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New lamellar phase with pores in the chain-melting regime of an anionic phospholipid dispersion

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Abstract. The anionic phospholipid DMPG (dimyristoyl phosphatidylglycerol) may exhibit in water, instead of a unique melting transition of the hydrocarbon chains, a “melting regime” for pH values above 5, where the phosphate groups are deprotonated, and for low ionic strength, where charge screening is weak. The chain-melting process of DMPG starts at \( T_{on} \) (onset of the melting regime at \( \sim 20^{\circ}\)C), but the complete fluid phase exists only above \( T_{off} \) (offset of the melting regime at \( \sim 30^{\circ}\)C). In a recent paper we developed a SAXS model for a bilayer with pores to explain SAXS results obtained for concentrations up to 70 mM DMPG (F. Spinozzi, L. Paccamiccio, P. Mariani, and L. Q. Amaral, \textit{Langmuir}, in print, 2010). A new lamellar phase with pores, starting 3\(^{\circ}\)C above \( T_{on} \) and existing up to 4\(^{\circ}\)C above \( T_{off} \), was also identified at the higher investigated DMPG concentrations (up to 300 mM DMPG). In this paper we focus in more detail the SAXS curves obtained in the concentration interval 70-300 mM DMPG. The slope of the scattering profile in the very small \( q \) range, as well as the anomalous increase in the intensity of the bilayer band centered around 0.12 \( \AA^{-1} \) after \( T_{off} \), have been in particular analyzed. By using a model of water-penetrated bilayers, the volume fractions of DMPG and water molecules inside the bilayer was derived as a function of temperature.

1. Introduction

Phospholipid molecules in aqueous dispersions may aggregate in the form of closed vesicles for small phospholipid concentrations (typically up to 100 mM or about 10% in weight). Vesicles can be unilamellar (a single bilayer) or multilamellar (several bilayers in an onion structure). Neutral phospholipids generally form multilamellar vesicles while charged phospholipids tend to form unilamellar vesicles due to electrostatic repulsion. Unilamellar vesicles of various sizes can be produced depending on the particular phospholipid and on the specific sample treatment used (agitation, sonication, extrusion, etc.) [1].

Phospholipids with two hydrocarbon chains and detergents with one hydrocarbon chain form respectively vesicular and micellar aggregates above a critical micellar concentration and above the so-called Krafft temperature, which defines the transition from ordered to disordered chains in the aggregates [2]. In biological membranes the order-disorder transition of the chains, called “main transition”, has received particular attention after the discovery that it is similar to the transition occurring in detergents [3].

In this paper we focus a particular system with an abnormal melting transition of the chains: dimyristoyl phosphatidylglycerol (DMPG), which may exhibit a “melting regime”, instead of a
unique melting transition. This abnormal behavior is observed for pH values above 5, where the phosphate groups are deprotonated, and for low ionic strength, where charge screening is weak. The chain-melting process of DMPG starts at $T_{\text{on}}^m$ (onset of the melting regime at $\sim 20^\circ C$), but the complete fluid phase exists only above $T_{\text{off}}^m$ (offset of the melting regime at $\sim 30^\circ C$). A review on the properties of DMPG can be found in [4].

DMPG dispersions (50 mM DMPG in 10 mM Hepes with 2 mM NaCl) were previously investigated as a function of temperature by small angle X-ray scattering (SAXS) down to $q \sim 0.03$ Å$^{-1}$ ($q$ being the scattering vector modulus given by $q = 4\pi \sin \theta/\lambda$, where $2\theta$ is the scattering angle and $\lambda$ is the X-ray wavelength) [5]. At all the investigated temperatures, SAXS profiles show a broad band centered at 0.12 Å$^{-1}$ (dependent on the bilayer electron density profile and called “bilayer band”) and a $q^{-2}$ behavior at lower $q$ values, typical of unilamellar vesicles. However, an anomalous decrease in electron density contrast in the bilayer band characterized the melting region. Additional X-ray results extended the $q$-range, from $q \sim 0.007$ Å$^{-1}$ to the wide angle region (WAXS), and DMPG concentration, from 10 to 80 mM DMPG [6]. WAXS analysis showed that the Bragg peak typical of ordered chains disappears above $T_{\text{on}}^m$, while the lower $q$ range revealed only in the intermediate phase the presence of a band (called IP band), which indicates the existence of a mesoscopic structure with a repeating of around 370 Å, interpreted as in-plane correlated cavities/pores on the vesicle surface. This proposal was corroborated by observation of DMPG giant unilamellar vesicles with phase contrast optical microscopy [6]. The effect of addition of salt to 50 mM DMPG dispersions was investigated in more two papers; the disappearance of the IP band in SAXS [7] was correlated with disappearance of transparency in the melting regime, as detected by light absorbance, DSC and fluorescence microscopy [8].

