DOUBLE-BILAYER: A NEW PHASE FORMED BY LYSOPHOSPHOLIPIDS AND THE CORRESPONDING FATTY ACID

Sérgio S. Funari*†
EMBL Outstation Hamburg, DESY
Gert Rapp‡
EMBL Outstation Hamburg, DESY
Frank Richter
EMBL Outstation Hamburg, DESY, Dept. of Physics, E22 Biophysics, TU Munich, D-85748 Garching, Germany

INTRODUCTION

Cell membranes are of crucial importance by providing the structural basis and the boundary for the cells. Their main components are functional proteins and lipids that self-assemble into bilayers where other components, as e.g. cholesterol, are incorporated. The membrane encapsulates important molecules such as DNA and RNA and protects them from foreign agents that could damage the cell components. On the other hand, the membrane must be permeable to nutrients and to the release of (toxic) products of its metabolism. Their remarkable properties are prime subjects in disease and drug therapy.

Secreatory phospholipase A2, sPLA2- has been successfully used to release drugs incorporated into degradable liposomes. It was shown that when loaded with doxorubicin (active agent in DOXIL®) these liposomes led a similar uptake of it into HT-29 colon cancer cells, compared to the drug as free doxorubicin. Interestingly, a similar experiment using cisplatin showed that the loaded liposomes were more cytotoxic than free cisplatin. This has been attributed to a possible additive effect of the sPLA2 catalyzed hydrolysis products to membrane perturbation, illustrating the importance of understanding the effect of these products on the cell membrane.

Here we are concerned with the products of phospholipid degradation, as in the case of their metabolism. The enzyme phospholipase A2, (PLA2) hydrolyses the ester bond of glycerol phospholipids at the C1 carbon, therefore its activity on dipalmitoylphosphatidylcholine (DPPC) produces palmitoyl-lyso-phosphatidyl choline (LPC) and palmitic acid (PA), in a 1:1 molar ratio. Both products are known as the basic active unit of toxins found in animal venoms. Therefore one could look for correlations between the structure of the products and symptoms occurring in persons or animals poisoned by snake bites or bee stings. Whereas there is agreement on the actual binding site and the catalytic mechanism of PLA2 activity, there is not much known about the structural behaviour of the reaction products upon their formation.

Many PLA2s show high sensitivity to surface topology, with regions of high curvature stimulating hydrolysis, despite displacing water from the lipid surface upon binding. The interface recognition is mediated by both electrostatic and hydrophobic components. PLA2 shows high activity toward zwitterionic phosphocholines, due to hydrophobic interactions and “both degradation products favour nonplanar geometries, namely normal micelles (lysophospholipids) and inverted hexagonal phases (fatty acids)”.

Differential Scanning Calorimetry -DSC- thermograms of our target system, LPC/PA 1:1 mixtures, using scan rates of 0.2 °C/min showed two endothermic transitions; one at about 42 and another at 48 °C (ref. 8 and Figure 1). On cooling one observes a different thermogram, resolving a well defined transition at 46.8 °C followed by a very broad transition spanned over ca. 15 °C. The phase transitions observed on heating and cooling are not in complete agreement with the previous DSC features of Jain and also do not agree either with each of the single components or with DPPC in excess water.

Others have employed electron and polarising optical microscopies, as well as 31P NMR, to investigate such mixtures. The data reported led to a bilayer structure. Those results were interpreted in terms of formation of a functional dimer that consists of 1:1 LPC/PA mimicking the DPPC phospholipid molecule, despite the headgroup of the lysolipid being much less constrained than the head group of the phosphatidyl lipid.

EXPERIMENTAL

The amphiphiles were obtained from Avanti Polar Lipids Inc., Alabaster, AL, USA. Samples were prepared by dissolving equimolar amounts LPC and PA in a Hepes buffer (150 mM NaCl, 1 mM NaN3, 1 mM EDTA, and 100 mM Hepes (pH 8) in milli-Q-water), to ob-
maintain a concentration of (LPC + PA) 0.2 M. The NaCl concentration simulates physiological conditions and screens possible Coulombic forces caused by the ionisation of PA molecules at pH 8. The x-ray measurements were performed at beamline X13 of EMBL at DESY, Hamburg. The following protocol was used: Half an hour equilibration at 35.5 °C, heating to 43.5 °C at a rate of 0.2 °C/min, equilibration for half an hour, heating at 0.2 °C/min to 53.5 °C, followed by the same protocol in reverse order on the cooling scan. The cooling was further extended to 23.3 °C, followed by a heating back to 35.5 °C (data not shown) to complete the thermocycle.

