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INVITED ARTICLE

Studies of some bio-inspired liquid crystals

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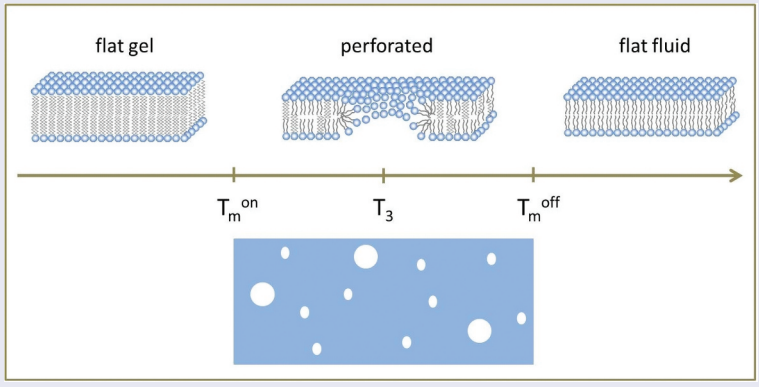
ABSTRACT

The main phase transition of lipid bilayers corresponds to a defined change from an ordered (gel) to a disordered (fluid liquid crystal) state of hydrocarbon chains, occurring in general at a defined temperature T_m. However, aqueous dispersions of pure anionic phospholipids, and particularly DMPG, may exhibit a 'melting regime', from T_m^{on} until T_m^{off}, over ~10°C. The melting process depends on pH, ionic strength and several details of sample preparation. This paper makes a review on seven papers of the author with different collaborations, with the original proposal of pores in the DMPG melting transition. The focus is on structural results obtained from X-ray scattering, varying concentration and temperature, but integrating with results from other experimental techniques. Initially the effect of salt addition was separated from the effect of DMPG concentration. At DMPG concentrations higher than 70 mM a lamellar phase starts and a detailed study of the temperature variation of 150 mM DMPG allowed arrival to detailed pore model, focused in the surface fractions of pores in the bilayers. The model admits large and small pores in the melting regime and agrees with the new Lamellar Phase with pores (Lp) starting at T_3. The large biological relevance is stressed.



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Introduction

Lyotropic systems (water/surfactant/cosurfactant/additives) have very complex phase diagrams and may classify as biological and bio-inspired liquid crystals, usually studied at a fixed temperature. They correspond to supramolecular aggregates, which at high water content may form micelles in isotropic phase, at a critical micellar concentration, in equilibrium with monomers of all constituent parts. At lower water content and above the Krafft temperature of fusion of the hydrocarbon chains, aggregates may self-organise with a long-range order, as lyotropic liquid crystals with hexagonal, lamellar and cubic symmetries [1]. Such macromolecular aggregates represent complex physical chemistry and biochemical problems, since they are transient and dynamical entities, even if their average structures may be considered thermodynamically stable. Studies developed since 1976 in our Crystallography Laboratory in systems with a single hydrocarbon chain were summarised in 2002 [2]. Analysis of the behaviour of the cell parameters as a function of volume concentration allowed the use of a simple basic principle from crystallography: filling of continuous space requires that the density of matter in the atomic level coincides with the macroscopic density of material. Such criteria allowed the determination of the size of aggregates as spheroids in the isotropic phase, as finite cylinders in hexagonal phases and detailed analysis of phase transitions with concentration involving lamellar, hexagonal and several cubic phases [2]. Our work in the nematic phases in the standard lyotropic

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micellar system water/sodium dodecyl sulphate (SDS)/decanol was used as basis in recent rheology studies of this system [3]. Some of our papers on nematic lyotropic phases were published in Liquid Crystals [4–8], including an invited review paper on micelles in biaxial phases [7]. Our work on phase transitions on nematic phases with temperature inspired also theoretical papers on new phase diagrams in the mixture of rods and plates [9].

In this paper the focus is turned to molecules with two hydrocarbon chains and their interest as bio-inspired liquid crystals. The basic principle of space filling was extended to analyse these complex phospholipids structures, but including the possibility of necessary empty spaces [10].

Focus is now on phospholipids with two hydrocarbon chains, and this means a change from Physical-Chemistry to Biochemistry, since water solutions depend strongly on pH and require the use of buffers in order to stabilise pH. The discussion here is on the melting transition between the ordered (gel) and the disordered (fluid liquid crystal) states of hydrocarbon chains in two specific phospholipids, in biomimetic membranes: the anionic phospholipid DMPG, a sodium salt with Na counter ions, (dimyristoyl phosphatidyl glycerol) and its zwitterionic analogue DMPC (dimyristoyl phosphatidyl choline). Such systems are usually studied at low concentrations (1 to 10 mM), when closed vesicles are formed, a subject of biochemistry [11]. The low intensity of our X-ray beam required instead concentrations of 50 mM and this lead to extend to even higher concentrations, aiming to study the cross over from vesicles in solution to bulk liquid crystalline phases.

My previous studies with several collaborators allowed defined results with the original proposal of pores in the DMPG melting transition, induced by changes in temperature, a non-trivial result, based on strong experimental evidence, here reported in detail.

The usual items on 'materials and methods' (MM) and 'results' are united, and a review is made on seven specific papers I made with different collaborations. Each of the considered papers has many details, and a synthetic review extracts the information considered necessary for a good understanding of the logics connecting the several steps of the research. A final discussion exposes new and non-trivial ideas to arrive to a conceptual understanding on the abnormal melting transition in DMPG.