Studies varying DMPG concentration indicated the existence of another type of structure above 70 mM, with possible destruction of the vesicles [6]. This made us start both to develop a SAXS model for a bilayer with pores and to study by SAXS higher DMPG concentrations, in the interval 70-300 mM. A model has been indeed constructed, able to explain SAXS curves in the melting regime up to 70 mM DMPG, while a new lamellar phase with pores was found at the higher concentrations, starting $3^\circ C$ above $T_{\text{on}}^m$ and existing up to $4^\circ C$ above $T_{\text{off}}^m$. Part of this study, focused in the SAXS pore model and on SAXS curves obtained with 70 mM and 150 mM DMPG, as well as WAXS, DSC and optical microscopy results with 150 mM DMPG, has been recently published in Langmuir [9].

In this paper we focus in more detail the SAXS curves obtained in the whole concentration interval (70-300 mM DMPG), and analyze changes in the slope of the curves observed in the very small $q$ range, as well as the abnormal behavior of the intensity of the bilayer band above $T_{\text{on}}^m$. The formation of pores in natural as well as model phospholipid bilayers is a key biophysical process, which may be associated with many phenomena, such as the anomalously large permeability of water, the formation of local defects that act as nucleation processes in membrane fusion and the permeability of ions through the membranes.

It should be noticed that the DMPG peculiar thermal and structural behavior at the transition from gel to fluid lipids opens several questions on the biological relevance. Indeed, about 10-40% of all naturally occurring lipids are negatively charged and in prokaryotes the most abundant anionic phospholipid is phosphatidylglycerol [10]. Such an intermediate regime, at least locally and characterized by perforated membrane microdomains, could also occur in natural lipid systems and cells could make use of a similar concept to activate structural changes and functionality in their membranes. Moreover, triggering similar membrane changes by controlling temperature, pH and salt concentration could potentially be used in targeted drug delivery [11].

2. Experimental

The sodium salt of DMPG (Avanti Polar Lipids, Birmingham, USA) and bidistilled water were used to prepare samples with DMPG concentrations in the interval 70-300 mM, as previously described [9]. The same buffer (10 mM Hepes pH 7.4 with 2 mM NaCl) was used throughout, but pH remained always above the apparent pK of DMPG, ensuring that DMPG can be assumed to be fully deprotonated and effects are due to changes in DMPG concentration.

Scattering curves were obtained at the SAXS beamline of LNLS Synchrotron (Campinas, Brazil) with \( \lambda = 1.608 \text{ Å}^{-1} \) and \( 0.008 < q < 0.25 \text{ Å}^{-1} \), using a linear position sensitive detector. A thermal bath was used for temperature variation from 12ºC up to 55ºC. Samples were conditioned in a sample holder with flat mylar walls and 1 mm thickness. Data, with acquisition time 10-20 minutes, were normalized for monitor integral counts (to compensate for oscillations in the beam intensity), sample attenuation and corrected for the SAXS detector response (measured with a radioactive source). The measured scattering due to the buffer, which has no structure in the SAXS region was subtracted from all scattering curves.

3. SAXS results at various concentrations

Visual observation of the DMPG dispersions under temperature variation showed that the melting regime is transparent only up to 70 mM DMPG, but for higher concentrations the sample is milky in all phases. SAXS data were obtained in different concentrations (70, 100, 150, 200 and 300 mM DMPG) varying the temperature. The whole ensemble of measured curves is shown in Figure 1 (note that in the previous paper [9] only representative results for 70 mM, 300 mM and 150 mM were displayed).

The broad band at around 0.12 Å\(^{-1}\), observed at all concentrations and temperatures, is related to the inner bilayer structure [5, 6], and therefore called “bilayer band”. The position, \( q_{\text{max}} \), and intensity, \( I_{\text{max}} \), of the bilayer band are shown in Figure 2 as a function of temperature, for all measured concentrations. As previously described, the complex thermal dependence of \( q_{\text{max}} \) and \( I_{\text{max}} \) can be explained in term of variations of the bilayer electron density profile [5], attributed to the appearance/disappearance of holes/pores in the melting transition [6], but a more careful analysis of these results will be presented later on.