RESULTS

In order to identify thermally induced structural changes provoked on the products of PLA activity on DPPC, time-resolved simultaneous small- and wide angle x-ray diffraction (SAXS and WAXS) measurements were performed using the same rate of temperature change as in the DSC by Lemmich et al.9

X-ray patterns collected on repeated heating and cooling scans gave qualitatively the same results, enabling us to check the reproducibility of our results and to identify the phase transitions, the structures formed and their lattice parameters.

The main results obtained are an unusually large lamellar lattice dimension of 11 nm with a second order diffraction peak stronger than the first and only a weak chain’s reflection appearing in the WAXS region of the scattering pattern. The formation of this structure could be seen following the structural evolution of the system upon a thermal scan. Initially, heating the LPC/PA 1:1 mixture from 37 up to 42 °C, we observe a weak SAXS-reflection corresponding to 4.4 nm accompanied by a WAXS signal typical of ordered chains, a peak at s = 1/0.41 nm⁻¹ characterizing a Lbβ phase. Above 42 °C, in a narrow temperature range (~1 °C), a sharp phase transition takes place, with the SAXS reflections associated with the Lα phase vanishing and new lamellar reflections, with corresponding lattice parameter 10.9 nm appearing, Figure 1. Simultaneously the intensity from the WAXS chains reflection decreases strongly. We shall refer to this rather large structure as double bilayer Lbββ.14

It is noteworthy that this happens at about the chain melting temperature of DPPC (41.5 °C, Lipidat).15 Also, the transition enthalpy for the LPC/PA system, 5.7 Kcal/mol (Jain et al.19) is smaller than for DPPC, 8.7 Kcal/mol (Boyano et al.16). This reflects a weaker interaction between the paraffinic chains in this large structure (the double bilayer) than in the corresponding phospholipid, with the phase transition caused by a thermally enhanced chain dynamics; therefore the weak signal appearing on the WAXS region of the scattering patterns (Figure 1).

Further heating to temperatures above 48 °C results in a phase transition to a single isotropic phase, evidenced by the absence of diffraction peaks (or reflexions) and an increase in scattered intensity, Figures 1 and 2. Attempts to fit the scattering curve to that for isolated micelles of different shapes failed. In order to unequivocally determine the existence of micelles and their respective size and shape requires the determination of the structure factor, due to strong interaction between the units. This is beyond the scope of this article, whose main aim is to describe the large lamellar phases observed.

On Figures 1 to 3, one should note that the apparent peak at very small angles, s ~ 0.016 nm⁻¹, is in fact part of the direct beam (the innermost part has been covered by the beam stop), therefore it is not due to the sample, but rather to the instrument.

![Figure 1. Contour plot of the time-resolved diffraction patterns and DSC from LPC/PA 1:1 molar mixture upon heating and cooling from 23.3 to 53.5 °C at 0.2 °C/min. At high temperatures one finds an isotropic phase that upon cooling forms the double-bilayer Lbββ with lattice parameters 10.9 and 8.49 nm respectively. The horizontal lines indicate temperatures at which the scan was halted to equilibrate the system for half an hour. Right: thermogram showing the different behaviour on heating and cooling, redraw from ref. 8. Note the broad transition on cooling](image)

![Figure 2. Experimental diffraction patterns observed at temperatures as indicated. The broad scattering pattern corresponds to the isotropic phase. The data at 43.5 °C (heating red and blue curve) have been multiplied by 1.2, for clarity. The peak at very low s values is the outer part of the direct beam](image)

![Figure 3. Diffraction patterns illustrating the improvement of resolution upon holding the sample at constant temperature (35.5 °C) for 30 min. The data at 35.5 °C before equilibrium (red curve) has been multiplied by 1.2, for clarity](image)
The cooling scan

On cooling, the isotropic-lamellar phase transition occurs around 47.4 °C giving rise to this large-spaced lamellar phase (L_{nop}) with SAXS-reflections slightly less intense than on heating, Figures 1 and 2. This reflects the time required for the system to reorganize enough to show long range order correlation. Upon further cooling, at 37.5 °C a very weak reflection develops indicating the formation of a transient and metastable phase with a reflection at s = 1/d = 0.12 = 1.85 nm^{-1} co-existing with the initial ones Figure 1. This phase is observed over only a short temperature range and vanishes at 35.5 °C. Note that the lattice parameter of such phase is also rather large, but not as much as the L_{nop} and we shall refer to it also as a double-bilayer, denoted L_{1op}.