MM and results of specific published papers in collaborations

A review is made on the results obtained, with different groups of collaborators, from projects using the Crystallography Laboratory of IFUSP and the Brazilian Synchrotron facility, under my responsibility [12]. X-rays are specially suited for structural studies of membrane bilayers, because the interaction is with electrons, via elastic Thomson scattering with no exchange time. Therefore, X-rays have much contrast between polar heads/paraffin moiety/water, and data integrate over the whole exposure time, averaging over fluctuations. Small-angle X-rays scattering (SAXS) detects the average structure, so that the typical behaviour q^{-2} at very small angles reflects an average planar structure, even with large fluctuations. A broad 'bilayer peak' gives information on the inner bilayer structure (around 50 Å), easily separated from q^{-2} planar behaviour in a log-log plot of SAXS curves. Wide-range X-rays scattering (WAXS) gives information around 4–5 Å on the structure of hydrocarbon chains. The combination of SAXS with WAXS allows the detailed investigation of changes in bilayer structures, as discussed in this paper. Neutrons and X-rays do not give the same information, since neutrons interact only with the nucleus (not with electrons) and exchange both energy and momentum, having defined interaction times with the system, with totally different contrast characteristics from X-rays. To study biological systems with neutrons, it is necessary to use deuterium oxide (instead of H_2O) to have coherent scattering in structural studies with momentum transfer. Hydrogen scatters neutrons incoherently, giving information only on energy exchanges (complementary to infrared spectroscopy). Furthermore, neutrons see a membrane without contrasts but are able to give information on dynamics of fluctuations.

The original contribution of this paper includes the review made in accessible language of the several specialised published papers, necessary to follow the logics of the structural research pursuit. The final discussion of the paper also gives original contribution on the proposal for formation of pores that are temperature dependent and their possible role in biology.

Abnormal melting transition of DMPG in vesicles

DMPG used in these measurements had sodium as counter ion and presents an 'abnormal' melting transition, as compared to DMPC, much studied previously in the low-concentration region with several experimental techniques: the melting transition, well defined at a melting temperature T_m in DMPC, extends over a wide temperature interval in DMPG. A review is made now on the collaborations made in Brazil, in a sequence of four papers [13–16] mainly with Karin A. Riske, a doctorate student in the Biophysical Group of IFUSP, continued after her PD period in Germany.

All the details of the samples (buffer, pH, ionic strength and vesicle preparation) and references on previous studies are in the papers, the review made here is focused on non-trivial SAXS structural analysis in systems with little available information, relying on physical intuition together with trust in experimental data.

Our initial paper [13] also presented data on differential scanning calorimetry (DSC) and electron spin resonance. SAXS results for temperatures between 10°C and 45°C [13] are seen in Figure 1 together with a good fit and show a broad peak (instead of Bragg peaks characteristic of multilamellar structures), indicating DMPG organisation in single bilayers. A quite unusual effect in the SAXS intensity was observed in three independent samples, starting to decrease at a temperature defined as T_m^{on} (~19°C), and presenting a sharp increase at a temperature defined as T_m^{off} (~30°C), coinciding with light transparency. Modelling the SAXS curves required a bilayer with three density levels (polar heads, paraffin moiety and middle CH_3) and evidenced structural changes in the bilayer thickness (40–50 Å) in the melting region, with a clear temperature-dependent decrease in contrast. The temperature variation of the peak position of the bilayer band (q_{max}) was expected, but variations of the maximum intensity (I_{max}) was unexpected. My interpretation: clear evidence of water inside the bilayer in the melting region.

These results lead to investigate the system further, with SAXS measurements at even smaller angles (to detect structures above 100 Å) and also WAXS (to detect the order of paraffin chains) [14]. At least three independently prepared samples were investigated in each experimental arrangement, showing clear data reproducibility, and all results were shown to be reversible upon heating and cooling cycles, with only small variation in the value of T_m^{off}. Besides the bilayer peak present in all phases, a SAXS peak corresponding to a mesoscopic structure at $d \sim 400$ Å $= 2\pi/q_1$ was detected for DMPG only in the melting region (called intermediate phase IP), as shown in Figure 2, where the q^{-2} behaviour in gel and fluid phases are also seen. A SAXS-WAXS camera was also used, and WAXS results (not shown) revealed a marked change at T_m^{on}, although smaller changes continue to occur until T_m^{off}.

The behaviour of the new peak IP was observed as a function of the lipid concentration cv, from 10 up to 80 mM DMPG, with an indication of superposition with other structures around 70 mM [14]. Results up to 70 mM were analysed with simple criteria of space filling: for a bilayer, the symmetry decouples the three-dimensional volume partition of the lipid in a one-dimensional factor (perpendicular to the bilayer) and a two-dimensional factor (in the bilayer plane). For infinite planar bilayers with thickness t, **cv** coincides with the linear fraction of lipid occupancy (t/d). When

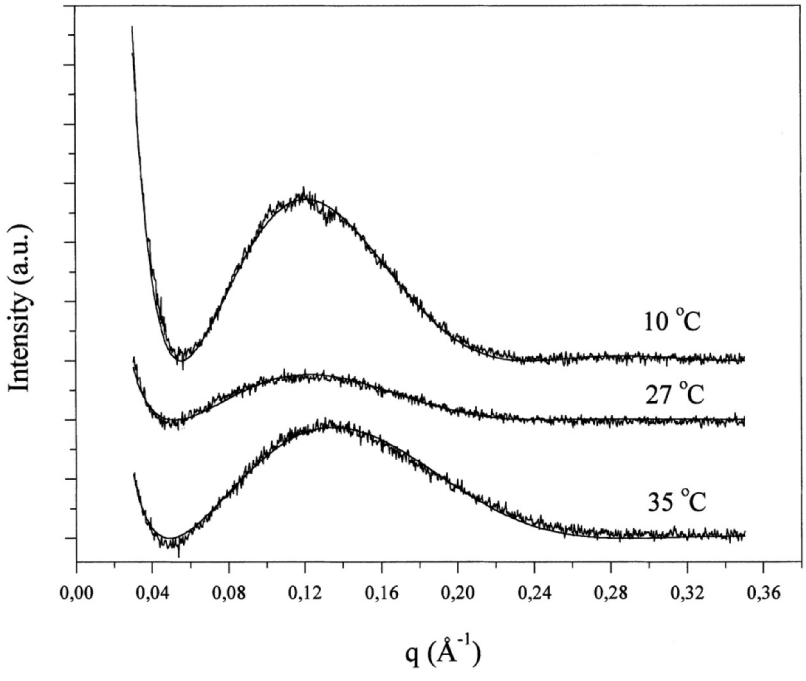

Figure 1. SAXS curves for samples with 50 mM DMPG with temperature variation, together with fits of the bilayer broad peak [13]. Reprinted from Biochimica et Biophysica Acta (BBA) –biomembranes, Vol 1511/2, Karin A. Riske, Lia Q Amaral, M. Teresa Lamy-Freund, Thermal transitions of DMPG bilayers in aqueous solution: SAXS structural studies, Pages 297–308, Copyright (2001), with permission from Elsevier (License 5,491,621,506,585).