Besides the bilayer band at \( \sim 0.12 \text{ Å}^{-1}\), at smaller \( q \) (around 0.01 Å\(^{-1}\)) correlation peaks, which change with both temperature and concentration, are observed. With 70 mM DMPG, the intermediate region starts at \( T_m^{\text{on}} \sim 20\text{ºC} \), where a broad band of the same type as the IP band observed at smaller concentrations occurs [6]. A model for the porous bilayers, in the form of bicelles with in-plane correlated pores (see Fig. 7), has been developed [9] and could fit the whole SAXS curves observed at 70 mM DMPG in the temperatures where the IP band exists. This confirms that the IP broad band is associated to the 2D correlation among pores, as proposed qualitatively in [6]. Still with 70 mM, the sharper peak detected above 23ºC is attributed to a new lamellar phase, called \( L_p \) (lamellar phase with pores) in the previous paper [9], which changes its characteristics above \( T_m^{\text{off}} \sim 30\text{ºC} \).

SAXS curves observed at DMPG concentration higher than 70 mM evidence that the sharp peak of the new \( L_p \) phase becomes well defined at 23ºC. Moreover, other peaks appear also in the gel and fluid phases, indicating the emergence of a correlation among bilayers in the direction perpendicular to their plane. The SAXS curves always change at \( T_m^{\text{on}} \sim 20\text{ºC} \), but the curves for 20 – 21ºC do not present the defined peak of the \( L_p \) phase, which exists above 23ºC.

The evolution of the position of this sharp peak with concentration was analyzed in [9] in terms of the lipid surface fraction parameter \( \alpha = c_V (d/t) \), where \( c_V \) is the lipid volume fraction in the sample, \( t \) the lipid bilayer thickness and \( d = 2\pi/q_{\text{peak}} \) the repeat distance from a correlation peak centered at \( q_{\text{peak}} \) [6]. In all cases, at 25ºC the parameter \( \alpha \) remains \( < 1 \), indicating the presence of bilayers with pores and/or with finite size. Calculations of \( \alpha \) for other temperatures showed that the value \( \alpha = 1 \) expected for a normal lamellar bulk phase \( L_\alpha \) is reached only for
Figure 1. Log-log plot of SAXS curves for DMPG samples at different concentrations (shown at the bottom of each column) and temperatures (reported on the left of each curve in °C). For 150 mM, a short (150 mM DMPG S) and a long (150 mM DMPG L) series of measurements with two independent samples are shown. Red lines refer to the determination of the initial slope up to $q = 0.012$ Å$^{-1}$. Green curves are the fits with a parabola used to obtain the position, $q_{\text{max}}$, and intensity, $I_{\text{max}}$, of the bilayer band.

300 mM DMPG in the gel phase ($T = 17.8$°C).

In the previous study [9] an extensive analysis was made on the different results obtained for 150 mM DMPG, in order to characterize the new $L_p$ phase. Several properties of the new
lamellar phase $L_p$ were indeed studied by SAXS, WAXS, DSC and optical microscopy. In particular, the temperature-induced phase sequence Ripple Gel - IP - $L_p$ - fluid was defined. Moreover, it was suggested that all the transitions to and out of the intermediate melting region should correlate with strong fluctuations of the bilayer, together with opening and closing of pores of yet unknown dynamics [9].

The variation of the position of the correlation peaks with temperature for all measured concentrations is shown in Figure 3. At temperatures where both the broad and sharp peaks are present, only the position of the sharper one was considered in Figure 3 (note that for concentrations larger than 70 mM there are peaks both below $T_{on}$ and above $T_{off}$, while they are not well defined at 70 mM).

The repeat distance $d$ decreases with concentration, from 300 Å at 70 mM to 160 Å at 300 mM within the melting regime, from 450 Å to 250 Å before $T_m^{on}$ and from 400 Å to 200 Å above $T_m^{off}$. The change at $T_m^{on}$ is sharp for 70, 100 and 150 mM, but not for 300 mM DMPG. Moreover, the melting interval becomes smaller with concentration, although not converging yet to $T_m = 23^\circ$C, the unique melting temperature observed for electrostatically screened DMPG dispersions with 500 mM salt [7], the same temperature where the new phase appears. Note that a decrease of the melting interval with temperature is also suggested by the changes in position and intensity of the bilayer band ($q_{max}$ and $I_{max}$), as reported in Figure 2, where $T_{off}$ approaches $T_{on}$ when the DMPG concentration increases.

