Sterically stabilized liposomes

Reduction in electrophoretic mobility but not electrostatic surface potential

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ABSTRACT The electrophoretic mobility of liposomes containing a negatively charged derivative of phosphatidylethanolamine with a large headgroup composed of the hydrophilic polymer polyethylene glycol (PEG-PE) was determined by Doppler electrophoretic light scattering. The results show that this method is improved by the use of measurements at multiple angles to eliminate artifacts and that very small mobilities can be measured. The electrophoretic mobility of liposomes with 5 to 10 mol % PEG-PE is ~ -0.5 $\mu ms^{-1}/Vcm^{-1}$ regardless of PEG-PE content compared with ~ -2 $\mu ms^{-1}/Vcm^{-1}$ for similar liposomes but containing 7.5% phosphatidylglycerol (PG) instead of PEG-PE. Measurements of surface potential by distribution of an anionic fluorescent probe show that the PEG-PE imparts a negative charge identical to that by PG, consistent with the expectation of similar locations of the ionized phosphate responsible for the charge. The reduced mobility imparted by the surface bound PEG is attributed to a mechanism similar to that described for colloidal steric stabilization: hydrodynamic drag moves the hydrodynamic plane of shear, or the hydrodynamic radius, away from the charge-bearing plane, that of the phosphate moities. An extended length of ~ 50 Å for the 2,000 molecular weight PEG is estimated from the reduction in electrophoretic mobility.

INTRODUCTION

The electrostatic properties of biological membranes are of great importance for a wide range of biological functions and have been investigated by a number of model studies relying on lipid vesicles or liposomes (see recent reviews by Matsumura and Furusawa, 1989; McLaughlin, 1989; Cevc, 1990). These studies have led to an understanding that the electrical dipoles and charges of phospholipids give rise to electrostatic potentials extending from the bilayer into the adjacent aqueous phase usually described by Gouy-Chapman theory. The electrostatic potential is important for many, if not all, types of interactions with membranes, for example, in calcium ion binding which has a major role in the initiation of many processes including pharmacological action (Bangham et al, 1965; Smejtek and Wang, 1990), exocytosis (e.g., McLaughlin and Whitaker, 1988), messengers and substrates binding with receptors (e.g., Bazzi and Nelsestuen, 1987; Newton and Koshland, 1989), and interactions with surfaces such as occurs in virus fusion or phagocytosis (MacDonald, 1988; Papahadjopoulos et al., 1988; Stegmann et al., 1989).

The Gouy-Chapman-Stern theory has been shown to successfully describe the electrostatic potential of many phospholipid bilayer model systems (see McLaughlin, 1989; Cevc, 1990). However, the combination of this theory with the Helmholtz-Smoluchowski equations, which relate the electrophoretic mobility to the potential at the hydrodynamic plane of shear (defined as the zeta potential), cannot account for observations made with red blood cells (Levine et al., 1983). The difference may be attributed to contributions either of a hydrodynamic drag due to the presence of bulky sugars or to differences in the location of the charge relative to the surface due to the position of the charged sialic acid groups on the glycolipids or both. Studies designed to address this issue using phospholipid bilayers containing G_{M1}, and other glycolipid components of the glycocalyx, showed that in these model bilayers the electrophoretic mobility (or zeta potential) was also lower than predicted by a factor of two (McDaniel et al., 1984; McDaniel et al., 1986). The results were interpreted as showing that both differences in the location of the charge (and thus the surface potential produced) and hydrodynamic drag by the bulky sugars are responsible (McDaniel et al., 1984; McDaniel et al., 1986; Pasquale et al., 1986; Langner et al., 1988).

Independently, in vivo studies of liposomes containing glycolipids revealed that incorporation of G_{M1} can prolong blood circulation (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen et al., 1989; Namba et al., 1990). These findings resulted from considerable effort by many laboratories to overcome the unexpected finding that liposomes are rapidly removed from blood, primarily by cells of the mononuclear phagocytic system (MPS) (see review by Papahadjopoulos et al., 1989). Given that liposomes are cleared quickly from blood

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suggests that despite their similarities with many biological structures, such as cell membranes and phospholipid coated lipoproteins, differences exist that lead to their recognition. Another lipid, hydrogenated phosphatidylinositol (PI), was also found to increase liposome blood circulation time, albeit not to the same extent (Gabizon et al., 1988). Consideration of these findings lead to the recent development of novel synthetic lipids with a large hydrophilic polymer headgroup formed of polyethylene glycol covalently coupled with phosphatidylethanolamine (PEG-PE) (Blume and Cevc, 1990; Klibanov et al., 1990; Woodle et al., 1990; Papahadjopoulos et al., 1991; Senior et al., 1991; Woodle et al., 1992).

