The peculiar thermo-structural behavior of the anionic lipid DMPG

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Abstract

Aqueous dispersions of the anionic phospholipid dimyristoyl phosphatidylylglycerol (DMPG), around 100 mM ionic strength, are known to exhibit a thermal behavior similar to that of the largely studied lipid dimyristoyl phosphatidylcholine (DMPC), which undergoes a gel to liquid crystalline phase transition at 23 °C, well characterized by differential scanning calorimetry (DSC), and other methods. However, at low ionic strength, DMPG has been 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. Structural properties of the DMPG gel–fluid transition region will be discussed, based on results obtained by several techniques: electron spin resonance (ESR) of spin labels at the membrane surface and intercalated at different depths in the bilayer; light scattering; DSC; small angle X-ray scattering (SAXS); and fluorescence spectroscopy of probes in the bilayer.

Keywords: DMPG; Gel–fluid transition; ESR; Fluorescence; DSC; Light and X-ray scattering

1. Introduction

Under physiological conditions, most cell membranes have a negative charge due to the presence of acidic lipid headgroups. In view of the potential importance of the membrane negative character in many biological processes, anionic phospholipids have been widely used as model systems for possible anionic domains in membranes. Phosphatidylglycerol (PG) is the most abundant anionic phospholipid headgroup present in prokaryotic cell membranes, and has been extensively studied as a model for negatively charged membranes (Seelig et al., 1987; Heimburg and Biltonen, 1994; Biaggi et al., 1997; Fernandez and Lamy-Freund, 2000). Due to the presence of an ionizable phosphate group, the thermo-structural properties of PG-lipids are not only dependent on the hydrocarbon chain length, but also strongly reliant on the pH of the medium and the presence of ions. Dimyristoyl phosphatidylylglycerol (DMPG), a saturated lipid with 14-C atoms in each hydrophobic chain, under physiological conditions presents a gel–fluid transition at 23 °C. Hence, it has been considered a rather suitable model system, for instance, for studying peptide–lipid interactions,
as, in the convenient temperature range of 15–40 °C, it would allow to monitor the bilayer in both the gel and fluid phases. The two phases could mimic acidic micro-regions of different fluidity, possibly present in biological membranes at physiological temperatures.

In general, in the study of proteins and peptides that remain at the membrane surface, partially penetrating the bilayer, the electrostatic interaction between their cationic groups and the anionic lipids is essential for increasing their local concentration at the membrane surface. Thus, for studying these systems, it may be necessary to use low ionic strength dispersions, to avoid the decrease in the membrane surface potential due to both counter ions surface binding and shielding, and allow a large protein/peptide concentration at the membrane surface. However, low ionic strength DMPG dispersions were found to present a thermal behavior rather different from that presented by dispersions under physiological conditions. DMPG in the presence of 100 mM NaCl presents a gel–fluid transition profile rather similar to that presented by the zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC), with a sharp differential scanning calorimetry (DSC) peak in the heat capacity profile at 23 °C. However, in a certain range of (low) salt concentration, DMPG displays a different and more complex thermal behavior. The low ionic strength DMPG thermal profile was first studied by Salonen et al. (1989), followed by Heimburg and Biltonen (1994). The first authors showed that DMPG in low ionic strength presented a rather unusual DSC profile, with two distinct peaks in the range of 20–40 °C. They interpreted their data as two phase transitions, identifying the first one with the main gel–fluid transition, \( T_m \), and the second one, not well characterized, was called post-transition, \( T_{post} \). Heimburg and Biltonen (1994) proposed the existence of a gel–fluid transition region between the two main DSC peaks. They observed that, for DMPG in low ionic strength media, there was a correlation between the DSC and the light scattering profiles: there was a sharp decrease in light scattering at the temperature of the first DSC peak, and an increase at the second main DSC peak. They also pointed out that DMPG dispersions were rather viscous in the gel–fluid transition region. Later on, Schneider et al. (1999) proposed the existence of an extended lipid network in the DMPG gel–fluid transition region.

Although the complete characterization of the gel–fluid transition region is far from achieved, the contributions we have made\(^1\) to the better understanding of the thermo-structural behavior of DMPG dispersions will be presented below.