The intensity of the bilayer band ($I_{max}$) merits to be further analyzed also as a function of DMPG molar concentration, $C$. The observed increase in intensity can be in fact partially explained by the trivial dependency with the number of scattering unities. This is shown in the Figure 4, where the relative intensity $I_{max}(C)/I_{max}(C = 70$ mM) is shown as a function of $C$ for several temperatures, together with the linear behavior expected for a change only in the number of scattering unities (with a constant form factor and interference function). At all temperatures, the intensity drops for the higher concentrations (200 mM, and specially 300 mM), indicating an increase of interference among scattering unities, consistent with the observed higher diffraction orders. For 70, 100 and 150 mM samples, a linear behavior similar to what expected is detected at $T = 28^\circ$C, near $T_{off}$, while for lower temperature the intensity is lower than expected, and for higher temperature it is higher than expected. This fact is indicative of changes in the bilayer structure while passing from 70 mM to 100 mM and 150 mM, specifically in the melting regime. This point will be further discussed.

Figure 2. Temperature dependence of position, $q_{max}$ (left panel), and intensity, $I_{max}$ (right panel), of the SAXS bilayer band for DMPG samples at different concentrations (reported in mM).
Figure 3. Peak positions \((d = 2\pi/q_{\text{peak}})\) as a function of \(T\) for all measured DMPG concentrations.

Figure 4. Relative intensity of the bilayer band with respect to the value for 70 mM DMPG as a function of DMPG concentration \(C\) for several temperatures. Black line refers to the behavior expected for a change only in the number of scattering unities.

4. Slope of the log-log SAXS curves at small \(q\)

All the measured SAXS curves have been analyzed in order to define the slope of the log\([I(q)]\) versus log\((q)\) at very low \(q\) (up to 0.012 Å\(^{-1}\)). Fitting lines are shown in Figure 1 while the changes in the measured slope with temperature and concentration are displayed in Figure 5.

It can be seen that there is a large variation of the slope with concentration. Values close to \(-2\), the fingerprint of large isolated bilayers, occur both for 70 mM DMPG in the whole temperature range and for 150 mM DMPG, but only in the low temperature region. Slopes from \(-4\) to \(-3\) characterize samples with the higher DMPG concentrations as well as the 150 mM DMPG sample at temperature above \(T_{m}\), indicating possible existence of correlated bilayers building an apparently more symmetrical object.

In order to better understand these results, extensive simulations have been performed with a structural model based on the presence of lamellar bicelles of radius \(R\), as used also in the model developed in [9] and elsewhere [12].

For a single bicelle with rims, simulations varying \(R\) are shown in Figure 6, where also the line with slope \(-2\) is reported for comparison. Very clearly, the slope \(-2\) (which is characteristic for an “infinite bilayer”) exists only for \(q\) values above a \(q^*\) value defined by the intersection of the line with \(q^{-2}\) behavior and the initial region with constant intensity. The insert of Figure 6 shows \(q^*\) as a function of \(R\) values from all the simulations. A clear straight line, also shown in
Figure 5. Slopes of the log-log SAXS curves obtained for the lowest $q$ region (from 0.008 to 0.012 Å$^{-1}$).

the insert of Figure 6, gives the relationship between $q^*$ and $R$, according to:

$$q^* = k \frac{R^\beta}{R^3}$$

where $k = 1.9 \pm 0.1$ and $\beta = 1.03 \pm 0.01$.

This result gives a practical way to obtain the $R$ value when the change in slope can be experimentally detected, and also a minimum value for $R$ when the slope $-2$ is experimentally observed in a defined $q$ range. The present experimental SAXS curves arrive only to $q = 0.008$ Å$^{-1}$: the $-2$ slope observed for example at 70 mM DMPG means that in this case the bicelle radius $R$ should be larger than 250 Å. However, it could be much larger than 250 Å, as only measurements at much smaller $q$ values could give the radius of a large bicelle.