The mechanisms for liposome phagocytosis in vivo are not well understood nor those for their reduced uptake when these specific lipids are incorporated. Since ultimately red blood cells are removed from circulation by similar cell populations, the surface characteristics of red blood cells may give insights into the factors responsible for liposome recognition and uptake. Red blood cells show a relatively low electrophoretic mobility of $\sim 1\ \mu ms^{-1}/Vcm^{-1}$ or $10^{-4}cm^2/Vs$ (Bangham et al., 1958). On this basis, the unusual relation between surface charge density and electrophoretic mobility (or zeta potential) of red blood cells, modeled by G_{M1}-containing bilayers, may be the determining factor. Since PEG-PE and to a lesser extent PI also show reduced uptake, their surface properties may show similar discrepancies between surface potential and electrophoretic mobility as well. Studies of electrophoretic mobility and surface potential produced by PI have shown that it behaves more like other negatively charged phospholipids, PG or PS, than like G_{M1} (McLaughlin and Whitaker, 1988; Langner et al., 1990). The only report of surface properties with PEG-PE concluded that it contributes a positive surface potential on the basis of an apparent positive electrophoretic mobility (Blume and Cevc, 1990). A recently proposed mecahnism for reduced uptake of these novel liposomes due to surface steric stabilization (Lasic et al., 1990; Needham and McIntosh, 1991; Martin and Lasic, 1991) would predict a reduction in electrophoretic mobility. Therefore, measurement of both properties, surface potential and electrophoretic mobility, of liposomes containing PEG-PE was undertaken.

Electrophoretic mobility, or zeta potential, measurements with small particles are not straightforward. Most studies reporting electrophoretic mobilities or zeta potential with liposomes have relied on optical methods that impose a limitation to relatively large particles ($>0.5\ \mu m$). While mobility is not normally affected by particle size, and preparations containing PEG-DSPE can be made sufficiently large to be observed microscopically, such large particles show increased uptake in vivo (Allen et al., 1989; Woodle et al., 1990; Klibanov et al., 1991; Papahadjopoulos et al., 1991; Woodle et al., 1992). Consequently, measurement of electrophoretic mobility with small particles was preferred for direct comparison with in vivo studies. Electrophoretic mobility measurements using a light scattering method (ELS) which is suitable for both cells and particles too small to be visualized microscopically has been described (Ware and Flygare, 1971; Uzgiris, 1981). Despite the development of instruments for measurements of the electrophoretic mobility by Doppler shift with lasers and initial reports of their successful application to liposomes (Schlieper et al., 1981; Plank et al., 1985) few further reports using this method have been made, perhaps due to a lack of commercial instruments until recently. The requirement for identification of the stationary layer also applies to this method which may determine the overall accuracy. In addition, another problem with the laser Doppler method not often described is the occurrence of multiple peaks in the frequency spectrum (Ermakov, 1990). One solution to this problem is to take advantage of the dependence of the frequency shift on detection angle. By eliminating artifacts, which don't show an angular dependence, the peaks due to electrophoretic motion can be identified. Evaluation of this multiangle method for determination of the electrophoretic mobility of small liposomes has been lacking.

In this work, we report studies of both surface potential and electrophoretic mobility on bilayers containing PEG-PE, a lipid with a large hydrophilic headgroup. The mobility results were obtained through the use of a commercial instrument providing Doppler ELS measurements simultaneously at multiple angles. Surface potential was determined from measurements with an ionic fluorescent probe (Langner et al., 1990). The results indicate that ELS measurements of liposomes are in good agreement with previous measurements and that use of multiple angles allows the elimination of apparent instrumental artifacts in the frequency spectrum. Bilayers containing PEG-DSPE exhibit a very low electrophoretic mobility despite a surface potential consistent with the mole content of the negatively charged lipid. These results are interpreted in terms of a large hydrodynamic drag due to the steric contributions of the large hydrophilic headgroup.

