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Ionization and Structural Changes of the DMPG Vesicle along Its Anomalous Gel−Fluid Phase Transition: A Study with Different Lipid Concentrations

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Dispersions of saturated anionic phospholipid dimyristoyl phosphatidylglycerol (DMPG) have been extensively studied regarding their peculiar thermostructural behavior. At low ionic strength, the gel−fluid transition is spread along nearly 17 °C, displaying several thermal events in the calorimetric profile that is quite different from the single sharp peak around 23 °C found for higher ionic strength DMPG dispersions. To investigate the role of charge in the bilayer transition, we carefully examine the temperature dependence of the electrical conductivity of DMPG dispersions at different concentrations, correlating the data with the corresponding differential scanning calorimetry (DSC) traces. Electrical conductivity together with electrophoretic mobility measurements allowed the calculation of the dependence of the degree of ionization of DMPG vesicles on lipid concentration and temperature. It was shown that there is a decrease in vesicle charge as the lipid concentration increases, which is probably correlated with the increase in the concentration of bulk Na^+. Apart from the known increase in the electrical conductivity along the DMPG temperature transition region, a sharp rise was observed at the bilayer pretransition for all lipid concentrations studied, possibly indicating that the beginning of the chain melting process is associated with an increase in bilayer ionization. It is confirmed here that the gel−fluid transition of DMPG at low ionic strength is accompanied by a huge increase in the dispersion viscosity. However, it is shown that this measured macroviscosity is distinct from the local viscosity felt by either charged ions or DMPG charged aggregates in measurements of electrical conductivity or electrophoretic mobility. Data presented here give support to the idea that DMPG vesicles, at low ionic strength, get more ionized along the temperature transition region and could be perforated and/or deformed vesicle structures.

Introduction

The main thermal transition (gel−fluid) of the saturated anionic phospholipid dimyristoyl phosphatidylglycerol (DMPG, 14 C atoms in the hydrophobic chains) at physiological conditions (pH 7.4 and 100 mM NaCl) is similar to that of the zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC): strongly cooperative behavior is seen in the narrow differential scanning calorimetry (DSC) peak at around 23 °C. However, at physiological pH but low ionic strength, DMPG exhibits a rather unusual thermal profile, with a main transition that extends over more than 10 °C. Its DSC trace displays a cooperative peak at the onset of the main transition ($T_m^{on} \approx 17$ °C), followed by several broad peaks, and a final small peak ($T_m^{off} \approx 35$ °C) sets the end of the gel−fluid transition. We shall refer to the temperature interval between T_m^{on} and T_m^{off} as the transition region because it separates the gel and fluid phases. This transition region displays several special properties, such as low turbidity,[1−5] high viscosity,[1,6,7] and high electrical conductivity.[2] The electron spin resonance (ESR) of a spin label located at the bilayer center revealed the coexistence of two structurally different microenvironments in the transition region: one of them resembles a gel bilayer, and the other is rather fluid and fairly hydrated, being compatible with a micellelike environment.[8] Spin and fluorescent probes indicate that the wide thermal transition happens at the bilayer level.[8−11] Small-angle X-ray scattering (SAXS) showed that DMPG at low ionic strength is not organized in multilamellar bilayers and revealed the emergence of a mesoscopic correlation at around 370 Å in the transition region.[12] Optical microscopy of DMPG giant vesicles showed that the bilayers lose their optical contrast in the transition region.[4,12] As the ionic strength is increased, the temperature extension of the transition region is reduced.[3,4]

On the basis of the above results, it has been proposed by some of us that DMPG would be structured as perforated vesicles over the temperature-transition region.[12] Thus, in-plane-correlated pores would be responsible for the mesoscopic correlation detected by SAXS, the decrease in the optical contrast, and the coexistence of two different lipid microenvironments corresponding respectively to the more rigid bilayer and the more fluid edges of

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the pores. This hypothesis was supported by fluorescence and light-scattering data,[5] which led the authors to propose the presence of tattered bilayer sheets over the transition region. Recently, compounds that enhance positive curvatures in lipid monolayers were shown to extend the DMPG temperature-transition region to higher temperatures. Considering that the rims of the holes in bilayers are positive-curvature structures, those experiments were discussed as indicatives of the presence of perforated bilayers along the transition region.[13] Moreover, optical and fluorescence microscopy indicated the presence of extensive bilayer perforation in DMPG giant vesicles over the transition-temperature interval.[4] Also supporting the perforation hypothesis, it was shown recently that the whole SAXS curve comprising the mesoscopic correlation peak and the bilayer band could be fit with a perforated bilayer model.[14]

Because of the high viscosity of DMPG in the transition region and on the basis of cryo-transmission and freeze-fracture electron microscopy, it has also been proposed that DMPG at low ionic strength forms a lipid network in that temperature interval, similar to the so-called sponge phase.[6] However, this possibility was ruled out on the basis of experiments that discarded lipid rearrangement between DMPG structures along the transition region.[5,10]

Though the structure of the DMPG aggregates over the transition region is still not completely understood, it is certain that the headgroup charges play a fundamental role. Actually, the characteristics of the transition region are due to a specific balance between headgroups and chains interactions in the DMPG bilayer: low ionic strength dispersions of dipalmitoyl phosphatidylglycerol (DPPG) consisting of the same PG headgroup but with 16 carbon atoms in its chains do not present the same complex thermal behavior.[6]

Considering that the high electrical conductivity displayed by dispersions of DMPG in the transition region at low ionic strength is probably related to the dissociation of sodium ions from PG headgroups,[2,3,15] an investigation of this property seems to be a good instrument for the evaluation of the charge of the DMPG aggregates. In the present work, we carefully study the conductivity of DMPG dispersions at different temperatures for lipid concentrations varying from 1 to 50 mM. Changes in electrical conductivity were correlated to DSC traces of the same samples. Moreover, measurements of the electrophoretic mobility of the DMPG aggregates allowed the calculation of the changes in the degree of ionization of PG headgroups over the temperature-transition region. Considering the possible influence of the medium viscosity on those dynamic properties (conductivity and electrophoretic mobility), the temperature dependence of the viscosity of the different DMPG dispersions was also measured and will be discussed here.

Materials and Methods

Materials. The sodium salts of phospholipids DMPG (1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-glycerol]) and DMPC (1,2-dimyristoyl-*sn*-glycero-3-[phosphocholine]) were purchased from Avanti Polar Lipids (Birmingham, AL) and used without further purification. Unless otherwise stated, the buffer system used was 10 mM Hepes (4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid) adjusted with NaOH to pH 7.4 ($[Na^+] \approx 4$ mM) + 2 mM NaCl. The data shown in Figure 10 were yielded by DMPG dispersions in 10 mM Hepes buffer at pH 7.4 with different concentrations of NaCl, as stated in the Figure. Milli-Q Plus water (Millipore) was used throughout.