In order to understand the larger (absolute) slopes observed in the lamellar $L_p$ phase, it should be reported that the fit to the $L_p$ peaks with Lorentzian interference functions gave a width compatible with lamellar correlation of around $N \sim 20$ bilayers [9]. The high absolute values for slopes then indicate that SAXS is sensing a global object whose shape is cylindrical or spheroidal, due to the presence of largely correlated bilayers. The indication is that the slope of the SAXS curve is sensible to the total size of correlation, which may go well over 1000 Å. On the other hand, the analysis of the experimental SAXS curves show that the very small slopes detected in few conditions are determined by the influence of nearby correlation peaks distorting the initial slope.

Therefore, the measured slopes support the conclusions that the IP phase corresponds to large uncorrelated bilayers, with the IP band due to two-dimensional correlation between pores in the bilayer surface, while the $L_p$ phase corresponds to lamellar correlation between bilayers. At 70 mM there is a match at 25°C between the IP band and the peak of the $L_p$ phase, as evidenced also by the values of the $\alpha$ parameter related to the IP band and the $L_p$ peak, displayed in [9].

5. Analysis of the bilayer band
The variations of $I_{\text{max}}$ with temperature for 150 mM DMPG have been analyzed in [9] in the context of the pore model. The formation of the $L_p$ phase is proposed to come from a balance between bilayer inflation, as visually observed in giant vesicles by fluorescence microscopy [8]
Figure 6. Simulated X-ray differential scattering cross sections for a single bicelle with rims, varying the inner radius $R$ reported on the left of each curve. Red arrows refer to the intersection, occurring at $q^*$, between the line with $q^{-2}$ behavior and the initial region with constant intensity, both shown as green lines. The insert shows the linear relationship between log($q^*$) and log($R$).

and available interlamellar space. The defined change of $I_{\text{max}}$ from the IP value at 20°C to the $L_p$ value at 23°C, as observed in Figure 2, is not directly associated with the parameters of the DMPG electron density function, and is probably connected to a change from the larger pores in IP to smaller pores in $L_p$.

A clear correlation also exists between the temperature variation of $I_{\text{max}}$ and of the intensity of the sharp lamellar peak of the new phase $L_p$. Since peak intensities are defined by the intensity of the form factor of the bilayer with pores in the $q$ region where there is the peak, a series of simulations were made in [9], showing the strong dependence on the electron density contrasts, particularly between the flat and pore regions of the bilayer. Such simulations indicate that the transition from the $L_p$ to the fluid state occurs via transfer of lipids from the pore borders to the flat region [9]. The complete study of 150 mM DMPG indicates clearly that pores open at $T_{m}^{\text{on}}$ and seal at $T_{m}^{\text{off}}$. On the other hand, the recovery of $I_{\text{max}}$ occurs only about 4°C above $T_{m}^{\text{off}}$. 
after the transition as detected by DSC has been completed [9].

The minimum of $I_{\text{max}}$ at $T_{\text{off}}^m$, followed by a maximum value only 4°C above $T_{\text{off}}^m$, was qualitatively ascribed to water remaining inside the bilayer [9]. Fits to $I_{\text{max}}$ with the pore model are however difficult, both because the number of model parameters and the fact that there is not a simple analytical expression for $I_{\text{max}}$. Simulations showed that $I_{\text{max}}$ depends directly on the size and number of pores, and particularly on the fraction of the bicelle surface occupied by pores, but depend also on the electron density distribution in the round parts of pores [9].

In this paper, the specific point of the recovery of $I_{\text{max}}$ after $T_{\text{off}}$ is focused, using a simple model of a planar infinite bilayer without pores. The idea is to analyze the volume fractions of DMPG and water molecules inside the bilayer, in order to define whether the recovery in $I_{\text{max}}$ is indeed due to water still remaining inside the bilayer above $T_{\text{off}}$, and being expelled as $I_{\text{max}}$ increases.

Tests were also previously made with a simple bicelle model without pores described by three levels of electron density contrasts. The region for $q > 0.05$ Å$^{-1}$ [9] was considered, which includes the minimum of the SAXS curve around 0.06 Å$^{-1}$ which is influenced by the bicelle radius $R$. Results showed that a bicelle radius $R = 953$ Å was obtained and the parameters of the bilayer profile remained practically the same as those of an infinite bilayer [9]. It can be then concluded that in the region where $I_{\text{max}}$ is measured, for $q > 0.07$ Å$^{-1}$, it is possible to use, for $T > T_{\text{off}}$ (= 28 ± 1°C), a simple model of bilayer to deal with the amount of water that could remain among lipid molecules after pore sealing. A cartoon representation of this simple model, compared with the pore model of Ref. [9] is reported in Fig. 8.

