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Polydispersity of liposome preparations as a likely source of peak width in capillary zone electrophoresis

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Abstract

Negatively charged liposomes consisting of phosphatidylcholine/phosphatidylglycerol/cholesterol in various ratios when subjected to capillary zone electrophoresis (CZE) in Tris–HCl (pH 8) buffer of different concentrations have been shown previously to exhibit a size-dependent migration rate at low ionic strength. The present study, focusing on the peak width under those conditions, shows that the polydispersity of liposomes correlated with, and appears to be a dominant source of, the peak width of the liposomes in CZE in a buffer of low ionic strength (2 to 5 m*M* Tris–HCl buffer, pH 8) at moderate electric field strengths (200 V cm^{-1} or less). This finding, beyond allowing for the analysis of liposome polydispersity by CZE, suggests that the size-dependent fractionation of liposome preparations by a preparative electrophoretic technique such as free-flow electrophoresis is potentially feasible. © 2001 Published by Elsevier Science B.V.

Keywords: Polydispersity; Liposomes

1. Introduction

The size-dependent electrophoretic migration of colloidal particles such as polystyrene latex (PS) microspheres, silica sols, and gold colloidal particles was demonstrated in capillary zone electrophoresis (CZE) in a number of publications over the last decade (reviewed in Ref. [1]). As has recently been shown [2], that size-dependent migration and, hence, the size-separation of colloidal particles (''microparticles'') is mainly a function of the ionic strength of the electrophoretic buffer (or, more rigorously, the ratio of particle radius to the characteristic thickness of the ionic atmosphere surrounding the particle). That electrophoretic behavior of colloidal particles in CZE was found to be consistent, at least qualitatively, with that expected from the Overbeek–Booth electrokinetic theory (exhaustive accounts of this theory can be found elsewhere, e.g., Ref. [3]). It is most likely that the so-called relaxation effect (distortion of the ionic atmosphere due to the Brownian motion of the particle [3]) is a dominant physical mechanism underlying the size-dependent electrophoretic migration of microparticles [2,4].

Liposomes, which are spherical (or near-spherical) artificial membrane vesicles, with diameters within

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the size-range of colloidal particles [5], also exhibited at low ionic strengths the size-dependent electrophoretic migration in CZE [4]. Hence, liposome preparations differing in size can be separated at an appropriate ionic strength of the background electrolyte [4].

When a population of particles migrates in a size-dependent manner in electrophoresis, one may expect a contribution of its polydispersity (size heterogeneity) to the peak width. Indeed, Ballou and co-workers [6,7] have concluded that electrophoretic heterogeneity (heterogeneity of mobility) is a dominant source of the zone broadening of PS microspheres in CZE. A similar conclusion was subsequently reached by this laboratory [2]. It was pointed out that the electrophoretic heterogeneity may reflect, in fact, the polydispersity of the PS microspheres [2]. Previously, Roberts et al. [8] had already postulated, on the basis of the observed size (measured by photon correlation spectroscopy) and mobility (the shape of liposome peak in CZE) distributions for a liposome preparation, that the peak width of liposomes in CZE originates from their size heterogeneity.

Polydispersity as a main source of the peak spreading would pose a necessary limit to resolution in the size-dependent electrophoretic separation of microparticles in general and that of liposomes in particular. However, there are reasons to expect [1,2,4] that one may be able to offset any peak spreading by an increase in selectivity gained by choice of an appropriate buffer ionic strength. The size-dependent electrophoretic migration of microparticles is not limited to CZE but presents a general electrokinetic phenomenon [2,4]. Accordingly, a mobility distribution originating from the size heterogeneity of a particle population at the appropriate ionic strength would allow the size fractionation of such population by an electrophoretic preparative technique such as free flow electrophoresis [9]. This fractionation in a size-dependent manner may be of practical interest in application to liposomes which are widely used in both research and industry [10].