Lipid Dispersion Preparation. A lipid film was formed from a chloroform solution, dried under a stream of N_2, and left under reduced pressure for a minimum of 2 h to remove all traces of the organic solvent. Dispersions were prepared by the addition of Hepes buffer followed by vortex mixing for about 2 min above T_m^{off} (~40 °C). The pH of the lipid dispersion was measured and found to decrease slightly as the DMPG concentration increased. However, the lowest pH value measured was 7.2 for the highest concentration of DMPG studied here (50 mM). Because the apparent pK of DMPG was reported to be 4.7 at low ionic strength (Figure 8 in ref 9), it is reasonable to assume that no significant protonation occurs when the pH decreases from 7.4 to 7.2. Therefore, we assume throughout our discussion that DMPG is fully deprotonated. The samples were kept at room temperature and used right after preparation.

Differential Scanning Calorimetry. DSC traces were obtained by heating the samples from 5 to 50 °C with a Microcalorimeter VP-DSC (MicroCal, Northampton, MA). Scan rates from 10 and 20 °C/h were used. (In this range, the DSC traces were found to be identical.) Baseline subtractions and peak integrals were carried out using MicroCal Origin software with the additional module for DSC data analysis provided by MicroCal, as described before.[16]

Electrophoretic Mobility. Electrophoretic mobility data were obtained through a combination of laser Doppler velocimetry and phase analysis light scattering in a patented technique called M3-PALS with the Zetasizer Nano ZS (Malvern, U.K.). After achieving the desired temperature, the sample was left for at least 5 min at each temperature before data acquisition.

The temperature dependence of the Na^+ electrophoretic mobility was calculated by the empirical equation[18]

$$\mu^{Na^+}(T) = \frac{\lambda^{Na^+}(T)}{N_A e}$$

where N_A is Avogadro's number and e is the unitary charge and the temperature dependence of the equivalent conductivity, λ^{Na+} (in $m^2\ M^{-1}C/V\ s$), is given by

$$\lambda^{Na^+}(T\ ^oC) = 50.11 + 1.0916(T-25) + 0.004715(T-25)^2 - 0.0000115(T-25)^3 \quad (1)$$

Electrical Conductivity. The conductivity data were obtained with an inoLab Cond 730 conductivity meter, with a TetraCon 325 conductivity cell (WTW, Germany) with a cell constant between 0.465 and 0.475 cm^{-1} adjusted with a standard solution of 0.1 M KCl. The temperature was controlled by a thermostated bath (Schott Instruments, Germany) and measured with a temperature sensor placed in the conductivity cell. After achieving the desired temperature, the sample was left for 10 min at each temperature before data acquisition.

To separate the solvent effect, conductivity data were analyzed via the so-called reduced conductivity, $\sigma_{red} = \Delta\sigma/\sigma^{buffer}(1-\phi)$ (Figure 1), where σ^{buffer} is the pure solvent conductivity, $\Delta\sigma$ is the difference between the dispersion and buffer conductivities, $\Delta\sigma = \sigma - \sigma^{buffer}(1-\phi)$, and ϕ is the volume fraction of buffer enclosed by the DMPG aggregates. (See, for example, ref 17.)

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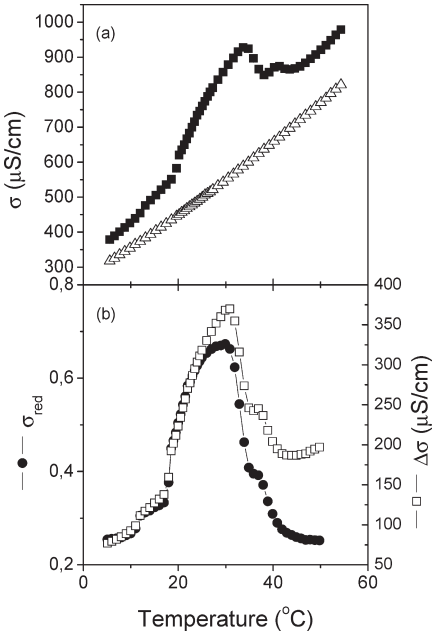

Figure 1. (a) Temperature dependence of the conductivity of the Hepes buffer (Δ) and the buffer with the addition of 20 mM DMPG (■). (b) Temperature dependence of the difference between the dispersion and buffer conductivities (from plot a), corrected by the volume fraction occupied by the DMPG aggregates, ϕ, $\Delta\sigma = \sigma - \sigma^{\text{buffer}}(1-\phi)$ (□), and the temperature dependence of the reduced conductivity, $\sigma_{\text{red}} = \Delta\sigma/\sigma^{\text{buffer}}(1-\phi)$ (●). Here $\phi = 0.050$, as discussed in the text.

The calculation of reduced conductivity demands the assumption of some form for the lipid aggregates in order to obtain the volume of buffer enclosed by aggregates. Unless otherwise stated, data displayed in this study correspond to the assumption of closed spherical vesicles. However, the possibility of open aggregates in the transition-temperature region is also discussed in the interpretation of some data. Thus, closed vesicles and the volume fraction of enclosed buffer, Φ, may be approximated by the volume fraction occupied by the lipid spheres, then $\Phi \approx \Phi_{\text{vesicles}}$. Considering that DMPG is organized as unilamellar vesicles of diameter $d = 100$ nm,[2,5] with equal amounts of lipids in both layers (internal and external) and with an area per lipid of $a = 0.50\,\text{nm}^2$, ϕ values for the DMPG dispersions of 1, 5, 10, 20, 30, 40, and 50 mM are 0.0025, 0.013, 0.025, 0.050, 0.075, 0.100, and 0.125, respectively.

Degree of Ionization. The electrophoretic mobility of a particle is defined as $\mu = v/E$, where v and E are the magnitudes of the velocity of the particle and the applied electric field, respectively. (See, for example, ref 19.) μ is positive if the particle is positive and the vectors **v** and **E** have the same direction. Because the electrical current density is defined as the electrical current per unit area, $J = I/A$, it is possible to show that $J = Nqv$, where N is the number of charged particles per liter and q is the charge of each particle. Using the above definition for electrophoretic mobility, the current density can be written as $J = Nq\mu E$, yielding the relation $\sigma = Nq\mu$ for the electrical conductivity of the medium.

In the case of the DMPG dispersion, both the DMPG aggregates and the counterions contribute to the electrical conductivity, σ, and we may write

$$\sigma - \sigma^{\text{buffer}}(1-\phi) = N^{\text{agg}}q^{\text{agg}}\mu^{\text{agg}} + N^{\text{counterion}}q^{\text{Na}^+}\mu^{\text{Na}^+}$$

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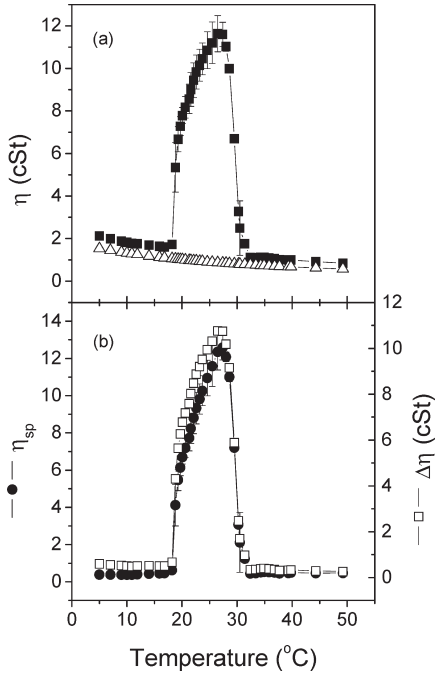

Figure 2. (a) Temperature dependence of the viscosity (η) of the Hepes buffer (Δ), and the buffer with the addition of 20 mM DMPG (■). (b) Temperature dependence of the difference between the dispersion and buffer viscosities (from plot a), $\Delta\eta = (\eta^{\text{dispersion}} - \eta^{\text{buffer}})$ (□); temperature dependence of the specific viscosity, $\eta_{\text{sp}} = \Delta\eta/\eta^{\text{buffer}}$ (●).

where the terms "agg" and "counterion" correspond to aggregates and sodium counterions in solution, respectively. The number of DMPG aggregates per liter is $(N^{\text{agg}}) = [\text{DMPG}]N_{\text{A}}/n$, where n is the number of lipids per aggregate.

