 125 to 488 nm in mean diameter. When liposomes of identical surface charge density were subjected to CZE in Tris-HCl (pH 8) buffers of various ionic strengths (0.001−0.027), they migrated in order of their size. Size-dependent electrophoretic migration and separation of liposomes in CZE can be enhanced or brought about by decreasing the ionic strength of the buffer. It was shown that size-dependent migration is primarily a function of κR, where κ^{-1} is the thickness of the electric double layer (which can be derived from the ionic strength, *I*, of the buffer) and *R*, the liposome radius. Liposome mobility depends on κR and surface charge density in a manner consistent with that expected from the Overbeek−Booth electrokinetic theory. Thus, the relaxation effect appears to be the physical mechanism underlying the size-dependent electrophoretic separation of liposomes.

Over the past decade, capillary zone electrophoresis (CZE) has increasingly been applied for analyzing/characterizing particulate species, including organic and inorganic colloids as well as biological and artificial membrane vesicles, which range in size up to several micrometers in diameter (reviewed in ref 1). CZE was demonstrated to provide electrophoretic mobilities of such microparticles in a good agreement with those determined by laser Doppler velocimetry.[2−4] It was also found that microparticles of a similar or identical chemical composition but differing in size (gold nanoparticles,[5] silica sols,[6] and polystyrene latex microspheres[7−10]) migrate in a capillary in a size-dependent manner and can be separated according to their size. Though McCormick[6] and, later, Hlatshwayo and Silebi[11] have pointed out that such size-dependent separation can arise from differences in electrophoretic mobilities of particles due to the so-called relaxation effect,[12−14] the exact mechanism of this separation was not clear.

The theoretical basis for expecting the size-dependent electrophoretic migration of microparticles is the classical electrokinetic theory. An exhaustive account of this theory can be found elsewhere (e.g., ref 12). Briefly, the electrophoretic mobility of a charged nonconducting spherical particle is governed by its ζ-potential and by a ratio of the particle radius, *R*, to the characteristic size of the ionic atmosphere surrounding the particle. This characteristic size, known as the thickness of the electric double layer (EDL), is a reciprocal of the Debye parameter, κ, and is an explicit function of the ionic strength of the electrolyte solution, *I* ($\kappa \sim \sqrt{I}$). The ζ-potential, in turn, depends on the surface charge density and the radius of the particle, as well as the thickness of the EDL. However, in practical terms, the dependence of ζ on *R* may be neglected for $\kappa R \geq 10$ (e.g., ref 12). It should be noted that, for any *R*, the ζ-potential is an inverse function of ionic strength.

The important feature of the ionic atmosphere surrounding the particle is its distortion (polarization). Once the particle moves, the atmosphere lags behind the particle, imposing an additional drag on particle motion, which is known as the "relaxation effect". The theoretical study of electrophoretic mobility of spherical nonconducting particles, taking into account the relaxation effect,

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(1) Radko, S. P.; Chrambach, A. *J. Chromatogr. B* **1999**, *722*, 1−10.
(2) VanOrman Huff, B. B.; McIntire, G. L. *J. Microcolumn Sep.* **1994**, *6*, 591−594.
(3) Fourest, B.; Hakem, N.; Guillaumong, R. *Radiochim. Acta* **1994**, *66/67*, 173−179.
(4) Fourest, B.; Hakem, N.; Perrone, J.; Guillaumg, R. *J. Radioanalyt. Nucl. Chem.* **1996**, *208*, 309−318.
(5) Schnabel, U.; Fischer, C.-H.; Kenndler, E. *J. Microcolumn Sep.* **1997**, *9*, 529−534.
(6) McCormick *J. Liq. Chromatogr.* **1991**, *14*, 939−952.
(7) VanOrman, B. B.; McIntire, G. L. *J. Microcolumn Sep.* **1989**, *1*, 289−293.
(8) Jones, H. K.; Ballou, N. E. *Anal. Chem.* **1990**, *62*, 2484−2490.
(9) Petersen, S. L.; Ballou, N. E. *Anal. Chem.* **1992**, *64*, 1676−1681.
(10) Hlatshwayo, A. B.; Silebi, C. A. *Polym. Mater. Sci. Eng.* **1996**, *75*, 55−56.
(11) Hlatshwayo, H. B.; Silebi, C. A. In *Methods of Particle Size Analysis III. ACS Symposium Series 693*; Provder, T., Ed.; American Chemical Society: Washington, DC, 1998; pp 296−310.
(12) Hunter, R. J. *Zeta Potential in Colloid Science: Principles and Applications*; Academic Press: London, 1981; pp 11−58, 98−123.
(13) Wiersema, P. H.; Loeb, A. L.; Overbeek, J. Th. G. *J. Colloid Interface Sci.* **1966**, *22*, 78−99.
(14) O'Brien, R. W.; White, L. R. *J. Chem. Soc., Faraday Trans.* **1978**, *77*, 1607−1626.