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\(^1\) M.Phil. and Ph.D. work of K.A. Riske at the Instituto de Fisica, USP-SP, Brazil (Riske et al., 1997, 1999, 2001).
1.1. General properties of DMPG dispersions

As shown in Fig. 1, the DMPG heat capacity ($C_p$) temperature variation is strongly dependent on the sample ionic strength\(^2\). Apart from the pre-transition ($T_p$) at around 12 °C (not the subject of the present work), the low ionic strength DMPG dispersion (in 10 mM Hepes + 2 mM NaCl, total ionic strength 6 mM) presents a rather complex calorimetric profile in the range of 17–35 °C, characterized by few broad peaks (Riske et al., 2001). For the reasons discussed below, we believe that the melting of the lipid chains starts at the narrow calorimetric peak at $T_m^{on}$ but is only complete at the somewhat broader peak at $T_m^{off}$ (the onset and the offset of the melting regime). This gel–fluid transition region will be called here intermediate phase (between gel and fluid phases). DSC clearly indicates that structural changes are occurring between $T_m^{on}$ and $T_m^{off}$, evidenced by the presence of broad $C_p$ peaks superimposed in this region. Upon increasing the sample salt concentration, the peaks tend to collapse and the main cooperative peak ($T_m$) shifts to higher temperature values. This latter effect is related to the known gel phase stabilization due to the screening of the surface charges by counter ions (Träuble et al., 1976).

Table 1 compares the transition molar enthalpies ($\Delta H$) of DMPG in high and low ionic strength and of DMPC. For all three systems, $\Delta H \sim$ 1 kcal/mol for the pre-transition. DMPC and DMPG at high ionic strength (100 mM NaCl) present very similar main transition molar enthalpies. It is noteworthy that if the complex calorimetric scan of the low ionic strength DMPG dispersion is integrated for temperatures above 15 °C (including $T_m^{on}$, $T_m^{off}$ and the region between them), a $\Delta H$ value rather similar to those yielded by the main transition of DMPC, or high ionic strength DMPG, is obtained. This strongly suggests that the chain melting of the anionic DMPG bilayers at low ionic strength is spread over a large temperature range (Riske et al., 2001).

The beginning and the end of the DMPG intermediate phase are also characterized by a sharp decrease in turbidity at $T_m^{on}$ and a sharp increase at $T_m^{off}$, as shown in Fig. 2. Contrary to the turbid gel and fluid phases (below $T_m^{on}$ and above $T_m^{off}$), the intermediate phase is optically transparent. The light scattering profiles are strongly dependent on the NaCl concentration, as in the DSC traces. A drop in light scattering at $T_m$ as observed for 100 mM NaCl DMPG sample (Fig. 2), has been attributed to a change in the refractive index increment of the solution per unit mass concentration of lipid (dn/dc), which is related to the known vesicle swelling and corresponding decrease in the bilayer density and thickness at the phase transition (Disalvo, 1991; Yi and MacDonald, 1973). Measurements of n and dn/dc for the low ionic strength sample showed that the variation of these parameters could not explain the large changes in light scattering at $T_m^{on}$ and $T_m^{off}$, indicating that they must have a different origin (Riske et al., 1997).

The light scattered at different angles by the low ionic strength sample displayed the same pattern as that observed at 90°: between $T_m^{on}$ and $T_m^{off}$ the scattering was significantly less intense than below $T_m^{on}$ and above $T_m^{off}$ (Fig. 3) (Riske et al., 1997). Results from Zimm plots (Zimm, 1948) evidenced a system with considerable polydispersity, detected by the strong scattering at low angles, and only angles from 45° and higher were used in the data analysis (collaboration with M.J. Politi and W.F. Reed; Riske et al., 1997). Though there was significant variation in the results when different preparations were measured, the overall trends were always the same. Below $T_m^{on}$, the weight average molecular weight of the scatterer ($M_w$) was higher, and the second virial coefficient ($A_2$) was very low and negative, indicating some net attraction, and, hence, a possible aggregation of the vesicles. Above $T_m^{on}$, $A_2$ becomes positive and large, indicating net repulsion, and $M_w$ drops significantly, while the scatterer $z$-averaged mean