The evaluation of the charge of the DMPG macroion depends on the model adopted for the aggregate. As stated previously, we have considered the possibility of both closed and open aggregates in some temperature region. Thus, we have considered three possible models: model 1 − closed unilamellar spherical vesicles and, following other authors, model 2 − perforated vesicles and model 3 − open sheets.

In the case of model 1, with an equal distribution of lipids in the internal and external layers, the charge of the vesicle would be $q^{\text{ves}} = n\alpha e/2$, where α is the PG degree of ionization and the concentration of counterions in solution is $(N^{\text{counterion}}) = [\text{DMPG}]N_{\text{A}}\alpha/2$. Hence, the degree of ionization would be

$$\alpha = 2\,\frac{\sigma - \sigma^{\text{buffer}}(1-\phi)}{eN_{\text{A}}[\text{DMPG}](\mu^{\text{ves}} + \mu^{\text{Na}^+})} \qquad (2)$$

In the case of model 2, all the counterions inside and outside the vesicles equally contribute to the conductivity measurements, $(N^{\text{counterion}}) = [\text{DMPG}]N_{\text{A}}\alpha$, and $\phi = 0$:

$$\alpha = 2\,\frac{\sigma - \sigma^{\text{buffer}}}{eN_{\text{A}}[\text{DMPG}](\mu^{\text{ves}} + 2\mu^{\text{Na}^+})} \qquad (3)$$

In the case of model 3, for open bilayer sheets, all PG^- would contribute to the total charge of the DMPG aggregate, $q^{\text{agg}} = n\alpha e$, and all counterions, $(N^{\text{counterion}}) = [\text{DMPG}]N_{\text{A}}\alpha$, with $\phi = 0$:

$$\alpha = \frac{\sigma - \sigma^{\text{buffer}}}{eN_{\text{A}}[\text{DMPG}](\mu^{\text{agg}} + \mu^{\text{Na}^+})} \qquad (4)$$

Actually, the expressions calculated correspond to effective degree of ionizations because the counterions that are strongly

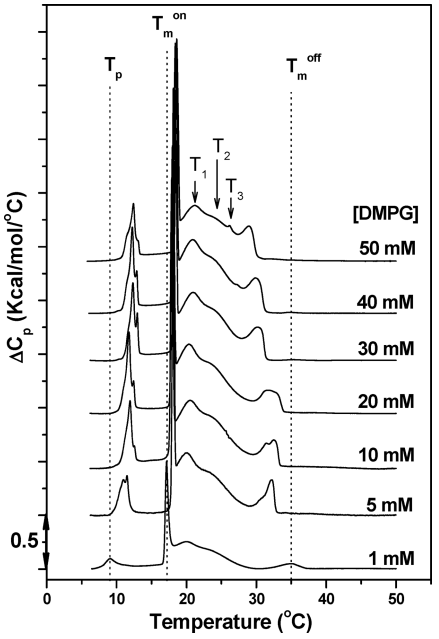

Figure 3. Excess heat capacity (ΔC_p) of DMPG dispersions in Hepes buffer at different lipid concentrations. The curves were shifted for clarity. The vertical dashed lines indicate the positions of three peaks on the thermogram of 1 mM of DMPG (T_p, T_m^{on}, and T_m^{off}). The other three peaks are indicated by arrows in the top trace corresponding to 50 mM DMPG.

bound to DMPG aggregates in the Stern layer will move with the aggregates and will be computed in the μ^{agg} data.

Viscosity. Viscosities were measured with an Ostwald viscometer (ViscoClock Unit from Schott Instruments, Germany) coupled to a thermostated bath (Schott Instruments, Germany). The temperature was measured with a Fluke 51 K/J thermometer that was immersed in the bath and allowed to equilibrate for 10 min before data acquisition.

To eliminate the solvent effect, the data were analyzed through the so-called specific viscosity $\eta_{sp} = \Delta\eta/\eta^{buffer}$, where η^{buffer} is the pure solvent viscosity and $\Delta\eta$ is the difference between the dispersion and buffer viscosities (Figure 2).

Data Analysis. All data presented here are average values of at least three measurements with different samples, and the uncertainties are standard deviations. When not shown, the uncertainty was found to be smaller than the symbols on the graphs.

Results

DSC Measurements. Figure 3 displays DSC traces for DMPG dispersions for different lipid concentrations ranging from 1 to 50 mM. All samples present the typical DSC profile of low ionic DMPG dispersions:[4] a pretransition (T_p) at around 10−12 °C, a sharp peak between 17 and 19 °C corresponding to the onset of the main transition (T_m^{on}), followed by a few broad peaks and ending with a well-defined small peak (T_m^{off}).[16,20,21] The total enthalpy of the transition region was found to be around 6 kcal/mol for all studied DMPG concentrations. It may be noted in Figure 3, as also pointed out in a previous study,[4] that it is possible to distinguish at least three other calorimetric events in the transition region (Figure 3), T_1, T_2, and T_3, with the latter only for 50 mM DMPG (Figure 3).

(20) Although the pretransition is probably associated with the whole process of chain melting in lipid bilayers (refs 16 and 21), here we will consider the "main transition region" to be the temperature interval between T_m^{on} and T_m^{off}.

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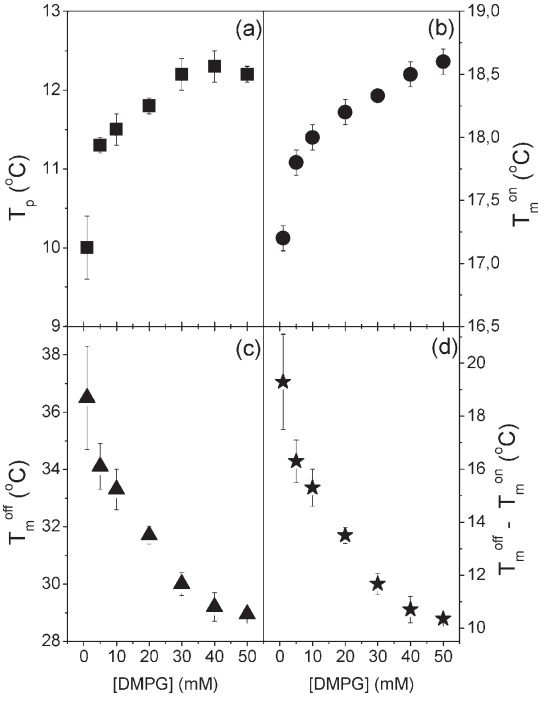

Figure 4. (a−c) DMPG concentration dependence of the three main thermal events observed by DSC: T_p (■), T_m^{on} (●), and T_m^{off} (▲). (d) Temperature difference between T_m^{off} and T_m^{on} as a function of DMPG concentration (★).