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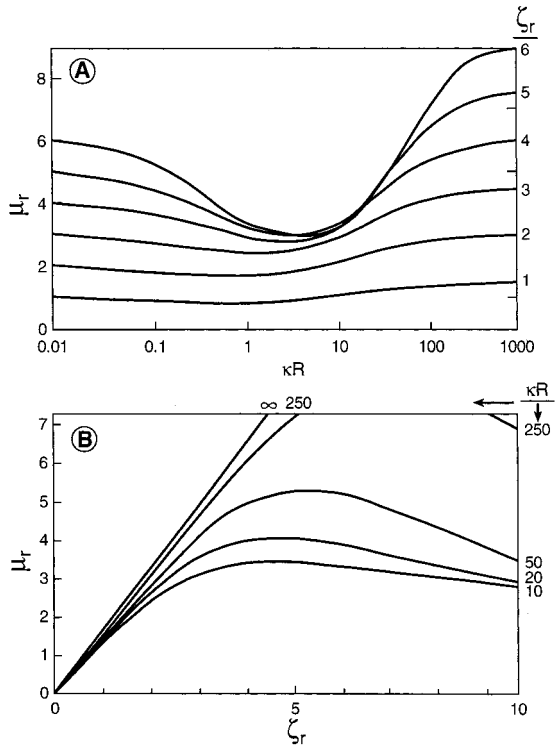

Figure 1. A schematic representation of the dependencies of reduced (dimensionless) electrophoretic mobility, μ_r, of nonconducting spherical particles on the product of the reciprocal of the thickness of electric double layer, κ, and particle radius, R (panel A), and that on the reduced (dimensionless) ζ-potential of the particles, ζ_r (panel B). The curves are drawn on the basis of the results of Wiersema et al.[13] (panel A) and O'Brien and White[14] (panel B). Corresponding values of reduced ζ-potential (panel A) and of κR (panel B) are indicated near the curves outside the panel.

has independently been carried out by Overbeek and Booth (the Overbeek−Booth theory). An analytical solution of this problem is not possible for a general case due to mathematical difficulties, but numerical solutions were later computed by Wiersema et al.[13] and O'Brien and White.[14] Figure 1 shows schematically the theoretically expected dependencies of electrophoretic mobility on κR and ζ-potential, based on computations of Wiersema et al. and O'Brien and White. Both mobility and ζ-potentials are expressed in terms of dimensionless quantities, reduced mobility, μ_r, and reduced potential, ζ_r ($\mu_r = \mu/(2\epsilon kT/3\eta e)$ and $\zeta_r = \zeta/(kT/e)$, where ϵ and η are dielectric permittivity and viscosity of the medium; e is the electron charge; k, the Boltzmann constant; and T, the absolute temperature. While contributing negligibly to mobility at small ζ-potentials ($\zeta \sim 25$ mV, $\zeta_r \sim 1$), the relaxation effect gives rise to a strong dependence of μ on κR with increasing ζ (Figure 1A). Another important consequence of the relaxation effect is that, at $\kappa R > 3$, the electrophoretic mobility undergoes a maximum as a function of ζ at a given κR (Figure 1B). Therefore, the particle mobility can in some cases be independent of, or even decrease with, ζ-potential.