The scattering intensity of a infinitely wide plane bilayer is given by the basic equation [13]

$$I(q) = \kappa r_e^2 c_V \frac{2\pi}{q^2} A_f(q) + B,$$

where $c_V$ is the volume fraction of the hydrated lipid molecules and $t$ is the bilayer thickness. $r_e$ is the classical radius of the electron, whereas $\kappa$ and $B$ are a scaling factor and a background, both due to the instrumental calibration. $A_f(q)$ is the Fourier transform of the electron density profile $\rho(z)$ along the direction $z$ perpendicular to the bilayer plane. According to [9], $\rho(z)$ has been modeled by assuming four domains (indexed with $i = 0, 1, 2, 3$) of constant electron density, corresponding to solvent, head group, alkyl chains and terminal domains, respectively, with smooth transitions between two adjacent levels. The hydrated lipid volume fraction is calculated as a function of the nominal lipid molar fraction $x_L$ of the sample,

$$c_V = \mu x_L \left( 1 - x_L \right) \rho_W + x_L \left( \nu - N_H \rho_W \right),$$

where $\mu$ is the volume of one hydrated lipid molecule, $\rho_W$ is the volume of a water molecule in the bulk solvent (with a well-known dependence on temperature [14]) and $N_H$ is the total number of hydration water molecules per lipid molecule dispersed in the bilayer. The main point of this simple model is an explicit determination of $\nu$ in terms of $N_H$, which is also used to calculate the domain electron densities $\rho_i$. Details are hereafter reported.

The volume of one hydrated lipid molecule is written as a sum of polar head, alkyl chains and terminal group volumes, $\nu = \nu_1 + \nu_2 + \nu_3$. Regarding the location of the $N_H$ water molecules associated to one lipid molecule, we make the hypothesis that $N_{H_{\text{pol}}}$ are in the polar head domain and $N_{H_{\text{par}}}$ are in the paraffinic moiety, with the condition $N_H = N_{H_{\text{pol}}} + N_{H_{\text{par}}}$. The paraffinic moiety of one lipid molecule is then constituted by $N_{CH_2}$ methylenes, $N_{CH_3}$ methyls and $N_{CH_2}$ water molecules. Each of these three groups (indexed by $k = CH_2, CH_3, H_{\text{par}}$) will be, on average, located in both paraffinic domains 2 and 3, according to three corresponding fractions, $x_k$, which are defined as the ratio between the number of $k$-groups in the domain 2
Figure 7. Geometry of the pore as used in the pore model developed in ref. [9].

Figure 8. Molecular representation of the bilayer (a) in the pore model from ref. [9] and (b) in the simple model presented in this paper. DMPC has red polar heads, green CH$_2$ groups and yellow CH$_3$ groups, while water is cyan. The bilayer is seen from its perpendicular axis in the upper part of the figure, in perspective in the middle part and its profile is seen in the bottom part of the figure, where bulk water molecules have been added for clarity. The bilayer profile in (a) has one pore, as sketched also in the TOC of [9].

The total number of \( k \)-groups in the paraffinic moiety. In this framework, the three domain volumes per lipid molecule can be written as

\[
\begin{align*}
\nu_1 &= \nu_{pol} + N_{H_{pol}} \nu_W / d_{pol,W} \\
\nu_2 &= N_{CH_2} \nu_{CH_2} x_{CH_2} + N_{CH_4} \nu_{CH_4} x_{CH_4} + N_{H_{par}} \nu_W x_{H_{par}} / d_{par,W} \\
\nu_3 &= N_{CH_2} \nu_{CH_2}(1 - x_{CH_2}) + N_{CH_3} \nu_{CH_3}(1 - x_{CH_3}) + N_{H_{par}} \nu_W(1 - x_{H_{par}}) / d_{pol,W}
\end{align*}
\]

In these equations, \( \nu_{pol} \), \( \nu_{CH_2} \) and \( \nu_{CH_3} \) represent the group volumes, whose values as a function of temperature and with the fraction of \( \alpha \) conformation in the chains \( (x_{\alpha}) \) are well known from literature data [15]. Moreover, \( d_{pol,W} \) and \( d_{par,W} \) are the relative mass density of hydration water molecules in the polar and paraffinic moieties.