\(^2\) In the experiments described here, freshly prepared DMPG dispersions were used. A lipid film was formed from a chloroform solution of lipids, dried, and vortexed with the desired concentration of NaCl in 10 mM Hepes buffer solution (4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid) adjusted with NaOH to pH 7.4. For the fluorescence and ESR measurements, the probes were added to the chloroform lipid solution.
square radius of gyration $R_{g}^2$ increases somewhat. Above $T_{m}^{on}$, $A_2$ becomes extremely small or even slightly negative, indicating a considerable decrease in the repulsive force between the vesicles, which parallels an increase in $M_w$. Apparently, between $T_{m}^{on}$ and $T_{m}^{off}$, a repulsive force between the charged vesicles dominates over the van der Waals attraction. This could be explained by an increase in the vesicle surface charge between $T_{m}^{on}$ and $T_{m}^{off}$, consistent with the observed increase in reduced solution conductivity in this range (Riske et al., 1997). The increase in the bilayer ionization degree $z$, at $T_{m}^{on}$, hence, the increase in the modulus of the electrostatic surface potential, could be related to the dissociation of the sodium ions from the PG headgroups, triggered by the beginning of the gel–fluid transition by some mechanism which has not yet been elucidated.

### 1.2. DMPG bilayer surface potential

(collaboration with O.R. Nascimento, B. Bales and M. Peric)

In an effort to better understand the role of surface electrostatics in the phase transition of DMPG vesicles, a small, highly aqueous soluble, deuterated (to reduce unresolved hyperfine splitting), cationic spin label, 4-trimethylammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (dCAT1), was used to directly monitor the negatively charged DMPG vesicle surface (Riske et al., 1999). Membrane electrostatic surface potential was calculated from the label water/bilayer surface partition ratios, based on a simple two-sites model,

<table>
<thead>
<tr>
<th></th>
<th>$T_p$</th>
<th>$T_m$</th>
<th>$T_{m}^{on}$</th>
<th>$T_{m}^{off}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC ($n_i = 6$ mM)</td>
<td>0.8 ± 0.2</td>
<td>5.0 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMPC ($n_i = 100$ mM)</td>
<td>1.0 ± 0.2</td>
<td>5.7 ± 0.8</td>
<td>1.0 ± 0.2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>From $T_{m}^{on}$ to $T_{m}^{off}$</td>
<td>5.2 ± 0.8</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1**

Transition molar enthalpies, $\Delta H$ (kcal/mol), of DMPC and DMPG in high and low ionic strength ($n_i$)
assuming that the electrostatic attraction dominates the interactions between the spin label and DMPG membrane surface. This strategy was based on those used for calculating surface potentials using amphiphilic fluorescent or spin probes, which insert into the bilayer (Castle and Hubbell, 1976; Hartsel and Cafiso, 1986; Khramstov et al., 1992; Franklin et al., 1993; Epand et al., 1996; Krasnowska et al., 1998), but which probe the membrane properties from the ‘outside’. Such an approach provides an alternative view and is likely to perturb the membrane less than a probe incorporated into the membrane, apart from yielding ESR signals typical of rapid movement in the ESR timescale, which can be accurately analyzed (Bales, 1989; Marsh, 1989). Moreover, due to the high negative surface potential of DMPG low ionic strength bilayers, an amphiphilic molecule (the same dCAT molecule bound to a 12-C chain, dCAT12) was found to completely partition into the membrane, not allowing the calculation of a surface potential through a water/bilayer partition ratio.

Fig. 4 illustrates how the dCAT1 surface partition ratios (surface label moles/total label moles) could be calculated. The ESR spectrum obtained with dCAT1 in DMPG dispersions (Fig. 4a) was different from that yielded by the label in buffer solution (Fig. 4b), the former clearly indicating the presence of dCAT1 in more than one microenvironment. For most temperatures and ionic strengths, it was possible to decompose the dCAT1 spectrum obtained in DMPG dispersions into two components, one of them corresponding to the label free in solution, here referred to as ‘free’ component. The other component was yielded by labels in a less polar and more packed microenvironment. Fig. 4c shows a typical spectrum obtained after subtracting a weighted free signal (Fig. 4b) from the composite one (Fig. 4a). The weight of the free signal was varied until the resulting spectrum looked like a one component signal and could be well fitted by a Voigt line shape (Bales, 1989). The fact that two well-separated spectra are obtained shows that the exchange rate between the two respective sites is slow on the ESR timescale. In the simple two-site model assumed, the resulting spectrum (Fig. 4c) was yielded by the population of spin label close to the DMPG surface (an average value), called ‘surface dCAT1’.