The present study aims at gaining insights into the sources of the mobility distribution (peak width) of liposomes in CZE. Liposome preparations composed of phosphatidylcholine/phosphatidylglycerol/cholesterol, with mean diameters and polydispersities controlled by extrusion, were employed. They were subjected to CZE in Tris–HCl buffers and peak width at half height was analyzed as a function of capillary effective length, electric field strength, buffer and sample concentrations, initial zone length, and liposome polydispersity.

2. Experimental

2.1. Materials

2.1.1. Buffer

Tris–HCl buffers ranging in concentration from 2 to 50 m*M* were obtained by diluting 1 *M* Tris–HCl solution, pH 8.0, (Quality Biological, Gaithersburg, MD, USA; catalog No. 351-007-100) with deionized water. Their pH values were within the limits of 7.97 to 8.10.

2.1.2. Liposomes

Distearoylphosphatidylcholine (DSPC), distearoylphosphatidylglycerol (DSPG), and cholesterol (Ch) were purchased from Avanti Polar Lipids (Alabaster, AL, USA; catalog Nos. 850365, 840465, and 700000, respectively). The lipids and cholesterol, dissolved in benzene–methanol (95:5) solution were mixed in molar ratios of 0.64/0.16/0.2, 0.6/0.2/0.2 or 0.3/0.5/0.2 of DSPC/DSPG/Ch. Liposomes were prepared and ''visualized'' by encapsulation of a fluorescent dye, fluorescein sodium salt (Polysciences, Warrington, PA, USA; catalog No. 16085), as described earlier [4]. Since DSPG is a negatively charged lipid, liposomes differing in DSPC/DSPG ratio possess different charge densities. Mean diameters and polydispersity were controlled by passing the liposomes through membrane filters of defined pore size (extrusion) [4]. After preparation, liposomes were stored in 2 m*M* Tris–HCl buffer at 4°C in the dark. The final lipid concentrations were not determined and only the upper limit of concentration, 0.32 m*M*, can be provided. Liposome preparations diluted 10-fold with Tris–HCl buffer of an appropriate molarity were used as samples for CZE experiments unless stated otherwise.

2.2. Photon correlation spectroscopy

Photon correlation spectroscopy was employed to derive the size characteristics of liposomes from autocorrelation functions. Measurements were performed on a Coulter Model N4 Plus Submicron Particle Sizer (Coulter, Miami, FL, USA) in accordance with the manufacturer's instructions. The mean diameter, $\langle d \rangle$, and standard deviation (σ) of the size distribution of liposome preparations were obtained by the unimodal data analysis provided by the N4 Plus software.

2.3. Capillary electrophoresis

CZE of liposomes was carried out by using the P/ACE 2200 capillary electrophoresis system (Beckman, Fullerton, CA, USA) equipped with a liquid-cooled capillary cartridge for temperature control and fluorescence and UV detectors. The procedure and conditions of CZE were specified earlier [4]. Fused-silica capillaries of 27, 37, and 67 cm total lengths× 100 μm I.D.×360 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA; catalog No. TSP100375), internally coated with uncrosslinked polyacrylamide were employed. The detection window was at 7 cm from the outlet (anodic end) of the capillary. Electroosmosis did not exceed 10^{-5} cm^2 s^{-1} V^{-1}. Details on the capillary coating and electroosmosis control can be found elsewhere [2,4]. Samples were introduced by pressure injection at 3.5 kPa for 1 to 4 s.

2.4. Data processing

The peak width at the half height, $\Delta X_{1/2}$ (cm), was derived from the data provided by the P/ACE Station software as specified in Ref. [2]. The relative peak width was calculated as $\Delta X_{1/2}/L_{\text{eff}}$, where L_{eff} is the effective capillary length (capillary length from the inlet to the detector position). The plate height, H, was obtained according to: $H=(\Delta X_{1/2})^2/5.54L_{\text{eff}}$. The relative standard deviation (RSD) of the liposome size distribution was calculated as RSD$=\sigma/\langle d \rangle$. The initial zone length, ΔL, was calculated as described in Ref. [11]. The liposome volume fraction, Φ, was estimated based on the upper limit of lipid concentration (see above), assuming that a lipid head occupies 0.7 nm^2 [3].

