Evidence for a Two-Dimensional Molecular Lattice in Subgel Phase DPPC Bilayers†

J. Katsaras,*,‡,§ V. A. Raghunathan, E. J. Dufourc, and J. Dufourcq

Centre de Recherche Paul Pascal−CNRS, Avenue Albert Schweitzer, F-33600 Pessac, France, and Atomic Energy of Canada Limited, Chalk River Laboratories, Chalk River, Ontario K0J 1J0, Canada

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ABSTRACT: Using a combination of X-ray diffraction data from oriented films and multilamellar liposomes of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) in the subgel phase, we have established the presence of a 2D molecular lattice containing two lipid molecules. The proposed 2D lattice is consistent with all the X-ray diffraction data on the subgel phase of DPPC available in the literature. In this phase, the DPPC molecules are ordered in the plane of the bilayer and are also found to be positionally correlated across a single bilayer but not with those in adjacent bilayers. We also present the possible molecular arrangements for the proposed lattice.

In 1980, Chen *et al.* observed a new phase transition, centered at about 18 °C, in a DPPC[1] multilamellar suspension using differential scanning calorimetry (DSC). Until then, DPPC suspensions were known to have only two thermotropic phase transitions; the main gel to liquid crystalline phase transition ($T_c \approx 41$ °C) and a broader so-called "pretransition" ($T_c \approx 35$ °C). However, this newly discovered phase, referred to as the subgel, was observed only after Chen and co-workers (Chen *et al.*, 1980) stored the multilamellar suspension of DPPC at ≈0 °C for several days. Since then, there have been many diffraction experiments carried out to characterize the structure of this phase (e.g., Tristram-Nagle *et al.*, 1994; McIntosh & Simon, 1993; Stümpel *et al.*, 1983; Ruocco & Shipley, 1982; Füldner, 1981).

The structural changes accompanying the subtransition, from the gel phase, have been well documented (e.g., Ruocco & Shipley, 1982; Tristram-Nagle *et al.*, 1994). There is a decrease in the lamellar periodicity and the appearance of a number of Bragg reflections in addition to the commonly observed lamellar and wide-angle reflections (1/4.2 and 1/4.1 Å^{-1} of the $L_{\beta'}$ phase). These "additional" reflections have been cited as evidence that the low temperature phase of DPPC has a much more ordered hydrocarbon chain structure than the $L_{\beta'}$ phase (Ruocco & Shipley, 1982). Also, ^{31}P nuclear magnetic resonance studies (NMR) have shown that in the subgel phase there is an incomplete motional averaging of the ^{31}P shift tensor not unlike dry DPPC samples (Füldner, 1981).

One of the most detailed X-ray studies of a subgel phase lipid/water system was performed using oriented dipalmitoylphosphatidylglycerol (DPPG) multibilayers (Blaurock & McIntosh, 1986). From the data obtained, Blaurock and McIntosh (1986) were able to state that the DPPG molecules, in one bilayer, crystallize in an oblique 2D lattice of dimensions $a = 5.50$ Å, $b = 7.96$ Å, and $\gamma = 100.5°$ containing one lipid molecule. Blaurock and McIntosh (1986) were also able to determine the hydrocarbon chain tilt angle θ to be between 30° and 35°.

In this study, using oriented films and multilamellar liposomes of DPPC, we find that the structure of the subgel phase is characterized by a 2D molecular lattice containing two lipid molecules. From the hydrocarbon chain reflections, we were able to precisely define the oblique hydrocarbon chain lattice from which we derived the 2D molecular lattice. The length of the hydrocarbon chain reflections from the oriented sample shows the subgel phase of DPPC to be formed by the stacking of 2D ordered bilayers with no out-of-plane correlations. In addition, the molecular lattices were found to be positionally correlated across the lipid bilayer. For the proposed 2D molecular lattice, six different molecular arrangements are possible. Three of these molecular arrangements belong to the plane group $p1$, while the rest belong to $p2$. Also, in the subgel phase the hydrocarbon chains have a tilt angle θ of 34.5° in a direction ≈5° off-nearest neighbor and an area/hydrocarbon chain of 19.1 Å^2. This analysis, for the first time, explains the X-ray diffraction data and possibly the NMR observations in the subgel phase of DPPC. Finally, this type of 2D molecular lattice may be present in other PC/water systems in the subgel phase.

