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Gel−Fluid Transition in Dilute versus Concentrated DMPG Aqueous Dispersions

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Aqueous dispersions of the anionic phospholipid dimyristoyl phosphatidylglycerol (DMPG) in 100 mM ionic strength, at pH values higher than 4, are known to exhibit a thermal behavior rather similar to that of the zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC), which undergoes a gel-to-liquid crystalline phase transition at 23 °C. However, in aqueous medium of low ionic strength, DMPG was shown to present a large gel−fluid transition region, ranging from 18 to 35 °C. This intermediate phase is optically transparent and characterized by a continuous change in membrane packing. The present work shows that, below a certain lipid concentration, which we denote c' ($c' = 0.4 \pm 0.2$ mM), this anomalous behavior vanishes, being replaced by a sharper phase transition centered at a unique transition temperature T_m. Interestingly, T_m increases as the lipid concentration is further decreased, reaching a limiting value of about 41 °C. Below c', opposite to the effect observed for DMPG at higher concentrations, the value of T_m decreases as the ionic strength is increased. Above 500 mM NaCl, the thermal behavior of DMPG aqueous dispersions is roughly independent of the lipid concentration. Furthermore, increasing sample pH significantly decreases T_m in the low concentration regime, whereas in concentrated DMPG dispersions, T_m is not affected by changes in pH above 6. The dependence of the thermal behavior of dilute DMPG aqueous dispersions on pH and ionic strength is discussed here in the light of the dependence of the headgroup protonation on DMPG concentration.

Introduction

The phospholipid dimyristoyl phosphatidylglycerol (DMPG) has an ionizable phosphate group, making its thermal behavior very sensitive to changes in external conditions, such as pH and salt concentration. It is well known that, under physiological conditions, DMPG has a thermal behavior quite similar to that displayed by the zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC), one of the most studied lipids in the literature. However, in a certain range of lipid and (low) salt concentrations, DMPG displays an interesting thermal behavior, as was pointed out in the past,[1−7] and, more recently, has become the subject of intense efforts.[8−12] Instead of undergoing the chain melting process in a narrow temperature interval, DMPG dispersions show an intermediate phase, as a large transition region between the gel and fluid phases, with specific properties, such as very low light and small-angle X-ray scattering,[9] high viscosity,[6] and conductivity.[7] A continuous decrease in membrane packing with temperature was measured using electron spin resonance (ESR) of spin labels intercalated into the bilayer.[6,9] Concomitantly, a continuous decrease in bilayer thickness was observed with small-angle X-ray scattering.[9] Differential scanning calorimetry (DSC) shows a complex calorimetric pattern, where broad peaks are present between a narrow peak, corresponding to the beginning of the chain melting process (T_m^{on}), and a broader peak, which marks the end of the main phase transition (T_m^{off}). The measurement of the membrane surface/water partitioning of a cationic aqueous soluble spin label revealed a high DMPG surface potential above T_m^{on} ($\Psi_o = -170$ mV at 6 mM ionic strength[8]).

The transition region narrows significantly as the ionic strength increases, until both temperatures, T_m^{on} and T_m^{off}, collapse into a single T_m value (23 °C), around 100 mM NaCl.[8] Further increase in salt concentration causes T_m to raise to 29 °C at 2 M NaCl.[13] A decrease in pH also induces the disappearing of the intermediate phase, probably with complete protonation of the phosphate groups, evidenced by a much higher bilayer transition temperature, $T_m = 42$ °C, related to a more stable gel state. Thus, the extent of the transition region and its characteristics are surely related to the presence of charged headgroups, which may cause a great repulsion between vesicles as well as between adjacent lipids at the bilayer surface, thus disturbing and altering its packing.

In this work we present a completely new aspect of low ionic strength DMPG dispersions. Below a concentration around 0.4 mM DMPG, denoted c', the transition region narrows and the DSC profile changes markedly, indicating a phase transition centered at a single calorimetric peak, rather than the sequence of Cp peaks observed between T_m^{on} and T_m^{off} in concentrated dispersions. The value of the transition temperature, T_m, significantly increases as the concentration is further decreased, reaching a limiting value of 41 °C for DMPG concentrations around 10 μM, in the buffer system used here (6 mM ionic

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strength, pH 7.4). This thermal behavior is highly dependent on the sample ionic strength and pH. Using DSC, fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), which intercalates in the bilayer core, and the observation of giant vesicles under a phase contrast light microscope, we compare the thermal behavior below and above c', as a function of the sample ionic strength and pH.

Apart from being a very interesting physicochemical system, DMPG has been extensively used as a model system for the lipid phase of negatively charged biological membranes.[6,14−18] Thus, the accurate characterization of DMPG dispersions is fundamental for the proper analysis of the structural alterations caused by various different molecules in the lipid bilayer. Indeed, this work shows that DMPG dilution may induce the gel−fluid transition, even at constant sample temperature, ionic strength, and pH.

Materials and Methods

Materials. The sodium salt of the phospholipid DMPG (1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-glycerol]) was purchased from Avanti Polar Lipids (Birmingham, AL). The fluorescent probe DPH (1,6-diphenyl-1,3,5-hexatryene) was purchased from Molecular Probes (Eugene, OR). The buffer system used was 10 mM Hepes (4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid) adjusted with NaOH to pH 7.4. The buffer ionic strength was calculated and measured to be 4 mM. Mille-Q Plus water (Millipore), pH $\sim$ 6, was used throughout. The total sample ionic strength mentioned throughout this work refers to the amount of added salt plus the buffer ionic strength (bulk ionic strength) only. The lipid counterion concentration is not included. Accordingly, whenever pure water is mentioned, it means water plus free counterions. Here, we make the assumption that there is a bulk phase where the lipid vesicle charge is neutralized by the counterions charge, as assumed in the Gouy−Chapman model.[19]

Lipid Dispersion Preparation (for Dsc and Fluorescence Anisotropy Measurements). A lipid film was formed from a chloroform solution of lipids, 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. Vesicles were prepared by the addition of the desired aqueous solution, followed by vortexing for about 2′ at 50 °C (above T_m^{off} or T_m). For all lipid concentrations and ionic strengths, the medium pH value was checked to be stable at 7.4. The samples were kept at room temperature and used within a few hours after preparation. For the fluorescence measurements, 0.5 mol % DPH was added to the chloroform lipid solution. The lipid concentration was checked by phosphorus analysis,[20] and the error in the values presented here is less than 6%.

