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X-RAY DIFFRACTION AND NMR STUDIES ON MIXTURES OF NON-IONIC SURFACTANT (C₁₂EO₂) AND PHOSPHOLIPIDS (POPC)

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We studied the temperature and concentration dependence of the POPC/C₁₂EO₂/water ternary system and identified and characterized the different phases occurring. The thermal behaviour is slightly dependent on the surfactant to lipid molar ratio $R_{s/l}$. At low surfactant content the behaviour is similar to the binary lipid/water system. Similarly at low lipid contents the system behaves similarly to the surfactant/water mixture. However, at molar ratios $R_{s/l}$ between 2 and 3 a new hexagonal phase forms which is peculiar to the mixture. ²H NMR measurements indicated the existence of a lamellar phase containing also curved surfaces additional to the flat ones. The surface area per surfactant molecule was calculated to 0.23 nm² which is in good agreement with the cross-section of a paraffinic chain.

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1. Introduction

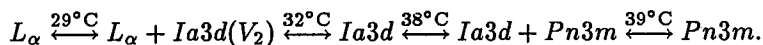
Phospholipids and surfactants are amphiphiles and self-aggregate due to the hydrophobic effect [1] when dispersed in water. The molecules constituting the aggregates have their paraffinic chains forming an inner core and the polar head groups are at the interface between the hydrophobic and hydrophilic environments. Depending on thermodynamic variables they show a rich variety of structures ranging from micellar, lamellar, hexagonal and cubic phases. These are composed of

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small isotropically distributed aggregates, stacked layers, infinite cylinders packed on a two-dimensional hexagonal array and three-dimensional networks of interwoven but otherwise unconnected systems of cylinders, respectively [2-4]. Amphiphile/water systems can also form the so-called inverted phases where the rods forming the inverted hexagonal H_{II} , or inverted cubic V_2 phases have the head groups facing inwards (negative mean curvature) [3]. Gel phases are composed of lamellar stacks of bilayers with the alkyl chains predominantly in the all-trans configuration which gives a characteristic X-ray diffraction peak in the wide angle (WAXS) regime at $(1/0.42 \text{ nm})^{-1}$ and thus enables to differentiate between the L_α phase, where the alkyl chains are liquid like.

Phospholipid dispersions are the basic model systems for cell membranes because of their tendency to forming bilayers. The incorporation of additives into the bilayer matrix modifies the interaction between the lipid molecules. Addition of non-ionic surfactant of the type C_nEO_m induces modifications mainly in the polar head group region and has the advantage of not introducing charges into the system. The extension of such modifications is strongly dependent on the structure of the additive and on the relative composition. It has been found that in the mixtures new phases can form which are not present in lipid/water or surfactant/water binary mixtures, respectively, alone. An example can be found in the POPC/ $C_{12}EO_2$ /water [5], which shows an inverted hexagonal phase which does not occur in aqueous binary systems of these amphiphiles [6].

In the present study a combination of 2H NMR and X-ray scattering was used to identify the phases and, moreover, their geometrical parameters in the ternary system POPC/ $C_{12}EO_2$ /water. The temperature and concentration dependence on the phases and their interconversion, respectively, were determined. Parts of this system have been studied before [7] to determine the phase transition temperatures when cubic phases and their respective structures occur. E.g. taking a sample with composition (in wt%) $H_2O(46.3)$, $C_{12}EO_2(46.0)$, POPC(7.6), $R_{s/l} = 17$, the thermal sequence showed the following phases and respective phase transition temperatures both on heating and cooling scans:



Starting from a lamellar L_α phase at $20^\circ C$ and raising the temperature two subsequent forming bicontinuous cubic phases were observed. The first cubic phase formed from the L_α phase was identified as $Ia3d$ which is of the gyroid type. The following cubic phase has a primitive unit cell and belongs to the space group $Pn3m$. The $Ia3d$ phase has a lattice parameter of $a = 15.0 \text{ nm}$ and the rod length and radius were calculated to 5.3 and 2.9 nm, respectively. For the $Pn3m$ phase the parameters were 9.3, 8.1 and 2.8 nm, respectively. It can be seen that the radius of the cylinder is similar in both cubic phases and corresponds to half of the bilayer thickness. We could also see that the three phases are epitaxially related through a repeat distance of $s = 1/d = 1/5.4 \text{ nm}^{-1}$, thus relating the (001) plane of the L_α to the (220) of the $Ia3d$ and the (111) of the $Pn3m$ phases, i.e., $d_{001}(L_\alpha) = d_{220}(Ia3d) = d_{111}(Pn3m)$.