Simultaneously we observe an intensifying of the the WAXS reflection, associated with organization of the carbon chains, in agreement with the DSC trace of Figure 1. Stopping the cooling scan at 35.5 °C for 30 min shows that this transient phase vanishes, giving rise to the gel phase with lattice parameter 4.2 nm, s = 0.24 nm^{-1} (Figure 3). The lattice parameters of the remaining phase (L_{nop} double-bilayer) and the gel lamellar structure increase slightly on further cooling.

X-ray diffraction pattern simulation

A successful attempt to understand the structure of such large lamellar phases was to propose some models and calculate their respective SAXS patterns. We simulated the intensity profile of the SAXS reflections for different configurations of the molecules assembly (Figure 4) and checked against the experimental patterns. We modelled the structure factor as described in Nallet et al. and took into account three possibilities, Figure 4I) single bilayers (dimers of LPC and PA intercalated) composed of two opposed monolayers and a large interlamellar water layer; II) a double bilayer formed such that identical headgroups face each other and III) the double bilayer with the choline and carboxyl head groups facing each other.

We simulated the scattering patterns from the three models proposed in Figure 4, using the box approximation, aiming to reproduce the relative intensities of the observed reflections (Figure 2). The different molecular configurations result in different electron density distributions.

The scattered intensity I(q) is given by:

\[ I(q) = \frac{N}{q^2} P(q) S(q) \]

\[ q = \frac{4 \pi \sin \theta}{\lambda} = \frac{2\pi}{d} = 2\pi s \]

N: number of unit cells within the coherently scattering membrane stack; P(q): form factor along the membrane normal; S(q): normalized structure factor of the stack, 20 the Bragg angle and \( \lambda \) the x-ray wavelength.

The normalized structure factor reads:

\[ S(q) = 1 + 2 \sum_{i=1}^{N} \left( 1 - \frac{R}{N} \right) \cos(nq d) \exp\left( -\frac{q^2}{2} \left( \langle u_n - u_o \rangle^2 \right) \right) \]

The trigonometric term within the summation describes the constructive interference from the N unit cells and the exponential takes into account (harmonic) fluctuations of an individual bilayer \( u_n \) relative to its equilibrium position \( u_o \).

The form factor P(q) contains the electron density distribution \( \rho \) over the unit cell

\[ P(q) = \int_0^\infty \rho(z) e^{i q z} dz \]

The electron densities of the various structural components (headgroups, hydrocarbon chains, etc.) relative to the solvent (water) are collected in Table 1 and were retrieved from the literature. They are independent of x-ray measurements and have been confirmed by x-ray data. It is assumed that the area per DPPC molecule is determined by the head group only. This is supported by the absence of non-lamellar phases in DPPC under excess water conditions. For the carbonyl group we have added 11 electrons to the 15 intrinsic due to the screening of the Na-ions in the buffer. The numbers for the mixed headgroups, i.e. carbonyl and (lyso)PC, are the sum of the two.

Our intensity simulation employs a variational approach with the carbon chain length as parameter, which is not known a priori. Figure 4 right shows the simulated intensities for the three models illustrated in Figure 4 left.

From the simulations performed, Figure 1S,II we could exclude the single bilayers (dimers of LPC and PA intercalated, Figure 4I) and the model proposed in Figure 4II because such structures yield first order diffraction reflections with by far the highest intensities. Moreover, the second model has similar head groups facing each other, prone to repulsion. Finally, the simulation shown in Figure 1SIII, corresponds to the structure of the double-bilayer seen in Figure 4IIII, validating our model for the double-bilayers structure L_{nop} proposed. It gives a pattern with stronger second order reflection, compatible with the experimental data obtained (see other patterns shown in Figure 2).

