Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl19

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Version of record first published: 24 Sep 2006

To cite this article: S. C. Costigan, P. J. Booth & R. H. Templer (2000): Swelling Series and Bilayer Defects Due to Sample Preparation, Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals, 352:1, 59-66

To link to this article: http://dx.doi.org/10.1080/10587250008023161

Mol. Cryst. and Liq. Cryst., 2000, Vol. 352, pp. 59-66
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Swelling Series and Bilayer Defects Due to Sample Preparation

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One well known method to establish important structural determinants of lipid bilayers, is the Luzzati method [1]. Here lipid molecular length and area are calculated from the excess water point and the unit cell spacing, determined by x-ray diffraction of a swelling series. It has been found that this method often results in larger area per lipid values than those found by other methods. Data are presented here demonstrating that this is at least partially due to the sensitivity of this method to sample preparation. Different sample preparation methods influencing bilayer alignment and bilayer defects, were investigated. Freeze-drying in the capillary with no or minimal subsequent mechanical mixing, lead to area per lipid values that are in general agreement with those found in literature: 78.7±2.0 Å^2 for dioleoylphosphocholine (DOPC) at 25°C, and 75.3±2.7 Å^2 for dipalmitoleoylphosphocholine (DPolPC) at 15°C.

Keywords: lecithin; aligned bilayers; X-ray diffraction

INTRODUCTION

Of fundamental importance in discussions about biomembranes and their interactions with proteins and other molecules, is an accurate knowledge of the structural parameters of the fluid bilayers themselves. Therefore the area per lipid, from which characteristics such as the average thickness of the lipid bilayer can be deduced, is of interest. One of the earliest methods used to estimate the average lipid area in bilayers is the Luzzati method [1, 2]. Here the unit cell spacing is determined by

x-ray diffraction of condensed lipid bilayer samples. It is then assumed that the lipid and water pack into completely separate layers (figure 1). With a knowledge of the molecular weights and densities of the lipids, the lipid thickness versus the water thickness can then be calculated. The generally accepted drawback of this method is that sample preparations at molar water to lipid ratios of approximately 20 and higher, have been shown to induce multi-layered vesicles (MLV's) and water pockets [3, 4], leading to an overestimation of the water contribution to the unit cell spacing. However, the occurrence of these defects is strongly influenced by the sample preparation method. Different sample preparation methods have been used in this study that are expected to influence bilayer alignment and thus the quantity and nature of bilayer defects in the sample. A sample preparation method is presented that results in area per lipid values that are in general agreement with those obtained with other methods.

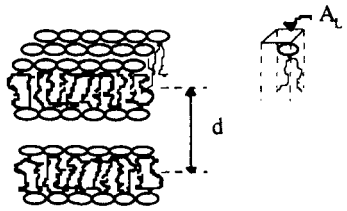

FIGURE 1. Model of a bilayer. d = unit cell spacing, A_L=area per lipid

MATERIAL & METHODS

Chemicals

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoleoyl-*sn*-glycero-3-phosphocholine (DPolPC) (Avanti Polar Lipids Inc., Alabaster, Alabama, USA) purity was given as >99%. Thin-layer chromatography, carried out using chloroform, methanol and distilled water (65:25:4) as eluting agents and iodine vapour as the locating agent, produced one spot for DOPC. For DPolPC a small second spot was seen in some of the plates. The high Rf value would suggest it might be triglycerides [5]. Given it's apparent low level of occurrence, no further purification was performed. Lipids were stored under nitrogen and freeze-dried from cyclohexane before use. The water used for hydrating the samples was distilled and Milli-Q (Millipore) purified.

Sample Preparation

1.5 mm diameter X-ray capillaries (W. Müller, Berlin) were weighed on a Sartorius microbalance (±3 μg precision) before and after addition of

lipid and water. The capillaries were sealed with heat shrink tubing (RS Components, Northants, UK). Final water contents were established immediately after an experiment. Unless mentioned otherwise, all samples were stored at 4°C until examination. Water content (C_w) is defined as: water / (water + lipid) [w/w].

Macro mixing - Lipid and the appropriate amount of water was placed in a small vial. The contents were mechanically mixed (vortexing, sometimes sonication, and centrifugation between vials through a 1.2 mm aperture) until they appeared homogeneous.

*Freeze-drying **on** a capillary* - Multiple layers of lipid were freeze dried from methanol onto the outside of a capillary, which was slipped into a 1.5 mm capillary containing the water. The sample was left to equilibrate at room temperature for at least 24 hours, in the dark.

*Freeze-drying **in** a capillary* - Lipid was freeze-dried in the capillary. Different amounts of water were added and the samples left to equilibrate overnight or longer at room temperature, in the dark. X-ray spectra were taken in 3 different parts of the capillary. If the repeat spacing differed by 1 Å or more, the capillary was centrifuged up and down a maximum of two times. Samples that were still not homogeneous were discarded on the assumption that too many defects in lamellar structure would be induced by too much mechanical mixing.