Large Unilamellar Vesicle (GUV) Preparation (for Light Microscopy). A chloroform lipid solution (10 mg/mL) was spread on the rough side of a Teflon disk, which stayed under reduced pressure overnight to remove all traces of organic solvent. 0.5 mol % DPH was added to the chloroform lipid solution to allow fluorescence anisotropy measurements in giant vesicles. The Teflon plate was accommodated inside a glass bottle, with the rough side containing the lipid film facing up. In a prehydration stage, the bottle was left in a humid atmosphere for 2 h at 37 °C. After that, 4 mL of the desired aqueous solution was gently poured inside the bottle. The bottle was closed with a cap, sealed with Parafilm and left at 37 °C for one or 2 days. The final lipid concentration could not be determined exactly, as it is not known whether all lipids left the Teflon surface to form vesicles. However, we estimated it to be smaller than 40 μM DMPG. The vesicle solution was gently transferred into a Teflon microchamber, thermally coupled to a water-circulating bath, covered with glass slits and sealed with silicon grease. The giant vesicles had diameters of about 10 μm.

Differential Scanning Calorimetry. The calorimetric data were obtained with a Microcal VP instrument. The scan rates used were 20 °C/h and 60 °C/h, for the concentrated and dilute samples, respectively. For samples above c' no significant changes in the DSC runs were observed for up scans obtained at rates between 1 and 60 °C/h. Below c', due to the low resolution of the DSC signal, it was only possible to ensure that the 60 °C/h runs were quite similar to those at 20 °C/h. Baseline subtractions and peak integrals were done using the Microcal DSC data analysis software.

Fluorescence Measurements. A Fluorolog 3 Jobin Yvon-SPEX spectrometer, equipped with a 4-sample holder, was used. Temperature was controlled by a water bath circulator Julabo HP 25, and the temperature inside the sample was measured with a thermocouple. The fluorescence anisotropy of DPH was measured using $\lambda_{ex} = 359$ nm, $\lambda_{em} = 427$ nm, 2 nm slits and 2 s acquisition time. Three measurements were performed at each temperature, and the error in measuring the anisotropy values was estimated to be roughly 1% in the gel phase and about 5% in the fluid phase. In all of the fluorescence anisotropy curves, the lines connecting the experimental points are for better visualization only.

Phase Contrast Light Microscopy. Visualization of the vesicles was done with an inverted Axiovert 135 Zeiss microscope equipped with a 40× Ph2 objective. Images of vesicles brought into focus were taken with a Hamamatsu C5985 CCD camera connected to a Vino video board of a Silicon Graphics Indy Workstation.

Results

Low Ionic Strength Dispersions. Figure 1a shows a typical DSC thermal profile of DMPG aqueous dispersions in low ionic strength medium (10 mM Hepes pH 7.4 + 2 mM NaCl, total ionic strength = 6 mM). This rather complex profile, presenting several peaks, is observed for DMPG samples in a large range of concentrations (1−500 mM), and has been reported and discussed before,[9,10] though it is far from being fully understood. Here, this will be called the DMPG high concentration regime, despite including concentrations as low as 1 mM. In Figure 1a, the small peak at 12.2 °C can be attributed to the pretransition (T_p), the sharp peak at 17.3 °C marks the beginning of the gel to fluid phase transition (T_m^{on}), and the small and broad peak at 34 °C indicates its end (T_m^{off}). In general, the temperature interval between T_m^{on} and T_m^{off} decreases as the lipid concentration increases.[6] Also shown in Figure 1a is the temperature dependence of the fluorescence anisotropy of the probe DPH incorporated in DMPG bilayers. A decrease in fluorescence anisotropy is related to a decrease in acyl chain order, as observed during the gel−fluid transition of lipid membranes.[21−24] It is clear from Figure 1a that the bilayer packing decreases gradually between T_m^{on} and T_m^{off}. The results obtained with the fluorescent probe used in this study are very similar to those obtained with spin labels.[6,9] As very dilute lipid samples are studied in the present work, the use of DPH is much more convenient, as this molecule is highly fluorescent and can be monitored at concentrations as low as 10 nM. Thus, it is possible to use DPH as a probe in dilute DMPG dispersions, never exceeding 0.5 mol % of the lipid concentration. Moreover, the excitation and emission wavelengths of DPH are relatively high