MATERIALS AND METHODS

Several methoxypolyethyleneglycol (M-PEG) derivaties of phosphatidylethanolamine (PE) were prepared by methods described elsewhere (Woodle et al., 1992). Briefly, the derivatives were prepared by first reacting M-PEG with a slight mole excess of carbonyldiimadazole in a dry organic solvent at elevated temperature, followed by addition of PE equimolar to the M-PEG and maintained at elevated temperature overnight. The product was purified by chromatography on reversed

phase silica gel by elution with methanol:chloroform mixtures. Structure and purity of the product was determined by NMR and TLC. Derivatives of 99% distearylphosphatidylethanolamine (Calbiochem, San Diego, CA) (PEG-DSPE) were prepared with M-PEG moieties of 1,900 molecular weight. This derivative, M-PEG carbamyl DSPE or PEG-DSPE, is a negatively charged molecule in aqueous solution at neutral pH due to ionization of the phosphate and has been purified as either the imidazole or sodium salts.

Extruded multilamellar liposomes were prepared according to procedures already described (Olson et al., 1979; Woodle and Papahadjopoulos, 1988). Briefly, thin films of lipids were prepared by rotoevaporation of lipid mixtures in chloroform using one or more of the following lipids: 99% partially hydrogenated egg phosphatidylcholine (PHEPC) with an iodine value of 40 (Asahi Chemicals, Tokyo, Japan) as described previously (Lang et al., 1990); 99% egg phosphatidylglycerol (EPG) (Avanti Biochemicals, Birmingham, AL); USP cholesterol (C) (Croda, Fullerton, CA); and PEG-PE. The lipid films were hydrated to give 10 μmol phospholipid per ml with 10 mM phosphate buffer by shaking above the phase transition temperature (22°C), followed by three cycles of freezing in a dry ice acetone bath and thawing in warm water before extrusion through defined pore filters (Olson et al., 1979). The extrusion was repeated until the mean diameter of a Gaussian particle size distribution was below 100 nm as determined by dynamic light scattering (Nicomp Instruments model 200, using "vesicle" mode [Santa Barbara, CA]).

Electrophoretic measurements

Electrophoretic mobility of liposomes was determined with a Coulter Electronics Inc. DELSA instrument (Hialeah, FL) for measurements of Doppler shift of scattered coherent light (Schlieper et al., 1981), but with the distinction that this instrument allows for measurements to be made at up to four angles simultaneously. With this method, electrophoretic motion was detected by the Doppler shift in frequency using a heterodyne method. Four frequency spectra were measured from four autocorrelation functions simultaneously determined with each heterodyne output. The mobility was determined from the Doppler frequency spectra according to the relations:

$$\Delta\omega = \mu(E \cdot K), \tag{1}$$

where $\Delta\omega$ is the Doppler frequency, μ the electrophoretic mobility (μm/s per V/cm), E the applied electric field strength, and K the scattering vector:

$$K = (4\pi n/\lambda) \sin(\Theta/2), \tag{2}$$

where λ is the wavelength of the laser, n is the refractive index of the medium, and Θ is the angle between the incident laser beam and the detector. These can be combined to give a single expression:

$$\mu = (\Delta\omega\lambda)/2nE \sin(\Theta/2). \tag{3}$$

Mobilities were converted to zeta potential by the assumptions of the Helmholtz-Smoluchowski equations:

$$\zeta = \mu\eta/(\epsilon_r\epsilon_o), \tag{4}$$

where η is the viscosity, ϵ_r and ϵ_o are the dielectric constants of the aqueous solution and vacuum, respectively.

For the results obtained here, the samples were diluted by 1:10 to 1:100 into the desired pH buffer prepared as mixtures of mono- and disodium phosphate (Kossovsky et al., 1991) and placed in the cell so that no air bubbles were visible. The cell was positioned in the instrument first with the laser beam at the edge of the cell by locating the point where the light intensity diminished and then moving the cell back so that the beam was positioned at the stationary layer. The applied field was adjusted to give a peak with a frequency shift of at least 20 Hz at the lowest angle of detection, ~8.6° as shown by representative data in Fig. 1 (*top*), in order to obtain mobilities within the linear region of operation (Harfield and Bunker, 1988). The frequency shift of the peak should increase by a factor equal to that of the increase in angle of detection. For this instrument a factor of two for increasing angles is used. The frequency spectra obtained were converted into electrophoretic mobility by the operating software on the basis of input parameters, as shown in Fig. 1 (*middle*). The data were converted to zeta potential using the dielectric constant supplied by the software for water, 78.36, as shown in Fig. 1 (*bottom*). Measurements of each sample at three field strengths were recorded and both electrophoretic mobility and zeta potential calculations were made for each angle and field strength. The results for each sample were expressed as the average ± the standard deviation of these measurements.