It appears that the systematic variation of both particle size and buffer ionic strength (thickness of the EDL), as well as particle surface charge density (ζ-potential), is the simplest way to reveal the physical basis of the size-dependent separation of microparticles that is observed in the CZE experiments. Recently,[15] we have undertaken such an attempt by using polystyrene size standards. However, a charged "hairy" layer present on the surface of polystyrene microparticles[16] renders a rigorous interpretation of dependencies of mobility on ionic strength difficult.[15]

Liposomes, which are spherical (or near-spherical) membrane vesicles with relatively smooth surfaces,[17] may be a convenient object to test the predictions of the electrokinetic theory in regard to the size-dependent electrophoretic migration. Liposomes can be sized by extrusion, that is, by passing them through membrane filters of defined pore size.[17] By changing the fraction of charged lipids in a lipid mixture composing a liposome, one can easily vary the surface charge density of liposomes and, thus, their ζ-potential at a given ionic strength. An applicability of CZE for analyzing/characterizing liposome preparations was demonstrated earlier.[18−20] Liposomes are increasingly being used in commercial products as well as in basic research (as a model for cellular membrane behavior) and in studies on drug delivery.[21] Hence, feasibility to analyze or to fractionate liposome preparations by electrophoresis in a size-dependent manner may be of practical interest.

The present study employs negatively charged liposomes composed of phosphatidylcholine/phosphatidylglycerol/cholesterol at different molar ratios. The mean size of the liposomes was controlled by extrusion and ranged from 120 to 490 nm mean diameter in various preparations. To test the theoretical predictions, preparations differing in the average size of liposomes were subjected to CZE in Tris-HCl buffers of various dilutions, which provided κR values in the range of 7−130.

EXPERIMENTAL SECTION

Reagents. Distearoylphosphatidylcholine (DSPC), distearoylphosphatidylglycerol (DSPG), and cholesterol (chol) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Fluorescein sodium salt was obtained from Polysciences, Inc. (Warrington, PA). *N*-octadecyl-*N*′-(5-(fluoresceinyl)) thiourea (F18) was purchased from Molecular Probes, Inc. (Eugene, OR). 1 M Tris-HCl solution (pH 8.0) was purchased from Quality Biological, Inc. (Gaithersburg, MD). Tris-HCl buffers ranging in concentration from 2 to 50 mM were obtained by diluting 1 M Tris-HCl solution with deionized water and were used in the preparation of the liposomes and as running buffers in the CE system. The pH values of the buffers were found to be within the limits of 7.97 to 8.10.

Preparation and Characterization of Liposomes. Chloroform solutions of lipids and cholesterol, dried under a stream of argon, were redissolved in benzene/methanol (95:5) and were used as stock solutions stored at −20 °C. After mixing in an appropriate ratio, solutions were dried under high vacuum for 4 h. Lipids were hydrated in 2 mM Tris-HCl buffer to give 1 mM multilamellar vesicle (MLV) suspensions. When fluorescein was

(15) Radko, S. P.; Stastna, M.; Chrambach, A. *Electrophoresis*, in press.
(16) Rasmusson, M.; Wall, S. *J. Colloid Interface Sci.* **1999**, *209*, 312−326.
(17) New, R. R. C. In *Liposomes: A Practical Approach*; New, R. R. C., Ed.; Oxford University Press: New York, 1990; pp 33−104.
(18) Roberts, M. A.; Locascio-Brown, L.; MacCrehan, W. A.; Durst, R. A. *Anal. Chem.* **1996**, *68*, 3434−3440.
(19) Tsukagoshi, K.; Okumura, Y.; Nakajima, R. *J. Chromatogr. A* **1998**, *813*, 402−407.
(20) Kawakami, K.; Nishihara, Y.; Hirano, K. *J. Colloid Interface Sci.* **1998**, *206*, 177−180.
(21) Philippot, J. R.; Schuber, F. *Liposomes as Tools in Basic Research and Industry*; CRC Press: Boca Raton, FL, 1995.

Table 1. Characteristics of Liposome Preparations Used in CZE Experiments

liposome preparation	DSPC/DSPG/chol (molar ratio)	mean diam[a] (nm)	SD[b,c] (nm)
L-161	0.64/0.16/0.2	161	38
L-126	0.64/0.16/0.2	125	29
L-172	0.64/0.16/0.2	172	48
L-225	0.64/0.16/0.2	225	66
L-386[d]	0.64/0.16/0.2	386	163
L-179	0.7/0.1/0.2	179	48
L-187	0.6/0.2/0.2	187	42
L-188	0.5/0.3/0.2	188	44
L-125	0.3/0.5/0.2	125	34
L-160	0.3/0.5/0.2	160	47
L-197	0.3/0.5/0.2	197	68
L-488[d]	0.3/0.5/0.2	488	219

[a,b] Average of three measurements. [c] Standard deviation corresponds to the polydispersity of the preparation. [d] Nonextruded liposomes.