In this system the transition between these two cubic phases is a Bonnet transformation, since it is adiabatic and has a metric relation between their lattice

parameters of $a_{Ia3d}/a_{Pn3m} = 15.0/9.3 = 1.61$, close to the theoretical value of $1.11\sqrt{2} = 1.57$ for a transformation from a cubic phase of the gyroid type to one of the diamond type, $C_g \rightarrow C_d$. We have argued [7] that lamellar and inverted cubic structures coexisting as a two-phase region supports the concept of transport across the bilayer (membrane in the case of cells) via a network of rods displaced in a cubic array. It also suggests that biological processes such as fusion between membranes have an intermediate state which shows cubic topology.

^2H NMR of $^2\text{H}_2\text{O}$ has shown compatible results and supports the X-ray observations [8]. In the lamellar phase they were Pake powder spectra with typical quadrupolar splittings of $\Delta\nu = 1.2$ kHz, increasing to $\Delta\nu = 1.4$ kHz in the $L_\alpha +$ cubic two-phase region. The cubic phase was characterized by a single peak at zero Hz, typical of $^2\text{H}_2\text{O}$ molecules isotropically distributed. For a sample containing surfactant to lipid molar ratio $R_{s/l} = 2.9$ the quadrupolar splitting decreased linearly with increasing water contents up to 29 water molecules per amphiphile, $R_{w/s+1} = 29$. Above that it was practically constant. For samples containing the same amount of water, i.e., 30 wt%, the quadrupole splitting increased nonlinearly with decreasing molar ratio $R_{s/l}$.

2. Materials and methods

X-ray experiments were done using either a pinhole camera in a commercial source or time-resolved at beam line X-13 of the EMBL Outstation at DESY, Hamburg. In both cases one-dimensional position sensitive detectors (PSD) were used. A detailed description of this beam line can be found elsewhere [8]. ^2H NMR spectra were obtained in a Bruker MSL 500 spectrometer operating at 11.74 T.

Palmitoyl-oleoyl-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids, USA and the surfactant di-ethylene-oxide-mono-dodecyl-ether (C_{12}EO_2) from Aldrich, Germany, or Nikko Chemicals, Japan, with purity higher than 97%. The α -deuterated surfactant ($\text{C}_{12}\text{EO}_2\text{-d}_2$) was home prepared and the purity was checked by NMR and chromatography, being higher than 97%. Other chemicals were used as supplied from the manufacturers.

For the static X-ray measurements at fixed temperatures samples were equilibrated for 30 minutes before the diffraction patterns were recorded. During time-resolved measurements, temperature scans of typically $0.5^\circ\text{C}/\text{min}$ were used where a 10 s exposure was recorded every 30 s. The temperature was controlled by a circulating water bath.

The samples were prepared by weighing the desired amount of each component into a tube and vortexed extensively at different temperatures. Later 1 mm diameter Lindeman capillaries were filled and flame sealed and then stored at 4°C .

3. Results and discussion

Initially samples were investigated in the concentration region where the phase transition between $L_\alpha + W$ and L_α was expected. ^{31}P NMR show typical spectra of the lamellar L_α phase with 47 ppm chemical shift. However, the ^2H NMR spectra of $^2\text{H}_2\text{O}$ did not show a quadrupolar splitting, but instead a triangular-like line shape. These results have been associated with the existence of highly curved surfaces on the mesogenic units. The morphology of these units can be thought of

either as small liposomes polydisperse in size [5, 9] or uncorrelated disruptions in multilamellae [10, 11].

4. Hydration

The addition of water to the system was followed in two ways: (i) either by preparing samples with the desired composition, or (ii) in the case of ^2H NMR from the α -deuterated surfactant by diluting the initial sample. This latter procedure has the advantage of keeping the molar ratio $R_{s/l}$ constant.