MATERIALS AND METHODS

1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC) was obtained from Avanti Polar Lipids Inc. (Birmingham, AL) and oriented on a 150 μm thick curved glass surface, using a concentrated solution of DPPC and methanol which was pipetted onto the glass surface. After evaporation of the methanol, a clear film of lipid was left adhering to the glass plate. The remainder of the methanol was evaporated

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‡ Centre de Recherche Paul Pascal−CNRS.

§ Atomic Energy of Canada Ltd.

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[1] Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; 2D, two-dimensional; $L_{\beta'}$ phase, gel phase; SFD, sample-to-film distance; RH, relative humidity; *d*-spacing, repeat distance.

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Table 1: Wide-Angle Repeat Spacings of Subgel DPPC Multilayers[a]

Füldner	McIntosh & Simon	Ruocco & Shipley	Stümpel *et al.*	Tris-Nagle *et al.*	present study (powder)	present study (oriented)
10.0	9.8	10.0	10.0	10.14	10.0[b]	10.0[b]
–[c]	–	9.30	–	9.36	9.4[b]	–
6.75	6.8	6.81	6.78	6.84	6.8[b]	6.8[b]
–	–	4.9	–	–	5.0[b]	–
–	–	4.52	–	–	4.5	4.5
4.4	4.4	4.43	4.40	4.46	4.4	4.4
–	–	4.2	4.20	4.25	4.2	4.2
3.85	3.9	3.83	3.88	3.92	3.8–3.9	3.9
						3.8

[a] These reflections are in addition to the lamellar reflections. All values are in Å. [b] Reflections due to the ordering of the DPPC molecules. [c] (–) Reflection not observed.

by placing the samples under a vacuum for 24 h, after which time they were hydrated in ≈100% relative humidity (RH) environment for a few days. To obtain oriented subgel DPPC bilayers, samples were kept at 4 °C for ≈5 days. The preparation of oriented bilayers on a curved glass surface produces a stack of ≈2000 highly oriented (<5° mosaic spread) bilayers and allows the simultaneous observation of both small- and wide-angle reflections. However, this diffraction geometry allows for only half the diffraction pattern to be recorded. Powder samples of excess water (75 wt % water) DPPC multilamellar dispersions were prepared and then transferred to X-ray diffraction capillary tubes which were flame sealed. The samples were then annealed at ≈50 °C for a few hours and subsequently stored at 4 °C for a period of 2 months.

The experiments were carried out with an 18-kW Rigaku Rotaflex RU300 rotating anode generator and a 2D Mar-research imaging plate detector having a plate diameter of 180 mm and pixel size of 150 μm × 150 μm. Monochromation of the Cu radiation was achieved using a flat graphite crystal having a mosaic spread of 0.4 ± 0.1° $FWHM_{(002)}$. The spot size, as defined by 3 sets of vertical and horizontal slits, was approximately 0.5 mm × 0.5 mm. The sample holder for the oriented samples (volume ≈300 cm^3) was designed to monitor and control both the RH and the temperature. The present data were obtained at a temperature of 7 ± 1 °C and a RH of 60 ±5%. The RH was monitored by a digital hygrometer (Quick RHT 300; Germany) and the temperature controlled by a Haake water bath (Berlin, Germany).

RESULTS AND DISCUSSION

Table 1 presents wide-angle X-ray diffraction data of DPPC multibilayers from various studies. Of the seven data sets presented, only the present experiment and the one by McIntosh and Simon (1993) used oriented multibilayers. The rest of the studies, including one presented in this paper, were performed using multilamellar liposomes. Although diffraction patterns from powder samples, as we shall see later on, lack a certain type of clarity when compared to data obtained from oriented multilayers, it is also evident that they are very sensitive in recording some of the weaker reflections (e.g., 9.4 and 5.0 Å reflections). In the present study, having data from both powder and oriented samples proved to be very useful.