Decomposition of spectra into free and surface components was carried out for DMPG samples with different ionic strengths at temperatures, between 5 and 45 °C. Membrane surface partition ratios of the probe dCAT1 were calculated from the double integral of the ESR signals (Fig. 5). The values were compared with those obtained with dCAT1 in the 12 mM SDS in water system. The partition ratios calculated for temperatures below 17 °C for DMPG-water, and 20 °C for DMPG-buffer at the various salt concentrations were not very accurate, due to the similarity between the
where the PG⁻ groups are partly shielded by the cations in solution, or in the neutral DMPG in low pH medium (pH 1), the dCAT1 spectra were almost identical to the free spectrum at all temperatures, that is, no surface label could be detected.

Surface electrostatic potential could be only accurately calculated for temperatures above $T_m^{on}$, namely, for the bilayer in the intermediate or fluid phases. Table 2 shows the calculated potentials for the different ionic strength samples at 30 °C. The values so calculated were compared with those yielded by the Gouy–Chapman–Stern model (see, for instance, McLaughlin, 1977; Evans and Wennebrock, 1994). Using a PG⁻–H⁺ binding constant $K_H = 15.8 \text{ M}^{-1}$ (Toko and Yamafuji, 1980; or calculated from Watts et al., 1978), it was found necessary to assume a PG⁻–Na⁺ binding constant, $K_{Na}$, different from zero. This is in contrast to the assumption made by some authors that monovalent cations do not bind to PG (Traub and Eibl, 1974; Traub et al., 1976; Cecc et al., 1980; Copeland and Andersen, 1982). Allowing for a PG⁻–Na⁺ binding constant, the Gouy–Chapman–Stern model predicts surface potentials similar to those measured with dCAT1, although the decrease in the surface potential with ionic strength was found to be somewhat steeper than that predicted by the model. The $K_{Na}$ value, estimated between 0.17 and 0.84 M⁻¹, is in the range of the sodium binding constants proposed in the literature (Eisenberg et al., 1979; Loosley-Millman et al., 1982; Lakhdar-Ghazal et al., 1983).

**Table 2**

<table>
<thead>
<tr>
<th>System</th>
<th>$n_i$ (mM)</th>
<th>$P$</th>
<th>$\psi_0$ (mV)</th>
</tr>
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<tbody>
<tr>
<td>DMPG in</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>0.1</td>
<td>0.95</td>
<td>-237</td>
</tr>
<tr>
<td>Buffer</td>
<td>4.0</td>
<td>0.75</td>
<td>-189</td>
</tr>
<tr>
<td>+2 mM NaCl</td>
<td>6.0</td>
<td>0.60</td>
<td>-171</td>
</tr>
<tr>
<td>+5 mM NaCl</td>
<td>9.0</td>
<td>0.40</td>
<td>-150</td>
</tr>
<tr>
<td>+10 mM NaCl</td>
<td>14.0</td>
<td>0.25</td>
<td>-132</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1</td>
<td>0.90</td>
<td>-220</td>
</tr>
</tbody>
</table>

![Graph](image)

**Fig. 5.** Partition ratios (surface label moles/total label moles) calculated from the second integral ratio between the surface and the composite spectra. (O) 12 mM SDS in water, 10 mM DMPG in (■) pure water, (○) Hepes buffer, buffer + (▲) 2 mM, (▽) 5 mM, and (♦) 10 mM NaCl (based on Riske et al., 1999).

1.3. Monitoring DMPG bilayer microenvironments: fluorescent and paramagnetic probes

A gradual decrease in the DMPG membrane packing between \( T_{m}^{on} \) and \( T_{m}^{off} \) was monitored with the fluorescence anisotropy of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) incorporated into the bilayers. Fig. 6 shows the fluorescence data for low ionic strength DMPG and DMPC membranes. A decrease in fluorescence anisotropy is related to a decrease in acyl chain order, as observed during the gel–fluid transition of lipid membranes. It is evident that the two lipids present similar packing in the gel and fluid phases. However, in contrast to the sharp decrease in the fluorescence anisotropy for DMPC at the gel–fluid transition, DMPG displays a gradual reduction in the bilayer anisotropy from \( T_{m}^{on} \) to \( T_{m}^{off} \).

