

ARTICLE

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Induction of a hexagonal phase in phospholipid-surfactant bilayers

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Abstract The possibility of modifying the curvature of lipid bilayers by mixing them with additives is demonstrated and the evolution of geometrical parameters with composition is discussed. X-ray diffraction patterns of the POPC/C₁₂EO₂/H₂O system were observed as a function of the relative humidity. The formation of an unexpected hexagonal phase indicates peculiar behaviour in these mixtures. The cylinder radius of this phase is considerably smaller than previously observed in cubic phases. A discussion of the head group interactions is presented. We have also been able to show that the uptake of water by the L_β gel phase is higher as a single phase than in the L_β+H_{II} two phase region. The water content is important for the stabilization of H_{II} phase and determination of its characteristic dimensions. However, it is argued that the interaction between the surfactant and the lipid is the key factor for its formation and that the EO₂ head groups displace water from the inner parts of the polar region of the mesogenic units.

Key words Gel phase · Micelles · Aggregation number · X-ray · Hydration · Geometrical parameters

Abbreviations L₂ isotropic inverted micellar · V₂ inverted bicontinuous · L_α lamellar liquid crystalline · L_β lamellar gel · L_δ lamellar with the hydrocarbon chains showing an orthorhombic arrangement · H_{II} inverted hexagonal · POPC 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylcholine · DSPE distearoyl-phosphatidylethanolamine · DHPE dihexadecyl-phosphatidylethanolamine · C₁₂EO₂ di-ethylene-oxide-mono-dodecyl-ether

Introduction

Lipid bilayers are the support matrix of different membranes in a living cell and contain many other functional molecules inserted in them, such as proteins and sugars (Cevc and Marsh 1987). They represent the simplest equivalent of a cell membrane. The inclusion of some additives modifies the balance of forces responsible for their stability. Depending on the nature of the additives, phases with different morphologies can be induced. Non-lamellar phases are the most interesting ones because the mesogenic units show considerable differences (Fig. 1). The tendency of bilayers to change into morphologies as in the hexagonal phase seems to activate membrane-bound proteins (Epand 1992) as well as to increase the rate of membrane fusion (Ellens et al. 1989). Cubic phases also play an important role in activated processes, such as fusion, due to curvature (Chung and Caffrey 1994 and Funari et al. 1996). A recent description of the biological significance of lipid polymorphism has been presented by Luzzati (1997).

POPC does not show curved morphologies at room temperature or at different levels of hydration. At 25 °C it forms different lamellar structures upon increasing the relative humidity RH, going from L_δ for dry lipid to L_β, and finally for RH>0.47 to L_α with interplanar repeat distance d₀=55 Å. In the L_δ and L_β phases the alkyl chains of the lipid molecules prefer to have the all-trans conformation (Katsaras et al. 1993, Volke et al. 1982, Mantsch and McElhaney 1991).

In contrast to the lipid, the surfactant C₁₂EO₂ at the same temperature shows a strong tendency to form curved surfaces, such as L₂ and V₂ (Conroy et al. 1990, Funari and Rapp 1997) and does not form gel or hexagonal phases.

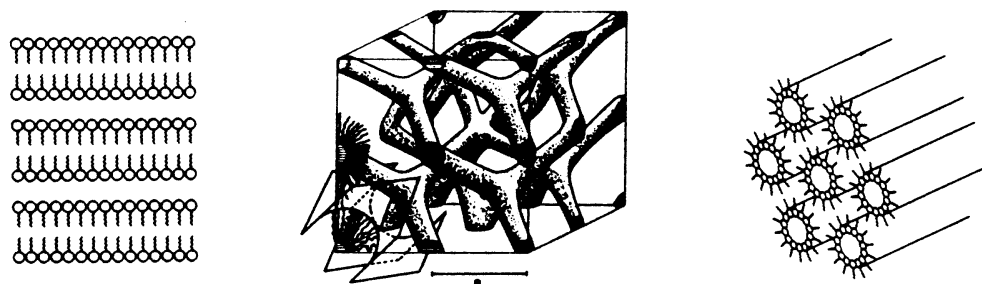
Materials and methods

POPC was purchased from Avanti Polar Lipids, USA and C₁₂EO₂ from Nikko Chemicals, Japan. Both components

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Fig. 1 The most frequent liquid crystalline phases, illustrating their respective morphologies: (left) lamellar phases L_α and L_β have planar surfaces while (middle) cubic and (right) hexagonal have curved surfaces



were of high purity and were used without further treatment. The desired amounts were weighed and dissolved in pure methanol. The latter was removed under vacuum $<10^{-4}$ Torr, for at least 4 hours.