In some cases, peaks that didn't show an angular dependency were observed. In one case this was anticipated: results obtained with ostensibly neutral samples, which should not move in an electrical field, are expected to give rise only to peaks in the frequency spectrum centered at or near zero Hz. Such peaks should not show any angular dependence and were observed with the ostensibly neutral samples, PC or PC:C. With these samples a frequency shift of 20 Hz could not be obtained at the highest fields possible, indicating both that the cell was positioned properly at the stationary layer and that charged contamination of these samples had not occurred. In another case, additional peaks were observed at or near zero Hz (and therefore also not exhibiting an angular dependence) as shown by the results presented in Fig. 1. Such observations can result either from particles adsorbed onto the surface of the cell, which therefore have no mobility, or from the presence of neutral particles, either aggregates or contaminating particles without charge. These two possibilities can be discriminated between on the basis of diffusion broadening which is a function of angle and can be easily detected by simultaneous measurements (S. Sugrue, personal communication). The peaks at zero Hz (those shown here are attributed to material adsorbed onto the cell reinforcing the importance of cell cleaning) are disregarded in the determination of electrophoretic or zeta potential.

Surface potential measurements

Surface potential was determined by fluorescence measurements of the distribution of the anionic fluorescent probe, TNS, as described (Eisenberg et al., 1979; McDaniel et al., 1984). Briefly, TNS was dissolved in distilled water and added to the liposome samples at a concentration of 1 μM TNS at five lipid concentrations by dilution over the range from 1:50 to 1:200. The fluorescence intensity was measured and the surface potential (ψ_o) calculated on the basis of the ratio of the fluorescence with that from an ostensibly neutral control formulation at the same lipid concentration using the relation:

$$f(-)/f(0) = \exp[F\psi_o/(RT)], \tag{5}$$

where F is the Faraday constant, R is the gas constant, T is the absolute temperature, and $f(-)$ and $f(0)$ are the fluorescense intensities of the negative lipid containing formulation and ostensibly neutral formulation, respectively. The results are expressed as the average of the surface potential determined at each lipid concentration.