used to "visualize" liposomes by encapsulating a fluorescent dye, the buffer was supplemented with 2 mM fluorescein sodium salt. When F18 was used to fluorescently label liposomes by incorporating the dye into the lipid bilayer, 1 mM solution of F18 in ethanol was added to the MLV suspensions (at 1:200 volume ratio), and the mixture was vigorously vortexed. Thereafter, the suspensions were subjected to five freeze−thaw cycles to ensure a homogeneous mixture of multilamellar vesicles. To produce liposome preparations differing in size, the MLVs were repeatedly extruded through two "sandwiched" polycarbonate filters of 100- (10 passages at 150 psi), 200- (10 passages at 100 psi), and 400-nm (5 passages at 50 psi) pore diameter (Osmonics, Inc., Minneapolis, MN), as described elsewhere.[17] Besides size reduction, the repeated extrusions are known to result in progressively more unilamellar liposomes.[17] A portion (0.8 mL) of each extruded as well as nonextruded (MLVs) liposome suspension was centrifuged at 300000*g* for 1 h, and pellets were re-suspended in 2 mL of 2 mM Tris-HCl buffer.

After preparation, the liposomes were stored at 4 °C in the dark. The size distribution of liposomes was determined by photon correlation spectroscopy on a Coulter model N4 Plus Submicron Particle Sizer (Coulter Corp., Miami, FL), using the manufacturer's software to derive size characteristics of liposomes from an autocorrelation function.

The characteristics of the liposome preparations used in CZE experiments are listed in Table 1. No appreciable alterations in size distributions of liposomes were observed during their storage (≤5 days). We did not determine the final lipid concentrations, and only the upper limit of concentration, 0.32 mM, can be provided. Liposome preparations diluted 10-fold with Tris-HCl buffer of an appropriate molarity were used as samples for CZE experiments. Some CZE experiments were carried out with liposomes labeled by entrapping fluorescein within the liposome interior during a liposome preparation. Some experiments were repeated using liposomes that were labeled with F18, a nonpolar neutral fluorescent dye, binding to membranes by insertion of its hydrophobic alkyl tail into the lipid interior. No appreciable differences in the electrophoretic patterns of liposomes labeled with either fluorescein or F18 were observed.

Capillary Zone Electrophoresis. The P/ACE 2200 capillary electrophoresis system (Beckman Instr., Fullerton, CA) equipped with fluorescence and UV detectors and with a liquid-cooled capillary cartridge for a temperature control was used. For fluorescence detection, the excitation/emission wavelengths were 488/520 (nm/nm). Data acquisition and processing used the Beckman P/ACE Station software. Experiments were performed in fused-silica capillaries of 27 and 37 cm total length, 100-μm i.d., and 360-μm o.d. (Polymicro Technologies, Inc., Phoenix, AZ), with the detection window at 7 cm from the outlet (anodic end) of the capillary. The capillaries were internally coated with 3% un-cross-linked polyacrylamide.[22] Electroosmosis was measured prior to CZE as the time of displacement from the anodic reservoir to the detector of buffer containing 0.05% mesityl oxide, detected by UV absorbance at 214 nm. Capillaries exhibiting electroosmosis in excess of 0.1 μmcm $s^{-1}V^{-1}$ were discarded. CZE was conducted at the electric field strength of 200 V cm^{-1}. The capillary cartridge was thermostated at 25 °C. The values of electric current did not exceed 52 μA, even for 50 mM Tris-HCl buffer. Samples were introduced by pressure injection at 3.5 kPa for 1 s.