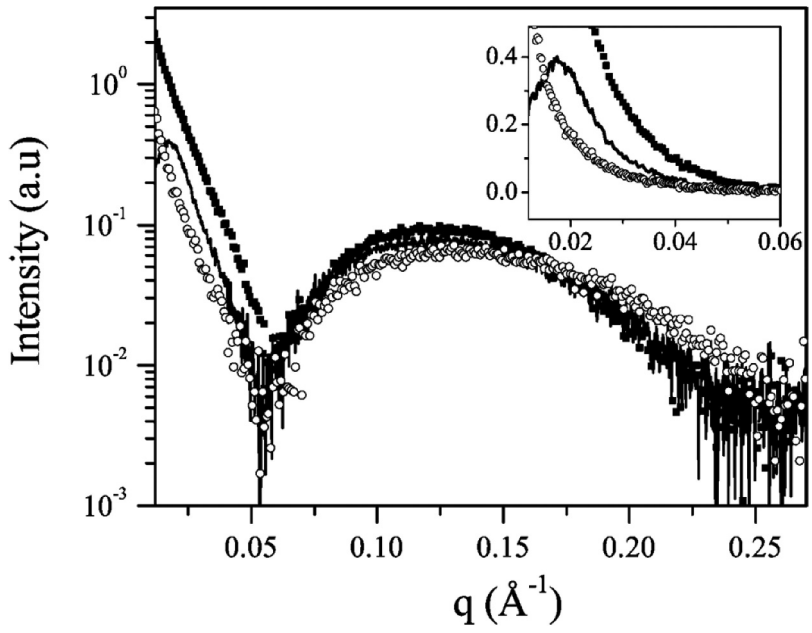

Figure 2. SAXS curves for samples with 50 mM DMPG with temperature variation, in semilog scale, showing in the insert the region of the IP mesoscopic peak in the melting region [14]. Reprinted from Biophysical Journal, Vol 86/6, K.A. Riske, L.Q. Amaral, H.-G. Döbereiner, M.T. Lamy, Mesoscopic Structure in the Chain-Melting Regime of Anionic Phospholipid Vesicles: DMPG, Pages 3722–3733, Copyright (2004), with permission from Elsevier (License 5,491,910,438,516).

the lamellae have fluctuations or are finite, it is possible to define a lipid surface fraction α, such that **cv** = α (t/d) [17]. The value α can be obtained from knowledge of **cv**, t and d, and it is <1, which is compatible with in-plane and not lamellar correlation, ruling out the possibility of a sponge phase (defined by α = 1.5).

This paper [14] included turbidity data evidencing a transparent intermediate phase IP, and also a new technique was used in Germany, with observation of giant unilamellar vesicles (GUV) with phase contrast light microscopy, showing that DMPG vesicles 'disappear' upon cooling below T_m^{off} and 'reappear' after reheating with several GUVs coexisting with a texture of small vesicles. This further proves that although vesicles cannot be visualised in IP, their overall structure is maintained. It was proposed [14] that the IP in the melting regime corresponds to unilamellar vesicles with perforations, a model which is consistent with all described experimental observations.

The third paper in this series [15] dealt with an increase in ionic strength investigated by SAXS and optical microscopy (OM), in 50 mM DMPG vesicles, with salt addition. The recipe for DMPG samples included 2 mM NaCl salt, to stabilise pH in the Hepes buffer, and further salt addition was made to detect the effect of differences in the ionic strength.

By SAXS, the broad bilayer peak, arising from the electron density contrasts within the bilayer was observed at all temperatures up to 100 mM NaCl addition. At higher ionic strength (250–500 mM NaCl), an incipient lamellar repeat distance around d = 90–100 Å is detected, superimposed to the bilayer form factor, indicating a loose multilamellar order of only four bilayers, accounted for by DLVO theory [15]. On the other hand, Figure 3 shows that the IP peak in SAXS curves at 25°C persists only up to 10 mM NaCl and disappears above. My conclusion: the ionic strength effect is more connected with salt addition than with DMPG concentration.

The final paper in this series [16] used DSC, turbidity and OM of giant vesicles, with phase and fluorescence microscopy to study the narrowing of the transition region in samples with 50 mM DMPG but with increase in ionic strength (salt addition). Figure 4 shows that it was possible to correlate the complex DSC profile with the vanishing of the bilayer optical contrast. The sequence of superimposed calorimetric peaks of DMPG, though complex, is characteristic for each salt condition. As seen in Figure 4a, upon the increase in ionic strength, T_m^{on} shifts to higher temperatures whereas T_m^{off} shifts to lower temperatures, and finally a single T_m (at 23°C) is reached, with 500 mM salt addition. But it is interesting to note that T_m also coincides with T_3, a temperature related to changes in transparency. Turbidity measurements (Figure 4b) evidenced that visual transparency corresponds to absorbance practically at the zero level. A transparent region between the turbid gel and fluid phases exists only in the presence of up to 20 mM NaCl, when T_m^{on} coincides with T_3. In the presence of 50–70 mM NaCl, sample turbidity shows a minimum, but the sample is visually turbid in all temperature range. The