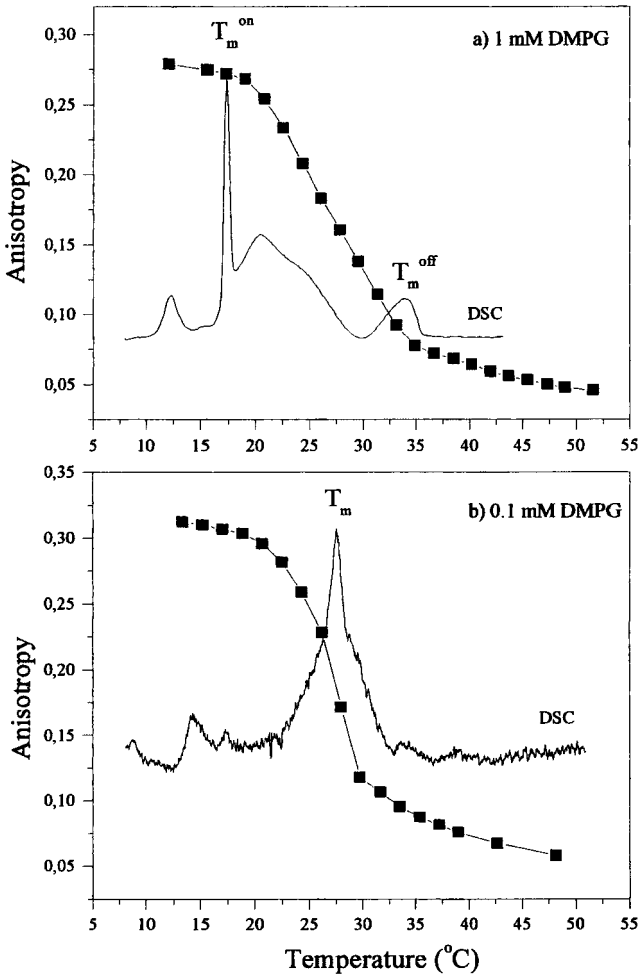

Figure 1. Fluorescence anisotropy of 0.5 mol % DPH and DSC traces of (a) 1 mM and (b) 0.1 mM DMPG in 10 mM Hepes pH 7.4 + 2 mM NaCl.

(λ_{ex} = 359 nm, λ_{em} = 427 nm), making the influence of light scattering negligible in the range of lipid concentrations used here.

In contrast to the results obtained with concentrated DMPG samples, dilute low ionic strength aqueous dispersions of DMPG yielded a simpler thermal profile, as illustrated for 0.1 mM DMPG in Figure 1b. The difference can be clearly seen by comparing the DSC runs of the two regimes. Whereas the DSC run in Figure 1a contains several calorimetric peaks, associated with the beginning and end of the main phase transition and with processes occurring between, the DSC run in Figure 1b is characterized by a calorimetric peak centered at T_m = 27.5 °C. The enthalpy of this peak was estimated to be around 5 kcal/mol. This value is comparable to the enthalpy per mol calculated over the whole transition region, between T_m^{on} and T_m^{off}, in the high concentration regime[9] (Figure 1a). A large decrease in DPH fluorescence anisotropy was observed at the transition temperature monitored by DSC, and with a much sharper profile, as compared to the steady decrease observed with the 1 mM DMPG sample (Figure 1a). The small peak at T_p = 14.2 °C (Figure 1b) can be attributed to the pretransition, occurring at a temperature higher than that found in the concentrated regime, T_p = 12.2 °C (Figure 1a). Even though the baseline at this low DMPG concentration is not well defined, it is possible to detect the pretransition at T_p = 14.2 °C, as a bump at that transition appeared in all runs, for all samples tested.

The change in the DMPG thermal behavior occurs over an interval of lipid concentration, rather than at a defined value.

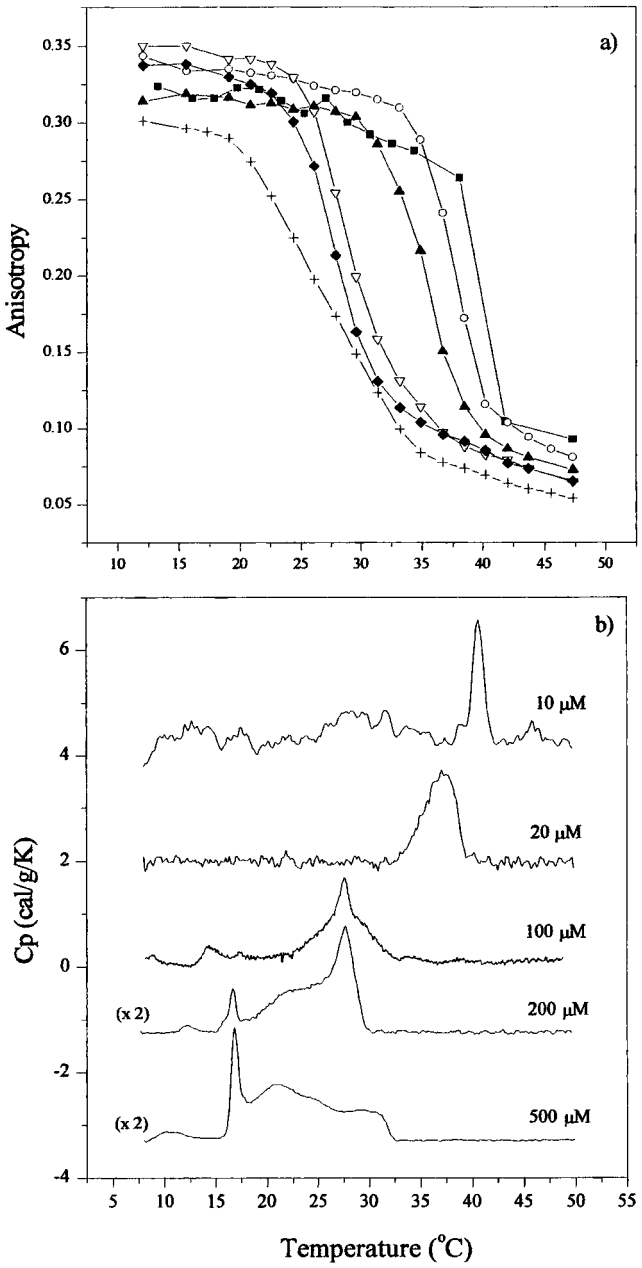

Figure 2. (a) Temperature dependence of the fluorescence anisotropy of 0.5 mol % DPH in (■) 5 μM, (○) 25 μM, (▲) 50 μM, (▽) 75 μM, (◆) 100 μM, and (+) 1000 μM DMPG in 10 mM Hepes pH 7.4 + 2 mM NaCl. (b) DSC traces of different DMPG concentrations in 10 mM Hepes pH 7.4 + 2 mM NaCl. The 200 and 500 μM traces were multiplied by 2 for better visualization. The scans are shifted from Cp = 0.