A sample containing the α -deuterated surfactant (composition in wt%: $\text{H}_2\text{O}(10.0)$, $\text{C}_{12}\text{EO}_2\text{-d}_2(57.7)$, $\text{POPC}(32.3)$, $R_{s/l} = 5.0$) prepared with deuterium depleted water gave a powder pattern with a quadrupolar splitting of $\Delta\nu = 5.0$ kHz at room temperature. The ^{31}P NMR spectra of the same sample under the same conditions showed a line shape typical of a hexagonal phase. Diluting this sample to $\text{H}_2\text{O}(15.1)$, $\text{C}_{12}\text{EO}_2\text{-d}_2(54.4)$, $\text{POPC}(30.5)$, $R_{s/l} = 5.0$ showed a spectrum with multiplets whose $\Delta\nu$ were 16.5, 11.2 and 5.7 kHz, respectively. This corresponds to a multiphase region. The smallest splitting is related to the hexagonal phase while the other values are attributed to the lamellar phase. We interpret the larger splittings as arising from molecules sitting in different parts of the lamellar mesogenic units containing different curvatures. The 16.5 kHz is associated with molecules in a planar surface. This can be an infinite bilayer or a bilayer from liposomes with large radius. The quadrupolar splitting of 11.2 kHz is associated with molecules diffusing in a curved surface. Again, size polydispersity of liposomes or disruptions in the lamellar bilayer structure can explain the existence of two splittings in a single phase. This shows that at compositions near the $L_\alpha + H_{II}$ and L_α phase boundary we have mesogenic units showing a morphology intermediate between flat and highly curved ones. Note however that this is not necessarily related to intermediate phases occurring between hexagonal and lamellar phases which are thermodynamically stable. Further increase in the water concentration to 50% H_2O and $\text{C}_{12}\text{EO}_2\text{-d}_2(32.1)$ and $\text{POPC}(18.0)$ ($R_{s/l} = 5.0$) shows a ^2H NMR Pake powder spectra with $\Delta\nu = 16.6$ kHz typical of a single lamellar phase. From this last sample spectra were recorded at different temperatures between 29 and 50°C. In all cases a single line was observed, indicating an isotropic structure (bicontinuous cubic phase in this case).

At a surfactant/lipid molar ratio $R_{s/l} = 2.0$ ($\text{H}_2\text{O}(10.0)$, $\text{C}_{12}\text{EO}_2\text{-d}_2(37.7)$, $\text{POPC}(52.4)$) the recorded ^2H and ^{31}P NMR spectra at room temperature are typical of a lamellar phase. The quadrupolar splitting was $\Delta\nu = 13.6$ kHz. Dilution to $\text{H}_2\text{O}(12.4)$, $\text{C}_{12}\text{EO}_2\text{-d}_2(36.6)$, $\text{POPC}(51.0)$ increases $\Delta\nu$ to 16.8 kHz. The quadrupolar splitting is not affected by further dilution to $\text{H}_2\text{O}(49.0)$, $\text{C}_{12}\text{EO}_2\text{-d}_2(23.0)$, $\text{POPC}(32.0)$. Since the quadrupolar splitting of ^2H is an indication for the average curvature in the system, i.e., small quadrupolar splitting indicates high curvature, these results also show that at low hydration the mesogenic units present higher curvature. The maximum value of the quadrupolar splitting for the lamellar phase is $\Delta\nu \approx 16$ kHz.

The structures observed are strongly dependent on the molar ratio between surfactant and lipid. At molar ratios $R_{s/l} \approx 0.5$ a lamellar gel L_β phase is found, while at $R_{s/l} \geq 3.8$ a so-called L_2 phase is observed, which we assume to formed

by inverted micelles. Intermediate $R_{s/l}$ values give rise to an H_{II} phase. In all cases addition of water leads to the formation of a lamellar phase, although not always as a single phase.

For a fixed molar ratio of $R_{s/l} = 0.28$ we evaluated the effect of hydration at room temperature (see Fig. 1). At low water content a hexagonal H_{II} phase is observed with spacings decreasing slightly with increasing water content. At 10.9 wt% ($R_{w/s+l} = 2.3$) $d_{10} = 3.76$ nm while at 15.5 wt% ($R_{w/s+l} = 3.5$) it is 3.67 nm. The hexagonal phase is associated with the inverse type because it was observed previously [3, 5] in samples where no water has been added. In such a situation the polyoxyethylene groups are expected to play the role of water. The corresponding lattice parameters are 4.34 and 4.23 nm, respectively. In the inverted hexagonal phases the lattice parameter is equal to both the diameter of the rods and their nearest neighbour distance.