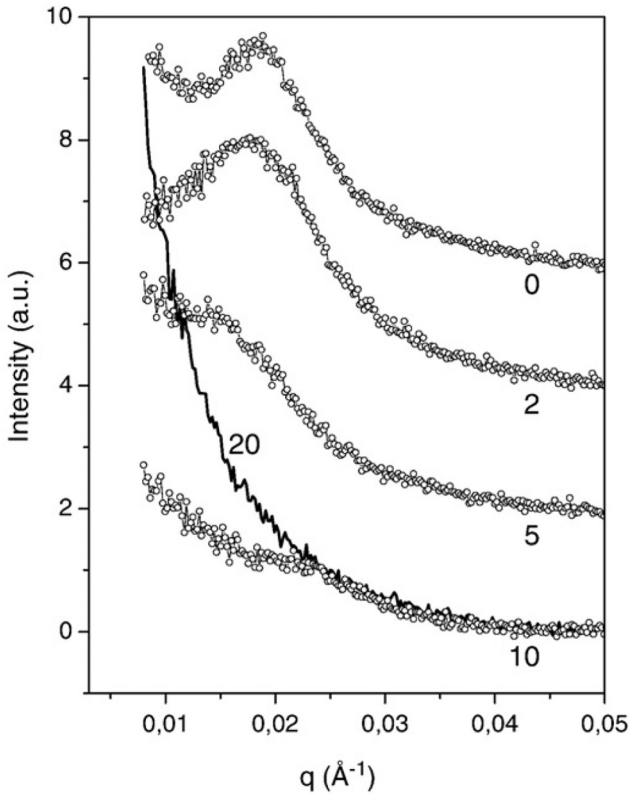

Figure 3. Details of SAXS curves for samples with 50 mM DMPG in the low q region (IP peak), with variation of added salt [15]. Reprinted from Biochimica et Biophysica Acta (BBA) –biomembranes, Vol 1778/4, Roberto M. Fernandez, Karin A. Riske, Lia Q. Amaral, Rosangela Itri, M. Teresa Lamy, Influence of salt on the structure of DMPG studied by SAXS and optical microscopy, 907–916, Copyright (2008), with permission from Elsevier (License 5,491,860,365,184).

combination of phase contrast and fluorescence microscopy in GUV vesicles gave very interesting results [16], showing a process of GUV elongation and ruptures connected with T_3.

This paper [16] closed the collaboration in Brazil, with the clear result that bilayers are perforated along the transition and the bilayer completely looses the optical contrast, directly related to turbidity. Furthermore, the effect of salt addition became clear. I decided then to turn to the study of the effect of DMPG concentration, but some initial experiments with DMPG in pure water showed completely different SAXS results, without trivial interpretation. Studies of phospholipids cannot follow the same rules used with detergents.

Combined effect of concentration and temperature on DMPG melting

This section reviews the three papers in the second collaboration [18–20], with the group at Ancona University (mainly with Dr. Francesco Spinozzi) with measurements in the concentration range 70–300 mM DMPG, with special attention to sample preparation, as described in the first paper of this series [18]. The same buffer (10 mM Hepes pH 7.4 with 2 mM NaCl) was used throughout, without any further adjustment of pH of the lipid dispersion, to ensure the same additional low ionic strength as in all the previously published results on DMPG at smaller concentrations. This is important because the change in ionic strength through addition of NaCl is known to destroy the intermediate region [14,16]. The pH of the DMPG dispersions was measured at room temperature (23°C). As expected, the pH decreased slightly with the increase in DMPG concentration but, even for the highest DMPG concentration, the pH is still above the apparent pK of DMPG, which is below 5. Therefore, DMPG can be assumed to be fully deprotonated in the full concentration range measured and possible changes in structure are to be attributed to the change in DMPG concentration.

A review is first made on two papers published in 2010 with the same group of Italian collaborators [18,19], showing experimental results from gel to fluid phases, investigated using SAXS, WAXS, DSC and polarised optical microscopy (POM). These papers also have further attempts of some modelling based on detailed X-ray scattering theory.

The first paper [18] details the measurements performed in the Brazilian syncrotron, with DMPG concentrations in the interval 70–300 mM, with temperature variation from 12°C up to 55°C. The reason for this choice was the fact that 70 mM is the crossover from 'independent vesicles' [14] to structures with other interactions. The result obtained previously for 70 mM [14] reproduced, with a mesoscopic peak IP in the melting region and the bilayer broad peak, while for 300 mM DMPG a very different SAXS curve presented several lamellar peaks superimposed to the broad bilayer peak. Analysis of the complete set of SAXS curves (70, 100, 150, 200 and 300 mM DMPG) leads to the decision to focus the intermediate concentration 150 mM DMPG, repeated slowly in intervals of 1°C. Figure 5 shows SAXS results for 150 mM DMPG in a log-log plot with a clear separation between the broad bilayer band and the mesoscopic IP region. It is possible to note a movement of the IP mesoscopic peak with temperatrure, first towards large q values, later towards smaller q values, with appearance of a new sharper peak.

My choice was to make a simple visual identification in terms of observed peaks value, and this subjective analysis leads to a proposal for the behaviour of the mesoscopic region with temperature, seen in Figure 6.

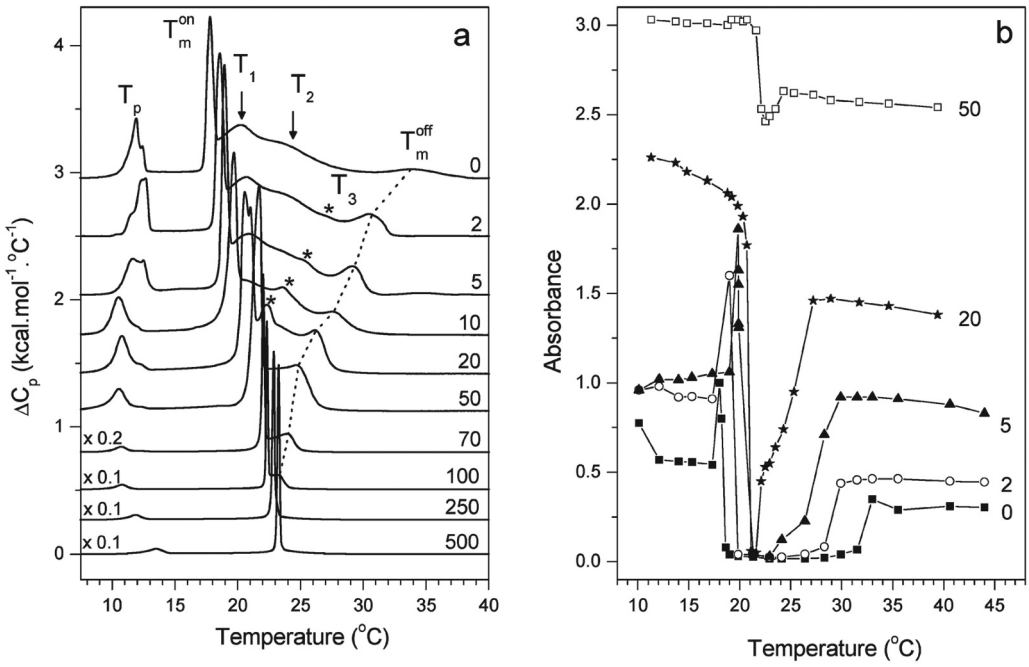

Figure 4. Results [16] of DSC (a) and turbidity (b) for samples with 50 mM DMPG with variation of added salt. Reprinted from Riske KA, Amaral LQ, Lamy MT. Extensive Bilayer Perforation Coupled with the Phase Transition Region of an Anionic Phospholipid. Langmuir. 25(17):10083 –10,091. With permission. Copyright {2009} American Chemical Society.