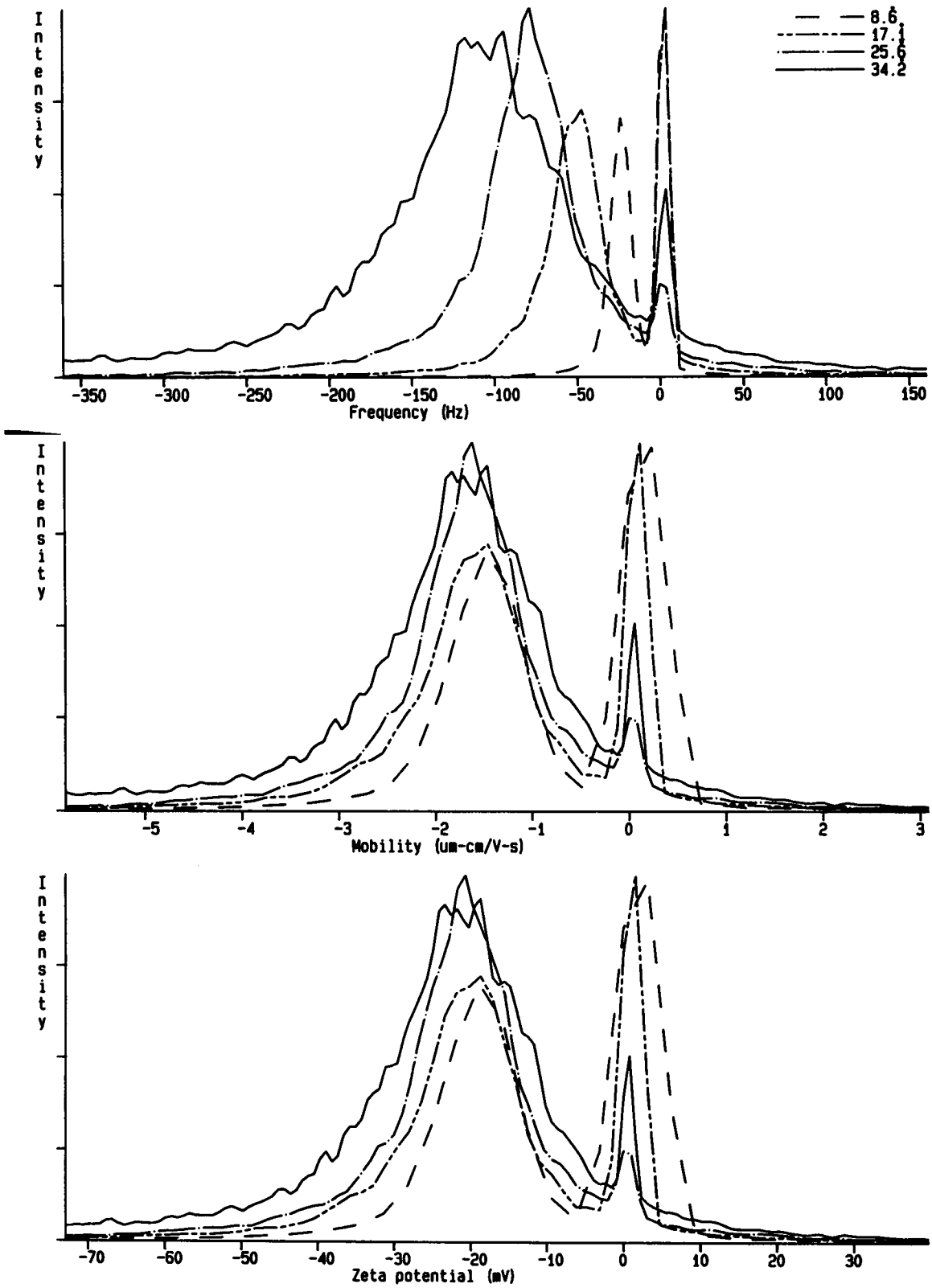

FIGURE 1 Results obtained by Coulter DELSA instrument for Doppler shift electrophoretic light scattering with PG:PC (0.75:9.25) in 10 mM phosphate buffer, pH 7.3. (*Top*) Frequency spectrum of Doppler shifts measured simultaneously at four angles. (*Middle*) Electrophoretic mobility determined from frequency shift. (*Bottom*) Zeta potential determined from frequency shift.

RESULTS

Electrophoretic measurements

Electrophoretic mobility of liposome samples was determined by Doppler ELS at several angles simultaneously. Measurements of ostensibly neutral liposomes gave Doppler shifts centered about zero Hz indicating proper cell alignment of the stationary layer in the light path. Only small differences over the pH range from pH 5.2 to 9.2 were observed in representative samples examined of each lipid composition as expected from earlier studies (Schlieper et al., 1981). Therefore, remaining studies were limited to a neutral pH of 7.3. The results obtained with ostensibly neutral liposomes and those containing the negatively charged lipids PG and PEG-DSPE are summarized in Table 1. The results obtained here with PG containing liposomes are in good agreement with literature and the predictions of Gouy-Chapman-Stern theory (McLaughlin, 1989). With liposomes containing PEG-DSPE, a very small electrophoretic mobility was observed (and therefore a small negative zeta potential) compared with the expectations from theory based on the mole content of PEG-DSPE.

Perhaps even more striking is the finding that the electrophoretic mobility of PEG-DSPE containing bilayers is constant over the range examined, 5 to 10 mol % in stark contrast with theory or previous results with any other liposome composition. Unfortunately, the limitation of making these measurements at such low electrophoretic mobilities, even at the low ionic strength used here, prohibits extending these measurements to lower levels of PEG-DSPE content. Increased mole content of PEG-DSPE also may prove unworkable due to the potential formation of nonbilayer structures (Lasic et al., 1991; Woodle et al., 1991).

In studies with a single level of either negatively charged component but including cholesterol, also shown in Table 1, a similar differences in the electrophoretic mobility was observed. The results show little effect of cholesterol on electrophoretic mobility in agreement with previous measurements with PG at up to 50 mol % (Plank et al., 1985).