3. Results and discussion

3.1. Conditions for maintenance of a single liposome peak in CZE at low ionic strengths

Fig. 1 shows representative electropherograms of two liposome preparations differing in size. A peak corresponding to the free fluorescein not trapped by the liposome interior [4] is marked as ''free dye''. When subjected to CZE in Tris–HCl buffer of relatively high concentration [50 m*M* in both background electrolyte (''running'' buffer) and sample buffer], liposome preparations provide a single, broad peak (Fig. 1, panels A and E, and Table 1). Upon similar dilution of both buffers (panels B and F – 25 m*M*; panels C and G – 10 and 5 m*M*, respectively), a peak ''splitting'' can be observed (see also Section 3.4). The peak ''splitting'' can be eliminated by increasing the concentration of the sample buffer by a factor of 2 to 5, compared to that of the ''running'' buffer (Fig. 1, panels D and H, and Table 1). Within the concentration range studied, elevating the concentration of the sample buffer by 2 to 10 times (compared to that of the ''running'' buffer) did not alter the relative peak width to any appreciable extent (Fig. 2A). Also, variation of liposome volume fraction in the sample by a factor of 5 did not result in an appreciable effect on the relative peak width under these conditions (Fig. 2B).

3.2. Operational conditions of CZE not affecting the relative peak width of liposomes at low ionic strengths

The axial diffusion, electrophoretic dispersion, thermal effects in the capillary, analyte–wall interactions, and a contribution from initial zone length are likely sources of peak spreading in CZE, depending on operational conditions (e.g., Ref. [12]). For a fixed capillary diameter, operational parameters such as electric field strength, conductivity of ''running'' buffer, capillary effective length, sample concentration, and initial zone length were varied.