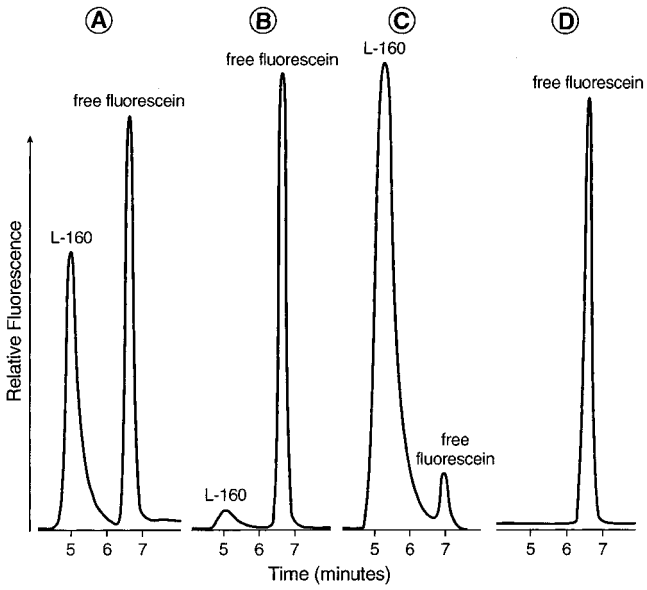

Figure 2. Electropherograms of liposome preparation L-160 (Table 1). The liposomes are fluorescently labeled by encapsulating fluorescein. Panel A: the original liposome preparation, L-160. Panel B: preparation L-160 sonicated for 10 min. Panels C and D: sediment and supernatant, respectively, of preparation L-160 centrifuged at 300000*g* for 1 h. CZE conditions: polyacrylamide-coated capillary of 100-μm i.d., 20-cm effective length; 200 V cm^{-1}; 25 °C; fluorescence detection (ex/em = 488/520); 50 mM Tris-HCl (pH 8, I = 0.0268).

RESULTS AND DISCUSSION

Identification of the Liposome Peak in Electropherograms. When liposomes fluorescently labeled by encapsulated fluorescein are subjected to CZE, two peaks are observed in electropherograms (Figure 2A). The peak corresponding to the free dye can easily be identified by running this dye alone, while the second peak is assumed to contain the liposomes. To verify this assumption, the liposome preparation was sonicated for 10 min in a sonicator bath (model G112SP1G, Laboratory Supplies Co., Hicksville, NY) at room temperature. This induced a leakage of fluorescein from the liposomes due to their "sealing-unsealing"

(22) Hjerten, S. *J. Chromatogr.* **1985**, *347*, 191−198.

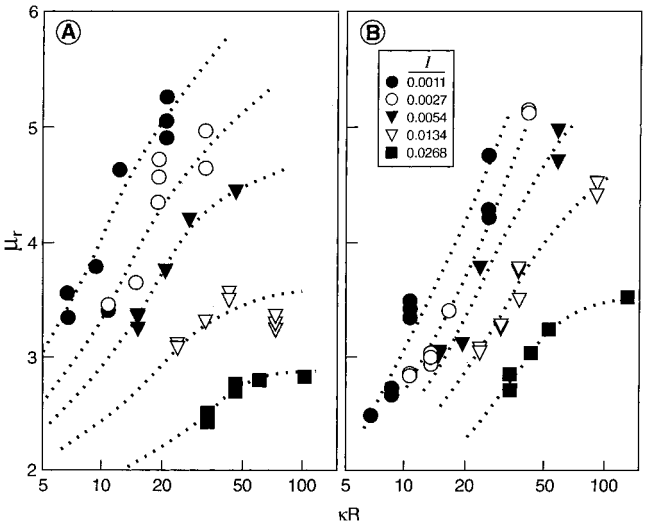

Figure 3. Dependence of reduced electrophoretic mobility of liposomes on κR. Liposome preparations are of various sizes (Table 1). Panel A: L-126, -172, -225, -386. Panel B: L-125, -160, -197, -488. Tris-HCl (pH 8) buffer is of various concentrations (Table 2). Ionic strength is defined by the symbols in the inner panel. CZE conditions: polyacrylamide-coated capillaries of 100-μm i.d., 30-cm effective length; 200 V cm^{-1}; 25 °C; fluorescence detection (ex/em=488/520). The dotted curves are drawn to guide the eye.

Table 2. Ionic Strength and Thickness of Electric Double Layer Calculated for Tris-HCl (pH 8) Buffers of Various Concentrations

	buffer concn, mM				
	2	5	10	25	50
I x 10^3 [a]	1.1	2.7	5.4	13.4	26.8
κ^{-1} (nm)[b]	9.3	5.9	4.2	2.6	1.9

[a] Ionic strength was calculated by means of the program REFFUB.[25] [b] The reciprocal of EDL thickness, κ, was calculated according to eq 2.3.18 of ref 12: $\kappa = 3.288\sqrt{I}$ (nm^{-1}).

during the sonication[17] and results in a decrease of the "liposome" peak area relative to that of free dye, which is indeed observed (Figure 2B). When a liposome sample is repeatedly centrifuged at 300000*g* for 1 h, the CZE analysis of the supernatant shows exclusively a peak of the free dye (Figure 2D). By contrast, a sharp drop in the amount of free dye can be found on electropherograms in CZE of the sediment containing the liposome (Figure 2C).