X-ray diffraction from powder samples of known amphiphile composition at defined relative humidity, RH, gives the lattice spacing, characteristic of each phase, while sorption isotherms indicate the amount of water hydrating the amphiphiles. These values enable one to calculate the geometrical parameters of the mesogenic units present in different phases. The measurements were performed using a commercial Philips source with a pin-hole setup and a one-dimensional position sensitive detector (PSD type OED-M50, from Braun, Germany). The temperature was controlled by a water bath with an accuracy of $\pm 0.1^\circ\text{C}$. The samples were hydrated in a vapour dense chamber in the x-ray beam path, by exposing them for at least 10 hours to saturated solutions of pure salts at a constant temperature of $25.0 \pm 0.1^\circ\text{C}$.

The sorption was measured in samples placed in sealed vessels, each with a different RH, adjusted using saturated salt solutions (Becker et al. 1969, Klose et al. 1992 and Rand and Parsegian 1989) and kept at $25.00 \pm 0.02^\circ\text{C}$. After equilibration for 72 hours the amount of sorbed water was determined gravimetrically (isopiestic method).

Results and discussion

NMR studies in mixtures of $\text{POPC}/\text{C}_{12}\text{EO}_2/\text{H}_2\text{O}$ identified a wide range of different phases. Large amounts of one of the components impose a predominant behaviour on the system, while at intermediate compositions the samples showed proper behaviour by forming an inverted hexagonal phase (Funari and Klose 1995). The interest in this structure derives from the observation that samples of neither lipid nor surfactant alone form an hexagonal phase in water, therefore its presence in this system is a consequence of interactions present only in the mixture.

The dependence of the lattice spacing on the relative humidity for different surfactant to lipid molar ratios, $R_{S/L}$ was determined and examples can be seen in Fig. 2. The structures observed and the transition between them have a strong dependence both on $R_{S/L}$ and RH.

Samples containing lipid to surfactant molar ratio $R_{S/L} = 0.3$, between $\text{RH} = 0.11$ and 0.33 show an L_β phase with

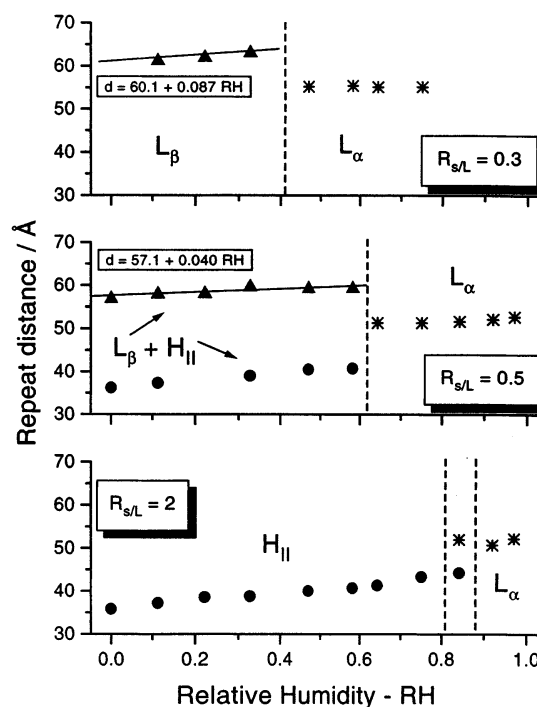


Fig. 2 Variation of the interplanar distance with the relative humidity RH for different surfactant to lipid molar ratios $R_{S/L}$ ($\text{C}_{12}\text{EO}_2/\text{POPC}$) at 25°C . The corresponding phases and molar ratios are indicated for each case. The dashed lines represent phase transitions whose position are estimated. For the gel, L_β , phase the water uptake is significantly higher when it occurs as a single phase than in a $L_\beta + H_{II}$ two phase region

small increasing repeat distances. It transforms into lamellar L_α at $0.33 < \text{RH} < 0.48$, with $d_0 = 55 \text{ \AA}$. The melting of the hydrocarbon chains on going from rigid, mostly all trans conformation, to liquid like is responsible for the phase transition.