The complex behaviour before T_m^{on} (~20°C) can be explained in terms of two gel phases: Gel I with two peaks, well separated at 12°C, that approach with temperature, coinciding into a Gel II ripple phase with a single peak. It is possible to follow a curve along the ripple phase interrupted by the intermediate region IP, but joining the fluid phase after T_m^{off} (~28°C). The even more complex behaviour in the melting region can be explained by the superposition of the broad IP peak starting at T_m^{on} and moving to larger q values, until about 23°C and a new sharper peak, assigned to a new lamellar phase L_p with pores, moving to smaller q values and entering at T_m^{off} in the fluid phase, when the peak starts moving to larger q values.

The sequence of phase transitions inferred from the position of IP peaks in SAXS curves is consistent with DSC results for the 150 M DMPG seen in Figure 7, with four peaks: the pre-transition (gel), a well-defined T_m^{on}, a new sharp peak at 23°C defining entrance in the new phase Lp = lamellar with pores, and a broad T_m^{off}, centred at ~28°C. Must be remarked that 23°C coincides with the temperature T_3 defined in [16]. Comparison of DSC results from Figures 4 and 7 reveals clearly the different effects of salt addition and of increase in DMPG concentration.

WAXS curves (not shown here) were fitted with sum of two Gaussian functions, also with a clear change in 23°C, but extending in the interval 22°C-28°C. The intermediate region has a milky visual aspect instead of the typical transparency observed at smaller concentrations. POM observations allowed characterisation only of black field (isotropy or pseudoisotropy) and anisotropy (white/coloured field) of unaligned 150 mM DMPG samples as a function of temperature. Birefringence appears only in the new lamellar phase Lp within the melting regime, and isotropy is recovered several degrees after T_m^{off}, in the fluid phase [18].

The observed intensities of all the mesoscopic peaks in SAXS curves of Figures 5 and 6 show a clear decrease at T_m^{on}, followed by an increase, reaching a maximum at the entrance in L_p, and going down until T_m^{off}, followed by a slower increase in the fluid phase. This curve of peak intensities [not shown] is trustable and asks for an explanation, obtained only in the last paper of this series [20]. Some attempts have been made to model peaks with Lorentzian functions [18], but no attempt of modelling the SAXS curves with 150 mM DMPG was possible in this paper.

For obtaining information on the water distribution in the sample, the lipid surface fraction α was calculated for all concentrations, from scattering data at 25°C, as done previously in [14]. Figure 8 shows the ensemble of all results [14,18], keeping $\alpha < 1$. The values obtained in [14] are seen in circles, while the values obtained in [18] are seen in squares. These α values obtained in [18] almost join the ones obtained in [14], evidencing an interlamellar distance in Lp almost matching the in-plane correlation at 70 mM

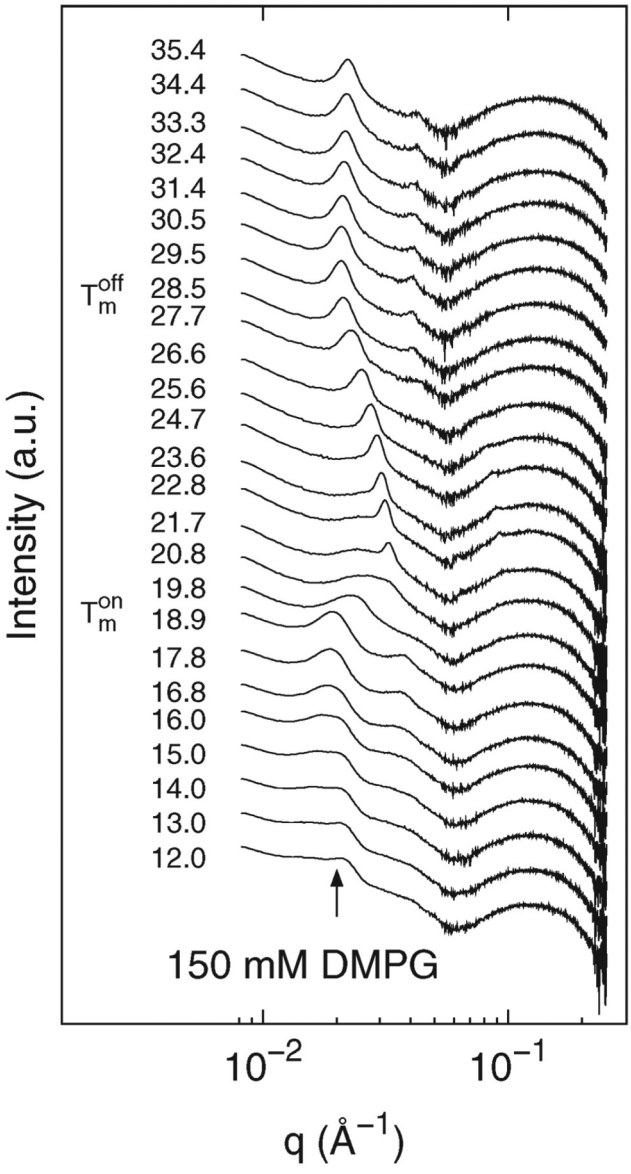

Figure 5. SAXS curves for 150 mM DMPG [18] with temperature variation, showing the complex behaviour of the peak IP in low q region. Reprinted (adapted) with permission from {Francesco Spinozzi, Lydia Paccamiccio, Paolo Mariani, and Lia Q. Amaral, Melting Regime of the Anionic Phospholipid DMPG: New Lamellar Phase and Porous Bilayer Model, Langmuir 26(9), 6484–6493}. Copyright {2010} American Chemical Society.