In this concentration range, here called c' for simplicity (c' = 0.4 ± 0.2 mM), the thermal behavior was found to be highly preparation dependent. The DSC runs could be either characteristic of the high (as in Figure 1a) or of the low (as in Figure 1b) concentration regimes. Some of the DSC runs were even a mixture of the two regimes, since they presented the peak centered at 27.5 °C superimposed to the complex profile found in the high concentration regime.

Figure 2 illustrates a typical result obtained with DPH fluorescence anisotropy and DSC measurements for different lipid concentrations. The 500 μM DMPG sample is clearly in the high concentration regime, displaying a complex DSC profile, similar to that observed for 1 mM (Figure 1a) or higher DMPG concentrations,[9] and a steady decrease in the DPH

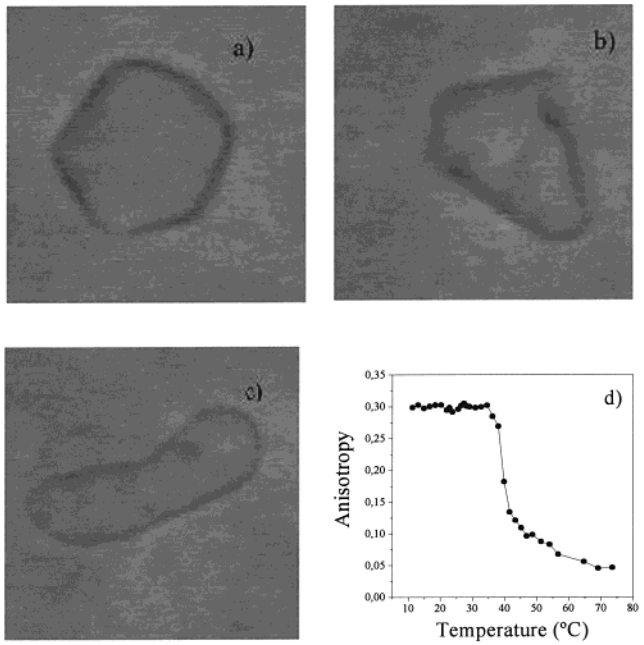

Figure 3. Images obtained from one giant DMPG vesicle (diameter ~10 μm) in the dilute regime in 10 mM Hepes pH 7.4 + 2 mM NaCl at three different temperatures: (a) 35 °C (below T_m), (b) 41 °C (at T_m), and (c) 43 °C (above T_m). (d) Temperature dependence of the fluorescence anisotropy of 0.5 mol % DPH incorporated in giant DMPG vesicles.

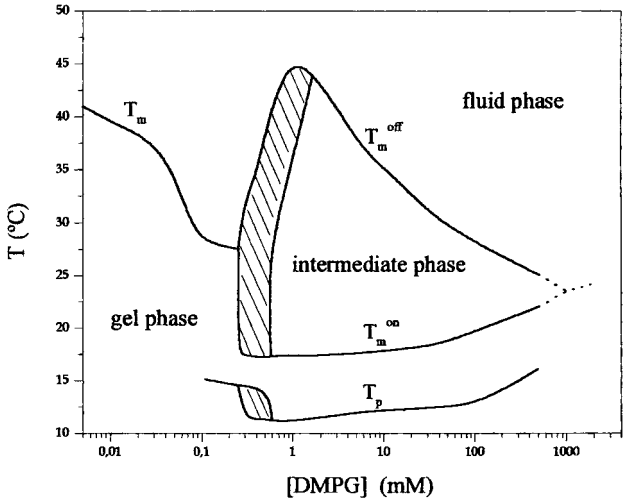

Figure 4. Schematic DMPG phase diagram at low ionic strength (10 mM Hepes pH 7.4 + 2 mM NaCl), obtained with DSC, fluorescence anisotropy, turbidity measurements, and ESR. The striped area represents c'. The dotted line at very high lipid concentration indicates that T_m^{on} and T_m^{off} might collapse into a single T_m value.

fluorescence anisotropy (Figure 2a). The 200 μM DMPG sample shows a DSC profile typical of a mixture of the two regimes. For concentrations lower than 100 μM DMPG, the samples were always found to be in the dilute regime, as shown in Figure 2b, presenting a transition centered at a single T_m value. Interestingly, as the lipid concentration decreases, the phase transition gets narrower and shifts to higher temperatures, reaching a limiting value of 41 °C, for 10 μM in the buffer system used. A good agreement between DSC traces and anisotropy measurements was found, as shown in Figure 2a,b. Due to the low resolution of the DSC runs, the presence of the pretransition could not be verified for concentrations below 100 μM. Although the enthalpies of the main transition could not be measured with high precision, due to the low signal-to-noise ratio of the very dilute samples, they were all found to be around 5 kcal/mol.

The dilute DMPG regime was also studied employing phase contrast video microscopy on giant vesicles. It is well known that lipid bilayers can only display visible shape fluctuations in the fluid phase, since the high shear modulus of the gel phase hinders fluctuations below T_m. Thus, the main phase transition of lipid bilayers can be clearly determined by optical observation of giant vesicles, as has been done, for instance, on giant vesicles of DMPC and DPPC.[25,26]

Giant vesicles were monitored over a large temperature range (10−50 °C) and were found to start fluctuating around 40−42 °C. The images of one typical giant DMPG vesicle undergoing the main transition are shown in Figure 3. At 35 °C (Figure 3a) the vesicle is in the gel state. Interestingly, in the gel phase, all DMPG vesicles presented polygonal nonfluctuating shapes, in contrast to DMPC vesicles, which always formed rigid spherical shapes (results not shown). Near T_m the vesicles present high curvature regions coexisting with flat ones as illustrated in Figure 3b (41 °C), presumably corresponding to liquid crystalline and gel domains, respectively. At 43 °C (Figure 3c) the vesicle displays large thermal shape fluctuations, indicating that the lipids are in the fluid state. The gel-to-liquid crystal transition was found to be reversible, since, by decreasing the temperature, the vesicles went back to the rigid state characteristic of the gel phase. It is interesting to note that increasing the temperature, just after crossing T_m, the vesicles often presented budding and other shape transitions.[27] This was not observed with giant vesicles of DMPC, which always presented spherical shapes in the gel phase and prolate shapes after crossing T_m[26] (data not shown).