It is interesting to point out that, whereas, the relatively large molecule DPH does not monitor any sharp structural transition in the DMPG bilayer, a paramagnetic lipid labeled at the 14th C-atom (1-palmitoyl-2-(14-doxyl stearoyl)-sn-glycero-3-phosphocholine; PCSL), containing the relative small label doxyl, detects a sharp decrease in the membrane packing at \( T_{m}^{on} \), followed by a continuous decrease till \( T_{m}^{off} \). Several parameters directly measured from the electron spin resonance (ESR) spectra can quantify chain mobility and/or disorder. We have chosen the ratio of the amplitudes of the central and low field lines (\( h_0/h_{-1} \)), due to its sensitivity in monitoring mobility changes and to the accuracy of the measurements.

![Fig. 6. Fluorescence anisotropy of 0.5 mol% DPH in 1 mM DMPG and DMPC in Hepes buffer plus 2 mM NaCl (based on Riske et al., 2002).](image)

![Fig. 7. Temperature dependence of the ratio between the amplitudes of the central and low field resonance lines (\( h_0/h_{-1} \)) measured on the ESR spectra of 0.3 mol% 14-PCSL incorporated in 50 mM DMPG in Hepes buffer plus 2 mM NaCl, and in 10 mM DMPC in buffer (based on Riske et al., 2001).](image)
(Fig. 7). This ratio will decrease tending to unity as the spin label mobility increases. Fig. 7a shows the parameter $h_0/h_{-1}$ measured from the spectra of 14-PCSL incorporated in DMPG and DMPC vesicles, the latter used as a reference. Similar to the results obtained with DPH, the spin label ESR spectra also indicate that the two lipids present similar packing in the gel and fluid phases. For better visualization, the data were also plotted as a function of $(T − T_m)$ for DMPG and $(T − T_m)$ for DMPC (Fig. 7b). It can be clearly seen that there is a great decrease in chain packing of DMPG bilayers at $T_m$, followed by a smooth decrease until $T_{m}^\text{off}$ is reached. Above $T_{m}^\text{off}$ the microviscosity of the fluid phases of DMPC and DMPG are quite similar. On the other hand, DMPC does not show this shoulder, going directly to a fluid phase after $T_m$. These results are in agreement with those obtained with DSC, and fluorescence anisotropy, indicating that, for low ionic strength DMPG, the melting process is only completed at $T_m^\text{off}$. Though less clear, the same behavior is indicated by lipids spin labeled at other positions in the hydrocarbon chain (Riske et al., 1997).

Spin labels also monitor the differences in the gel–fluid transition profile yielded by DMPG dispersions at different ionic strengths, as shown in Fig. 8 for 5- and 12-PCSL (the outer hyperfine splitting $A_{\text{max}}$ and the linewidth of the low field line are the empirical parameters used; Riske et al., 1997). It is interesting to point out that the packing of the lipids in the different ionic strength dispersions are very similar for all samples in both the gel (low temperatures) and fluid (high temperatures) phases. Also important, is the information given by the highly anisotropic ESR signal of 5-PCSL (spectra not shown), indicating that DMPG, at all salt concentrations, is organized in bilayers in the whole temperature range studied (for instance, amphiphiles labeled at the 5th C-atom yield a quite different ESR spectrum in micelle-like environment; Benati et al., 2001).

Very recently, we have found an interesting fingerprint for the DMPG intermediate phase. In this range of temperature, the ESR spectra of phospholipids labeled close to the acyl chain end, at the 16th carbon atom, are likely to be a composition of two signals. One signal is typical of the nitroxide in a bilayer core (low order, low polarity), and the other one is rather unusual for a bilayer, also corresponding to a low order microenvironment but with a much higher polarity (typical of the 5th C-atom position in a bilayer). Therefore, the experimental results suggest the presence of two structurally different membrane domains in the DMPG intermediate phase (manuscript in preparation). It is important to point out that although the other spin labels (5- to 14-PCSL)
could be partitioned between the two possible different membrane patches, two distinct signals could have been only detected with 16-PCSL due to the faster movement of the nitroxide moiety in this probe, which yields narrower lines in the ESR spectra (Marsh, 1989).