By the knowledge of the volumes \( \nu_i \) of the domains, it is straightforward to calculate the domain thicknesses \( R_i \) as a function of the bilayer thickness \( t \):

\[
R_i = \frac{\nu_i t}{\nu} \quad 2
\]

The area per lipid molecule is easily calculated by \( A = 2\nu / t \). The domains’ electron densities are \( \rho_i = e_i / \nu_i \) where the domain number of electrons \( e_i \) are calculated on the basis of the domain
Figure 9. Global fits of the SAXS curves for 150 mM DMPG, long series, at \( T > 28^\circ C \) using the model of a water-penetrated infinite bilayer. Temperature values are reported on the left of each curve in °C. Curves are stacked by a factor 1.2.

Figure 10. Temperature dependence of the number of water molecules associated to each DMPG molecule in the polar, \( N_{\text{Hpol}} \) (a), and paraffinic, \( N_{\text{Hpar}} \) (b), moieties obtained by the analysis shown in Figure 9. In both graphs, the relative intensity of the bilayer band, \( I_{\text{max}}(T)/I_{\text{max}}(19.8^\circ C) \) is also reported in green for comparison.

composition (see Eqs. 4-6),

\[
\begin{align*}
\epsilon_1 &= \epsilon_{\text{pol}} + N_{\text{Hpol}} \epsilon_{W} + x_{\text{Na}^+} \epsilon_{\text{Na}^+} \\
\epsilon_2 &= N_{\text{CH}_2} \epsilon_{\text{CH}_2} x_{\text{CH}_2} + N_{\text{CH}_3} \epsilon_{\text{CH}_3} x_{\text{CH}_3} + N_{\text{Hpar}} \epsilon_{W} x_{\text{Hpar}} \\
\epsilon_3 &= N_{\text{CH}_2} \epsilon_{\text{CH}_2}(1 - x_{\text{CH}_2}) + N_{\text{CH}_3} \epsilon_{\text{CH}_3}(1 - x_{\text{CH}_3}) + N_{\text{Hpar}} \epsilon_{W}(1 - x_{\text{Hpar}})
\end{align*}
\]  

where \( \epsilon_{\text{pol}}, \epsilon_{\text{CH}_2}, \epsilon_{\text{CH}_3}, \epsilon_{\text{Na}^+} \) and \( \epsilon_{W} \) are the number of the electrons of DMPG\(^{-}\), \( \text{CH}_2, \text{CH}_3, \text{Na}^+ \) and water groups, respectively. Here \( x_{\text{Na}^+} \) is the fraction of \( \text{Na}^+ \) counterion dissolved in the domain 1. Notice that the possible electrostriction effect of \( \text{Na}^+ \) is indirectly taken into account by variations of the relative mass density of water in the domain 1, \( d_{\text{pol,W}} \).

In summary, the free parameters of the model, which will be dependent on temperature and
then specific of each SAXS curve, are the bilayer thickness, \( t \), the number of water molecules in the polar and in the paraffinic moieties, \( N_{\text{Hpol}} \) and \( N_{\text{Hpar}} \), and the \( x_{\text{CH}_2} \), \( x_{\text{CH}_3} \) and \( x_{\text{Hpar}} \) parameters which define the fraction of \( \text{CH}_2 \), \( \text{CH}_3 \) and water groups located in the paraffinic domain 2. Note that for \( T > 28^\circ \text{C} \), the fraction of lipids in \( \alpha \) conformation can be reasonably fixed to \( x_\alpha = 1 \). Moreover, a few tests have shown that the sodium fraction \( x_{\text{Na}^+} \) does not significantly change with temperature. Hence, a unique average value \( x_{\text{Na}^+} \) can be determined by fitting all the curves to the model. Other common parameters, that can be considered independent on temperature, are the relative mass densities of the water in the polar and in the paraffinic moieties \( d_{\text{pol},W} \) and \( d_{\text{par},W} \). The variances of the error function that smoothes the transitions between two adjacent levels of electron density [9] have been fixed to 1 Å. Finally, a unique instrumental calibration constant, \( \kappa \) (Eq. 2) should be found by the analysis. Notice that all the other parameters of the models, such as thicknesses and domain electron densities are calculated from the fitting parameters.