The Oriented Diffraction Patterns. Figure 1 shows diffraction patterns arising from the bilayer repeat structure (Figure 1a) and the hydrocarbon chains (Figure 1b) whose repeat spacings are the same as those obtained from the

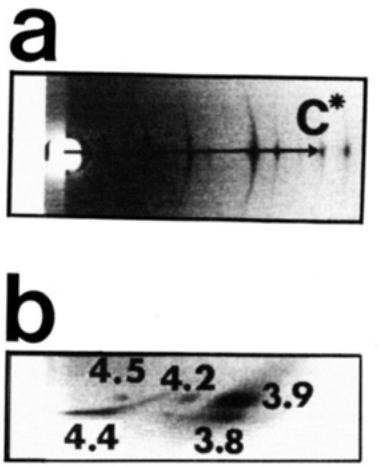

FIGURE 1: Diffraction patterns showing the lamellar reflections (a) and the hydrocarbon chain reflections (b) from oriented subgel phase DPPC multibilayers having a repeat spacing of 56.4 ± 0.3 Å. The calculated tilt angle θ is 34.5 ± 1.0° while the splitting angles measured directly from the diffraction pattern of the (2 0), (1 1), and (1 1) reflections are 5°, 29°, and 32°, respectively. The area/hydrocarbon chain in the subgel phase of DPPC is 19.1 ± 0.2 $Å^2$. SFD was 185 ± 1 mm.

powder data (Table 1 and Figure 4a). The lengths of the 3.8, 3.9, and 4.4 Å (Figure 1b) hydrocarbon chain reflections are approximately equal to the separation between successive lamellar reflections, giving an out-of-plane correlation length comparable to the bilayer thickness (Leadbetter *et al.*, 1979). This means that the subgel phase of DPPC multibilayers is not a 3D structure, but rather is composed of 2D structures with no out-of-plane correlations. This also has been shown to be the case in oriented multibilayer samples of DPPG in the subgel phase (Blaurock & McIntosh, 1986). In addition, the fact that the 4.4 Å [(0 2) lattice plane of the hydrocarbon lattice] reflection is practically centered on the equatorial axis (perpendicular to c^*) and the 3.8 ($\bar{1}$ 1) and 3.9 (1 1) off-equatorial reflections do not have the same d-spacings proves that the DPPC molecules are tilted approximately toward nearest neighbor and form a hydrocarbon lattice which is oblique ($\gamma = 94°$) (Leadbetter *et al.*, 1979; Smith *et al.*, 1988). The hydrocarbon chains are tilted by 34.5° with respect to the bilayer normal.

In Figure 2, we present a 2D diffraction pattern which shows weak reflections parallel to c^* and whose projections onto the equatorial axis (perpendicular to c^*) correspond to lattice spacings of 6.8 and 10.0 Å. These two reflections are commonly observed in powder patterns (please see Table 1 and Figure 4b). The fact that these two reflections extend past the thirteenth lamellar reflection indicates that they are due to in-plane ordering of some moieties that are short

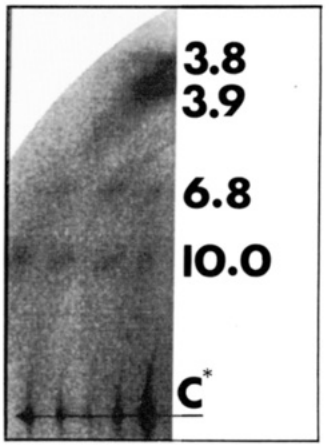

FIGURE 2: 2D oriented diffraction pattern of 56.4 Å bilayers in the subgel phase showing the 10.0 and 6.8 Å reflections parallel to the c^* axis. These reflections, due to the ordering of the DPPC molecules, are split up into discrete equidistant spots whose separation corresponds to ≈40 Å, which is typically the distance between the phosphoryl groups across the bilayer. Therefore, the modulation in intensity of the 10.0 and 6.8 Å reflections indicates the presence of correlations in the headgroup ordering across the bilayer. The 3.8 and 3.9 Å hydrocarbon chain reflections are shown only partly.