The midpoint temperatures of the three main peaks, corresponding to T_p, T_m^{on}, and T_m^{off}, were found to be dependent on the lipid concentration (Figure 4a−c). Both T_p and T_m^{on} increase with lipid concentration, whereas the last event, which signals the end of the main transition, is the most sensitive: T_m^{off} significantly decreases from 35 to 29 °C as the DMPG concentration increases from 1 to 50 mM. Hence, the temperature interval for the transition region ($T_m^{off} - T_m^{on}$) decreases as the lipid concentration increases (Figure 4d). This feature is in contrast to the behavior of neutral lipid dispersions, such as DMPC, for which the pretransition and the main transition temperatures may be considered to be independent of lipid concentration in the range of 1−50 mM (results not shown).

Electrical Conductivity. Detailed measurements of electrical conductivity as a function of temperature are shown in Figure 5a (σ_{red}, as discussed in Materials and Methods), for the same DMPG concentrations studied with DSC (1−50 mM). Clearly, the conductivity increases with the DMPG concentration. For a better comparison of the different samples, Figure 5b shows the electrical conductivity normalized by the lipid concentration (σ_{red}/[DMPG]). The data were found to be quite reproducible, as evidenced by the very small uncertainties in Figure 5.

Similar to the DSC profiles, the thermal dependence of the electrical conductivity was found to follow the same trend for all studied DMPG concentrations. The conductivity is much higher along the DMPG transition region, as seen before,[2] but other interesting features could now be detected. Figure 6 shows the conductivity data for 1, 10, and 50 mM DMPG, together with the DSC traces. For all samples, it is possible to distinguish (i) a clear, sharp increase in σ_{red} at a temperature close to T_p (~11 °C), (ii) a smooth increase from T_p to T_m^{on}, and (iii) a sharp increase at T_m^{on} (~18 °C), followed by a continuous increase up to a temperature close to T_m^{off}, at which the conductivity decreases abruptly. An additional bump was observed at higher temperatures not associated with any peak on the DSC trace. Different

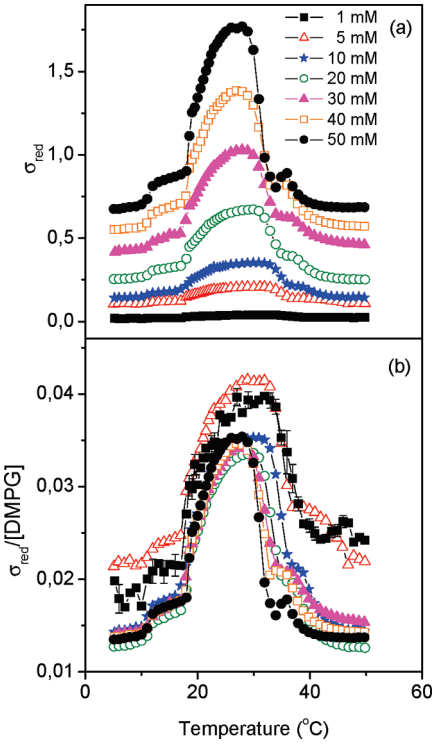

Figure 5. (a) Temperature dependence of the reduced electrical conductivity of DMPG dispersions (Figure 1) in Hepes buffer at various lipid concentrations. (b) Data presented in plot a normalized by the lipid concentration.

from the other features, this bump is not observable in cooling scans (data not shown). This extra feature above T_m^{off} was also detected by light scattering[5,13] and needs more investigation.

Electrophoretic Mobility. In the analysis of the conductivity data, it is important to account for the contribution of both charged lipid aggregates and counterions, apart from buffer ions, to the electrical conductivity of the charged lipid dispersion (σ). As shown in Materials and Methods, the electrical conductivity σ can be written as a function of the DMPG apparent degree of ionization, α, and the electrophoretic mobility of both DMPG aggregates (μ^{agg}) and Na^+ (μ^{Na+}) counterions (eq 2). Accordingly, the electrophoretic mobility of DMPG aggregates at different temperatures was measured as described in Materials and Methods, and μ^{Na+} as a function of temperature was calculated (eq 1 in Materials and Methods, dashed line in Figure 7). Figure 7 shows the measured electrophoretic mobility for 1, 5, 10, and 20 mM DMPG. The data are average values of at least five different samples with the corresponding standard deviations. The deviations are relatively large, possibly because of the polydispersity of the samples (above 20 mM DMPG, measurements were found unreliable). In spite of considerable scattering in the data, it is possible to see an anomalous increase in the electrophoretic mobility of DMPG aggregates over the lipid transition region, between T_m^{on} (~18 °C) and T_m^{off} (~30 °C), mainly for 5, 10, and 20 mM DMPG (Figure 7).

Viscosity. Considering that one of the fingerprints of the anomalous behavior of a low ionic strength DMPG dispersion is the high viscosity observed along the temperature-transition region[1] and the possible relationship between the sample viscosity and the other dynamic measurements presented here, viscosities of the same DMPG dispersions (1, 5, 10, 20, 30, 40, and 50 mM) were measured. The anomalous increase of the dispersion viscosity along the temperature-transition region was observed for all

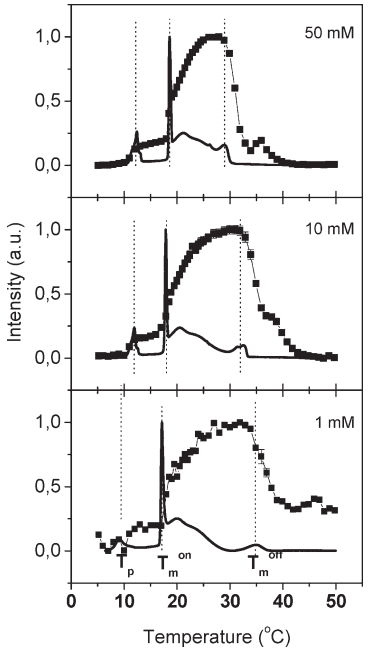

Figure 6. Comparison between DSC traces (—) and electrical conductivity (■) for 1, 10, and 50 mM DMPG dispersions in Hepes buffer. For each DMPG concentration, the maximum values in Figures 3 and 5a were normalized to unity.

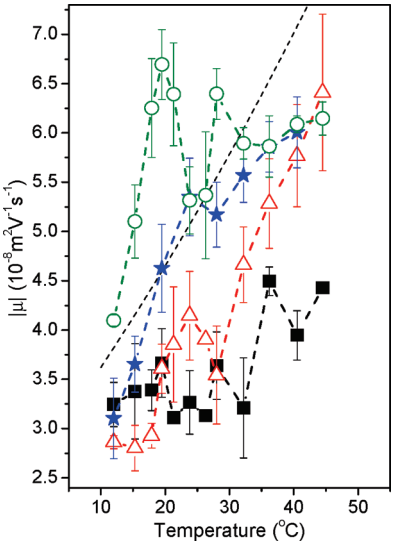

Figure 7. Temperature dependence of the electrophoretic mobility (absolute values) of 1 (■), 5 (red △), 10 (blue ★) and 20 (green ○) mM DMPG in Hepes buffer and sodium ion in water (---) as calculated from eq 1.