DPH fluorescence anisotropy measurements were also done on dilute giant DMPG vesicles, yielding a thermal profile similar to that shown for the low concentration samples in Figure 2a, with T_m = 40 °C (Figure 3d). This is a strong indication that differently prepared dilute DMPG dispersions, in the same low ionic strength medium, present a similar thermal behavior. It is important to point out that the thermal behavior observed for giant vesicles grown in the high concentration regime (manuscript in preparation) is completely different from that shown here for dilute giant vesicles.

The optical observation of the fluctuations of giant vesicles is crucial to ensure that the gel−fluid transition is really happening at such a high temperature. The calorimetric peak obtained by DSC is not conclusive to define which transition it refers to, even though the enthalpy calculated is consistent with the one expected for the gel−fluid transition. The high fluorescence anisotropy values measured at low temperatures strongly indicate the existence of a gel phase. Therefore, the combination of these three techniques definitely shows that the main phase transition temperature assumes this very high value in dilute dispersions. It is important to remember that, in concentrated samples, such a high T_m value is only observed at low pH values, when PG is in its protonated state.

Based on the results presented here, and on previous studies at higher concentrations, a phase diagram of DMPG at low ionic strength (10 mM Hepes buffer pH 7.4 + 2 mM NaCl) could be outlined (Figure 4). The striped area between 0.2 and 0.6 mM DMPG corresponds to the c' region, where composite DSC runs may occur. Below c', a single T_m value is observed. The transition temperature increases as lipid concentration is decreased. The region above c' is marked by the presence of a large transition region, the intermediate phase, that tends to

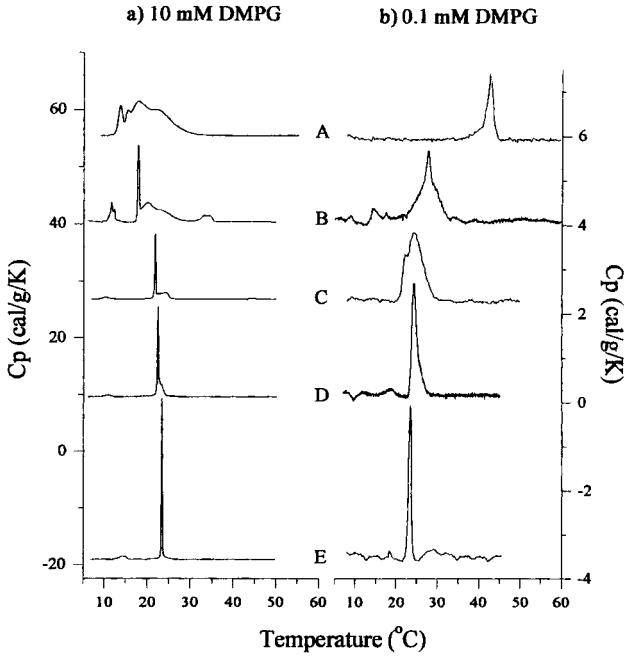

Figure 5. DSC traces obtained with (a) 10 mM and (b) 0.1 mM DMPG in (A) pure water, and 10 mM Hepes pH 7.4 + (B) 2 mM NaCl, (C) 50 mM NaCl, (D) 100 mM NaCl, and (E) 500 mM NaCl. For better visualization, the scans were shifted from Cp = 0. The traces A and B in (a) were multiplied by 4 and 8, respectively, and the traces D and E in (b) were multiplied by 0.5.

disappear as the concentration is further increased. We monitored the thermal behavior of DMPG up to 500 mM, where the intermediate phase has almost completely vanished. Preliminary results indicate that by further increasing the lipid concentration the intermediate region is replaced by a single T_m value that increases with the lipid concentration (dotted line). This very high lipid concentration limit, however, will not be discussed in the present work.

Below c', DMPG dispersions were stable over a period of 24 h, after which some samples presented a broader and less defined phase transition. Above c', the measurements were quite reproducible even after incubating the samples for 3 days at room temperature. No relevant temperature hysteresis was found.

It is interesting to point out that, though we have not determined a value for the cmc (critical micelle, or vesicle, concentration) of DMPG, the high DPH fluorescence anisotropy observed with DMPG at low temperatures clearly indicates the presence of bilayer structures in the gel phase for lipid concentrations down to 5 μM. Unfortunately, below that concentration, both the DSC and fluorescence data were no longer reliable.

Ionic Strength Dependence. In the high concentration regime, at neutral pH, the temperature interval between T_m^{on} and T_m^{off} decreases with the increase in ionic strength until the intermediate phase completely vanishes and a single T_m value is observed.[3,8] A further increase in the sample ionic strength causes an increase in T_m, as the screening of the surface charges stabilizes the close packing of the gel phase.[3,13] The above-described behavior can be monitored by ESR of spin labels incorporated in the bilayer or light scattering[7] and by DSC, as shown in Figure 5a. As mentioned before,[9] if the complex calorimetric scans of the low ionic strength DMPG dispersions are integrated for temperatures above 15 °C (including T_m^{on}, T_m^{off}, and the region between them), the enthalpies of the main transition are found to be around 5 kcal/mol for all samples.