compared to the hydrocarbon chains, like the headgroups. The modulating intensity along the 6.8 and 10.0 Å line reflections corresponds to a repeat spacing of ≈40 Å (separation between maxima) and is the result of the phosphate groups being positionally correlated across the bilayer. This spacing is therefore a measure of the phosphate-to-phosphate separation within one bilayer and is in agreement with the value obtained from the powder data (Figure 4b) and 1D electron density profiles of subgel phase DPPC multibilayers constructed to a resolution of ≈4 Å (unpublished data). In the oriented sample we could not follow the modulation in intensity at smaller Bragg angles due to the high background (please see Figure 1a) contributed by the glass substrate. This was not the case in the powder sample (Figure 4b). Due to our diffraction geometry, we were only able to detect half of the diffraction pattern. Figure 3 is a composite of Figures 1 and 2 and shows the positions of the various reflections with respect to one another.

The Powder Patterns. Figure 4 shows diffraction patterns of the hydrocarbon chain region (Figure 4a) and the region containing the much observed 10.0 and 6.8 Å reflections (Figure 4b) from DPPC liposomes having a *d*-spacing of 58.6 Å in the subgel phase. The lamellar reflections in Figure 4a,b are labeled *h*/*d*, where *h* is the order number. Figure 4a is equivalent to the diffraction pattern in Figure 1b which was obtained using oriented multilayers. The only difference between the two diffraction patterns is that the 3.8 and 3.9 Å reflections in Figure 1b are not resolved in the diffraction pattern from the lipid dispersion (Figure 4a).

In the diffraction pattern from oriented multibilayers, the 6.8 and 10.0 Å reflections (Figure 2) contain maxima whose separations correspond to the thickness of 1 bilayer. In the powder pattern, each of the discrete maxima along the 10.0 and 6.8 Å reflections gives rise to a ring, with the separation between adjacent rings increasing as we go further from the origin. This also has the effect of making the rings with larger radii more diffuse. Thus we can only see the first few rings in the powder, unlike the oriented pattern. The discrete maxima along the 10.0 Å reflection in the oriented

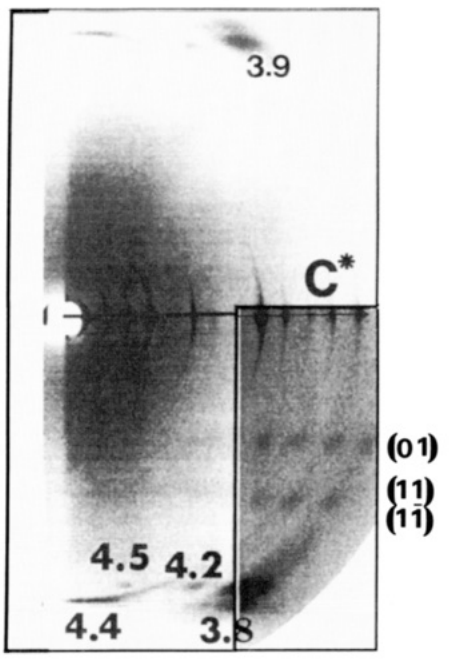

FIGURE 3: Composite diffraction pattern of Figures 1 and 2. In this figure, one can clearly see the positional relationship between the various Bragg reflections. The 10.0 and 6.8 lattice line reflections arising from the ordering of DPPC molecules in two dimensions have been labeled (0 1) and [($\bar{1}$ 1), (1 1)], respectively.

pattern (Figure 2) which are separated by ≈0.025 $Å^{-1}$ will then fall in the powder pattern at positions 1/10.0, 1/9.8, and 1/9.0 $Å^{-1}$ and at 1/6.8, 1/6.7, 1/6.4, and 1/6.1 $Å^{-1}$ for the 6.8 Å reflection. Of these weak reflections, the ones which are clearly observed in the powder pattern (Figure 4b) are the 1/10.0, 1/9.0, 1/6.8 and 1/6.1 $Å^{-1}$ reflections while the rest fall close to other maxima. These reflections are in agreement and also complement the data from the oriented DPPC sample (Figure 2). In Figure 5 we present a schematic diagram of the diffraction maxima from the oriented and powder data.