Increasing the water concentration drives the system into an $H_{II} + L_{\alpha}$ two-phase region and upon further addition of water a single lamellar phase [5] is reached. As shown in Fig. 1, at 34.4 wt% of water ($R_{w/s+l} = 10.0$) a lamellar phase forms with a repeat distance $d_{001} = 5.04$ nm which increases proportionally with water concentrations up to $d = 5.49$ nm at 51.6 wt% ($R_{w/s+l} = 20.3$), see Fig. 1.

Assuming the molecular volumes to be additive and taking the densities of heavy water, lipid and surfactant as 1.0, 1.0 and 0.90 g/ml respectively, we estimated the surface area per molecule and the hydrophobic core thickness of the L_{α} phase. The surface area per amphiphile (1 POPC+0.28 $C_{12}EO_2$) was estimated

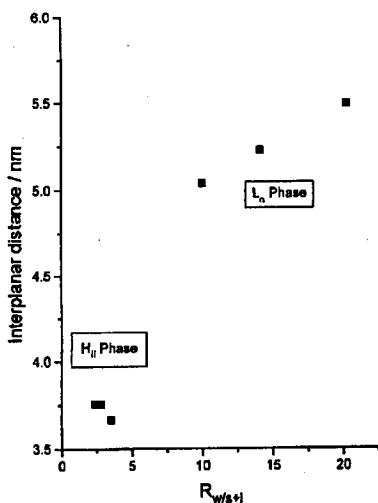


Fig. 1. Hydration dependent phase sequence for a sample containing surfactant $C_{12}EO_2$ to lipid POPC molar ratio $R_{s/l} = 0.28$. The change in the interplanar distance reflects a phase transition from an inverted hexagonal H_{II} phase to a lamellar L_{α} phase. There is a continuous uptake of water in the lamellar phase as seen by the increase in the interplanar distance.

to $S = 0.71 \text{ nm}^2$ and the hydrophobic core to $d_{hc} = 3.89 \text{ nm}$. Taking the area of a single POPC lipid molecule to $S = 0.65 \text{ nm}^2$ [12] the area per surfactant molecule is $S = 0.23 \text{ nm}^2$. This value compares well with the area of a hydrocarbon chain.

5. Thermal behaviour

Heating has shown the same qualitative effect on the phase behaviour as addition of water. Samples showing gel or hexagonal phases at around 15°C transformed into the lamellar L_α phase upon heating. In Fig. 2 the thermal evolution of the repeat distance d of the phases present is shown. For $R_{s/l} = 2$, below room temperature a gel and an inverted hexagonal phase is observed. The spacing of the gel phase decreases while the spacing of the hexagonal in the two phases increases continuously until the transition to a single H_{II} phase. For $R_{s/l} = 3$ no gel phase was observed and the behaviour of the interplanar distance measured with temperature is different, showing a minimum at $d = 3.45 \text{ nm}$. Such a behaviour requires further investigation to be fully elucidated. It is possible that also at $R_{s/l} = 3$ at low temperatures a two-phase system exists, however, with only a small and not detectable proportion of the gel phase. Alternatively, the system forms an intermediate structure. This is supported by the observation that samples containing higher surfactant molar ratios $R_{s/l}$ (see below) show isotropic phases with average distances of the same magnitude.

Depending on the surfactant to lipid molar ratio $R_{s/l}$ the inverted hexagonal phase shows no or only little temperature sensitivity. Qualitatively, it was observed

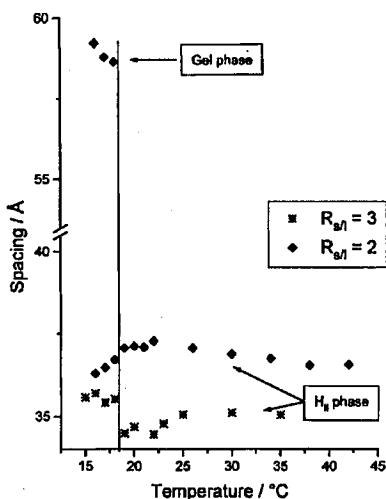


Fig. 2. Interplanar distance variation with temperature for samples with molar $R_{s/l} = 2$ and 3. $R_{s/l} = 2$ shows a lamellar gel + inverted hexagonal phase at lower temperatures which changes to single hexagonal upon heating. For the molar ratio $R_{s/l} = 3$ we only observe distances typical of the hexagonal phase although it shows an unexpected minimum (see text).