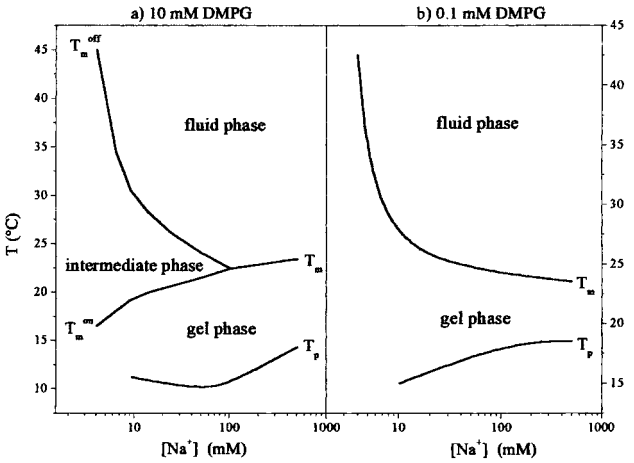

Figure 6. Schematic DMPG phase diagram as a function of sodium ion concentration, [Na^+], in (a) 10 and (b) 0.1 mM DMPG, obtained with DSC, fluorescence anisotropy, turbidity measurements, and ESR.

The calorimetric pattern shown in Figure 5a for DMPG in pure water in the high concentration regime is different from that in the presence of buffer. The sharp peak that characterizes T_m^{on} seems to be broader and the small and broad T_m^{off} peak is not present in the temperature range studied, in accordance with previous light scattering data.[7]

Opposite to the increase in T_m with salt concentration observed in concentrated samples and in many other charged lipid systems,[28−31] the transition temperature in dilute DMPG dispersions was found to decrease as the ionic strength increases, as illustrated in Figure 5b with DSC traces. Similar results were obtained with DPH fluorescence anisotropy (not shown). Comparing the high (a) and low (b) lipid concentration regimes, we clearly see that the increase in sample ionic strength weakens the dependence of the thermal behavior on the lipid concentration. In the presence of 500 mM NaCl, no significant difference is observed between the 10 and 0.1 mM DMPG thermal profiles, similar to what we found for DMPC dispersions (not shown). At 100 mM NaCl, there is still a slight dependence of T_m on the lipid concentration, since the T_m values were found to be 22.4 °C, 24.0 °C, and 25.5 °C at 10 mM (Figure 5a), 100 μM (Figure 5b), and 10 μM DMPG (not shown), respectively. Thus, above a certain ionic strength, the DMPG phase diagram is concentration independent, presenting a single T_m value.

At low concentrations, the behavior of DMPG in pure water seems to be rather similar to that in buffer, but with an even higher T_m value. At 0.1 mM DMPG in pure water (Figure 5b) a single peak at T_m = 42.5 °C is observed. This value increases up to 44 °C as the lipid concentration goes down to 10 μM DMPG, with a significant broadening of the Cp peak (not shown). It is interesting to point out that the DSC trace clearly indicates that 0.1 mM DMPG in pure water does not present the pretransition, which would be still detectable at this lipid concentration (as seen in Figure 1b).

Figure 6 shows a schematic phase diagram of DMPG as a function of sodium concentration above (Figure 6a) and below (Figure 6b) c'. Below c', T_m decreases with an increase in ionic strength. Above c', T_m^{on} increases while T_m^{off} decreases with an increase in ionic strength, until they collapse into one single phase transition temperature, T_m, which raises with further increase in ionic strength. The dependence of c' on the sample ionic strength is under investigation.

pH Dependence. It has been shown previously that in the high concentration regime the gel−fluid transition temperature value of DMPG dispersions increases with the decrease in pH.[32]

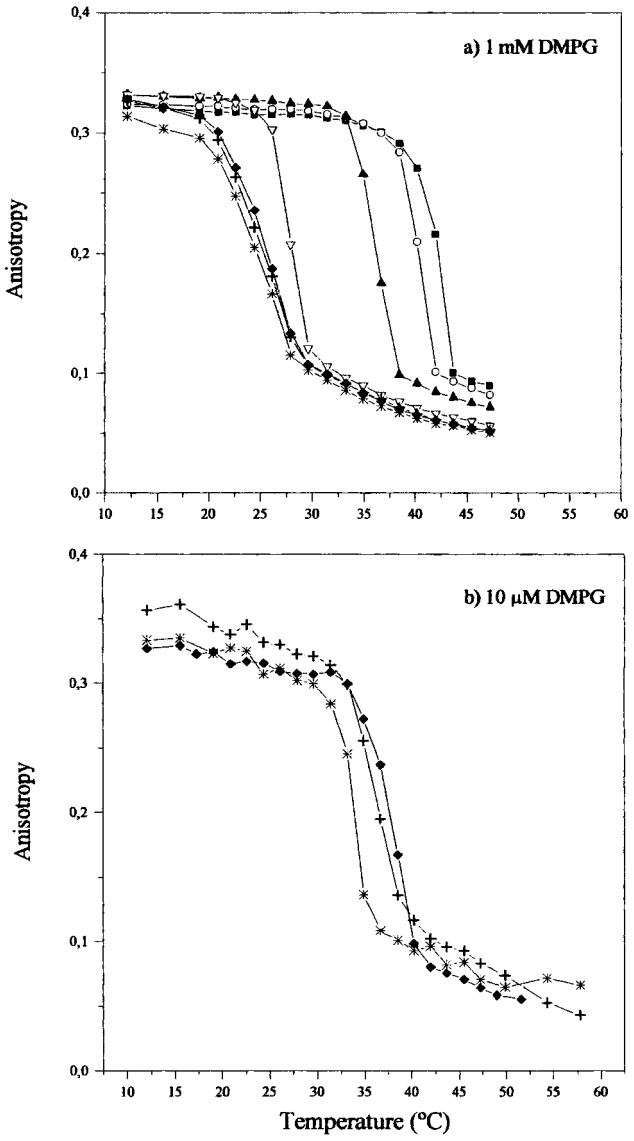

Figure 7. Temperature dependence of the fluorescence anisotropy of 0.5 mol % DPH in (a) 1 mM and (b) 10 μM DMPG in water + 6 mM NaCl at pH = (■) 2.7, (○) 3.3, (▲) 4.0, (▽) 5.0, (◆) 6.5, (+) 9.1 and (*) 11. The sample pH was adjusted by addition of HCl below pH 7 and of NaOH above pH 7.

