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Inverse cubic liquid-crystalline phases of phospholipids and related lyotropic systems

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Abstract. We have previously found that saturated phospholipids such as phosphatidylethanolamines can, in certain cases, adopt as many as three different inverse bicontinuous cubic phases in water, of probable space groups *Ia3d* (No 230), *Im3m* (No 229) and *Pn3m* (No 224). We found that these cubic phases could be induced to appear by reducing the chain length, or by increasing the hydrophilicity of the headgroup of the phospholipid molecule. All of these cubic phases are located in the phase diagrams between the lamellar and the inverse hexagonal (H_{II}) phases. We now report the observation of a novel inverse face-centred cubic phase, of probable space group *Fd3m* (No 227), in two different systems of hydrated binary lipid mixtures. One of these systems consists of mixtures of phosphatidylcholine with diacylglycerol; the other is an acid-soap mixture of an unsaturated fatty acid with its alkali salt. This *Fd3m* cubic phase in both systems occurs between the inverse hexagonal (H_{II}) phase and the inverse micellar solution (L_2), with increasing concentration of the lipid component with the less strongly hydrophilic headgroup. We surmise that the average mean curvature of the polar/non-polar interface in this *Fd3m* cubic phase is more negative than that of the neighbouring H_{II} phase; this is quite different from the inverse bicontinuous cubic phases, where it has a value intermediate between those of the lamellar and H_{II} phases. We conclude that the structure of this *Fd3m* cubic phase most probably consists solely of closed inverse micellar aggregates.

1. Introduction

This paper extends our earlier work on phospholipids [1, 2].

There are four principal locations in the phase diagram where lyotropic cubic phases may be found (for recent reviews, see [3–6]). Normal topology (oil-in-water) cubic phases may be found either adjacent to the micellar solution (often between the normal micellar solution (L_1) and the hexagonal H_I phase) or between the H_I phase and the lamellar L_α phase. In the former case the structures probably consist of anisotropic micellar aggregates [7–9]. In the latter case the structures appear invariably to be bicontinuous. Cubic phases may also be found between the L_α and H_{II} phases. In this case, the structures are inverse (water-in-oil), and are also invariably bicontinuous. Most of our knowledge about the structures of bicontinuous cubic phases stems from the pioneering x-ray diffraction studies of Luzzati and co-workers [10, 11]. Cubic phases occurring in the fourth likely location of the phase diagram, namely between the H_{II} phase and the inverse micellar solution (L_2) have until now never been clearly identified [3, 5].

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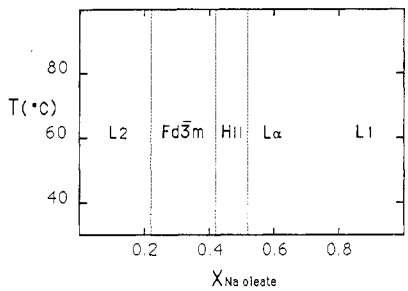

Figure 1. Schematic pseudo-binary phase diagram for oleic acid/sodium oleate in excess water. The phase boundaries are approximate, and phase coexistence regions are not indicated.

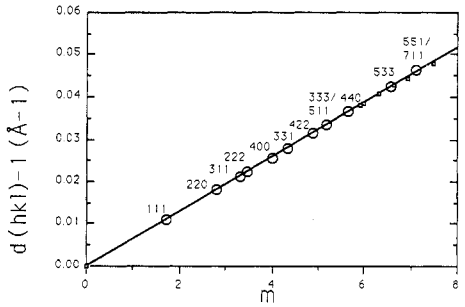

Figure 2. Indexing of the x-ray diffraction data (not shown) from a dioleoylglycerol/dioleoylphosphatidylcholine (DOG/DOPC) mixture ($c_{DOG} = 0.70$) in excess water at 30 °C. For a cubic phase, plots of $(1/d_{hkl})$ versus $m = \sqrt{(h^2 + k^2 + l^2)}$ should pass through the origin, and be linear with slope $1/a$, where a is the unit cell lattice parameter. The data (open circles) index perfectly as cubic aspect 15 (space groups *Fd3m* and *Fd3*) with a lattice parameter of $a = 153 \pm 2$ Å. The first unobserved reflection (small open squares) has indices 531.

We propose that the *Fd3m* cubic phase—at least in the two systems discovered by us—has a location between H_{II} and L_2, and that it has a structure based solely upon a packing of discontinuous inverse micellar aggregates.

The *Fd3m* cubic phase was first discovered in a lipid extract from *Pseudomonas fluorescens* [12]. Only recently, however, has a structure been proposed, on the basis of a crystallographic analysis [11, 13]. In addition to the two systems described here, one further example of an *Fd3m* cubic phase has been found, in hydrated monoolein/oleic acid mixtures [11].