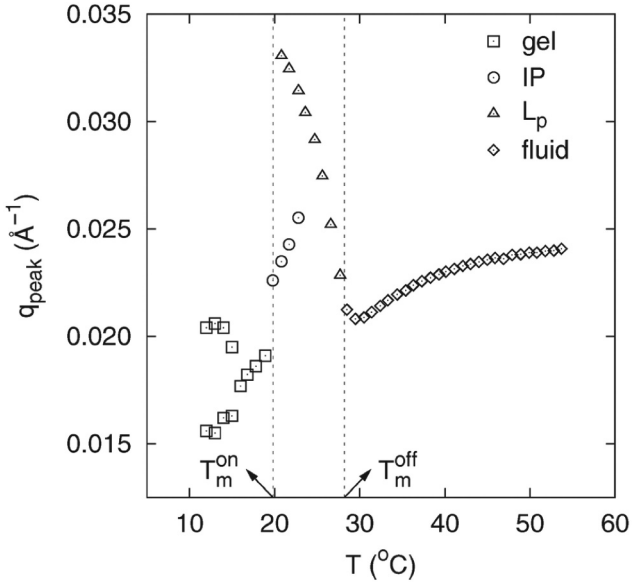

Figure 6. Observed peak values [18] in the SAXS curves with temperature variation showing new phase Lp. Reprinted with permission from {francesco Spinozzi, Lydia Paccamiccio, Paolo Mariani, and Lia Q. Amaral, Melting, Regime of the Anionic Phospholipid DMPG: New Lamellar Phase and Porous Bilayer Model, Langmuir 26(9), 6484–6493}. Copyright {2010} American Chemical Society.

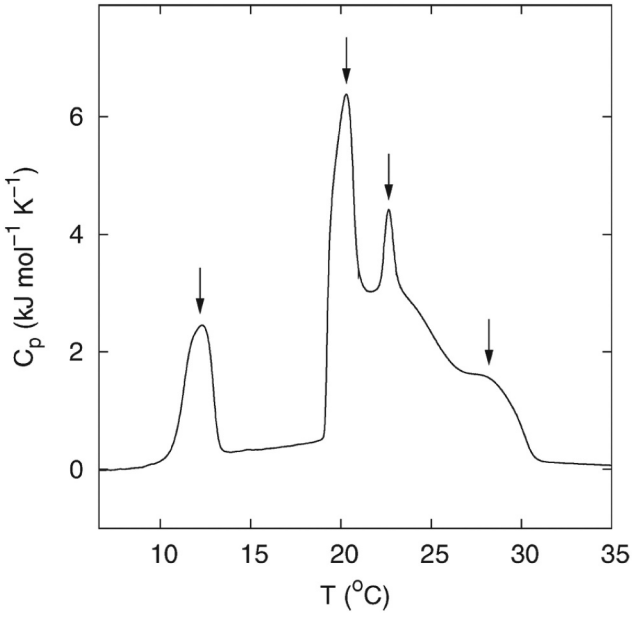

Figure 7. DSC results [18] for the 150 M DMPG showing new phase Lp at 23°C. Reprinted with permission from {Francesco Spinozzi, Lydia Paccamiccio, Paolo Mariani, and Lia Q. Amaral, Melting Regime of the Anionic Phospholipid DMPG: New Lamellar Phase and Porous Bilayer Model, Langmuir 26(9), 6484–6493}. Copyright {2010} American Chemical Society.

DMPG. This result strongly suggests that pores existing until 70 mM lead at higher concentrations to breakage of many vesicles into pieces, giving origin to the lamellar Lp phase, with lamellar distance of the order of the inter-pore distance.

A porous bilayer model was presented in [18] and calculations were performed for a finite bicelle with pores. Several simulations were made to localise conditions for appearance of a peak due to 2D correlation between pores, resulting in pores of radius ~150 Å in bicelles of radius ~1000 Å, which could give origin to an in-plane correlation peak at the position of the IP band, with a reasonable fit to 70 mM SAXS curves [shown in [18], but not here].

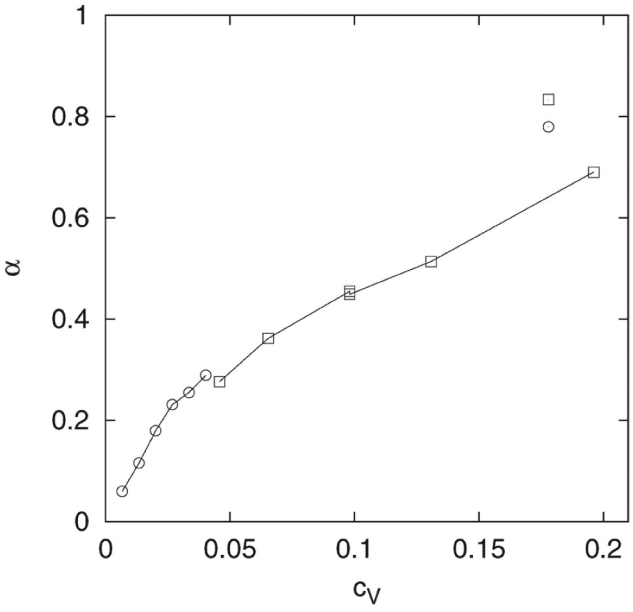

Figure 8. Lipid surface fraction α: values in circles [14] compared with values in squares [18], see text for discussion [18]. Reprinted (adapted) with permission from {Francesco Spinozzi, Lydia Paccamiccio, Paolo Mariani, and Lia Q. Amaral, Melting Regime of the Anionic Phospholipid DMPG: New Lamellar Phase and Porous Bilayer Model, Langmuir 26(9), 6484–6493}. Copyright {2010} American Chemical Society.

This paper [18] is a superposition of my physical intuition with strong experimental data plus detailed X-ray scattering theory showing the possibility of in-plane correlation with 70 mM DMPG, justifying all the qualitative analyses made to explain the very complex temperature behaviour. But it should be noticed that the pre-transition region seen by SAXS is not really explained by the DSC curves, evidencing a very complex evolution of changes with time, not just fluctuations around fixed positions.