Increasing surfactant to $R_{S/L} = 0.5$ produces considerable changes in the system, which acquires a proper behaviour. For low water content one finds a two-phase region $L_\beta + H_{II}$, see Fig. 2. The hexagonal phase, H_{II} , can be classified as such because it occurs even in dry samples; no water present in the system. It is composed of a two-dimensional hexagonal array of long rods (or cylinders) with the hydrophobic part containing the polar head groups (and water) pointing towards the centre. These phases coexist at $\text{RH} \leq 0.58$ and at higher humidities a phase transition to

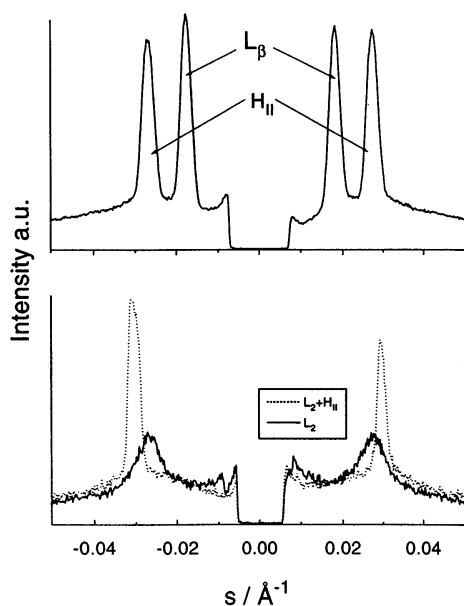


Fig. 3 Small angle x-ray diffraction patterns. Top. The L_{β} and H_{II} phases for surfactant to lipid molar ratio $R_{S/L}=1$ at 25°C and $\text{RH}=0.32$. Bottom. The L_2+H_{II} biphasic region phase (dotted line) and single L_2 phase (solid line) measured at 25°C . The dotted pattern corresponds to, $R_{S/L}=3$ and $\text{RH}\sim 0$. The sharp and intense peak is associated with the H_{II} phase and overlaps a broad and weak one associated with the L_2 phase. The solid line is associated with the L_2 phase and shows that although it consists of a broad peak, a repeat distance corresponding to the average distance between micelles can be measured. The sample had a molar ratio composition of $R_{S/L}=5.6$ and $\text{RH}\sim 0$.

a single L_{α} , $d_0=52\text{ \AA}$ takes place, demonstrating a lateral extension of the bilayer with the increase in the amount of water sorbed.

At $R_{S/L}=1$ we observed a gel and hexagonal two phase region for $\text{RH}\geq 0.48$, see Fig. 2. At $\text{RH}=0.58$ an additional peak is seen corresponding to a repeat distance of 57 \AA which lies between those of the gel and lamellar L_{α} phases. Most likely it reflects thermodynamic non-equilibrium. One must note that slow kinetics for hydration of lipid systems have long been reported (Finner and Darke 1974, Gawrisch et al. 1985). Increasing the humidity further, at $\text{RH}\geq 0.84$, only a single lamellar L_{α} phase is observed up to $\text{RH}=0.97$ with repeat distance of 52 \AA . Alternatively it could be due to the formation of an intermediate phase (Funari et al. 1994, 1997).

A single hexagonal phase is seen from $\text{RH}=0$ until $\text{RH}>0.75$ for $R_{S/L}=2$ (1:1 in terms of alkyl chains) when it transforms via a two phase region into a single L_{α} . This indicates that around this molar ratio the balance of interactions between the amphiphiles present provides the optimum conditions for the formation of the hexagonal phase in this system, where none of the components exerts a predominant behaviour. The repeat distance of the H_{II} phase changes from 36 \AA at $\text{RH}\sim 0$ to 44 \AA at $\text{RH}=0.75$. The L_{α} phase shows a repeat distance of 52 \AA .

Higher surfactant content, $R_{S/L}=3$, leads to a biphasic region, $H_{II} + L_2$, for $0 < \text{RH} < 0.58$. Above this humidity,