Axial diffusion: It has been shown previously that

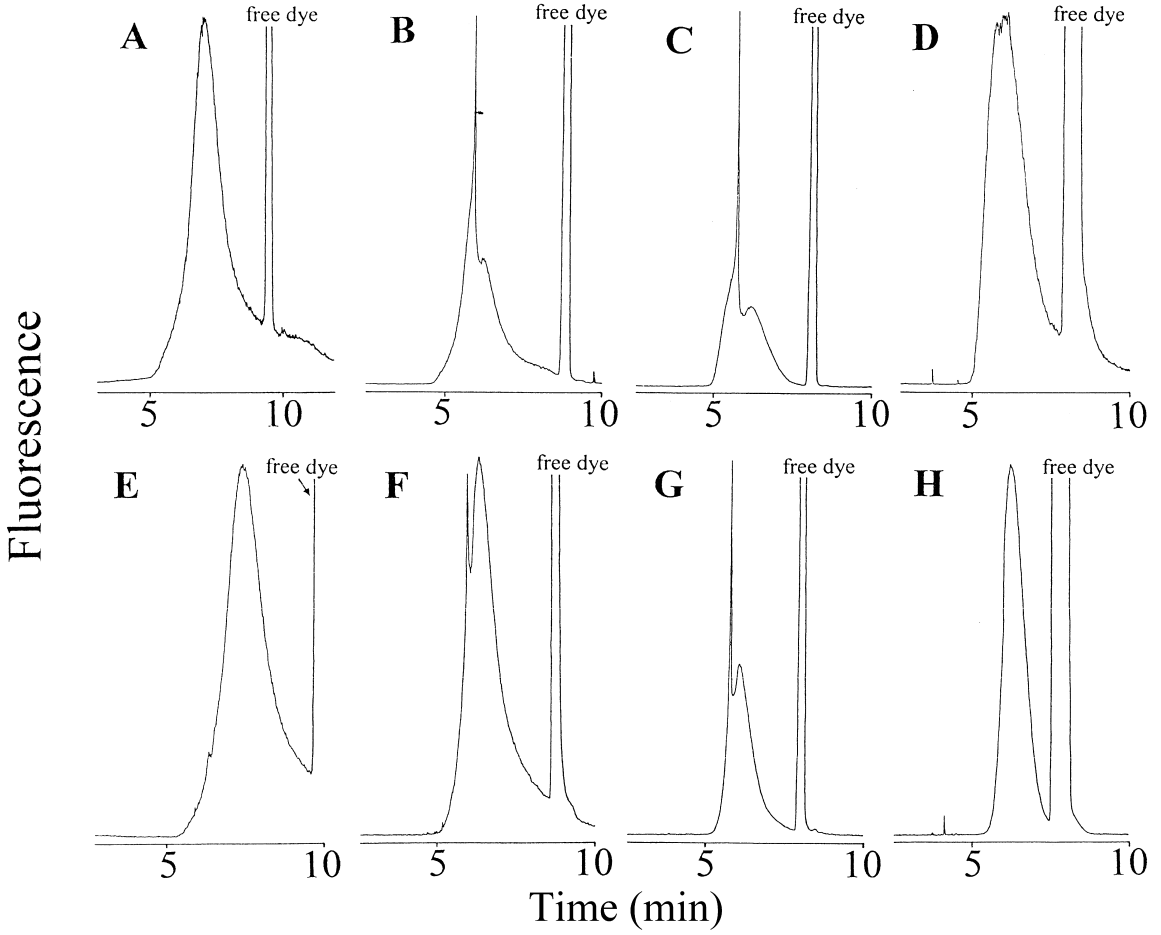

Fig. 1. Representative electropherograms of liposome preparations (DSPC/DSPG/Ch=0.64/0.16/0.2) subjected to CZE. Panels A to D corresponds to the liposome preparation with a mean diameter ($\langle d \rangle$) of 172 nm and a relative standard deviation of variation of liposome size distribution (RSD) of 0.28; panels E to H – to that with $\langle d \rangle$ of 125 nm and RSD of 0.23. Tris–HCl solutions (pH 8) are used as both ''running'' and sample buffers at the concentrations shown in Table 1. CZE conditions: capillary of 30 cm effective length×100 μm I.D., 200 V cm^{-1}, 25°C, 1 s pressure injection.

contribution to the peak width due to the axial diffusion is negligible for particles of sub-micron sizes similar to those of liposomes [2,6,7]. For the liposomes of the size under study, the maximal estimated contribution from axial diffusion to the plate height, H_D, is 10^{-8} m.

Electrophoretic dispersion: The contribution to the plate height from the electrophoretic dispersion is known to be proportional to Δk^2, where Δk represents the discrepancy in conductance between sample zone and the ''running'' buffer [12]. Therefore, electrophoretic dispersion can be tested by varying the concentration of the analyte. A 25-fold variation of liposome volume fraction in the sample (from $3 \cdot 10^{-5}$ to $7 \cdot 10^{-4}$ compared to that of $1.4 \cdot 10^{-4}$ used routinely in the CZE experiments) did not give rise to an appreciable change of the relative peak width, $\Delta X_{1/2}/L_{eff}$ (Fig. 2B). Thus, electrophoretic dispersion appears not to contribute to the peak width of liposomes under conditions of the study.

Thermal effects: The contribution to the final peak height from the thermal effects, H_t, has been estimated as being proportional to k^2E^5 where k is the buffer conductivity [12]. Fig. 3A demonstrates the

Table 1
Concentrations of background electrolyte (''running'' buffer) and sample buffer used for CZE experiments in Fig. 1 (see also Sections 3.1 and 3.4)

Panel	Buffer concentration (Tris–HCl, pH 8.0) (m*M*)	
	''Running'' buffer	Sample buffer
A	50	50
B	25	25
C	5	5
D	5	25
E	50	50
F	25	25
G	10	10
H	10	15