Rapid thawing - Capillary samples prepared by 'macro mixing', of which the unit cell spacing had already been determined, were frozen at -20°C overnight. They were put straight from the freezer in the x-ray sample holder and held at 15°C.

Slow thawing - The 'rapidly thawed' samples were again frozen at -20°C overnight. They were subsequently placed in a brass block, initially at -20°C, in a cool box, in a refrigerator and thus slowly brought to 2°C in the course of approximately 24 hours. From 2°C they went directly into the sample holder.

X-ray Diffraction

X-ray diffraction was used to establish the repeat unit, or unit cell, spacing, of the lipid bilayer samples. An Elliot GX20 rotating anode x-ray generator (Nonius, Netherlands) was used, equipped with double-mirror of Franks x-ray optics producing a point focussed beam of 190 μm diameter [6]. The detection system consisted of an image intensified x-ray detector coupled to a CCD [7]. Temperature control was achieved by means of computer controlled Peltier devices, with temperature control of ±0.03°C. DPolPC scans were taken at 15°C, DOPC at 25°C.

Data analysis

Water excess points were determined as the water content where the linear increase in the unit cell spacing versus the water content, reached

the water excess equilibrium value. As re-written from [2], lipid areas were then calculated according to:

$$A_L = 3.32113 * MW_L * \left((1 - C_{w,0})/\rho_L + C_{w,0}/\rho_w\right) / \left((1 - C_{w,0}) * d\right)$$

with A_L = area per lipid, MW_L = lipid molecular weight , $C_{w,0}$ = water excess point, ρ_L = lipid density, ρ_w = water density, d = unit cell spacing.

RESULTS

Different sample preparation methods have been used in the literature concerning the determination of structural parameters of bilayers. A swelling series of DOPC at 25°C prepared by 'macro mixing' (see methods section) is presented in figure 2. This procedure results in

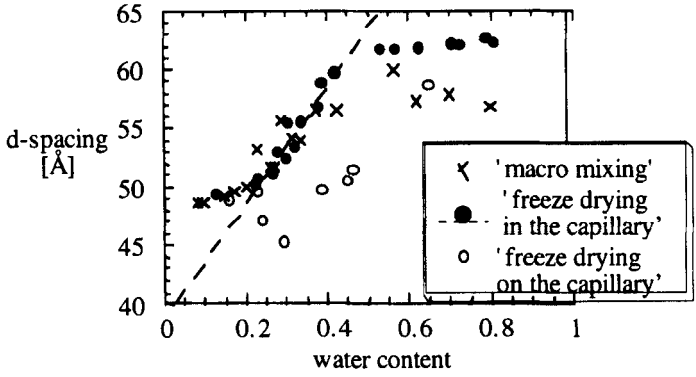

FIGURE 2 DOPC swelling series. Samples prepared in three different ways. See methods section for more details. The dashed line represents a linear fit to the unit cell spacing values of the samples freeze dried in the capillary, obtained at water contents between 0.2 and the water excess point.

unoriented bilayers, simulating the various forms of mechanical mixing performed in different studies, such as e.g. in [3, 4, 8-12]. These results are compared to those obtained by 'freeze drying on the capillary' (figure 2), an adaptation of a method used to produce aligned bilayers on silicone surfaces [13]. Lastly, in analogy with lipids freeze dried onto surfaces and hydrated from humid air to produce oriented

bilayers (e.g. in [14, 15]), samples were prepared by 'freeze drying in the capillary'. From the 'freeze dried in a capillary' data, the water excess point and area per lipid were determined as described in the material and methods section. For DOPC (25°C) this was 78.7 ±2.0 Å^2, and for DPolPC (15°C, data not shown) 75.3 ±2.7 Å^2.

Annealing is generally believed to iron out defects in bilayers. There is a large variation in the temperatures between which samples have been annealed, the number of cycles applied and the speed of temperature changes. As model representations of the annealing process, swelling series of DPolPC at 15°C were prepared by 'macro mixing' followed by 'rapid thawing' of the same samples, and subsequent 'slow thawing'. Results are presented in figure 3. The data obtained with samples 'freeze dried in a capillary' have been left out for the sake of clarity, however, the general trend of the samples made by 'macro mixing' is followed, including the average unit cell spacing found under excess water conditions, but with less variation in the data and closer adherence to a linear increase of unit cell spacing with increasing water content, below the water excess point.

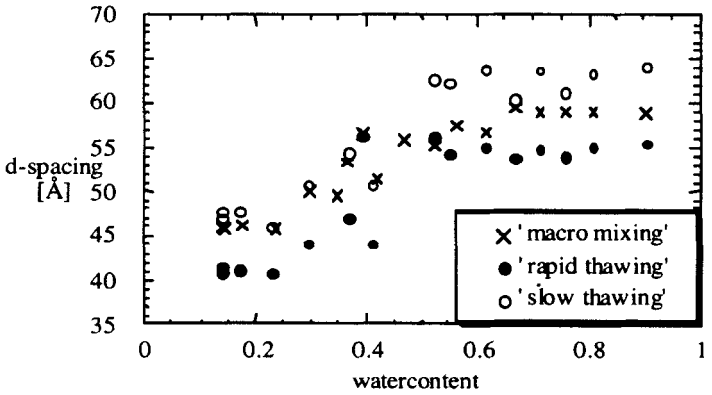

FIGURE 3 Swelling series of DPolPC demonstrating a significant effect of sample temperature history. The samples were treated in three different ways. See methods section for more details.