Surface potential

Most reports simply apply Gouy-Chapman theory to estimate either surface charge or electrophoretic mobility from measurements of the other rather than obtaining a direct measurement of both which may only be valid for some lipid compositions. For liposomes composed of PEG-DSPE or PG mixtures with PC, measurement of the surface potential was made by observations of an anionic fluorescent probe distribution. The results, in Table 2 and Fig. 2, show that the surface potential increases from −14 to −32 mV when the PEG-DSPE content is increased from 5 to 10 mol %. Also shown is that simple mixtures of either PEG-DSPE or PG with PC at the same mole content produce an identical surface potential, as expected for phospholipids with the charge located at the phosphate in contrast with the differences observed in electrophoretic mobility (Table 1).

Surprisingly, addition of the sterol cholesterol had a substantial effect on the ratio of the observed fluorescence of the probe and thus the calculated surface potential, contrary to results obtained by extrapolating from electrophoretic mobility (zeta potential) (Plank et al., 1985). If this result is not due to an artifact such as an

TABLE 1 **Electrophoretic mobility and zeta potential of PEG-DSPE containing liposomes**

Lipid composition*	Electrophoretic mobility			Zeta potential		
	pH 5.2	pH 7.3	pH 9.2	pH 5.2	pH 7.3	pH 9.2
		μm-cm/V-s			*mV*	
PC	0.05 ± 0.04	0.09 ± 0.01	−0.06 ± 0.02	0.6 ± 0.2	1.3 ± 0.2	−0.8 ± 0.26
PEG:PC (1:9)	−0.54 ± 0.02			−7.0 ± 0.12		
PEG:PC (0.75:9.25)	−0.60 ± 0.03	−0.45 ± 0.04	−0.45 ± 0.03	−7.4 ± 0.5	−5.4 ± 0.4	−5.7 ± 0.7
PEG:PC (0.5:9.5)		−0.65 ± 0.20			−7.5 ± 2.6	
PG:PC (0.75:9.25)	−1.94 ± 0.07	−1.65 ± 0.06	−1.69 ± 0.05	−24.9 ± 0.8	−21.2 ± 0.8	−21.6 ± 0.5
PC:Chol (10:5)		0.03 ± 0.02			0.8 ± 0.3	
PEG:PC:Chol (0.75:9.25:5)		−0.50 ± 0.06			−6.6 ± 0.8	
PG:PC:Chol (0.75:9.25:5)		−1.96 ± 0.04			−25.2 ± 0.5	

*PC refers to phosphatidylcholine; PG to phosphatidylglycerol, a negatively charged lipid; PEG to PEG-DSPE, a negatively charged lipid; and Chol to cholesterol.

TABLE II **Surface charge of PEG-DSPE containing liposomes**

Lipid composition*	Surface potential
	mV
PC	0‡
PEG:PC (1:9)	−31.8 ± 6.2
PEG:PC (0.75:9.25)	−24.8 ± 4.7
PEG:PC (0.5:9.5)	−14.4 ± 2.4
PG:PC (0.75:9.25)	−25.4 ± 5.8
PC:Chol (10:5)	0‡
PEG:PC:Chol (0.75:9.25:5)	−36.9 ± 7.4
PG:PC:Chol (0.75:9.25:5)	−51.3 ± 4.9

*PC refers to phophatidylcholine; PG to phosphatidylglycerol, a negatively charged lipid; PEG to PEG-DSPE, a negatively charged lipid; and Chol to cholesterol. ‡By definition (f(0), see Eq. 5).

unexpected interaction with the probe dependent on the surface potential, then this would represent an interesting effect of a neutral molecule on the surface potential.

DISCUSSION

Surface potential measurements of PEG-DSPE mixtures with PC (Table 2 and Fig. 2) indicate that PEG-DSPE conveys a negative surface potential to bilayers whose amplitude is dependent on the PEG-DSPE mole content. This is inconsistent with a recent suggestion, based on extrapolation of surface potential from zeta potential results (obtained with a slightly different PEG-PE derivative, but which would be expected to have a similar structure and charge contribution), that incorporation of PEG-PE results in a positive surface potential (Blume and Cevc, 1990). The conclusion that this lipid contributes a negative charge seems more reasonable according to consideration of the molecular structure of the lipid; the amino moiety of the DSPE is derivatized and cannot be protonated, leaving an ionized phosphate moiety with a negative charge at neutral pH.