DMPG concentrations (Figure 8). Figure 8a,b display data for the specific viscosity, η_{sp}, and the specific viscosity normalized by the lipid concentration, η_{sp}/[DMPG], and make evident the strong dependence of the sample viscosity on the lipid concentration along the transition region. The sample viscosity for lipid concentrations from 1 to 50 mM DMPG varied significantly along the temperature-transition region for the different preparations assayed, though the very high values obtained for 40 and 50 mM DMPG (Figure 8, $\eta_{sp} > 20$), inside the transition region, are probably beyond the precision of the Ostwald viscometer used.

Figure 9 compares the data obtained with the three techniques, namely, DSC, electrical conductivity, and viscosity, for DMPG

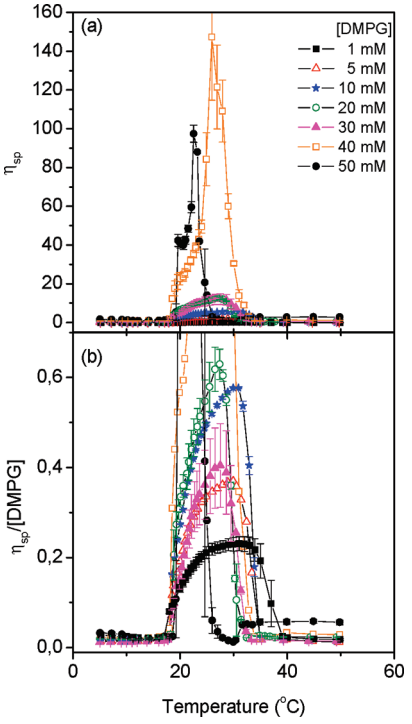

Figure 8. (a) Temperature dependence of the specific viscosity of DMPG dispersions (Figure 2) in Hepes buffer at various lipid concentrations. (b) Data presented in plot a normalized by the lipid concentration.

dispersions at 1, 10, 30, and 50 mM.[22] It can be seen that analogous to the conductivity behavior there is a sharp increase in the medium viscosity at T_m^{on} for all studied lipid concentrations. Except for 50 mM DMPG, both viscosity and electrical conductivity display similar smooth increases up to around 28 °C. However, the sharp drop in the medium viscosity does not correlate to the drop in the electrical conductivity, especially for the highest lipid concentrations.

It may also be noted that two features of the electrical conductivity measurements (Figure 5), the shoulder at the pretransition (T_p) and the shoulder that remains after the end of the transition region at T_m^{off}, are entirely absent in the viscosity data.

Discussion

Correspondence between Increasing Lipid or NaCl Concentrations. Figures 3 and 4 show that as the DMPG concentration increases, the onset of the main transition (T_m^{on}) increases and the offset (T_m^{off}) decreases. Similar behavior was obtained by fixing the DMPG concentration and increasing the medium ionic strength.[4] Indeed, increasing DMPG concentration yields an augmented concentration of Na^+ in solution because of diminished solvent volume. The equivalence of the two regimes, the increase in either macroion concentration or sample ionic strength, has been pointed out previously with respect to the electrophoretic mobility for macroions of fixed form and dissociation.[23]

One might rationalize the lipid−salt equivalence in the case of lipid dispersions under study as follows: the total concentration of Na^+ in solution is given by

$$[Na^+] = [Na^+ \text{ bulk}] + [\text{counterion}] \quad (6)$$

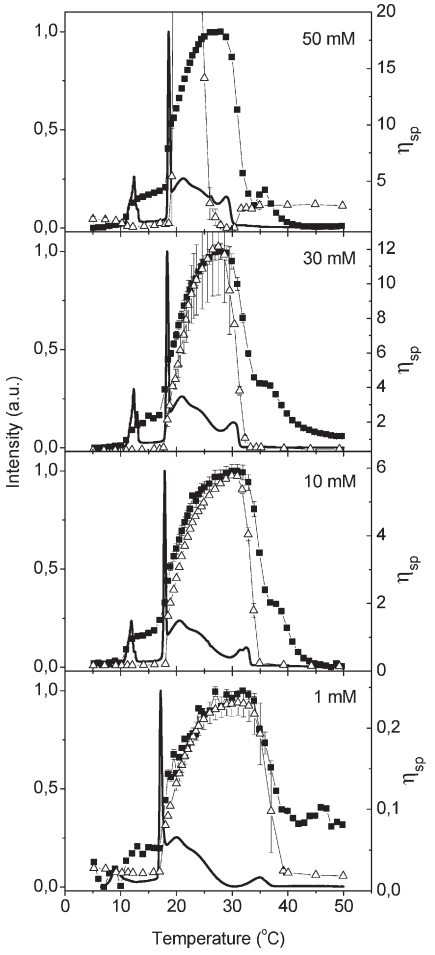

Figure 9. Comparison among DSC traces (—), reduced electrical conductivity, σ_{red} (■), and specific viscosity, η_{sp} (Δ), for 1, 10, 30, and 50 mM DMPG dispersions in Hepes buffer. For each DMPG concentration, the maximum values of DSC and σ_{red} (from Figures 3 and 5a) were normalized to unity (left scale). The η_{sp} values (from Figure 8) are shown on the right scale.

and [counterion] = α[DMPG], with α being the effective PG degree of ionization. Thus, the ion concentration in solution increases both with added [Na^+] and with [DMPG].

To examine the effect of increasing either the lipid or salt concentration on T_m^{on} and T_m^{off}, Figure 10 displays the variation of T_m^{on} and T_m^{off} with added [NaCl] for 10 mM DMPG in Hepes buffer at pH 7.4. (The corresponding DSC traces are not shown here.) If we compare this data with those for 50 mM DMPG (T_m^{on} and T_m^{off} in Figure 4b,c, shown in Figure 10 as dotted lines) under the hypothesis that T_m^{on} and T_m^{off} depend on [Na^+] in solution, then we may suggest that the 50 mM DMPG dispersion (in 10 mM Hepes buffer at pH 7.4 + 2 mM NaCl; see Materials and Methods) is equivalent to the 10 mM DMPG dispersion in 10 mM Hepes buffer at pH 7.4, with ∼6 mM added NaCl (dotted lines in Figure 10).

One may compare ion concentrations for the two dispersions by assuming that DMPG is organized as unilamellar closed vesicles occupying volume fractions ϕ such as those listed in Material and Methods. Considering the degree of ionization α of the external monolayer, the dissociated counterions in solution will be dissolved in a volume $V(1-\phi)$. Therefore, the Na^+ concentration in solution (eq 6) is

$$[Na^+]_{[Na^+ \text{bulk}],\,[\text{DMPG}]} = [Na^+ \text{ bulk}] + \frac{\alpha[\text{DMPG}]/2}{(1-\phi)}$$

(22) Viscosity values are shown up to $\eta_{sp} = 20$ only because higher values are probably unreliable.

(23) Lobaskin, V.; Dunweg, B.; Medebach, M.; Palberg, T.; Holm, C. *Phys. Rev. Lett.* **2007**, 98.