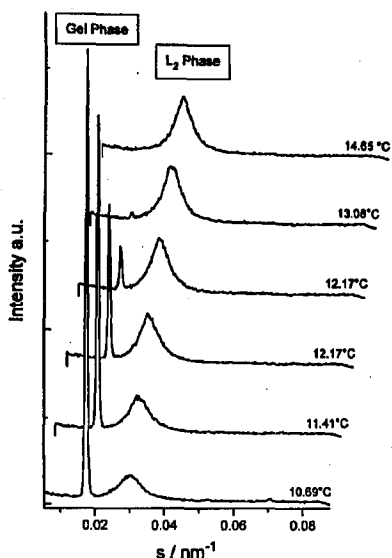


Fig. 3. Time resolved X-ray diffraction patterns on a fast cooling scan for a sample with composition in wt%, $C_{12}EO_2(58.7)$, $POPC(41.3)$ $R_{s/1} = 4.0$. Initially one observes a broad peak related to the isotropic L_2 phase. Cooling slightly sharpens and shifts them towards larger interplanar distances. The final frames show a sharp peak associated with the gel phase, whose interplanar distance corresponds to 5.70 nm. The gel phase is insensitive to changes in temperature.

that with increasing $R_{s/1}$ the thermal sensitivity decreases. As shown in Fig. 2, only small differences can be seen between $R_{s/1} = 2$ and 3, where the sample with the higher lipid concentration shows larger changes with temperature.

For $R_{s/1} = 4$ at low temperatures a phase showing a sharp and intense peak was found whose interplanar distance corresponds to $d = 5.69$ nm which most likely originates from a three-dimensional structure, Fig. 3. Heating leads to the appearance of a broad peak associated with an L_2 phase. The corresponding average distance in this phase decreases slightly with increasing temperature from 3.36 nm at 14.7°C to 3.27 nm at 64.3°C. Assuming this phase to be formed by spherical micelles, this distance can be associated with their diameter. At $R_{s/1} = 5.6$ we observed only a broad reflection centered at $1/3.60$ nm $^{-1}$ which corresponds most likely to an isotropic L_2 phase.

For much higher surfactant molar ratios in hydrated ternary systems, e.g. $R_{s/1} = 17$, no gel or hexagonal phases were observed in the range from 15°C to 65°C. A lamellar L_α phase was found from below room temperature to 29°C where it transforms into a cubic $Ia3d$ structure [7]. In both phases the lattice parameters did not show a measurable temperature dependence.

6. Conclusion

The aim of the present study was to elucidate the phase behaviour of ternary system of water, lipid and surfactant. For high lipid concentration, lamellar gel and

liquid crystalline L_α phases were found. At the other extreme of high surfactant concentration lamellar L_α and cubic phases were found. In both cases the phases formed and their thermal evolution closely matches the phase behaviour of the respective compound in a binary system with water. However, at intermediate concentrations the behaviour is much more complex. As demonstrated by the formation of an inverted hexagonal phase, which is not present in either pure binary system with water, this is a clear example of this structural behaviour peculiar to the mixtures.

^2H NMR of deuterated surfactant showed results compatible with ^{31}P NMR, X-ray diffraction, and the previous ones observed with heavy water in samples with compositions near the $L_\alpha + W$ or $L_\alpha + H_{II}$ to L_α phase transition. They gave further evidence for the concept of a lamellar phase containing different curvatures in their mesogenic units.

X-ray measurements identified the phases formed and allowed estimation of geometrical parameters. The surface area per molecule calculated for the C_{12}EO_2 in the lamellar phase assuming that the molecular volumes of the amphiphiles are additive is in very good agreement with the expected values for the single paraffinic chains.

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