That has been attributed to the phosphate group protonation, therefore to the neutralization of the PG headgroups, stabilizing the highly packed gel phase. Accordingly, an apparent pK (pK_a) of 2.9 was estimated for DMPG in 100 mM salt, using water−lipid partitioning of spin labels.[32] Figure 7a shows that the DPH fluorescence anisotropy can also be used to monitor the dependence of T_m on the degree of DMPG protonation. Using the T_m values obtained from the curves in Figure 7a, an apparent pK of 4.7 can be estimated from the dependence of T_m on the sample pH value, as shown in Figure 8. This value is rather close to the one that can be calculated from the literature[32] (pK_a = 4.1), considering the variation of the apparent pK_a with the bilayer surface potential, therefore, with the sample ionic strength.[33] Figure 7a shows that there is a limiting value for T_m, around 42 °C, close to pH 2.7, which should correspond to the nearly fully protonated DMPG bilayer surface. Above pH 6 the DMPG thermal profile is independent of the medium pH, showing that the phosphate groups are fully deprotonated. It is interesting to note that for protonated samples (at low pH) the phase transition is sharp, whereas for pH values higher than

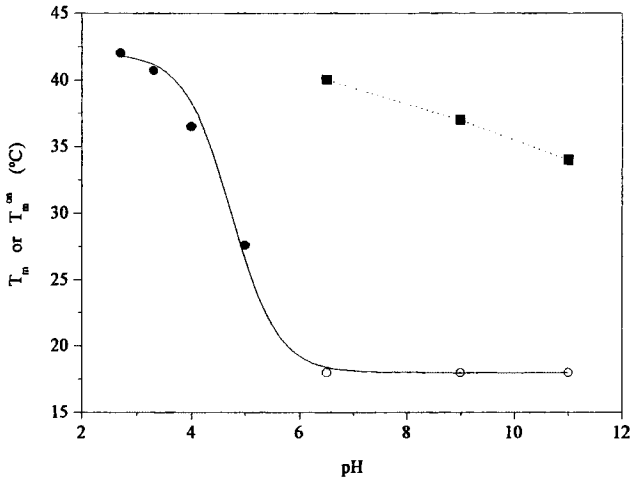

Figure 8. Dependence of T_m on the medium pH for 1 mM (● and ○) and 10 μM (■) DMPG. T_m was measured at the midpoints of the curves in Figure 7a (pH ≤ 5) and in Figure 7b. The open circles (○) indicate the values of T_m^{on} in Figure 7a (pH ≥ 6.3). The theoretical curve (—) is a proton titration curve assuming T_m proportional to (1 − α).

6.3 the transition gets broader, as observed before (Figure 1a),[34] suggesting the presence of the intermediate phase.

The results obtained with dilute DMPG samples (10 μM), shown in Figure 7b, are very different from those described above. Whereas, in the high concentration regime there is no significant change in the thermal profile for pH values higher than 6.3, in the low concentration regime the gel−fluid transition temperature T_m decreases as the pH is raised to pH 11. This suggests that there are still protons associated to the phosphate groups at pH values as high as 9 or 11.[35] For low DMPG concentrations, at pH values below 6, the gel−fluid transition temperature does not change much but there is a clear broadening of the lipid main transition (data not shown). It was not possible to determine a complete titration curve for dilute DMPG,[35] see Figure 8.

Discussion

In charged bilayer systems, an increase in the gel−fluid transition temperature, T_m, is usually attributed to either a decrease in the bilayer ionization degree α[36], by counterion binding, or to a screening of the surface charges, by the increase in the sample ionic strength. This has been observed with several charged phospholipids and is explained in terms of a decrease in the electrostatic repulsion between adjacent charged headgroups, thus stabilizing the gel phase.[28−31]

In the high concentration regime, DMPG dispersions are found to present a complex thermal transition profile (Figure 1a), with a large gel−fluid transition region. That happens in conditions where DMPG is fully deprotonated and under reduced ionic strength, that is, when the lipid bilayer surface potential is relatively high. The intermediate phase vanishes when either the ionic strength is raised above 100 mM NaCl or the pH is lowered below 5. Concentrated DMPG samples seem to be nearly fully protonated at pH values around 2, presenting a high gel−fluid temperature transition, at approximately 42 °C (Figure 7a). The $PG^- - H^+$ binding constant was estimated to be K_H = 16 M^{-1} (pK_i = 1.2 or calculated from the literature).[32,37] On the other hand, the increase in ionic strength, at neutral pH, increases T_m up to ∼29 °C, in 2 M NaCl.[13] Therefore, the different sizes, hydration radii, association and screening characteristics of proton and sodium ions must bring different characteristics to the bilayer headgroup region, there-

fore explaining the different T_m values found for saturating amounts of each ion. At neutral pH, the phosphate groups are deprotonated, but not fully ionized, as the $PG^- - Na^+$ binding constant (K_{Na}) must be considered to account for the measured bilayer surface potential.[8] K_{Na} was estimated to be 1 order of magnitude smaller than that for protons: $K_{Na} \sim 0.6$ M^{-1}.[8,30,31,38−41] Thus, according to the Gouy−Chapman model,[19] we estimated $\alpha \sim 0.5$, due to the binding of sodium ions only, for 10 mM DMPG at neutral pH and 6 mM ionic strength.[7,8]

The present work clearly shows that dilute DMPG dispersions at low ionic strength (below 100 mM NaCl) display a rather peculiar thermal behavior. The very high T_m value obtained for the 10 μM DMPG gel−fluid transition temperature ($T_m = 41$ °C in Figure 2) suggests that this dilute lipid system is nearly fully protonated at pH 7.4, a condition at which DMPG is certainly deprotonated in the high concentration regime. Accordingly, a decrease in T_m is observed upon increasing the sample pH (Figure 7b), showing that there are still protons associated to the phosphate groups up to pH 11. Moreover, we observed a decrease in T_m upon addition of NaCl (Figure 5b). This could be a direct consequence of the decrease in the amount of protons at the membrane surface (a raise in the bilayer surface pH, therefore a decrease in pK_a) due to the reduction in the electrostatic surface potential caused by the increase in the medium ionic strength. Thus, according to Figure 5b, 0.1 mM DMPG in pure water would be almost completely protonated ($\alpha \sim 0$) and the increase in ionic strength would significantly increase the ionization degree, decreasing the value of T_m to the limiting value of 500 mM NaCl, where no more significant binding of protons would occur. The absence of the pretransition in the DSC run of 0.1 mM DMPG in water would support the hypothesis of nearly full protonation, since protonated DMPG membranes do not present a pretransition,[32] possibly due to the smaller size of neutral PG headgroups.