Oriented Sample Reflection Morphology. It is evident by now that oriented samples have important advantages over powder samples. For example, from the visual examination of a diffraction pattern from oriented multilayers one can easily and quickly identify the reflections resulting from structure parallel and perpendicular to the plane of the bilayer. This cannot be done with a powder pattern. Of importance is that the 4.2 and 4.5 Å reflections, which lie in a straight line along with the 3.9 Å reflections parallel to the c^* axis (Figures 2 and 3), are due to the form factor of the finite length hydrocarbon chains (Smith *et al.*, 1990). As a result, they do not arise from lattice spacings. This type of "reflection morphology" information is not available from powder samples on which the majority of the X-ray diffraction experiments in the subgel phase have been carried out (e.g., Tristram-Nagle *et al.*, 1994; Stümpel *et al.*, 1983; Ruocco & Shipley, 1982; Füldner, 1981).

Except for the present experiment and that of McIntosh and Simon (1993), all data concerning the subgel phase of DPPC comes from powder samples (Table 1). Because the hydrocarbon chains are tilted the *d*-spacings (e.g., 3.9 and 3.8 Å) we measure do not correspond directly to the spacings of the different lattice planes; only their projections (e.g., 4.65 and 4.4 Å) onto the equatorial axis (perpendicular

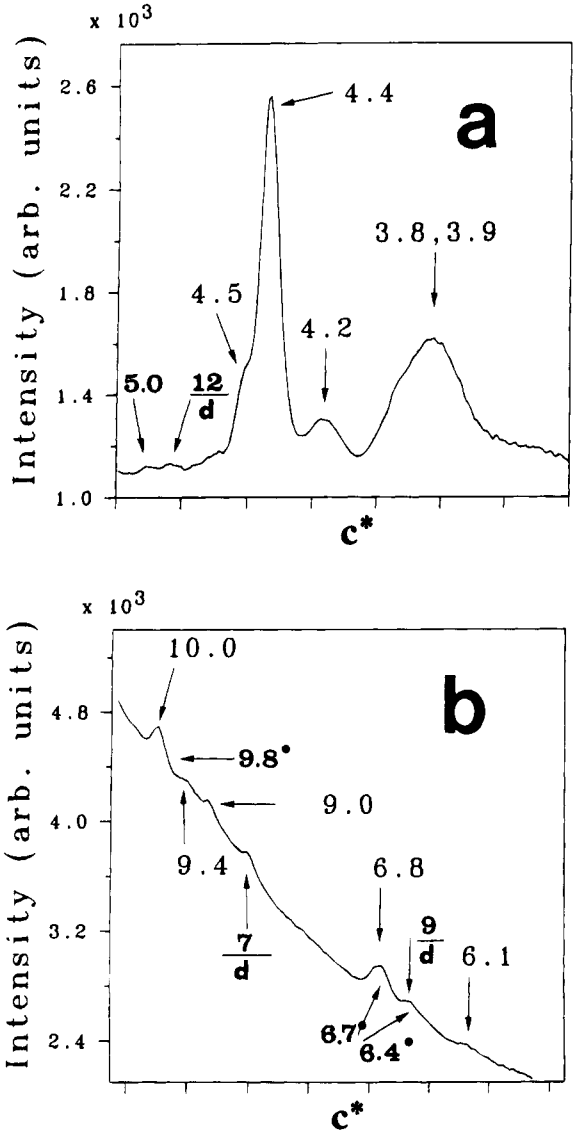

FIGURE 4: Circularly averaged X-ray diffraction patterns of 58.6 ± 0.2 Å repeat spacing DPPC liposomes at 7 °C which were annealed for 2 months at 4 °C. (a) Wide-angle region showing the hydrocarbon chain reflections accompanied by their corresponding repeat distances. The broad peak contains both the 3.8 and 3.9 Å reflections seen in Figure 1b. The two weak reflections labeled 5.0 Å and 12/*d* are due to the molecular lattice [(0 2) planes] and the lamellar repeat distance (12th order Bragg reflection), respectively. The sample-to-film distance (SFD) was 177.3 ± 1.0 mm. (b) the 10.0 and 6.8 Å regions at large SFD. The two lamellar Bragg reflections are the 7th and 9th orders. The 10.0, 6.8, and 9.4 Å reflections correspond to lattice planes of the 2D molecular lattice. The remaining reflections are a result of the molecular lattices being positionally correlated across a single bilayer. The ones which fall close to other maxima and, as such, do not show up as single peaks are accompanied by a • (e.g., 9.8, 6.7, and 6.4 Å). The SFD for this diffraction pattern was 290.5 ± 1.0 mm.

to *c**) do. In the case of a powder pattern, all the information regarding the chain tilt becomes obscured, and thus it is extremely difficult to assign the satellites (the 4.2 and 4.5 Å reflections) as coming from the form factor of the hydrocarbon chains. Therefore, using the 4.4, 3.9, and 3.8 Å reflections along with their splitting angles, we calculated a hydrocarbon chain tilt angle θ of 34.5°, an area/hydrocarbon chain of 19.1 Å^2, and hydrocarbon chain lattice parameters a = 5.25 Å and b = 8.8 Å.