Dependence of Liposome Mobility on κR. Figure 3 demonstrates dependencies of reduced mobility, μ_r, on κR for liposomes differing in surface charge density (panel A: DSPC/DSPG/chol = 0.64/0.16/0.2, $\sigma_s = 4.57$ μC cm^{-2}; panel B: DSPC/DSPG/chol = 0.3/0.5/0.2, $\sigma_s = 14.29$ μC cm^{-2}). Surface charge density of liposomes was calculated on the basis of the molar ratio of lipids and assuming that a lipid head occupies 0.7 nm^2.[17] Values of κ used in the calculation of κR are listed in Table 2. Experimental points for liposome preparations L-126, -172, -225, and -386 and those for L-125, -160, -197, and -488 (Table 1) are presented in panels A and B of Figure 3, respectively. Each curve corresponds to liposomes of a constant σ_s at a given ionic strength for various liposome sizes. The absolute mobility of negatively charged liposomes can readily be calculated from reduced mobility as $\mu = \mu_r \times (-1.34\ \mu\text{m cm s}^{-1}\ \text{V}^{-1})$ for 25 °C.[12] Here ϵ and η are taken as those of water.

The patterns of liposome mobility as a function of κR (Figure 3) are similar to those theoretically expected for species with a uniform ζ-potential in the respective κR range (Figure 1A). Though some reduction of ζ-potential for liposomes with decreasing R can be expected at low κR,[12] it would not necessarily result in a decrease of mobility. As seen in Figure 3 (compare panels A, B), mobility decreases at a given I in the range of small κR, despite an elevation in ζ-potential due to the increasing surface charge density. Such behavior is a manifestation of the relaxation effect.[12,14] Together with dependencies of μ_r on κR, it clearly indicates that the relaxation effect is, indeed, responsible for the observed size-dependent electrophoretic migration of liposomes.

Dependence of Liposome Mobility on Calculated ζ-Potential. Although ζ-potential can be derived from the mobility rate by using the numerical algorithm of O'Brien and White,[14] the derivation becomes ambiguous when the ζ-potential approaches or crosses the maximum of the plot of μ vs ζ.[12,14] Thus, to investigate how mobility depends on ζ-potential, we have calculated the values of ζ from known surface charge densities and ionic strength of electrolyte solutions at a given R, assuming a flat plane geometry. By solving eq 2.3.23 of ref 12

$$\sigma_s = 11.74c^{1/2}\sinh(19.46\psi_s) \qquad (1)$$

as a quadratic equation in relation to $e^{19.46\psi_s}$, the surface potential of particles can easily be calculated. Here c is the concentration of the ionic species of the electrolyte (in mol^{-1}) and ψ_s the surface potential (in volts). To convert the surface potential into ζ-potential, eq 2.3.16 of ref 12 has been employed

$$\tanh(\psi_r/4) = \tanh(\psi_{rs}/4) \times \exp(-\kappa x) \qquad (2)$$

Here ψ_r is the reduced potential at a distance x from the surface and ψ_{rs}, the reduced surface potential. A reduced potential is related to a potential of a negatively charged particle as $\psi = -25.7\psi_r$ (mV) at 25 °C.[12] Assuming a shear plane located at 2 nm from the surface, the reduced ζ-potential was calculated as $\zeta_r = \psi_r$., taking $x = 2$ nm. Although the flat plane geometry appears to be a reasonable approximation for $\kappa R > 20$, it is rather a rough one for $\kappa R \cong 10$. However, because we are concerned with a relative change in mobility with ζ-potential varied by a surface charge density at constant κ and R, rather than with absolute values of the mobility, such approximation appears acceptable for all κR under study.