The second paper [19] was published in the Proceedings of a SAS conference, and attempts were made there to analyse SAXS curves for all measured concentrations, not only 150 mM as done in [18], in particular considering the possibility of trivial differences in intensity related to the number of scattering unities.

Fits with a parabola were used to obtain the position and intensity of the bilayer band and of the IP peaks for all concentrations, with their temperature variation. The relative intensity of the bilayer band compared with the expectation with only change in number of scattering unities confirms that above 150 mM the interference increases. The initial slope up to $q = 0.012$ Å^{-1} was determined for all SAXS curves. However, conventional SAXS analysis on the very complex ensemble of SAXS curves with varying concentration and temperature was not able to give satisfactory answers. Several simulations were then made trying to advance on the model of the bicelle shown in [18].

A model of water-penetrated bilayers (instead of pores) for concentrations above 70 mM DMPG was used, together with volume fractions of DMPG and water molecules inside the bilayer as a function of temperature. Calculations did not show good agreements with the details of the variations observed in the SAXS curves, but some information on existence of water in the bilayer above T_m^{off} was obtained.

To sum up, this paper [19] eliminated trivial solutions, but the direction of chemical analysis of existing atoms in the sample together with detailed X-rays theory was promising.

Such promising direction was followed during the next years and finally the paper by Spinozzi and Amaral [20] was published in 2016, with a very detailed pore model, focused on the lipid surface fractions of pores in the bilayers and detailed analysis of distribution of matter with temperature, together with a robust process of data analysis based on the GENFIT software [21]. The present Graphical Abstract is a sketch of the pore model in the intermediate melting region.

The assumption of existence of large and small pores was necessary to explain the SAXS results with 150 mM DMPG. Both pores have toroidal geometry and contain DMPG in the fluid conformation. The flat region of the bilayer can contain DMPG molecules in fluid and in gel conformation. The fraction of pores projected in the bilayer plane is calculated with a normalisation condition, and the 'effective' electron density profiles in the direction perpendicular to the bilayer is a linear combination of the contribution of all types of DMPG regions. The vertical correlation in the lamellar phase is modelled with a Caillé structure factor, taking into account the poly-dispersion in the number of correlated bilayers. This model is used to fit in a unique calculation all the ensemble of SAXS curves, with several fitting parameters.

The electron density continues to be modelled with few electron density levels (3 for gel and 2 for fluid), the simplest model able to account for large contrasts in a system with large fluctuations. But instead of few parameters (as done before in the series with collaboration in Brazil), the electron densities now consider all molecular and ionic species that characterise the system and the temperature dependency of their volumes. In other words, the electron density has 'real' values, considering all the atoms present in the sample. But no attempt is made to model the interactions between such atoms and results are obtained from the fit of SAXS curves to the many parameters existent on the model, which are free under defined limits.

Usual X-ray atomic structure determination requires the existence of many diffraction peaks, possible only with crystalline samples. The solution presented in [20] uses

instead all the ensembles of temperature-dependent SAXS curves as 'data' and a very large number of parameters in a global fit of the pore model. Such global fit of all 37 experimental SAXS curves for 150 mM DMPG sample in the temperature interval 18.9°C (still in the gel state) up to 53.7°C (in the fluid state) gave as the defined result [20]. The gel phase transforms initially, at 19.4°C, in uncoupled bilayers with large pores (radius 93.2 ± 0.5 Å, with water channel diameter 137 ± 1 Å), which transform into small pores along the lamellar phase L_p (defined change in ~28°C). The minimum intensity of the SAXS bilayer peak corresponds to a maximum number of small pores and change in the fractions of molecules in the flat region of the bilayer, with decay of the gel and increase of the fluid conformations. Above 35°C, the system enters into the normal lamellar fluid phase, without pores. The Graphical Abstract of [20] displays the variation of calculated surface fractions in the bilayer, considering the gel and fluid flat parts and small and large pores. All the details regarding the theoretical model of the lipid bilayer, geometry of pores, scattering model considering all atoms in the sample, X-ray theory, parameter control and data analysis together with results must be seen in [20].

Here, Figure 9 reproduces some selected relevant results of the pore model [20] as a function of temperature: Figure 9(a) displays the charge density in bulk water, showing that the regions with pores contain fewer Na+ ions per polar head; hence, when they are forming, there is a release of Na+ ions towards the bulk. Figure 9(b) displays the average area per polar head, with a peak of ~80Å^2 and a base line of 60 Å^2. Such results are clearly of considerable biological relevance.

The final discussion in [20] quotes a theory on the formation of stable pores in low ionic strength limit [22], which mentions our results with DMPG [16] as an experimental basis to the theory. The curvature of the surfactant parameter is also discussed, in terms of a theory for change in micellar form [23]. However, the inexistence of any statistical mechanical theory, predicting pore formation in the abnormal melting transition, precluded the recognition of the real importance of solving the DMPG problem with this pore model in terms of a detailed theory of X-ray scattering coupled with chemical sample composition.

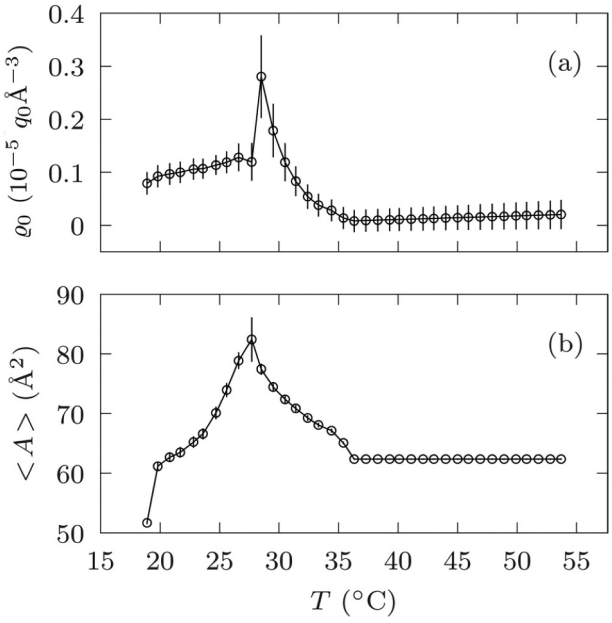

Figure 9. Reprinted (adapted) with permission from {Francesco Spinozzi and Lia Q. Amaral, Pore Model in the Melting Regime of a Lyotropic Biomembrane with an Anionic Phospholipid, Langmuir 32 (50), 13556 –13,565}. Copyright {2016} American Chemical Society. 9(a) displays the charge density in bulk water; 9(b) displays the average area per polar head [20].