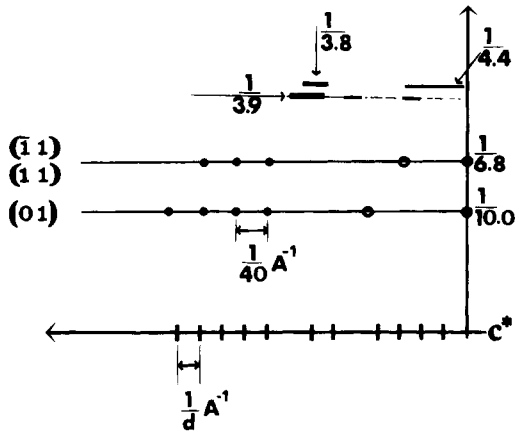

FIGURE 5: Composite schematic diagram of the oriented and powder data (○). Reflections which cut across the equatorial axis are directly meaningful since their *d*-spacing corresponds to a lattice parameter (e.g., 4.4, 6.8, and 10 Å). Reflections which are off-equatorial give rise to lattice parameters by projecting them onto the equatorial axis. The 1/3.8 and 1/3.9 Å^{-1} reflections thus correspond to 4.4 and 4.65 Å lattice spacings. The (0 1) and [(1̄ 1), (1 1)] reflections have their intensity modulated along their length. The separation of the most closely spaced maxima corresponds approximately to the thickness of one bilayer.

The 2D Molecular Lattice Model. At high hydrations, the "disordered" gel phase $L_{\beta'}$ is characterized by lamellar and wide-angle reflections at 1/4.24 and 1/4.1 Å^{-1} (Smith *et al.*, 1988; Hentschel & Rustichelli, 1991). Therefore, in the gel phase, the hydrocarbon chains form a centered rectangular lattice with lattice parameters a = 5.45 Å and b = 8.5 Å. These lattice parameters are not much different than the ones calculated for the hydrocarbon chain lattice of the "ordered" subgel phase (a = 5.25 and b = 8.8). As such, there is no qualitative change in the hydrocarbon chain region between the gel and subgel phases of DPPC, indicating that the additional reflections result from the ordering of the DPPC molecules themselves. This is a classic disorder−order transition, whereby there is little alteration to the parent lattice formed by the hydrocarbon chains and the extra lines which appear are due to the ordering of the lipid molecules.

It is possible to construct a variety of 2D molecular lattices (not shown). However, of all the possibilities, only the one shown in Figure 6 is in agreement with all of the observed reflections in Table 1. Using the chain lattice parameters as a starting point, we calculated those of the molecular lattice to be a = 9.3 Å, b = 10.0 Å, and γ_S = 90°. Table 2 lists the calculated reflections due to the molecular lattice presented in Figure 6. Clearly, the agreement between the calculated and observed reflections presented in Table 1 is excellent. As mentioned earlier, the 2D molecular lattices on either side of the bilayer are correlated, giving rise, in the oriented pattern, to lines with modulating intensity parallel to the *c** axis (Figure 2). The separation between the equidistant maxima along the 10.0 and 6.8 Å reflections corresponds to a distance of 40 Å, the approximate separation between phosphoryl groups across the bilayer.