The mobility of liposomes with differing surface charge densities but similar mean sizes (preparations L-179, -187, and -188 (Table 1); $\sigma_s = 2.86$, 5.71, and 8.57 μC/cm^2) was plotted vs the ζ-potential calculated according to eqs 1 and 2 (Figure 4). As seen, the observed dependencies are consistent with behavior expected for the case of a profound relaxation effect (Figure 4 vs Figure 1B). The important fact is that, indeed, electrophoretic mobility of liposomes can became near-independent of, or even decrease with, the surface charge density at low ionic strengths (Figure 4). Thus, electrophoretic migration of relatively highly charged liposomes under such conditions will be a function of liposome size rather than charge-to-size ratio, as commonly assumed.[18]

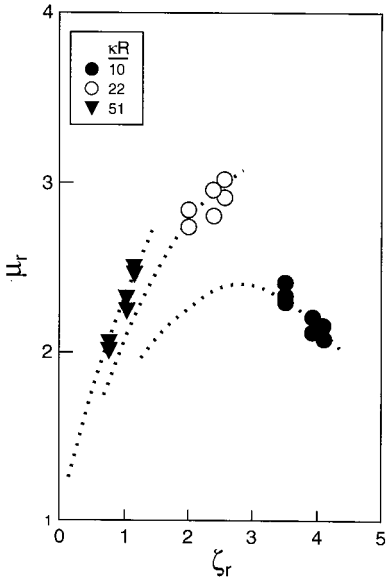

Figure 4. Dependencies of reduced electrophoretic mobility of liposomes on reduced ζ-potential calculated on the basis of the surface charge density of the liposomes. Liposome preparations are L-179, -187, and -188 (Table 1). Tris-HCl buffer (pH 8) is of ionic strengths of 0.0011, 0.0054, and 0.0268 (Table 2). CZE conditions as in Figure 2, except for the ionic strength.

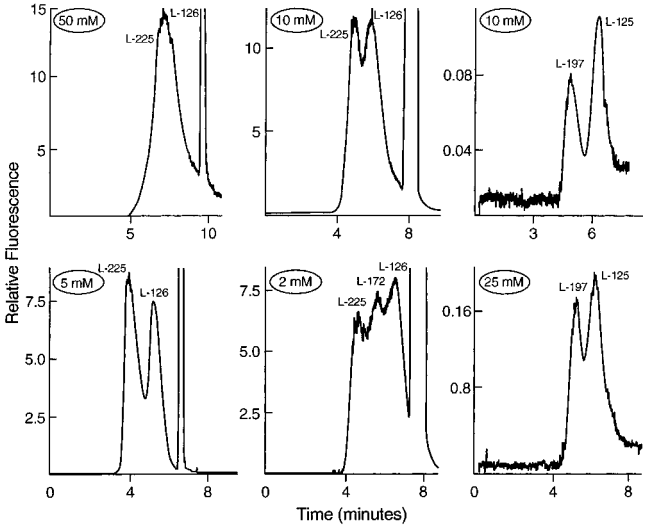

Figure 5. Representative electropherograms demonstrating separation by CZE of liposome preparations differing in size. Electrophoretic buffer concentrations and liposome preparations are indicated in the panels. CZE conditions are as in Figure 3.

Separation by CZE of Liposome Preparations Differing in Size. Figure 5 shows the representative electropherograms for mixtures of liposome preparations differing in size that were subjected to CZE in Tris-HCl buffer of various concentrations. Although, in 50 mM Tris-HCl buffer, liposome preparations of 126- and 225-nm mean diameter exhibit completely overlapping peaks, they become progressively separated upon decreasing the buffer concentration. In 2 mM Tris-HCl buffer, even three mixed preparations, L-126, -172, and -225, can be resolved. For the preparations with higher surface charge density such as L-125 and L-197 (Table 1), the same trend holds (compare electropherograms in 25 and 10 mM buffers for these preparations). In 10 mM buffer, a better separation (in terms of selectivity) is achieved for L-125 and L-197 than that for L-125 and L-225, despite the smaller size difference (Figure 5 and Table 1). The fact that size-dependent electrophoretic separation is a function of the ionic strength of the buffer, which is enhanced by an increase in particle surface charge density, directly points to the relaxation effect as the mechanism governing such separation (Figure 1).