### Table 1. Parameters used and respective values of the calculated electron density profile used for the x-ray scattering pattern simulation

<table>
<thead>
<tr>
<th>Molecular Unit</th>
<th>Electrons</th>
<th>Length/ nm</th>
<th>Volume/ nm^3</th>
<th>( \rho )</th>
<th>( \rho_{min} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
<td></td>
<td>0.30°</td>
<td>0.33</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>LysoPC-head</td>
<td>164</td>
<td>0.11</td>
<td>0.33</td>
<td>0.50</td>
<td>0.17</td>
</tr>
<tr>
<td>Carbonyl head</td>
<td>26</td>
<td>0.17</td>
<td>0.05</td>
<td>0.51</td>
<td>0.18</td>
</tr>
<tr>
<td>Acyl chain</td>
<td>120</td>
<td>Variable</td>
<td>0.39^*</td>
<td>0.31</td>
<td>-0.02</td>
</tr>
<tr>
<td>Methyl group</td>
<td>9</td>
<td>0.25</td>
<td>0.05^*</td>
<td>0.17</td>
<td>-0.16</td>
</tr>
<tr>
<td>Mixed headgroups</td>
<td>190</td>
<td>1.07</td>
<td>0.38</td>
<td>0.50</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* data from Petrache et al.
DISCUSSION

In order to explain and compatibilize both the thermodynamic and the x-ray signature of the system, we propose the formation of a new organization for the supramolecular self-assembled mixture of equimolar LPC and PA amphiphiles. We named it a double-bilayer.

The diffraction patterns upon the lamellar-isotropic phase transition (Figure 2) are reproducible experimentally, including the scattering associated with the isotropic phase, excluding a dissociation into single mixed bilayer domains, as in pure DPPC L phase. This observation contrasts with results observed by Allegreni reporting an anisotropic liquid crystalline phase up to 55 °C, using NMR in similar mixtures.11 Looking at the DSC trace in Figure 1, one sees the first transition as a sharp endothermic peak but the second signal is much broader, spread over a wide temperature range.

Cooling from the isotropic phase leads to the formation of the double bilayer L_{βββ}. Even above 42 °C one can already see the onset of a chain reflection whose intensity and definition rise upon further cooling, indicating improved chain ordering, until reaching the formation of the L_{iββ} phase, with a shorter lamellar, lattice (Figure 1). This is a metastable phase coexisting with the L_{ββ} until it vanishes after the equilibration at 35.5 °C. Note that the reflection on the WAXS does not change position, indicating a common organization of the chains in both phases.

As we have modelled it, the L_{βββ} phase is composed of two distinct bilayers – a LPC plus a PA bilayer – in direct contact, i.e. the cholines of the LPC face the carboxyl groups of the PA, see Figure 4III. The remarkable feature of such structure is the separation of each component into two homogenous bilayers that together form the double-bilayer.

Assuming all-trans configuration for both amphiphiles forming the double-bilayers and taking the lengths of LPC and PA to be 3.1 and 2.2 nm respectively, we can predict very well the measured spacing of both lamellar phases; The double-bilayer L_{βββ} with 10.9 nm and the L_{iββ} with 8.5 nm assuming it to show interdigitation of the fatty acid chains, i.e. a PA-interdigitated double-bilayer.

The lysolipid and the palmitic acid have the same chain lengths, so the two bilayers forming the double-bilayer should have similar response to thermal activation. Moreover, the thin water regions i.e., the head group interfaces, are also equivalent; Figure 4III, contributing to the stability of this double-bilayer arrangement.

The isotropic-lamellar phase transition involves a drastic change in the topology of the mesogenic units, therefore equilibration time affects the stability of this double-bilayer arrangement.

Preparation of LPC/PA mixtures in excess water conditions and room temperature, leads to a initial behaviour indeed similar to that of DPPC. This implies that the main transition of the LPC is overlaid by the interaction with PA which in counterpart inhibits its micellization. As a result one has the formation of the lamellar gel structure. Increasing the temperature overpowers the interaction between the components of the mixture, leading initially to the formation of the double-bilayer and later to a lamellar-isotropic phase transition. This overpowering is best seen on slow cooling, Figure 1: At the phase transition, the bilayers form and organize into the double-bilayer arrangement, setting the basis for the PA interdigitation seen upon further cooling.