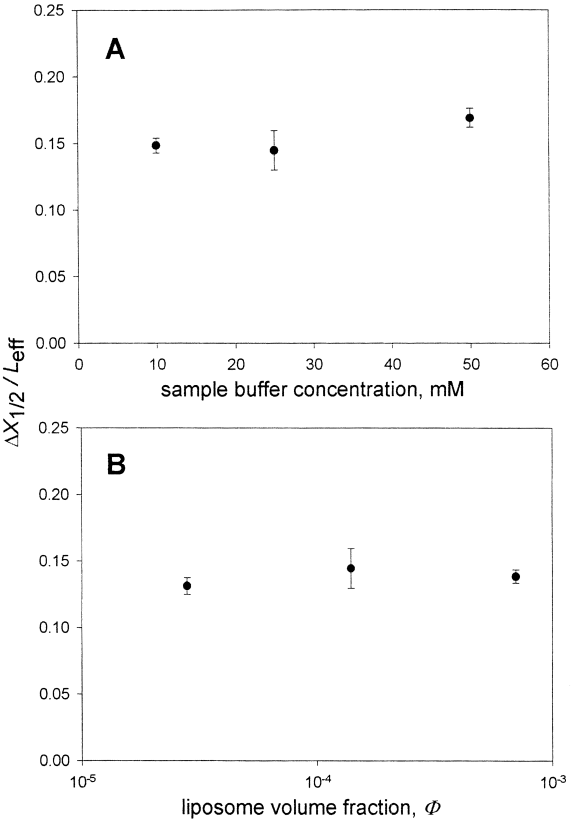

Fig. 2. Relative peak width ($\Delta X_{1/2}/L_{eff}$) as a function of sample buffer concentration (panel A) and liposome volume fraction (panel B). Liposome preparation: DSPC/DSPG/Ch=0.64/0.16/0.2, $\langle d\rangle$=125 nm, RSD=0.23. ''Running'' buffer: 5 m*M* Tris–HCl (pH 8). Samples: panel A – liposomes in Tris–HCl buffer of different concentrations; panel B – liposomes of various concentration in 25 m*M* Tris–HCl buffer. Other CZE conditions as in Fig. 1.

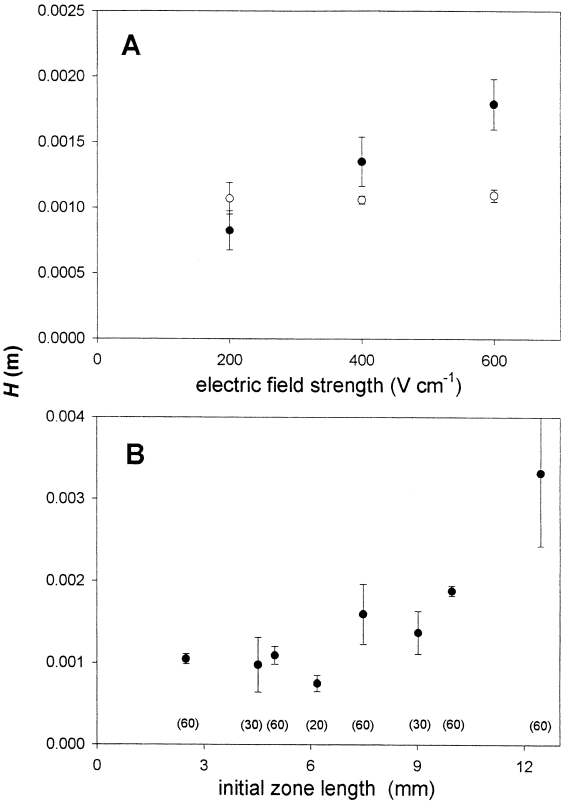

Fig. 3. Plate height, *H*, vs. electric field strength (panel A) and initial zone length (panel B). The numbers in parentheses in panel B indicate the effective length of the capillary (cm). Closed circles: liposome preparation – DSPC/DSPG/Ch=0.6/0.2/0.2, $\langle d\rangle$=187 nm, RSD=0.22; ''running'' buffer – 10 m*M* Tris–HCl; sample buffer – 50 m*M* Tris–HCl. Open circles: liposome preparation – DSPC/DSPG/Ch=0.64/0.16/0.2, $\langle d\rangle$=125, RSD=0.23; ''running'' buffer – 5 m*M* Tris–HCl; sample buffer – 25 m*M* Tris–HCl. Other CZE conditions as in Fig. 1 except for the electric field strength (panel A) or capillary effective length and injection time (panel B).