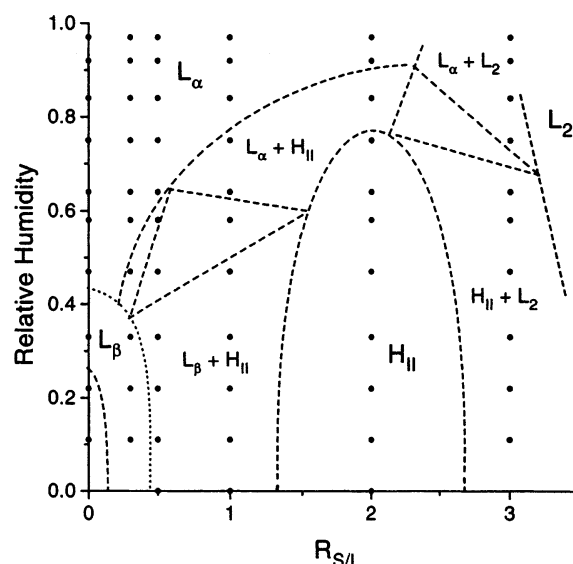


Fig. 4 Partial phase diagram at 25°C from mixtures of POPC and C_{12}EO_2 in relation to relative humidity, for the range of molar ratios $R_{S/L}\leq 4$. The estimated borders between different phases are drawn with dashed lines

until $\text{RH}=0.84$, only the hexagonal phase occurs. The characteristic x-ray diffraction pattern, Fig. 3 (bottom), shows a maximum whose position is related to the average distance between two micelles in contact. Both H_{II} and L_2 phases have a similar spacing, with the hexagonal showing a sharp peak overlapping the broad one associated with the L_2 phase. The spacing observed for the hexagonal phase in the two phase region changes with increasing humidity, going from 33 to 42 \AA . The swelling is attributed mainly to increasing numbers of water molecules at the centre of the rods of the hexagonal phase. This is supported by the observation of the same swelling behaviour obtained for $R_{S/L}=1$ where no L_2 phase was present.

At $R_{S/L}=4$ and $R_{S/L}=5.6$, Fig. 3 (bottom, solid line) at $\text{RH}\sim 0$ only broad peaks were seen, corresponding to 33 \AA . Assuming that the components are incompressible and the micelles are distributed in a face centred cubic lattice, we estimate their diameter and aggregation number. Taking the volume of POPC (König 1993) and C_{12}EO_2 molecules to be 1260 \AA^3 and 504 \AA^3 respectively, we find 50 amphiphile molecules per micelle; of which 10 are lipids and 40 surfactants, with diameter of 40 \AA .

Phase diagram

Figure 4 shows that at low surfactant contents the lipid behaviour predominates while at high amounts the system's behaviour approximates the behaviour of the surfactant. This is not the case for intermediate compositions.

For $\text{RH} < 0.47$, increasing surfactant leads to an increase in the curvature of the phases formed. This can be attributed to a combined effect of the smaller size of the

surfactants' head group together with the mismatch in size of the alkyl chains from the surfactant and lipid. The hexagonal phase occurs as a single phase at RH below 0.84 and $R_{S/L}=2$. At this amphiphile molar ratio the mixture reaches a balance of interactions favouring the formation of the hexagonal phase and the mixture behaves as a pseudo single component. Increasing the water contents enhances the general tendency to form flat surfaces as in the lamellar L_α phase, at least up to $R_{S/L}=2$, from where the L_2 phase predominates.

It is worth noting the phase transition between the L_β and H_{II} phases, as well as their coexistence in the two phase region. Intuitively the melting of the hydrocarbon chains in the L_β phase would lead to a lamellar L_α phase. However, an L_β to H_{II} phase transition is less common and less intuitive. Nevertheless, it has been seen in saturated NaCl solutions of DSPE and DHPE where it has been demonstrated that the alkyl chain melting and the phase transition L_α to H_{II} occur coincidentally (Marsh and Seddon 1982).

The formation of the hexagonal phase can be explained by considering that the EO_2 head group of the surfactant binds to the lipid molecules, replacing water. The combination of dipolar and van der Waals interactions enables the system to overcome the high lateral pressure among the individual molecules, intrinsic to the inverted hexagonal phase, stabilizing it at low amounts of water in the system.

The observed sequence of phases upon hydration of L_β corresponds to an initial increase in the surface area occupied by the molecules, releasing the packing constraints in the hydrophobic core, therefore favouring the formation of phases with "melted chains". The extent of expansion is larger in the hydrophobic moiety because of the stronger interaction between the head groups, leading to a transition to the H_{II} phase. A similar degree of expansion in the hydrophobic and hydrophilic moieties would lead to the formation of an L_α phase. For the same RH, at low $R_{S/L}$, one does not see the H_{II} phase or it occurs in a two phase region. For $R_{S/L}=1$, upon hydration of the two phases the gel vanishes at lower RH than the H_{II} phase. A single H_{II} phase is only observed at $R_{S/L}\sim 2$.