1.4. No membrane fusion

Spin labels were also used to test whether there was vesicle fusion at either \( T_{m}^{on} \) or \( T_{m}^{off} \). The following experiment was carried out. A phospholipid spin label (16-PCSL), which shows negligible partition into the aqueous medium, incorporated in 10 mM DMPG dispersion was used in a concentration high enough to yield a spectrum broadened by spin exchange (3 mol% of the lipid concentration). One volume of that dispersion was mixed with two volumes of a DMPG sample in the same concentration, but without the spin label. Therefore, if fusion occurred, a significant line-width decrease would be expected, corresponding to the dilution of the spin label to 1 mol%. No decrease in the spin exchange was observed during and after the following temperature cycle: 30 \(^\circ\)C, 30 \(^\circ\)C, 40 \(^\circ\)C and back to 40 \(^\circ\)C. Fig. 9 compares the ESR spectra obtained with 1 and 3 mol% of spin label at 40 \(^\circ\)C to evidence the remarkable difference between them. The ESR spectrum obtained after mixing and going through the temperature variations described above (dashed line) is very similar to that obtained for the original sample (3 mol% of label). That experiment indicated that the spin labeled phospholipid could not spread out through the DMPG vesicles added afterwards, which would be expected if fusion had occurred.

1.5. DMPG dispersions monitored by SAXS (collaboration with L.Q. Amaral)

Small angle X-rays scattering (SAXS) has been widely used to characterize the structure of amphiphiles in aqueous media (see, for instance, McIntosh and Simon, 1986; Wiener et al., 1989, 1991; Wiener and White, 1992; Kodama et al., 1997). Many lipids are known to arrange themselves in multibilayer structures with a repeat distance of few nanometers, thus, giving rise to Bragg diffraction in the small angle region. This is illustrated in Fig. 10a for DMPC at various temperatures, indicating a repeat distance of 66 Å in the fluid phase, consistent with previously reported values (Marsh, 1990). The repeat distance corresponds to the bilayer thickness plus the water layer between the lamellae. In contrast to the results obtained with DMPC, low ionic strength DMPG dispersions presented only a broad peak around 0.12 Å\(^{-1}\) for the range of lipid concentration (10–50 mM), temperature (10–45 \(^\circ\)C) and scattering vector \( \mathbf{q} \) (0.03 to 0.35 per Å) studied (Riske et al., 2001), shown in Fig. 10b. Such a broad peak is typical of a single bilayer and arises from the electron density contrast between the bilayer and the solvent (Glatter and Kratky, 1982).

The temperature variation of the peak position (\( q_{\text{max}} \)) and the maximum intensity (\( I_{\text{max}} \)) for three different preparations are shown in Fig. 11. In the gel phase the peak was centered at \( q_{\text{max}} \approx 0.12 \) Å\(^{-1}\). Above \( T_{m}^{on} \), the peak slowly shifted to higher \( q \) values, reaching \( q_{\text{max}} \approx 0.13 \) Å\(^{-1}\) above \( T_{m}^{off} \). On the other hand, on increasing the temperature the peak intensity started to decrease at \( T_{m}^{on} \) and reached its minimum value just below \( T_{m}^{off} \), after which the peak intensity increased abruptly. The shift in \( q_{\text{max}} \) (associated to a decrease in bilayer thickness) is expected at a gel–fluid transition (McIntosh and Simon, 1986). However, it occurs

![ESR spectra of 16-PCSL incorporated in DMPG bilayer at (---) 3 and (...) 1 mol% of the DMPG concentration, and (---) obtained after mixing samples and going through temperature variations, as described in the text. Total spectra width 80 G, \( T = 40 \) \(^\circ\)C.](image-url)
sharply at $T_m$ for neutral phospholipids while for DMPG it is spread between $T_m^\text{on}$ and $T_m^\text{off}$. Hence, in agreement with the DSC, fluorescence anisotropy and ESR results, the SAXS data indicate that a complete fluid phase exists only for temperatures above $T_m^\text{off}$. The decrease in the X-rays scattering intensity in the melting regime is a remarkable and unusual result.