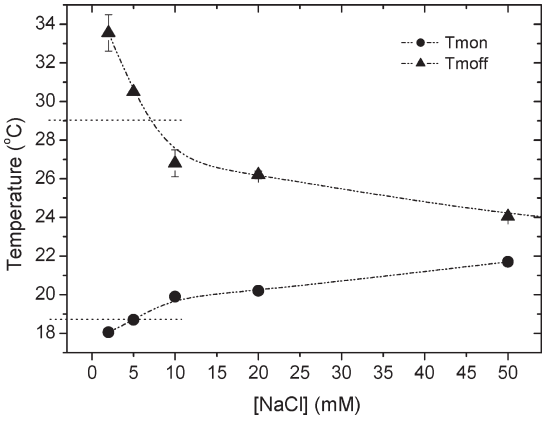

Figure 10. Dependence of the onset (T_m^{on}, ●) and offset (T_m^{off}, ▲) of the main transition of 10 mM DMPG dispersions with different concentrations of added NaCl to 10 mM Hepes buffer at pH 7.4. Dotted lines correspond to T_m^{on} (lower value) and T_m^{off} (higher value) of 50 mM DMPG in 10 mM Hepes buffer at pH 7.4 + 2 mM NaCl (values from Figure 4). Lines (-·-) are guides for the eye.

where [Na^+ bulk] = [Na^+ due to 10 mM Hepes buffer at pH 7.4] + [added Na^+] = 4 + [added Na^+], where 4 is the [Na^+] due to 10 mM Hepes buffer at pH 7.4. All concentrations are in mM.

Hence, for 50 mM DMPG with added 2 mM NaCl (Figure 4) we have

$$[Na^+]_{6,50} = 6 + \frac{\alpha 25}{0.875} \quad (7)$$

whereas for 10 mM DMPG with added 6 mM NaCl (Figure 12) one gets

$$[Na^+]_{10,10} = 10 + \frac{\alpha 5}{0.975} \quad (8)$$

Under equilibrium, for noninteracting vesicles, one expects the same ionization constant α for the same concentrations of ions in solution. Considering that [Na^+] is the same for the two lipid dispersions, they present similar values of T_m^{on} and $[Na^+]_{6,50} = [Na^+]_{10,10}$ (eq 7 = eq 8) and a value for the degree of ionization of the DMPG bilayer surface can be calculated, α = 0.17. Interestingly, even with these very simple hypotheses, one arrives at a value that is rather similar to the α values calculated for the gel and fluid phases of the 10 mM DMPG dispersion using electrical conductivity and electrophoretic data (Figure 12, discussed below). Hence, the dependence of the DSC profile with the DMPG concentration does seem to be related to the degree of ionization of the DMPG bilayer.

Charge and Structure of DMPG Vesicles. For "well-behaved" spherical macroions with constant charge and radius, the electrophoretic mobility is expected to increase steadily with temperature, as happens to Na^+ because of the decrease in the medium viscosity upon heating. (See eq 1 in Material and Methods or the dashed line in Figure 7.) However, for lipid vesicles or other colloid particles, temperature-phase transitions may yield a break in the temperature dependence of mobility μ, indicating either a change in particle charge or a change in its dimensions. (See, for example, refs 24 and 25.) Thus, the anomalous increase in mobility observed, especially for 5, 10, and 20 mM DMPG dispersions, over the transition region (Figure 7) is an indication that the DMPG aggregate itself must be changing with respect to charge, size, or form or in more than one of these properties as the temperature varies over that region.

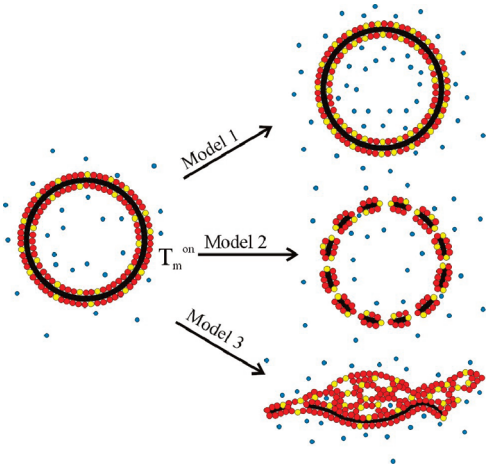

Figure 11. Cartoon of DMPG vesicles in the gel−fluid transition region for the three models discussed. On the left is the sketch of the DMPG vesicle before the transition. Red and yellow circles represent neutral and ionized PG headgroups, respectively. Apart from DMPG, only Na^+ counterions are represented (blue circles).

The electrophoretic mobility, together with the electrical conductivity, can be used in the calculation of the temperature dependence of the apparent degree of ionization (α) of the DMPG aggregate. However, the calculated α values for the phase-transition region of DMPG aggregates depend on the model chosen for the aggregate form along the transition (Materials and Methods). To gain insight into the form and charge of the aggregates, we consider below the three different models presented in Materials and Methods for the colloid particles in the transition region (Figure 11).

Model 1. If closed unilamellar vesicles are considered over the whole range of temperature, from 10 to 45 °C, then only the counterions in solution and PG^- groups from the external layer of the vesicles, apart from buffer ions, would contribute to the electrical conductivity (Material and Methods, eq 2). Accordingly, Figure 12 shows that the vesicle degree of ionization increases considerably over the transition-temperature region, with the gel and fluid phases displaying rather similar α values (apart from 1 mM DMPG).

Model 2. This model was proposed in previous work,[4,8,12] where the EPR of spin probes, SAXS, and optical microscopy of giant vesicles suggested that pores would open in the DMPG bilayer along the temperature transition region, possibly leading to highly perforated vesicles. Recently, this hypothesis was supported by experiments with molecules that stabilize the positive curvature of bilayer holes.[13] To probe the effect of perforation upon the degree of ionization, we have calculated the corresponding α in the middle of the transition region at 28 °C, as displayed in Figure 12. The hypothesis of complete permeability of vesicles to ions was adopted in the calculation of α (eq 3) at the midpoint of the transition region, at which the largest effect of perforation would be observed.[4,12] This result implies a small increase in α along the transition region.

Model 3. For the extreme case that perforation turns vesicles into bilayer sheets, not only all the buffer ions but also all PG^- ions from both the internal and external membrane layers contribute to α (eq 4). The corresponding α value in the midtransition region, 28 °C, is also shown in Figure 12. Under this condition,

(24) Tatulian, S. A. *Biochim. Biophys. Acta* **1987**, *901*, 161–165.
(25) Sierra-Martin, B.; Romero-Cano, M. S.; Fernandez-Nieves, A.; Fernandez-Barbero, A. *Langmuir* **2006**, *22*, 3586–3590.