2. Results and discussion

Figure 1 shows a schematic pseudo-binary phase diagram for the acid-soap system oleic acid/sodium oleate in excess water. With increasing concentration of oleic acid (the component with the less strongly hydrophilic headgroup), the lamellar L_α phase transforms first to the inverse hexagonal H_{II} phase, then to a viscous isotropic phase, then to an inverse micellar (L_2) solution. Qualitatively similar behaviour has previously been observed [14] for the hydrated double-chain lipid mixture diacylglycerol/phosphatidylcholine. We find that the diffraction pattern from the viscous isotropic (cubic) phase of the latter system [15] and the former system (data not shown) are extremely similar, in terms of the relative intensities of the various Bragg reflections. This is a striking result, given that one system is a pseudo-binary mixture of double-chain lipids, one of which contains electron-dense phosphate atoms in the headgroup, whilst the other system is a pseudo-binary mixture of single-chain lipids, with lower electron density in the headgroup region.

The indexing of the diffraction data from the viscous isotropic phase of the hydrated diacylglycerol/phosphatidylcholine system is shown in figure 2, as a plot of the reciprocal d-spacings ($1/d_{hkl}$) versus $m = \sqrt{(h^2 + k^2 + l^2)}$. For a cubic phase, such a plot should pass through the origin, and be linear with slope $1/a$, where a is the cubic unit cell lattice parameter.

A total of 11 Bragg peaks are observed, which index as the 111, 220, 311, 222, 400, 331, 422, 511/333, 440, 533 and 711/551 reflections of a cubic phase of cubic aspect 15 [16], with a lattice parameter of $a = 153 \pm 2$ Å, with no unobserved reflections below $hkl = 531$. There are only two space groups posible for this cubic aspect, namely *Fd3m* (Q^{227}), and *Fd3* (Q^{203}). In accordance with the suggestion of Luzzati and co-workers [11], we assume that the more symmetrical space group, *Fd3m* (Q^{227}), is the correct one. The difference between *Fd3m* and *Fd3* is that for a reflection with $h \neq k \neq l$, $I(hkl)$ and $I(khl)$ are equal for the former spacegroup, but different for the latter one. In order to measure these intensities separately, it would be necessary to have an aligned sample of the cubic phase. However, none of the observed reflections actually fit the criterion of $h \neq k \neq l$, and so even if a monodomain sample were available, it would only permit the discrimination to be made if further reflections such as 531, 620 or 642 could be detected. It should be emphasized that, for liquid-crystalline phases, the Bragg reflections always tend to become very weak for high *hkl* indices, because of the short-range disorder inherent in fluid phases.

3. Location of the *Fd3m* cubic phase in the phase diagram

The location of this phase in the pseudo-binary phase diagram (in the presence of an excess aqueous phase) appears to lie between the H_{II} phase and the inverse micellar solution (L_2): x-ray diffraction of pure diacylglycerol in water shows a diffuse maximum in the region of $1/d = 28$ Å^{-1} [14], indicative of micellar aggregates, undoubtedly of inverse topology. A strong argument in support of this view is that the effect of incorporating the weakly amphiphilic diacylglycerol (oleic acid) into the strongly amphiphilic phosphatidylcholine (sodium oleate) should be to reduce the effective hydrophilicity of the lipid headgroup region, and hence tend to reduce the hydration. Reducing hydration will tend to drive the phase equilibrium towards inverse phases of increasingly negative interfacial mean curvature (i.e. curvature of the interface towards the aqueous region). The fact that the cubic phase appears for higher concentrations of the weakly amphiphilic component than the H_{II} phase implies that the average interfacial mean curvature of the cubic phase must be more negative than that of the H_{II} phase, and must therefore lie 'beyond' the H_{II} phase, rather than between the lamellar and the H_{II} phase.

A noteworthy feature of this *Fd3m* cubic phase is that, like the inverse bicontinuous cubic phases *Pn3m* and *Im3m*, it can coexist with an excess aqueous phase, and thus may be of more direct relevance to biological systems than those cubic phases which have a normal (type I, oil-in-water) topology, and are not normally stable in the presence of an excess aqueous phase.

It seems likely that the requirement to form this phase is for an amphiphilic component which is weakly hydrophilic, yet which is able to interact (probably via hydrogen bonding) with the phospholipid headgroups, effectively reducing their hydration.

The bicontinuous cubic phase *Ia3d* (Q^{230}) is commonly observed with both normal and inverse topologies. However, all (or nearly all) *Im3m* (Q^{229}) and *Pn3m* (Q^{224}) cubic phases observed so far are inverse structures. It is not yet clear whether there is some

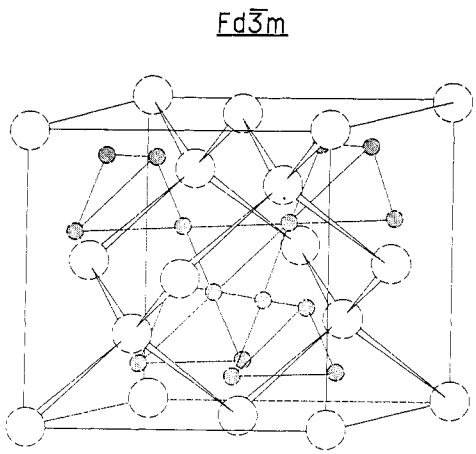

Figure 3. Drawing of the probable structure of the inverse *Fd3m* cubic phase, composed solely of inverse micellar aggregates. The dark and light shaded circles represent the polar cores of the smaller and larger inverse micelles, respectively. The remaining volume is filled by the liquid hydrocarbon chains of the lipids. The connecting lines are drawn merely to guide the eye.

fundamental underlying reason for this topological asymmetry. As regards the micellar cubic phases, *Pm3n* (Q^{223}) has so far only been observed with a normal topology: conversely, the examples of *Fd3m* (Q^{227}) are all inverse.