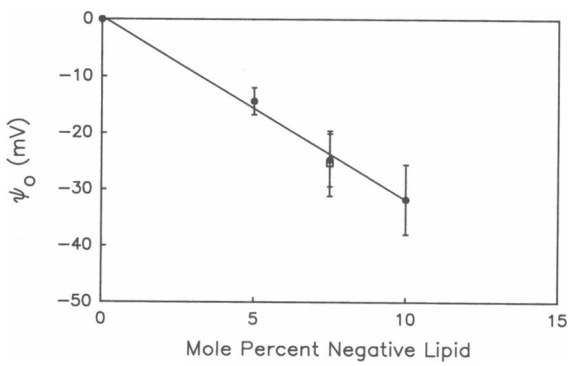

FIGURE 2 Dependence of surface potential on mole percent negative lipid with PC in 10 mM phosphate, pH 6.4. The solid line represents a least square fit to the closed circle points. (*Closed circles*) PEG-DSPE. (*Open square*) PG.

The surface potential imparted by PEG-DSPE as a function of mole content (Fig. 2) agrees well with that of PG (McDaniel et al., 1984), indicating that the charged nature of PEG-DSPE is electrostatically identical with that of the other negatively charged phospholipids: PG, PS, and PI (Langner et al., 1990). Nevertheless, confirmation of this by complete measurements of the surface potential profile in the aqueous phase by the use of probes with defined locations would be desirable (Langner et al., 1990). Such a conclusion is reasonable because for all these lipids the negative charge should be located at the same plane as it originates from the same phosphate moiety. Therefore, even if this method averages the electrical field at the position of the fluorescent probe, which may give either an over or under estimate of the field, the effect is similar for all these lipids. The findings of surface potential imparted by G_{M1}, on the other hand, are in stark contrast (McDaniel et al., 1984; Pasquale et al., 1986; Langner et al., 1988). In this case, a lower surface potential appears to result from a difference in location of the charged sialic acid on the glycolipid compared with the phosphate of the phospholipids (McDaniel et al., 1984; McDaniel et al., 1986).

A limiting problem with the laser Doppler method not often described is occurrence of multiple peaks in the frequency spectrum (Ermakov, 1990). One solution to this problem is to take advantage of the dependence of the frequency shift on detection angle and make measurements at several angles. By eliminating peaks that don't show the expected angular dependence, those resulting from the electrophoretic motion can be identified. Results obtained here with such a method indicate that reliable measurements down to at least 0.5 $\mu ms^{-1}/Vcm^{-1}$, or $5 \times 10^{-5} cm^2/Vs$ which translates into a zeta potential of ~5mV, can be made. However, to obtain such results, repeated measurements and data analysis should be made. Importantly, the electrophoretic mobility is dependent on the applied field up to a threshold level, above which linear performance results in constant values. This implies that a minimum velocity must be achieved.

Electrophoretic mobility of bilayers containing PG obtained in this work are in good agreement with previous results obtained by either microscopic observation or ELS (Schlieper et al., 1981; McDaniel et al., 1984). When PEG-DSPE was incorporated into PC bilayers at the same mole percentage negative lipid, the resulting electrophoretic mobility was greatly diminished despite equivalent surface potential (Table 1 vs.

Table 2 and Fig. 2). A similar reduction in mobility also observed with G_{M1} had been explained by hydrodynamic drag of the bulky sugars despite a difference in location of the charged sialic acid residue, which might be expected to increase the mobility (McDaniel et al., 1986; Pasquale et al., 1986; Langner et al., 1988). For PEG-DSPE, though, the location of the charge, and thus the surface potential, is similar to that of other phospholipids. Observations of bilayers containing a small (eight ethylene oxide units) PEG derivative of a single acyl chain are in agreement (Arnold et al., 1990). However, conflicting evidence of a similar effect by a single acyl chain derivative also exists (Moser et al., 1989).