effect on the peak width of electric field strength. As seen, in 5 m*M* Tris–HCl buffer, values of the plate height, *H*, do not change over the wide range of the electric field strength, *E*, from 200 to 600 V cm^{-1} while they are approximately doubled over that range in 10 m*M* Tris–HCl buffer. Taking into account the doubling of *H* upon threefold increase of *E*, one may conclude that, at moderate *E* (200 V cm^{-1} or below), the thermal effects could not account for most of the final peak width (Fig. 3A).

Liposome–wall interaction: Another possible

cause of peak spreading is the analyte–capillary wall interaction, which is known to contribute to peak spreading (in terms of H) in proportion to electric field strength (e.g., Ref. [13]). For the same reasons stated above in reference to thermal effects and based on the same evidence (Fig. 3A), a significant contribution of liposome–wall interaction to the peak spreading can be ruled out.

Initial zone length: Fig. 3B demonstrates the effects of initial zone length and capillary effective length on the peak width of liposomes. The number in parentheses below each experimental point indicates the effective length (cm) of the capillary used to perform the experiment. Within the limits of experimental scatter, there is no effect of the capillary effective length on the plate height. The length of the initial zone appears not to affect the peak spreading under conditions used if that length, ΔL, is less than approximately 6 mm (Fig. 3B). When the absolute length of the initial zone exceeds ~6 mm, the plate height starts to increase. Note that the direct contribution from the initial plug length to the plate height is negligible: if one estimates such contribution as $H_{inj}=(\Delta L)^2/12L_{eff}$ [12], the maximal value of H_{inj} (under the conditions used) is about $2\cdot10^{-5}$ m, i.e., two orders of magnitude less than the experimentally observed values of H (Fig. 3B). It is thought that the increase of the plate height at the greater lengths of the initial zone may be due to a higher buffer concentration in this zone when the zone length exceeds some critical value. However, at ''small'' ΔL corresponding to 1 s pressure injection under the experimental conditions of this study, both the initial zone length and capillary effective length appear not to affect the peak width of liposomes.

3.3. Correlation between peak width of liposomes and their polydispersity

As was recently shown, the size-dependent electrophoretic migration of liposomes of an identical surface charge density can be brought about by appropriately decreasing ionic strength of the ''running'' buffer [4]. While being relatively slight or absent in 50 m*M* Tris–HCl (pH 8) buffer, such migration was greatly enhanced in 2 or 5 m*M* buffer [4]. Therefore, one may expect a correlation between peak width and liposome polydispersity (size heterogeneity) at low ionic strength when the peak spreading due to operational conditions appears to be negligible (see Section 3.2).

Fig. 4 shows values of the relative peak width of liposome preparations plotted against values of their polydispersity expressed as a coefficient of variation