To support this concept, we observe the role of curvature, e.g. rim of defects in lamellae, in fat metabolism. The formation of synaptic-like microvesicles - SLMV- is affected by PLA2. It was concluded that this enzyme reduce the basal SLMV formation when antagonizing the activity of endophilin 1 in the conversion of lysophosphatidic acid to phosphatidic acid, condition for the SLMV formation.22 Paton observed the formation of a lamellar phase followed by an appearance of a viscous isotropic phase,23 most likely of cubic symmetry, upon hydrolysis of emulsified fat droplets. In terms of kinetics, it has been observed that the activation rate of the PLA2 enzyme during the initial phase increases with decreasing phospholipid vesicle size.24

We suggest that due to the strain from curvature, vesicles or micelles, can only temporarily accommodate the products of fat digestion, prior to the formation of a double-bilayer. Indeed, the formation of this phase, with very similar characteristics to the double-bilayer in LPC/PA-mixtures, due to PLA2 activity on DPPC at 42.1 °C has been observed,25 Thus, either reaction products or a simple mixture of components yields the same phases - the formation of double-bilayer structures, L_{iββ} and L_{ββ}. Moreover, Jain et al have observed that the incorporation of products from PLA2 activity in substrate dispersions increases the binding affinity rather than increase the rate of binding.26 These observations were consistent with their hypothesis that pancreatic enzyme binds to defect sites at the phase boundaries in substrate bilayers induced by the PLA2 activity products.

We may speculate about the biological significance of the double bilayer structure since the vast majority of organelles indeed have a two bilayer membrane. The inner and the outer membranes are known to be of different lipid composition. The double bilayer discussed here may thus be further used when a simple model of a cell membrane is required.

CONCLUSION

We consider the interdigitation of the fatty acid to be a slow process, reflecting the time necessary to build up the long range order, characteristic of both lamellar double-bilayers L_{iββ} and L_{ββ}. Comparing the WAXS and SAXS (I_{ββ}) scattering peaks can make this point clearer. The I_{ββ} SAXS peaks are relatively weak and broad throughout, while upon the bilayers formation one sees a quicker rising of the WAXS peak that is associated with the chain ordering common to both double-bilayer phases, see Figures 1, 3, 4III and 1S (Supplementary Material). The relative contribution of each phase can be followed examining the increase in the ratio between intensities of the peaks in the WAXS and SAXS regions, I_{WAXS}/I_{SAXS}. Figure 1S. The excess increase from the WAXS intensity represents the contribution from the non-interdigitated L_{iββ} double bilayer.

Comparing the temperature range of changes in the WAXS d-spacing and the integral intensity of this reflection allows us to look at two independent processes. A small decrease in d-spacing reflects a thicker packing of the paraffinic chains (Figure 1) and being independent of the increasing integral intensity, associated with an improved long range order of these chains (Figure 2S), shows that the two processes are decoupled.
SUPPLEMENTARY MATERIAL

In http://quimicanova.sbq.org.br

ACKNOWLEDGEMENTS

We thank Drs V. M. Haramus and L. A. S. A. Prado for the support in the attempt to identify and characterize micelles we believed to exist at high temperatures.

REFERENCES AND NOTES

15. The nomenclature proposed for this phase is based on Luzatti’s nomenclature for liquid crystals [see Luzatti, V.; In Biological Membranes, D. Chapman Ed.; (Academic Press, London, 1968)] and considers the following. L indicates a lamellar structure. A subscript b indicates a bilayer (b) and (β) the paraffinic chains with all-trans configuration. Similarly the subscript i indicates a layer of interdigitated (i) paraffinic chains also with all-trans configuration (β). The first pair is assigned to the LPC bilayer and the second to the PA (bi)layer. We would like to remark that the term double bilayer has already appeared in the literature (Bouwstra, J. A.; Thewalt, J.; Gooris, G. S.; Kitson, N.; Biochemistry 1997, 36, 7717) but not related to a lamellar structure possessing two different bilayers per unit cell.
DOUBLE-BILAYER: A NEW PHASE FORMED BY LYSOPHOSPHOLIPIDS AND THE CORRESPONDING FATTY ACID

Sérgio S. Funari*#  
EMBL Outstation Hamburg, DESY  
Gert Rapp#2  
EMBL Outstation Hamburg, DESY  
Frank Richter  
EMBL Outstation Hamburg, DESY, Dept. of Physics, E22 Biophysics, TU Munich, D-85748 Garching, Germany

Figure 1S. Ratio between the integral intensities of the WAXS and SAXS peaks during the temperature scan. Note the effect of 30 minutes equilibration at 35.5°C, changing from a predominantly forming process to an ordering one. At the same time the interdigitated double-bilayer becomes visible (see Figure 1)

#Present address: HASYLAB at DESY, D-22607, Notkestr. 85, Hamburg, Germany.
*e-mail: sergio.funari@desy.de
#2 Present address: Rapp OptoElectronic, Gehlenkamp 9a, 22559 Hamburg, Germany