For a given ionic strength (κ) and a given pair of particles differing in size ($R_1 > R_2$, $\zeta_1 = \zeta_2$), differences in particle mobilities as a function of $\Delta R = R_1 - R_2$ are expected to be the strongest when both κR_1 and κR_2 are within the range of approximately 5−100 (Figure 1A). Indeed, a liposome separation not observed in 50 mM Tris-HCl buffer ($\kappa R = 66$ and 118 for L-126 and L-225, respectively) was achieved in 10 mM ($\kappa R = 23$ and 42 for L-126 and L-225, respectively) and 2 mM ($\kappa R = 14$ and 24 for L-126 and L-225, respectively) buffers (Figure 5 and Tables 1 and 2). However, if the relaxation effect does operate here, the size-dependent separation of liposomes achieved by reducing the ionic strength of the electrophoretic buffer has to be naturally limited. At some point, the reduction of I should decrease selectivity because the mobility difference will decrease upon approaching the mobility minimum (Figure 1A, κR values approaching approximately 5). Earlier, a maximum in selectivity as a function of the ionic strength was, indeed, observed for polystyrene microspheres of differing sizes subjected to CZE in Tris-borate buffer.[15] Unfortunately, our attempt to test for the existence of such maxima for the liposome preparations by lowering the concentration of Tris-HCl buffer below 2 mM (Table 2) failed due to an unstable current.

Previously, Roberts et al.[18] suggested that the width of a liposome peak originates from the size heterogeneity of a liposome preparation. The suggestion was based on the likelihood of size (derived by means of photon correlation spectroscopy) and mobility (the shape of liposome peak in CZE) distributions for a liposome preparation. However, a firm conclusion regarding the origin of peak width can only be drawn by ruling out contributions to the final peak width due to intra- and extracolumn spreading (thus, requiring data on peak spreading as a function of E, capillary effective length, sample concentration, and initial zone length)[23] and by showing the correlation between the peak width and the width of size distribution for various liposome preparations. If size heterogeneity of liposomes is the major source of their electrophoretic heterogeneity, that will have two practical consequences which should be pointed out. First, in terms of resolution, separation of liposome preparations differing in size would be limited by an increase in peak width parallel to the increase in selectivity. Second, the size-dependent electrophoretic migration of liposomes is clearly not restricted to the case of CZE but reflects a general electrokinetic phenomenon. Thus, if electrophoretic heterogeneity of liposomes results from their size heterogeneity, that would render the size fractionation of a liposome preparation by a preparative electrophoretic technique such as, for instance, free-flow electrophoresis at least feasible.

It should be noted that the wide-spread accounting for electrophoretic mobility by the "charge-to-size ratio" mechanism[23] is a particular case of electrokinetic theory, namely, $\zeta_r \sim 1$ and

(23) Grossman, P. D. In *Capillary Electrophoresis: Theory and Practice*; Grossman, P. D., Colburn, J. C., Eds.; Academic Press: San Diego, CA, 1992; pp 3−43.

$\kappa R \leq 1$ or, more rigorously, $\kappa R \ll 1$ (the so-called Hückel limit).[12] In this case, the particle translational motion results from the balance of two forces: the driving electric force, $F_e = Q_e E$, where Q_e is a net (electrokinetic) charge and E, the electric field strength, and the frictional force, the so-called Stokes drag $F_s \sim \mu E R$. After a rearrangement, one obtains: $\mu \sim Q_e/R \sim \sigma_e R$, where σ_e is the net surface charge density. In fact, the condition $\zeta_r \sim 1$ and $\kappa R \leq 1$ covers most cases of protein electrophoresis. However, the electrophoretic migration of microparticles, to which liposomes belong, would not obey the charge-to-size mechanism in many, if not most, practical cases. Though a dependence of mobility on buffer ionic strength can be incorporated into the net charge,[12] this cannot be done for the size-dependent separation as a function of ionic strength. Indeed, for the selectivity of separation,[24] one may write: $\Delta\mu/\mu_{av} \equiv |\mu_1 - \mu_2|/[(\mu_1 + \mu_2)/2] = \sigma_e \Delta R/\sigma_e R_{av} = \Delta R/R_{av}$. Thus, for particles of identical surface charge density, the selectivity is independent of the ionic strength, which is contrary to observation (Figures 3 and 5).

CONCLUSIONS

The size-dependent electrophoretic migration and separation of relatively highly charged liposomes is primarily a function of κR, where κ and R are the reciprocal of the thickness of the electric double layer and liposome radius, respectively. The electrophoretic behavior of liposomes in CZE is consistent with that expected due to a strong relaxation effect, in accordance with the Overbeek-Booth theory. This finding makes that theory a useful guide for electrophoretic analysis and fractionation of liposomes in a size-dependent manner.

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(24) Giddings, J. C. *Unified Separation Science*; John Wiley: New York, 1991.
(25) Chrambach, A. *The Practice of Quantitative Gel Electrophoresis*; VCH: Weinheim; 1985, Appendix 7.