From the results obtained here, the extent of the *increase* in the hydrodynamic radius by the PEG coating, that is the *increase* in the distance from the surface to the region of hydrodynamic flow as described by the electrophoretic mobility or zeta potential, can be estimated from the *decrease* in the zeta potential, given that the actual surface potential is constant. The approximation is based on the decrease in electrostatic potential as a function of the distance from the surface:

$$\psi_x = \psi_o \exp(-\kappa X), \quad (6)$$

where ψ_x is the surface potential at a distance x from the surface, ψ_o is the surface potential, and κ^{-1} the Debye length. This implies the usual assumptions of Gouy-Chapman theory and that the dielectric of the PEG coating is not different from the aqueous phase that it displaces. In addition, this approximation is based entirely on the definition of zeta potential as being that potential at the hydrodynamic radius, meaning the plane at which hydrodynamic flow occurs. Therefore, the use of these potentials permits estimation of the distance to the plane at which hydrodynamic flow occurs. From this simple approximation, the distance calculated from the observed zeta potentials increases from 5.2 Å with PG to 48 Å with PEG-PE (from the observations of 21 and 5 mV, respectively). Furthermore, this estimate of the PEG coating thickness of ~50 Å is very similar with other estimates of ~ 60 Å for the extended length of the PEG from the surface by independent methods (Lasic et al., 1991; Needham and McIntosh, 1991; Woodle et al., 1991).

One aspect of the results obtained here cannot be explained easily: the lack of a dependence of electrophoretic mobility on PEG-DSPE mole content over the range from 5 to 10 mol %. That the electrophoretic mobility is reduced, compared with PG, at a single mole content can be attributed to an *increase* in the hydrodynamic radius due to the bulky PEG coating. However, the lack of a dependence on PEG-DSPE mole content is not consistent with the results with PG nor double layer theory. One possibility is simply that the dependence exists but it could not be resolved by the instrumentation. Because of the very small mobilities with PEG-DSPE, accurate measurements are difficult and smaller differences over the relatively narrow range of PEG-DSPE content might not have been detected. Studies over a broader range in PEG-PE content, at lower ionic strength, or using microscopic measurements with a large particle size might address this possibility by improving the signal to noise. An alternative explanation is that the PEG coating thickness may be a function of PEG-PE content, but this hypothesis could be difficult to test especially because increasing the range of PEG-DSPE mole content is limited by the potential for formation of nonbilayer structures (Lasic et al., 1991; Woodle et al., 1991).

The addition of cholesterol was found to increase the apparent surface potential on the basis of distribution of the anionic TNS probe. The effect was observed with both PEG-PE and PG but is greater with PG. If the probe has a reduced bilayer interaction due to the presence of cholesterol, this effect should be similar for both charged and neutral bilayers. Since these results were obtained by measurements relative to bilayers with cholesterol but lacking the charged lipid, such an effect would not be expected to influence the surface potential determination. However, other unexpected interactions with the probe, which are dependent on the surface potential, may be responsible and this issue should be examined further. For example, full calibration of the TNS probe, such as investigation of the fluorescent lifetime as a function of cholesterol and surface potential and the use of alternative surface potential probes (Cafiso et al., 1989), could give insight as to the actual effect of cholesterol on the surface potential. A similar effect on the electrophoretic mobilities was not observed (Table 1), in agreement with previous results with PG (Plank et al., 1985). The lack of an effect on the electrophoretic mobility is consistent with evidence that cholesterol has little effect on phospholipid headgroups, and therefore the surface charge distribution. This may indicate that, in the presence of cholesterol, an interaction of the TNS fluorescent probe exists not entirely dependent on electrostatic surface potential. Nevertheless, if real, this result is surprising and would be of interest because cholesterol is neutral.

In conclusion, incorporation of PEG-DSPE into bilayers produces a substantial negative surface potential but a lower than expected electrophoretic mobility due to an increase in the hydrodynamic radius from steric effects of the PEG. A simple model is described for movement of the hydrodynamic plane of shear outward from the ionic surface to the edge of the PEG layer giving an estimate of 50 Å for the PEG coating. A similar

reduction in mobility by G_{M1} has been attributed to the same mechanism of frictional drag, but may be complicated by other effects due to changes in location of the charge (McDaniel et al., 1984; McDaniel et al., 1986; Pasquale et al., 1986; Langner et al., 1988; Beitinger et al., 1989). Regardless of the mechanism responsible, low electrophoretic mobility may be at least partly indicative of the surface properties responsible for controlled MPS uptake of particulates such as liposomes, lipoproteins, and red blood cells.

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