The SAXS curves (Fig. 10b) can be analyzed using a simple model for the unusual decrease in intensity: a decrease in the bilayer electron density contrast (Riske et al., 2001). The electron density profile $\rho(x)$ of the bilayer is modeled by a three level function, representing the headgroups ($\rho_1$, $R_1$), the acyl chains ($\rho_2$, $R_2$), and the methyl groups ($\rho_3$, $R_3$), and has the meaning of an ‘effective profile’. The determination of such $\rho(x)$ does not imply a hypothesis of a single phase in the melting regime. For the understanding of the structural changes involved at $T_m^\text{on}$ and $T_m^\text{off}$, the three SAXS curves showed in Fig. 10b, corresponding to DMPG in the gel, intermediate and

![Fig. 10. (a) DMPC in Hepes buffer +2 mM NaCl SAXS curves at 10 °C (below $T_m$), 27 and 35 °C (above $T_m$). (b) DMPG in Hepes buffer +2 mM NaCl SAXS curves, and corresponding theoretical fits at 10 °C (below $T_m^\text{on}$), 27 °C (between $T_m^\text{on}$ and $T_m^\text{off}$) and 35 °C (above $T_m^\text{off}$), for one of the preparations. Curves are shifted for clarity (based on Riske et al., 2001).](image)

![Fig. 11. Temperature dependence of the peak position (top) and maximum intensity (bottom) of the SAXS curves for three different samples ($\bullet$, $\circ$ and $\times$) of 50 mM DMPG in Hepes buffer +2 mM NaCl. The error in measuring the peak position was estimated to be about $1.5 \times 10^{-3} \text{Å}^{-1}$ and was omitted for a better visualization of the data. The error in the intensity maximum measurement was less than the symbol size (Riske et al., 2001).](image)
fluid phases were fitted. By using specific constraints to avoid physical inconsistent solutions, the different parameter sets that gave reasonable fit showed only slight changes in their values, always preserving the same trend. The best fits are shown in dashed lines in Fig. 10b and the corresponding \( \rho(x) \) functions are presented in Fig. 12.

The main differences between the 10 °C (gel) and 35 °C (fluid) bilayer density profiles are the ones expected to occur between a gel and a fluid phase: the curve at 35 °C was characterized by a clear decrease in the methylene chain thickness as compared with the one found for 10 °C. In the intermediate phase, between \( T_{m}^{\text{on}} \) and \( T_{m}^{\text{off}} \), a marked decrease in electron density contrast for the bilayer occurred, both in the headgroup and CH₃ regions. In general, the fits at 27 °C yielded intermediate \( R_2 \) values. The electron density contrast in the headgroup region (\( \Delta \rho_1 \)) decreases by almost a factor of two, going from 0.13 at 10 °C to 0.07 at 27 °C. The decrease in electron density absolute values is, however, much smaller (0.46 to 0.40 e/Å³). Such a decrease could be related to a local increase in the surface area, due to separation between polar headgroups. For a constant \( R_1 \), the drop of 13% in \( \rho_1 \) corresponds to an increase in headgroup area of the same percentage, meaning that a 6.5% increase in headgroup separation would be enough to explain the drop in intensity. An increase in the methyl group packing at 27 °C is also seen, since \( R_3 \) decreases and \( R_3 \) increases. This could indicate a small degree of interdigitation between the monolayers. The acyl chain density, \( \rho_2 \), practically does not change, whereas, \( R_2 \) decreases. The abrupt increase in \( I_{\text{max}} \) at \( T_{m}^{\text{off}} \) could correspond to a final rearrangement of the chains in a packed but fluid state, with a decrease in the mean area per headgroup, in view of the increase in \( \rho_1 \) observed after \( T_{m}^{\text{off}} \) (35 °C in Fig. 12). It is seen, therefore, that the decrease in \( I_{\text{max}} \) can be explained in terms of reasonable structural changes at the bilayer level. The separation between charged headgroups could also correlate with local fluctuations in membrane curvature and disruptions with water penetration.

Hence, for DMPG at low ionic strength, apart from the finding that a single bilayer is the basic structure over the whole temperature interval studied, other conclusions emerged from the discussed SAXS analysis: (i) the dominant process seems to be the separation of the charged headgroups, initiated at \( T_{m}^{\text{on}} \), not accompanied by complete melting of the chains, since only a small contraction of the thickness occurs, with some interdigitation at the CH₃ position; (ii) only when the headgroup separation reaches a maximum value, near \( T_{m}^{\text{off}} \), is complete melting achieved and electron density contrast partially recovered.