Further sorption of water increases the rods' diameter, decreasing the lateral pressure, finally promoting the phase transition from H_{II} to L_α , Fig. 2. A strong dependency of the interplanar distance on the water content has been assigned as typical of hexagonal phases of the inverted type and can be used to identify them (Seddon 1990).

Increasing the humidity of samples containing the L_β phase at different $R_{S/L}$ increases the interplanar distance linearly, Fig. 2. The slope for a single L_β phase is higher than in the $L_\beta + H_{II}$ two phase region. In this region the water sorbed has to be distributed between the L_β and H_{II} phases and the latter shows higher affinity for it. The H_{II} phase has the polar head groups in the centre of the rods subject to a higher lateral pressure than in the L_β phase. Therefore the hydration of the hexagonal phase is favoured compared to the gel phase.

The realization that all phases showing curved surfaces have liquid-like chains indicates that they also play an im-

portant role in the stabilization of the H_{II} phase, owing to the mismatch between the lengths of alkyl chains from the lipid and surfactant. Upon melting, the longer alkyl chains of the lipid can fill the voids due to the shorter chains of the surfactant. In this respect the molar ratio $R_{S/L}$ also plays an important role in promoting the transition.

The hexagonal phase is also observed in the neighbourhood of the L_2 phase; indeed a two phase region was observed. Although there are no scattering planes in the L_2 phase, we can see in Fig. 3 (bottom) that peaks in this and in the hexagonal phase correspond to similar repeat distances, relating the centre of two spherical micelles to the centre of two neighbouring cylinders in the hexagonal phase. This concept has not been definitely proved, although it is in agreement with the work of Amaral et al. (1992) where they proposed the formation of rod shape micelles in the micellar phase near the transition to hexagonal. In this approach, the formation of the H_{II} phase corresponds to a unidirectional growth of micelles from the L_2 phase.

Knowing the sorption isotherms and using the repeat distance measured for the H_{II} phase of $R_{S/L}=2$ at RH=0.11, we calculated the lattice parameter (Marsh 1990) according to $a=2*d_0/\sqrt{3}=43 \text{ \AA}$ which in this case equals the diameter of the cylinders, using a weighted average for the molecular weight of the amphiphiles and assuming the density of the lipid to be 1. These results compare well with the hexagonal phase (Gawrisch et al. 1992) of DOPE, 22 to 48 \AA^2 for 5...30 wt% water. This behaviour, i.e. small areas and strong dependence on the water content, illustrates that the geometrical parameters of the H_{II} phase are mainly determined by the amount of water present in it, in particular at low humidities.

Using the same formalism we calculated the area occupied by an "effective molecule of amphiphile" at the water interface in the lamellar L_α phase, obtaining 105 \AA^2 for $R_{S/L}=2$ and RH=0.97. Considering volume additivity and taking 65 \AA^2 for POPC in this phase (König 1993) we obtain 40 \AA^2 for the surfactants' surface area, which gives 20 \AA^2 per $C_{12}EO_2$ molecule. These parameters are similar to the ones obtained for the L_α phase (Klose et al. 1992) of egg phosphatidylcholine and $C_{12}EO_2$ at $R_{S/L}=0.07$.

For all molar ratios $R_{S/L}$ studied the repeat distance of the lamellar L_α phase is practically constant until RH=0.97. Above that there is a vertical swelling of the lamellar stacks (Klose et al. 1988) with incorporation of water. The EO_2 groups enhance this effect by competing for the binding sites of the lipid molecules, restricting the access of the water molecules to the hydrophilic region. This way, as more water is added to the system the larger becomes the aqueous layer separating two consecutive lamellae.

POPC assemblies illustrate that lipids in general can acquire almost any morphology desired, ranging from flat surfaces to spheres, via cylinders of different characteristics. Obviously a compromise between temperature and chemical purity has to be found. For example, POPC/ $C_{12}EO_2$ mixtures provide a wealth of curved surfaces with radius from 20 (micelles) to 29 \AA (cubic Ia3d)

(Funari and Klose 1995), depending on the structure and its water content.

It is interesting to note that the surfactant is apparently not important for the characteristic geometric parameters of the H_{II} phase. It plays a definite role in whether this phase forms or not, but water seems the decisive factor in the extension of its stability range and the characteristic dimensions of the mesogenic units, specifically the radius of the cylinder.

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