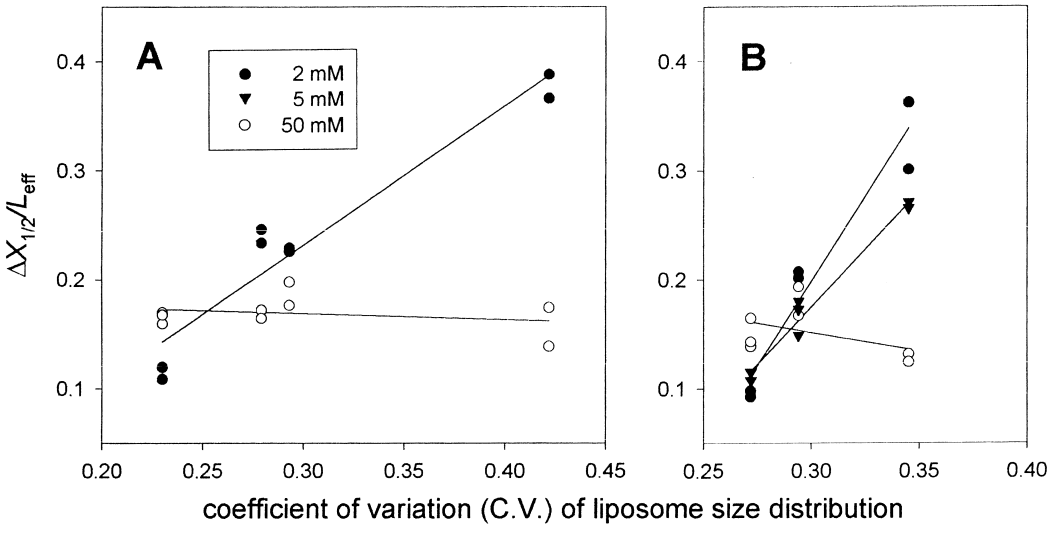

Fig. 4. Correlation between the relative peak width ($\Delta X_{1/2}/L_{eff}$) and the polydispersity of liposomes. DSPC/DSPG/Ch=0.64/0.16/0.2 (panel A) and 0.3/0.5/0.2 (panel B). Concentrations of the ''running'' buffer (Tris–HCl, pH 8) are shown in the insert. Concentrations of the sample buffer were 10, 25 and 50 m*M* for 2, 5 and 50 m*M* ''running'' buffer, respectively. CZE conditions as in Fig. 1.

of liposome size distribution (RSD, see Section 2.4). Results for two different lipid compositions (DSPC/DSPG/Ch=0.64/0.16/0.2, panel A, and 0.3/0.5/0.2, panel B) in 2, 5, and 50 m*M* Tris–HCl buffers are shown. There is no correlation between $\Delta X_{1/2}/L_{eff}$ and RSD in 50 m*M* buffer (correlation coefficient, r^2, equals 0.074 and 0.218 for panels A and B, respectively), whereas the peak width strongly correlates with the RSD in 2 m*M* (r^2=0.933 and 0.935 for panels A and B, respectively) and 5 m*M* (r^2=0.974, Fig. 4B) buffers.

3.4. Distortion of the shape of the liposome peak in the electrolyte solutions of low ionic strengths

When both ''running'' buffer and sample buffer are low in ionic strength (<50 m*M*), the liposome peak splits into two (Fig. 1, Table 1, and Section 3.1). The mechanism underlying the observed phenomena is unclear. The procedure for liposome preparation used in the study is well established [3,4]. The multiple freeze–thaw cycles followed by liposome extrusion (passing through polycarbonate filters under a high pressure) make it unlikely that two liposome populations well distinguished in charge or size would exist in the preparation. Under identical conditions, the ''splitting'' was found to depend on liposome size and charge in a complicated manner (data not shown although panel B vs. panel F indicates it). At that point, we are unable to arrive at a hypothesis which could account for the observed phenomena. One may speculate that a non-even distribution of co- and counter-ions in a colloidal suspension [3] and, thus, in the sample zone, could lead to a peculiar electric process at the boundary of the initial sample zone and background electrolyte, resulting in a peak distortion. It can be expected that a thorough study on peak distortion (including systematic variations in both ''running'' and sample buffer concentrations as well as initial zone length, liposome size and charge characteristics, and liposome concentration) might provide the experimental data needed for elucidating the mechanisms responsible for the peak distortion. However, such study is beyond the scope of the present work. A simple practical solution to eliminate the peak ''splitting'' was provided by increasing the concentration of the sample buffer as described (Fig. 1, Table 1, and Section 3.1).

4. Conclusion

The size heterogeneity (polydispersity) of liposomes appears to be a dominant source of the peak width in CZE conducted at low ionic strength (corresponding to that of 2 to 5 m*M* Tris–HCl buffer, pH 8) and moderate values of the electric field strength (200 V cm^{-1} or less). This finding suggests that both the analysis of liposome polydispersity by CZE and the size-dependent fractionation of liposome preparations by a preparative electrophoretic technique such as free-flow electrophoresis are potentially feasible.

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