Discussion

My long-lasting interest in the melting transition of DMPG (more than 15 years, in parallel with other research activities) is due to my belief in its importance as a basic problem in biology, for several reasons:

* DMPG is the most abundant anionic phospholipid present in prokaryotic cell membranes, and the large effect observed in its abnormal melting region may represent a model of what occurs in other charged membranes in eukaryotes.

* A theory on membranes [24] discusses the large peak in passive permeability at the bilayer phase transition temperature due to cavities in the headgroup region.

* The role and function of DMPG in prokaryotes are possibly connected with bacterial capacity of exchanging DNA material. DMPG in real systems may have different counter ions than the Na salt used in these seven papers, since the charge behaviour of DMPG depends on the chemical ambient. But a real understanding of the pore process with anionic DMPG studied here will certainly help to understand the more complex behaviour.

I must now give arguments to bring this discussion on DMPG to present days. A step further must come now from the recognition that exact mathematical physical theories were unable to arrive even to a real definition of life. Difficult biological problems require a clear understanding of the hypothesis of existing theories, and insightful changes in such hypothesis, in order to define new starting points, possible to lead to more effective directions of research. Simplified hypotheses are necessary to construct theories, but it is time now to realise

some limitations. The Theory of Lipid Bilayer Phase Transition (LBPT) by Nagle [25] was a fundamental achievement in the understanding of the bilayer melting transition T_m, but it has not much to say on the abnormal DMPG melting regime. His theory on lateral compressibility connected to permeability [24] is more useful regarding the existence of cavities.

The fundamental theory on membrane fluctuations by Helfrich [26] is the basis for all the work done on shape fluctuations in biological membranes, but it is strictly valid only in the fluid phase, its application to the DMPG abnormal melting regime is not trivial.

The opening of pores in giant vesicles has been verified experimentally in the case of tensed unilamellar synthetic vesicles [27], when pore opening is driven by the membrane tension, and pore closure by the line tension. Only a single pore is observed at a time in a given vesicle, and a cascade of transient pores appears in the same vesicle [27]. The whole process is quite different than what was verified with DMPG [14,16,18]. Studies with pores in biomembranes refer in general to pores connected to membrane proteins and do not show temperature dependence related to the transition in lipid membranes.

A recent article on passive water transport in biological pores [28] analyses pores in specific membrane proteins. A molecular-kinetic description of water transport in pores is compared to analytical models based on macroscopic parameters such as pore diameter and length, without much success. The data point towards models in which the chemical bonds between water and the pore are of central importance, as well as the interaction between pore wall and solutes. Finally, it must be remembered that the functioning of real biological cells do not depend only on the lipid biomembrane, there exists a cytoskeleton responsible for the mechanical dynamics of real cells. A very recent article [29] analyses the regulation of GUV mechanics via actin network architectures. Also, cell-mimicking would benefit from the comparison of basic properties of liposomes (spherical vesicle with lipid bilayers) and polymersomes (self-assembled block-copolymer vesicles) [30].

Simple arguments on the dimensional dependence of matter, as used here and in our previous work [2], together with more sophisticated and exact experimental results can lead to good science. A recent paper on intermicellar interactions [31] comes after (and quotes) our work on the structure of the hexagonal phase in the binary SDS/water system [32]. A recent paper on lyotropic bicontinuous cubic phases [33] is based in our previous studies on such phases [34]. The mainstream tradition follows directions where there are already solid theories, avoiding the risks inherent to deviations from known routes. But some space must be open to explore non-trivial alternatives, especially in cases when exact solutions are not available, as is the case of soft lyotropic liquid crystals, with very little available information.

My point is that a novel hypothesis must then rely on simple physical intuition, guided by basic arguments on dimension and scaling laws (as occurred previously with fractals and quasi-crystals).

While writing this paper (presented orally online in ILCC 2022) I came across a new article on DMPG melting regime, from 2020, using neutron techniques (neutron spin echo spectroscopy and neutron scattering, besides DSC and light scattering) with deuterium oxide instead of water [35], so that it is focused on dynamics, not on structure. The exact procedures to obtain a solution of relatively monodisperse lipid vesicles, as used in biochemistry research, were followed in [35], with concentrations of 3 and 30 mM DMPG. Their result on dynamics shows remarkable effects in the melting regime, with a dramatic increase in fluctuations in the transition region, with a very large softening, as compared to the higher temperature fluid phase, and mentions [35] the possibility of porous and/or perforated bilayers in the DMPG transition region, in agreement with all of our proposals. But it is not possible to make any trivial comparison with our structural results, especially regarding higher DMPG concentrations aiming to understand the gap between vesicles and bulk liquid crystals.

It seems to me that the direction of physical insights together with chemical intuition and good knowledge on the samples, together with control on experimental data, are necessary to advance in interesting non-trivial biological problems. In the case of DMPG melting regime, the pore model able to give a good fit to the experimental data [20] required pores of different sizes, large ones after T_m^{on}, transforming into smaller, possibly transient pores in the intermediate region, closing only after T_m^{off}. Such a model is based in rigorous X-ray scattering theory and detailed account of all atoms present in the sample, with particular attention to water. It is hoped that the experimental agreement obtained with the pore model in samples with 150 mM DMPG [20] might inspire further work on the melting regime of DMPG. It is possible to mention which hypothesis could bring a theoretical advance:

- use of the concept of lipid surface fraction α in a generalised way, to account for the existence of stable pores coexisting with transient pores, connected to regions of co-existence of chains in gel and fluid conformations and

- electroporation is still the only process well studied and applied in biophysics for changing membrane permeability; the qualitative idea of bilayer perforations due to curvature defects, valid for the fluid phase [22], must be extended to the melting transition.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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