DISCUSSION

Careful sample preparation is often crucial for successful experiments. Many papers reporting on work performed with lipid bilayers in the

fluid Lα-phase have mentioned that results were sensitive to temperature variations experienced, mechanical treatment, equilibration time, or just more generally sample history, e.g. [2-4, 8, 16]. However, despite this acknowledgement of the importance of sample preparation, the description of what was actually done is often too general for an exact reconstruction. A thorough study by Klose and colleagues [3] using x-ray diffraction and electronmicroscopy, reports on the effect of different morphologies found when swelling lipids, on the unit cell distance. In short, at very low water contents only large extended planar sheets of lipid bilayers were found. As the water content increases, the level of defects in the bilayers increases and multilamellar vesicles (MLV's) appear, even before the water excess point has been reached. Above the water excess point, MLV's with a large distribution of size exist. It has been reported [4] that the diameter of the MLV's formed decreases as the water content increases. The presence of different sized MLV's is the most likely reason for the variation found even in water excess unit cell spacing by us and others (e.g. [2, 16], figures in [8]). Recently the hypothesis was presented that the different unit cell spacings observed in sample containing MLV's were due to a greater freedom for bilayer undulations in the outer layer(s) of the vesicle, versus the inner layers, and thus larger bilayer separations [17], and indeed, confirmatory experimental data has been found [18]. Obviously, the presence of bilayer defects, MLV's and waterpockets below the water excess point will lead to an overestimation of the water contribution to the unit cell spacing. Since in the Luzzati method all this water is assumed to be homogeneously distributed amongst the bilayers, this results in too large an area per lipid. It is therefore imperative that our sample preparation method should minimise the opportunity for MLV- and bilayer defect-formation, or iron them out.

The two methods attempted in this study to minimise the defects in the first place, are the 'freeze drying on the capillary' and the 'freeze drying in the capillary'. The results are compared to the traditional 'macro mixing' method in figure 2. Both are adaptations of existing methods to prepare oriented bilayers. The large variation for 'freeze drying on a capillary' is presumably due to the difficulty in accurately determining the water content. Because the lipid is on the outside of the first capillary, all handling between removal out of the freeze dryer and insertion into the second capillary, creates the possibility of rubbing off some of the lipid, or of the lipid hydrating from the air. Surprisingly, the decision to not use samples that need to be spun up and down more than twice to hydrate homogeneously seemed to affect samples at all hydrations equally, except in excess water. With this requirement in place, the 'freeze drying in the capillary' method, showed a clear improvement in reducing variation in the data versus the traditional

'macro mixing' and in maintaining the linearity in the increase in unit cell spacing below the water excess point. Based on the swelling curves obtained by this method, the lipid area values found for DOPC and DPolPC were respectively 78.7 ±2.0 Å^2 (25°C) and 75.3 ±2.7 Å^2(15°C). The range of lipid areas published for DOPC spans from 70 to 82 Å^2 with most of the values being in the lower half of this range [2, 8, 10, 11, 19]. Thus the value obtained here is in reasonable agreement with the published values. This also lends support to the value reported here for DPolPC, obtained by the same method. As this is the first report on DPolPC the authors are aware of, this extends the current data base of structural parameters of different lipids.

Once bilayer defects have already been formed, it is a well known practise to anneal the sample to try and iron out these defects. However, no common protocol exists and often the temperatures between which is annealed, the number of cycles and the speed of temperature changes is not regulated or reported. Heating to 60°C and slowly cooling to room temperature, resulted in the appearance of bigger liposomes [4]. A rapid, repeated freeze thaw procedure between liquid nitrogen and 50 - 60°C resulted in initially tightly packed 'onion skin' MLV's changing to lamellar structures with significantly increased interlamellar distances and even with closed lamellar systems intercalated between bilayers [20]. This could well be the reason for the increase in the variation found in the unit cell spacing after the different freeze-thawing regimes applied, and for the 'slow thawing' even resulting in a significant increase of the water excess unit cell spacing over and above the normal equilibrium unit cell spacing reported in this study. The individual defects in bilayer structure could well be decreased, but the spacing between the bilayers in the vesicular structures is now more variable. Naturally, many other hypotheses are also imaginable, such as the formation of water crystals below 0°C that, at different thawing rates, might well do different damage to the bilayer structures.

CONCLUSION

The main aim of this study was to find a reliable and reproducible method of sample production to establish structural parameters of the lipid bilayer. It has been found that freeze drying the lipid in the capillary, checking homogeneity of the sample throughout the capillary, discarding samples that required too much mechanical mixing, and checking the water content after x-ray diffraction, is an effective means of obtaining reliable structural data that is in reasonable agreement with published values.

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