4. Structure of the *Fd3m* cubic phase

Electron density maps have been calculated using the intensity data from the diffraction pattern of the *Fd3m* cubic phase plotted in figure 2 [17]. Although we are not yet able to deduce the most probable phase combination using the pattern recognition approach [11], the most plausible maps clearly show a structure consisting of a packing of two types of closed, discontinuous aggregates, undoubtedly of inverse topology.

Figure 3 shows a drawing of the proposed structure of the inverse *Fd3m* cubic phase, composed solely of inverse micellar aggregates. The structure is essentially similar to that of the cubic Laves phase C_{15} found in binary metal alloys of composition AB_2, such as $MgCu_2$ [9]. The unit cell contains two kinds of inverse micelle, 8 larger ones arranged tetrahedrally on a diamond lattice, and 16 smaller ones grouped in tetrahedral clusters, occupying the four 'vacant' octants of the unit cell. The polar cores containing the water and the lipid polar headgroups are not necessarily spherical as drawn, although should be nearly so. This unit cell structure also has a fundamental similarity to that of the 17 Å cubic crystalline clathrate hydrates [9]. In such crystals, water molecules form space-filling assemblies of polyhedral cages around solute molecules [18]. In the *Fd3m* structure, there are 16 small cavities formed by slightly distorted dodecahedral cages, and 8 larger cavities formed by hexakaidecahedral cages (16-hedra with 12 pentagonal, and 4 tetrahedrally arranged hexagonal faces). In the lyotropic liquid-crystalline *Fd3m* structure described here (figure 3), the locus of the methyl end groups of the hydrocarbon chains of the lipids should essentially map out these polyhedral surfaces.

It is fascinating to note that such a structure has recently been predicted in a topological/geometrical study of the possible structures formed by periodic systems of frustrated fluid films [9]. When the films are constrained to meet so that the dihedral and edge angles stay close to 120° and 109°28′, which are the optimal values to balance the film tensions, then only two space-filling solutions are found which have cubic symmetry.

These two structures are analogous to those of the water crystalline cubic clathrates, consisting of close-packed assemblies of two types of polyhedra. One has space group *Pm3n* (Q^{223}), and the other has space group *Fd3m* (Q^{227}). A type I (oil-in-water) version of a *Pm3n* (Q^{223}) cubic phase has in fact been observed, as previously noted, in the region of the binary phase diagram adjacent to the normal micellar solution, in systems such as lysophosphatidylcholine [8, 19, 20]. In the geometrical view of Charvolin and Sadoc, the 'frustrated interfacial film' in this case would correspond to the locus of mid-points of the aqueous regions separating two sets of non-equivalent micelles (2 of one type and 6 of the other) within the cubic unit cell [9]. It is very interesting that the other possibility has spacegroup *Fd3m*, with a structure that is identical to that which we propose for the two lipid systems described in this manuscript.

An *Fd3m* cubic phase has previously been observed in two other hydrated lipid systems: mixtures of monoolein with oleic acid, and in a lipid extract from the micro-organism *Pseudomonas fluorescens* [11]. For the latter case, a structure has been proposed, consisting of four groups of tetrahedrally arranged clusters of inverse micelles (16 micelles per unit cell) surrounded by a continuous cage of tetrahedrally-connected inverse water/lipid channels [13]. For the former system, it now appears likely that the structure may be the same as that reported here, i.e. based solely on inverse micellar aggregates [21]. It thus seems that there may be two distinct types of *Fd3m* inverse cubic phase, with different, yet closely related structures.

It should be noted that the inverse micellar *Fd3m* cubic phase structure is not based upon any underlying periodic minimal surface; this implies that there is a greater frustration in the packing of the hydrocarbon chains, than exists in the inverse bicontinuous cubic phases. This may be seen if one considers the variation in average molecular length for lipids in the smaller inverse micelles, in different directions. The distance to the (hydrophobic) midpoint of the tetrahedral cluster from the centre of one of the smaller micelles is 6 Å greater (based upon a lattice parameter of 153 Å) than the half-distance to its nearest (smaller) neighbours. Assuming the polar cores to be spherical, this difference has to be accommodated by a variation in average length of the hydrocarbon chains, which costs free energy (note that for both the systems described here, both lipid components have the same chain length).

Self-diffusion measurements by pulsed field gradient NMR should provide a clear test of the inverse micellar structure for the *Fd3m* cubic phase: neither the lipid nor the water components should be free to diffuse over macroscopic distances (as they can in the inverse bicontinuous cubic phases), but rather should be restricted to diffusing over distances not greater than 30 Å (at the polar cores). We are about to carry out such experiments.

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