The dataset of \( N_C = 27 \) curves of the 150 mM DMPG sample (\( x_L = 0.00298 \)), long series, at increasing temperature from 28.5 to 53.7°C, in the range \( q > 0.07 \) \( \text{Å}^{-1} \), has been fitted with the method reported above. In order to avoid dramatic variations or oscillations with temperature of the six single curve fitting parameters, \((t, N_{\text{Hpol}}, N_{\text{Hpar}}, x_{\text{CH}_2}, x_{\text{CH}_3}, x_{\text{Hpar}}, \) here indicated as \( X_j \)), a regularization method has been exploited [16]. The merit functional to be minimised is constituted by a standard reduced \( \chi^2 \) of the dataset and a regularization term,

\[
\chi^2 = \frac{1}{N_C} \sum_{c=1}^{N_C} \sum_{q=1}^{N_q} \left( \frac{I_c,\text{exp}(q_i) - I_c(q_i)}{\sigma_c(q_i)} \right)^2 + \alpha_{\text{reg}} \sum_{c=1}^{N_C} \sum_{j=1}^{6} \left( 1 - \frac{X_{c+1,j}}{X_{c,j}} \right)^2
\]

where \( N_q \) is the number of \( q \)-points of each curve, \( \sigma_c(q) \) is the experimental uncertainty of the \( c \)-curve and \( \alpha_{\text{reg}} \) is a regularization parameter whose value is chosen in order to get a relative weight of the second term of 10-20% with respect to the first term.

**Table 1.** Parameters obtained by the analysis of the bilayer band. 27 SAXS curves at 150 mM DMPG, long series, for \( T > 28.5\)°C have been considered.

| \( \kappa \) (a.u. cm\(^{-1} \)) | \( x_{\text{Na}^+} \) | \( d_{1,W} \) | \( d_{\text{W,}\alpha} \) |
|-------------------------------|-----------------|-----------|-----------|
| 25.5 ± 0.3 | 0.25 ± 0.07 | 1.076 ± 0.002 | 1.08 ± 0.02 |
| \( x_{\text{CH}_2} \) | \( x_{\text{CH}_3} \) | \( x_{\text{Hpar}} \) | \( t \) (Å) |
| 0.71 ± 0.03 | 0.46 ± 0.03 | 0.60 ± 0.08 | 45 ± 1 |

Figure 9 shows the fits obtained with the SAXS curves of 150 mM DMPG for \( T > 28^\circ \text{C} \) using such a model. The quality of the fit is very high. The common parameters are reported in Table 1, together with the average value of the single curve fitting parameters which resulted practically independent on temperature. The more interesting results of this analysis are the number of water molecules associated to each DMPG molecule in the polar, \( N_{\text{Hpol}} \), and paraffinic, \( N_{\text{Hpar}} \), moieties. Their temperature dependence, shown in Figure 10, suggests that at \( T_{m}^{\text{off}} \) water is still inside the bilayer, probably reminiscent of the pores present in the phases occurring at lower temperatures. On heating up to around 40°C, water molecules are expelled from the paraffinic region, so that they may contribute to the observed increased hydration of the polar heads. At the higher temperatures, water molecule are also released from the polar region, probably due to the increased thermal disorder.
6. Conclusions
In solution SAXS data, obtained at different DMPG concentrations (from 70 to 300 mM) and as a function of temperature across the main transition, have been analyzed to derive information on the structural modifications accompanying the changes from vesicles to bulk lamellar structures induced by concentration and/or temperature.

At one side, simulated and measured slopes of the SAXS curves at very low $q$ support the conclusions that the IP phase corresponds to large uncorrelated bilayers, characterized by two-dimensional correlated water pores within the DMPG lamellae, while the $L_p$ phase is characterized by largely correlated bilayers. On the other side, the model fitting of the bilayer band centered at 0.12 Å$^{-1}$ after $T_{m}^{off}$ showed that at this temperature water is still inside the DMPG bilayer, probably due to a defective pore closing. On heating, water molecules are released first from the paraffinic region and then from the polar heads, probably due the increased thermal disorder. It is well known that in concentrated solutions, amphiphiles may form bulk lamellar structures, and other complex liquid crystalline structures, largely studied in the case of detergents and soaps [17]. The change from vesicles to bulk lamellar structures is expected to occur with increase in phospholipid concentration, but this transformation is in general not studied in detail mostly because the region of smaller concentration is a physico-chemical problem, while the higher concentration region is a condensed matter problem. We are persuaded that this paper will give some contribution to the understanding of the transformations occurring on this charged phospholipid system, which may have biological significance.

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