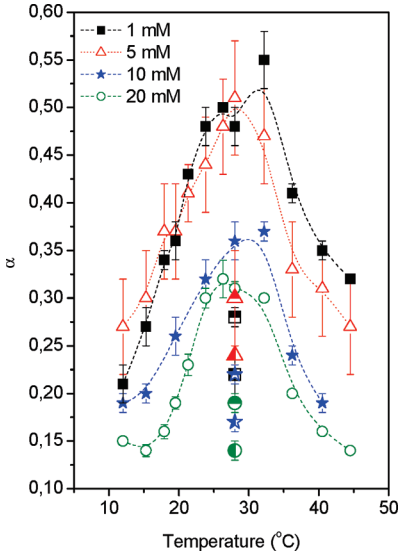

Figure 12. Temperature dependence of the calculated effective degree of ionization α of 1 (■), 5 (red △), 10 (blue ★), and 20 (green ○) mM DMPG in Hepes buffer, assuming the presence of closed unilamellar vesicles (model 1, eq 2). Lines were drawn to guide the eye. At 28 °C only, data for perforated vesicles for 1 (⬒), 5 (red triangles, top half solid), 10 (blue stars, top half solid), and 20 (green ◓) mM DMPG (model 2, eq 3) and data for opened bilayer sheets for 1 (◧), 5 (red ▲), 10 (blue stars, left half solid), and 20 (green ◐) mM DMPG (model 3, eq 4).

there would be no significant increase in the vesicle degree of ionization over the transition region.

To analyze the consequences of the different models, one must consider the effect of charge and friction upon mobility. Electrophoretic mobility behavior may be discussed in terms of the drag force on a slowly moving particle in the solution, $F = -bv$, where v is the particle velocity and b is the frictional coefficient. Because $\mu = v/E$ (Materials and Methods) and $bv = qE$, we can write $\mu = q/b$. Thus, mobility increases with the charge of the particle (q is proportional to the degree of ionization α) and decreases with the particle frictional coefficient b.

Our data for electrophoretic mobility (Figure 7) and the vesicle degree of ionization (Figure 12) shows that they depend both on lipid concentration and on temperature. Let us analyze these two dependencies separately.

In relation to the concentration dependence, Figure 12 shows that the bilayer ionization decreases as the lipid concentration increases for the three models considered (though 1 and 5 mM DMPG present similar values). This is in accord with the discussion in the previous section where it was considered that an increase in the DMPG concentration was similar to an increase in the sample ionic strength, hence leading to a decrease in the bilayer degree of ionization due to larger screening and less effective ionization. Also, in the previous section we mentioned the similarity between the α value calculated for 10 mM DMPG (0.17) and the one obtained from the experimental data for the gel and fluid phases of 10 mM DMPG (unilamellar closed vesicles), ~0.18 in Figure 12. It must also be noted that mobility (Figure 7) and vesicle charge (Figure 12) display opposite behavior: the DMPG vesicle electrophoretic mobility data display a clear increase with lipid concentration (Figure 7). If q diminishes with rising lipid concentration, then the magnitude of frictional coefficient b must decrease sufficiently to allow an increase in $\mu = q/b$ as [DMPG] increases. Therefore, frictional coefficient b must be a decreasing function of lipid concentration. Setting aside its dependence on viscosity, to be discussed in the next subsection, this coefficient b is known to be related to the size and form of the particle (b reduces to the Stokes relation in the case of spheres, $b = 6\pi\eta R$, where η is the medium viscosity and R is the radius of the particle[26]). Additionally, the effective size may depend on hydration, which is also charge-dependent. If the colloid charge diminishes with lipid concentration as a result of charge screening, then the effective radius due to solvation also diminishes, contributing to decreasing b. However, q and b cannot be linearly related.

As for the temperature dependency, the anomalous increase in electrophoretic mobility along the transition region (Figure 7) has implications for the particle charge q and frictional coefficient b in that region. Because the particle mobility may be written in terms of the ratio of the particle charge and frictional coefficient, $\mu = q/b$, the colloid particle properties of the three models presented above can be rationalized as follows. In the case of model 1 for closed vesicles, α increases considerably with temperature at fixed concentration along the transition region (Figure 12), hence the total lipid charge q increases. Maximum α (Figure 11) at 28 °C implies that q increases approximately 2-fold. The approximate 20% increment of measured mobility (Figure 7) yields an approximately 70% increase in b. If the Stokes relation is considered to be valid, then model 1 for closed spheres of a fixed radius and higher charge presents increased b in the transition region, in accordance with the suggested behavior proposed in the theoretical literature. (See, for example, ref 27.) In the case of model 2 for spheres with completely permeable pores in the middle of the transition region (~28 °C), the small increase in α (around 10% at 28 °C, Figure 12) is of the same order of magnitude as the increment in particle mobility (near 10%, see Figure 7), implying nearly constant friction b. This result is consistent with theoretical predictions in the related literature, according to which the presence of pores, for constant charge and radius, would reduce the viscosity,[27] whereas augmented charge increases b. Thus, the two effects may compete to maintain b unaltered. However, one may relax conditions on model 2 such that highly charged regions in the lipid bilayer might favor the opening of pores of limited permeability to ions, which would lead to a somewhat larger degree in ionization than for model 2 (Figure 12), accompanied by some increase in b. Finally, in the limiting case of completely open vesicles in the middle of the transition region (model 3), there would be no change in the bilayer degree of ionization (Figure 12), but the actual charge q of the DMPG aggregate would double, with the exposition of the internal PG^- groups. Analogous to the case of model 1, frictional coefficient b should suffer an increment of 70%, but in this case, it would be related to the change from spheres in the gel and fluid phases to open sheets around the middle of the transition region.

The three models demand an increase in friction coefficient b (although smaller in the case of "soft" model 2) in the transition region. However, in the case of model 1, this change is due to charge, whereas in model 3 it is due to form. Model 2 lies midway, and both charge and form contribute to produce a variation of b.

Macroviscosity vs Local Viscosity. Viscosity measurements are difficult to interpret and will not be discussed in detail in the present work because they are the subject of an upcoming paper (Barroso et al., in preparation). Here, the viscosity data will be focused only as far as they are relevant to the discussion of the electrical conductivity and electrophoretic mobility data. We will argue below that the measured sample viscosity (macroviscosity)

(26) Landau, L. D.; Lifshitz, E. M. *Fluid Mechanics*; Pergamon Press: Oxford, U.K., 1959.
(27) Natraj, V.; Chen, S. B. *J. Colloid Interface Sci.* **2002**, *251*, 200–207.

has little or no influence on either the conductivity or the electrophoretic mobility data. That is, frictional coefficient b has no relation to the macroviscosity measured in the presence of lipid vesicles, though the high viscosity measured along the DMPG transition region is certainly related to other anomalous properties of this region.

To gain some insight into the relation between conductivity and viscosity, we have investigated other systems for control.

As discussed in Materials and Methods, the conductivity is given by $\sigma = Nq\mu = Nq^2/b$, where $\mu = q/b$. In accordance with the proportionality between b and η (generalized Stokes relation[26]), the conductivity of a NaCl solution was found to vary with $1/\eta$, when η was increased by raising the glucose concentration (data not shown). Similarly, the electrophoretic mobility of a charged DMPG dispersion was found to be very sensitive to the presence of glucose, decreasing as the glucose concentration increases (data not shown). In this case, both small and macroion mobilities are sensitive to the measured macroviscosity, when the sample viscosity is increased by the addition of small molecules such as glucose.