1.6. Concluding remarks

The presence of an intermediate phase between the gel and fluid phases of freshly prepared DMPG at low ionic strength is well established. This phase appears over a wide lipid concentration range (at least 1–50 mM; Riske et al., 2002) under conditions at which the DMPG surface potential is high: pH values above 6 and salt concentration below 100 mM. Fluorescent and spin probes tell us that for temperatures below \( T_{m}^{\text{on}} \) the DMPG bilayer is highly packed, in a gel phase, and above \( T_{m}^{\text{on}} \) the bilayer is fluid. The packing of the gel and
fluid phases is similar for DMPG at all ionic strengths studied (from pure water to 100 mM NaCl) and similar to that of DMPC. At \( T_{m}^{\text{on}} \) there is a sharp decrease in membrane packing (monitored by spin labels only), followed by a gradual increase in the membrane fluidity till \( T_{m}^{\text{eff}} \). Therefore, the intermediate phase is a gel–fluid transition region, over which the melting of the acyl chains occurs. This is also confirmed by the presence of several calorimetric peaks in the DSC profile between \( T_{m}^{\text{on}} \) and \( T_{m}^{\text{eff}} \). On the other hand, the intermediate phase clearly has specific properties, such as very low turbidity and high viscosity (Heimburg and Biltonen, 1994) and electrical conductivity (Riske et al., 1997), and is delimited by defined calorimetric peaks at \( T_{m}^{\text{on}} \) and \( T_{m}^{\text{eff}} \). The existence of the intermediate phase was shown to be strictly related to the presence of negative phosphate groups at the DMPG surface, since by increasing the salt and/or proton concentrations the intermediate phase vanishes (Riske et al., 2002), and a highly cooperative gel–fluid transition occurs, similar to ubiquitous PC-lipids.

Even though the existence of the intermediate phase is well accepted, its structural characteristics are still the subject of controversy. One hypothesis, raised by Schneider et al. (1999), proposes that this intermediate phase consists of an extended threedimensional bilayer network. However, the fact that no vesicle fusion, necessary for the formation of an extended lipid network, was observed at any temperature, do not support this hypothesis. Other proposed model (Goldman et al., 1999; Goldman, 2001), a statistical approach considering surface fluctuations, also assumes lipids redistribution.

The results obtained so far could be rationalized considering that, in low ionic strength DMPG bilayers, the lipid gel–fluid transition does not occur abruptly, but there is a competition among the different temperature dependent lipid–lipid interactions. At \( T_{m}^{\text{on}} \) a \( \text{Na}^+–\text{PG}^- \) dissociation process would be triggered by some mechanism still not understood, increasing the membrane surface potential, with correlated changes in viscosity and light scattering. Thus, a strong repulsion between adjacent headgroups would start the melting regime, monitored as a sharp event in DSC and ESR of spin labels. From \( T_{m} \) onward there would be a competition among different interactions, namely headgroup repulsion, van der Waals inter-chain attraction and intra-bilayer repulsion between monolayers, resulting in a large gel–fluid temperature region. This could bring a coexistence of domains with different packing characteristics, which could explain the gradual variation of the several parameters measured with the different techniques described here, over the temperature interval \( T_{m}^{\text{on}}–T_{m}^{\text{eff}} \). This would certainly induce membrane deformations imposed by the coexistence of more rigid and flat patches with softer and highly curved regions, similar to morphological observations of giant vesicles at the main phase transition (over a much narrower temperature interval though; Sackmann, 1995; Bagatolli and Gratton, 1999). At \( T_{m}^{\text{eff}} \), marked by a broad calorimetric peak, there would be a rearrangement of the lipid packing bringing the membrane to a homogeneous fluid phase.

It is interesting to point out that DSC traces indicated that, whereas, the saturated PG lipid with 12-C chains (DLPG) presented an intermediate phase over an even larger temperature interval than that of DMPG, the longer chain lipid DPPG (16-C) displayed a sharp DSC peak (Schneider et al., 1999), typical of a highly cooperative gel–fluid transition. That confirms the critical role played by the different lipid–lipid interactions in the existence of the gel–fluid transition region: different chainlengths would alter the balance among the different interactions.

Though much has been found about the DMPG intermediate phase, a comprehensive explanation of the anomalous behavior of low ionic strength PG lipids is still necessary. That would contribute to the better understanding of the interesting physicochemical problem concerning the interactions present in a charged aqueous lipid dispersion, both inter and intra bilayers.

**Acknowledgements**

The authors would like to thank all their collaborators in the works cited here. The work was supported by USP, FAPESP, CNPq, and CAPES/DAAD.
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