It is interesting to point out that in a study with dipalmitoyl phosphatidylglycerol (DPPG), working with a fixed low lipid concentration (1.3 mM), varying the medium pH and ionic strength, it was observed that even at pH 8.5 DPPG could not be completely deprotonated.[42,43] A high value for the phosphate group dissociation ($pK_a \sim 7.0$) in low lipid concentration (0.1 mM), and low ionic strength, was also observed with dimyristoyl methyl phosphatidic acid.[28]

Our experimental data clearly indicate a much stronger association of the DMPG headgroups to protons for concentrations below c'. The ionization degree α would be around zero for 10 μM DMPG at 6 mM ionic strength (or 100 μM DMPG in water), but with relevant contributions from the proton binding only. Therefore, the $PG^- - H^+$ binding constant below c' is certainly much higher than the value estimated previously for concentrated samples ($K_H = 16$ M^{-1}, corresponding to $pK_i = 1.2$). To explain $\alpha \sim 0$ at 6 mM ionic strength, K_H would have to increase several orders of magnitude ($K_H \sim 10^7$ M^{-1}, corresponding to $pK_i \sim 7$). So far, we have no explanation for that huge increase in K_H around c'.

Alternatively, the data presented here could be discussed in the light of the ion-exchange model developed by Quina and Chaimovich, for micelles.[44] The advantage of this model, over the Gouy−Chapman model presented above, would be the explicit dependence of the amount of protons bound to the bilayer surface on the lipid concentration, as this model assumes an ion-exchange constant ($K_{H/Na} = K_H/K_{Na}$) at the interface, and the Na^+ concentration is lipid dependent (the sodium salt of DMPG is used here). However, in this model, the ionization degree is a parameter, independent of the amphiphilic concentration (a reasonable assumption for micelles[44]), and, as pointed out before, we observe a great decrease in α for DMPG concentrations below c'. Thus, the exchange model can fit the experimental data shown here with the assumption that α changes from 0.5 above c' to roughly zero below c' (curves not shown). Furthermore, a huge ionic exchange constant between sodium ions and protons is necessary ($K_{H/Na} \approx 10^7$) to achieve a complete protonation in the dilute regime.

In summary, below c' we have to impose, in both models, an enormous affinity of the bilayer to protons to overcome the extremely low proton concentration at pH 7.4 and be able to explain the observed very low α value. Though the experimental findings clearly point to a protonation process that starts at c' and is complete around 10 μM DMPG (for 6 mM ionic strength), as the lipid concentration is lowered, the mechanisms for that process are still unclear. Possibly, a more comprehensive model has to be developed, including the balance among added salt, lipid counterions, and protons upon lipid dilution. Otherwise, we can speculate that, at c', the structure of the bilayer surface markedly changes, altering the bilayer affinity to different ions. This could be caused by the complete vanishing of the electrostatic interaction between vesicles, thus changing the balance of forces present in the system. For instance, the interaction between opposed monolayers could have relevant contributions only when the interaction between vesicles is no longer present. For future discussions, it is important to have in mind that even in the very diluted DMPG systems used here the lipids are organized in bilayers, since a main phase transition is detected by DSC, fluorescence anisotropy measurements, and light microscopy of giant vesicles.

From a physicochemical point of view, the different thermal behavior presented by dilute low ionic strength DMPG dispersions is certainly very important to the complete characterization of this system, either to be used as a model for biological membranes, or as a possible drug carrier. This behavior may have some biological relevance, since a small increase in T_m was observed even upon the addition of only 10 mol % DMPG to DMPC dilute vesicles (results not shown). Natural membranes of prokaryotes generally have 10 mol % PG in their composition, though not only DMPG. It would be very interesting to search for similar behavior in other charged systems.

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References and Notes

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(33) Considering a Boltzmann distribution for the protons, $pK_a = pK_i - e\Psi_o/2.3kT$, where pK_i is the intrinsic pK (DMPG $pK_i = 1.2$, calculated from the literature,[32] assuming the electrostatic bilayer surface potential $\Psi_o = -100$ mV [13]) and $\Psi_o = -170$ mV for DMPG in Hepes + 2 mM NaCl.[8]
(34) The small difference observed between the DPH thermal profile presented in Figure 1a and those shown in Figure 6a, for high pH samples, can be possibly attributed to the somewhat different aqueous systems used in the two experiments: in Figure 1b the 6 mM ionic strength is due to 10 mM Hepes buffer pH 7.4 + 2 mM NaCl, whereas in Figure 6a, to facilitate the pH variation, no buffer was used and the ionic strength was set upon addition of 6 mM NaCl.
(35) The addition of NaOH, necessary to increase the medium pH, also increases the sample total ionic strength. For instance, pH = 11 corresponds to 1 mM sodium ions added to the system. It is important to note that the decrease in the T_m value observed for that sample (at pH 11, in Figure 6b) is not caused by the increase in Na^+ in solution, since T_m had very similar values for samples in the range of 4−6 mM added salt. Unfortunately, higher pH samples would correspond to higher ionic strengths (10 mM Na^+ for pH = 12, or 100 mM Na^+ for pH 13), changing completely the DMPG thermal behavior (see Figure 5b).
(36) $\alpha = [PG^-]/[PG]_{tot}$, the ratio between the ionized and total DMPG concentrations.
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