This is completely distinct from the results obtained when the sample viscosity was varied by the addition of neutral vesicles: as the lipid concentration is increased, the sample viscosity increases whereas the electrical conductivity does not change. The sample specific viscosity ($\eta_{sp} = (\eta - \eta^{buffer})/\eta^{buffer}$, see Material and Methods) was found to go up to 0.14 in the presence of 20 mM DMPC (20 times extruded through a 100 nm filter), which is about the expected value using the Einstein relation, $\eta_{sp} = 2.5\phi$.[28] (ϕ was calculated by assuming unilamellar vesicles of 100 nm diameter, with equal amount of lipids in the internal and external layers and a 0.50 nm^2 area per lipid headgroup for the fluid-phase bilayer.) However, the electrical conductivity of 1 mM NaCl was found to be the same in the presence or absence of 20 mM DMPC. This experiment clearly indicates that the conductivity of small ions (and hence their electrical mobility) is insensitive to the increased sample viscosity due to neutral colloid particles. Similar results will be discussed below and are related to the conductivity of macroions.

In the case of charged DMPG dispersions, the conductivity and measured macroviscosity dependence on lipid concentration are very different. The reduced conductivity/[DMPG] is nearly independent of [DMPG] from 10 to 50 mM (Figure 5b), even in the lipid transition region, whereas the values of the measured specific viscosity/[DMPG] are strongly dependent on the DMPG concentration (Figure 8). We must conclude that the measured macroviscosity cannot be the local viscosity felt by the colloid particle, proportional to the frictional coefficient.

The latter conclusion is made stronger by the analysis of the temperature dependence of conductivity and viscosity, fixing the DMPG concentration. As discussed above, σ may be taken to be roughly proportional to q^2/η. If we take closed vesicles (model 1) and the 10 mM DMPG sample, we have the following: in the temperature interval from 10 to 28 °C, while the conductivity increases 2.3-fold (Figure 5) the charge increases nearly 2-fold (Figure 12) and the viscosity increases by a factor of 35 (Figure 8). Thus, the conductivity, σ, increases roughly 2-fold, whereas the ratio q^2/η decreases approximately 9-fold . This is clear evidence that the increased sample macroviscosity cannot affect colloid mobility or conductivity because of either small ions or macroions.

Our results strongly suggest that the macroviscosity measured by the flow of a lipid dispersion is different from the local viscosity felt by a microion or macroion in solution. This result might have some relation to results presented by Horn et al.[29] from experiments on latex particles in which the steady shear viscosity is always found to be greater than the viscosity measured under an oscillatory driving force. In our study, electrophoretic mobility and conductivity data were obtained from the particle response to an oscillatory field, whereas the viscosity was measured with an Ostwald viscometer under steady flow.

The above arguments are strongly supported by the data obtained with 50 mM DMPG, where a clear disconnect between the drop in the sample viscosity and conductivity is observed (Figure 9). The 50 mM DMPG dispersion clearly shows that viscosity can drastically drop (at T_3 in Figure 9), with no change in the sample electrical conductivity.

Temperature T_3 (Figure 9) was the temperature at which the sample turbidity was found to start increasing, upon heating a 50 mM DMPG dispersion.[4] Accordingly, it was suggested that above this temperature the DMPG bilayer would become less perforated, with the membrane starting to recover its normal structure. Indeed, such a drop in the medium viscosity does suggest that DMPG aggregates are somehow restructuring themselves. However, no evident change in the sample electrical conductivity is observed at T_3 (Figure 9), indicating that whatever is responsible for the high conductivity is little affected by the possible reorganization of the vesicles at T_3. Hence, model 3 presented above, which assumes totally opened bilayer sheets along the transition region and correlates the increase in the DMPG electrophoretic mobility (and hence in the sample conductivity) to the exposition of internal and external bilayer PG^- groups, does not seem to be appropriate. That does not rule out the possibility of semiopened perforated bilayer sheets along the temperature-transition region, but the accompanying increase in conductivity should be due to an increase in the bilayer degree of ionization, α, and not to the total exposition of internal and external lipid ionized lipids, as used in model 3 (eq 4).

Supporting the above discussion against totally opened bilayer sheets, there are experiments which show that no lipid exchange between DMPG vesicles occurs along the transition region (with spin and fluorescence probes[5,10]), even with a 50 mM DMPG dispersion (ESR experiments). Considering 100-nm-diameter vesicles in a 50 mM DMPG dispersion (unilamellar vesicles with equal amount of lipids in both layers, internal and external, and with a 0.50 nm^2 area per lipid), the distance between them (distance between their surfaces) would be around 60 nm. Hence, if the vesicles are completely open (open sheets), then one would expect some fusion among them, which may be discarded from ESR experiments.

Conclusions

It was shown here that the dependence of the DSC profile of low ionic strength DMPG dispersions on the lipid concentration is probably due to the different degrees of ionization of the lipid bilayers. As the DMPG concentration increases, the bilayer becomes less ionized because of the increase in the concentration of bulk Na^+. The effect on the DSC profile of increasing DMPG concentration was found to be similar to the effect of increasing the NaCl concentration and allowed the calculation of the degree of ionization for 10 mM DMPG vesicles. The degree of ionization calculated from electrical conductivity and electrophoretic mobility data (0.17) agrees well with the value calculated from DSC

(28) Larson, R. G. *The Structure and Rheology of Complex Fluids*; Oxford University Press: New York, 1999.

(29) Horn, F. M.; Richtering, W.; Bergenholtz, J.; Willenbacher, N.; Wagner, N. J. *J. Colloid Interface Sci.* **2000**, *225*, 166–178.

data (0.18) and also indicates that DMPG vesicles are less charged in higher lipid concentration dispersions.

A sharp increase in σ_{red} at the bilayer pretransition was observed (Figure 6). Though the electrophoretic mobility data are not precise enough to allow us to draw a conclusion about an increase in the vesicle degree of ionization at this temperature, this seems to be a sound and interesting hypothesis, considering that the pretransition has been identified as the beginning of the bilayer melting process[16] and the gel−fluid transition region was characterized here by higher α values.

Following previous discussions[4,13] and in light of the new experimental results presented here, it could be rationalized that by increasing the temperature at and immediately after T_m^{on}, DMPG bilayers would be more ionized, possibly perforated, maybe somehow deformed vesicles (models 1 and 2). That would be related to the measured high viscosity, high electrical conductivity, and low turbidity. At T_m^{off}, vesicles would get less charged and less perforated, hence the optical turbidity would increase and the sample viscosity would decrease. At higher temperatures, "normal" fluid vesicles would be present and the sample turbidity and conductivity would go back to normal values found for the DMPG fluid phase.[4]

The measured macroviscosity of a DMPG dispersion was shown to be distinct from the local viscosity felt by either charged ions or DMPG charged aggregates in measurements of conductivity or electrophoretic mobility. However, whatever brings on the lipid transition at T_m^{on} is responsible for the huge increase in the sample viscosity, possibly related to an increase in the interaction among DMPG aggregates while flowing.

We believe that the work presented here contributes to a better understanding of charged DMPG dispersions. However, several open questions remain. Certainly, a comprehensive thermostatistical model to explain the experimental data is missing. It should take into account the different interactions present in the system between DMPG charged headgroups and hydrocarbon chains in a water medium. It is important to keep in mind that whatever happens in the transition region is fairly reversible, as far as DSC, viscosity, and conductivity data can detect.

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