For a given 2D molecular lattice, there are many ways of packing the lipid molecules. The six possible packings for the proposed molecular lattice are shown in Figure 6. The •'s represent the hydrocarbon chains, while the ■'s represent the phosphorylcholine headgroups. One lipid molecule is made up by connecting two nearest neighbor hydrocarbon chains and placing a headgroup. For the various molecular

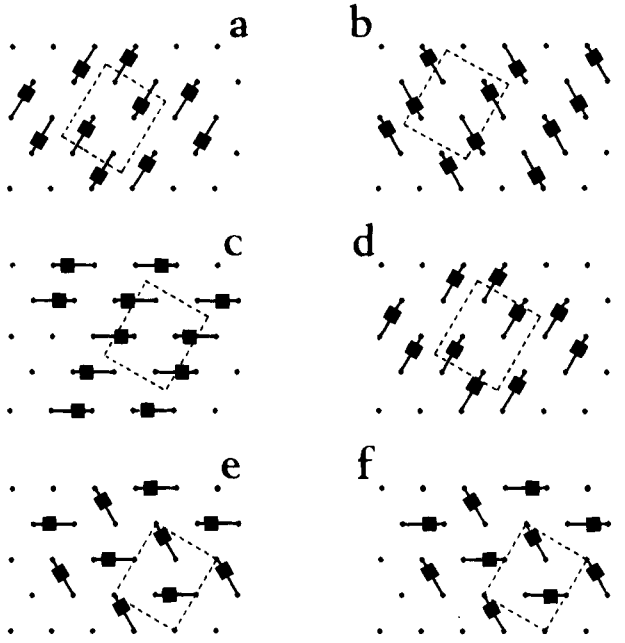

FIGURE 6: The possible molecular arrangements in the 2D molecular lattice obtained by connecting the hydrocarbon chains in various ways. The •'s represent hydrocarbon chains while the ■'s represent phosphorylcholine headgroups. The molecular lattice is represented by a series of dashed lines and has dimensions of a = 9.3 Å, b = 10.0 Å, and γ = 90°. The origin of the unit cell is at the lower left-hand side. By connecting two nearest neighbor hydrocarbon chains with one headgroup, we obtain a lipid molecule. The molecular lattice, which contains two lipid molecules, was constructed from a hydrocarbon lattice of dimensions a = 5.25 and b = 8.8 with an angle γ = 94°. The molecular arrangements shown in a–c belong to the plane group $p2$, while the rest (d–f) belong to $p1$.

Table 2: d-Spacings of the Various Lattice Planes of the 2D Molecular Lattice

(h k)	d-spacing (Å)	(h k)	d-spacing (Å)
0 1	10.0	$\bar{0}$ 2	5.0
1 0	9.3	$\bar{1}$ 2	4.4
$\bar{1}$ 1	6.8	1 2	3.9
$\bar{1}$ 1	6.8	2 0	3.8

packings presented in Figure 6 the molecular lattice is denoted by dashed lines and is identical in all of the six molecular arrangements. The molecular arrangement of DPPC molecules presented in Figure 6a–c belongs to the plane group $p2$ while the rest belong to $p1$. At present, we are not able to determine which one of these molecular arrangements makes up the subgel phase of DPPC.

The DPPC studied here presents many similarities with the structure of the subgel phase of DPPG multibilayers (Blaurock & McIntosh, 1986), but shows one major difference. Both these systems are characterized by the presence of a 2D molecular lattice, with the lattices in the two monolayers constituting each bilayer being positionally correlated. The tilt of the hydrocarbon chains with respect to the bilayer normal is also comparable in the two systems. The main difference arises from the fact that the chain lattice is not significantly altered across the gel–subgel phase transition in DPPC. On the other hand, the DPPG molecules in each bilayer crystallized in a 2D oblique lattice of dimensions a = 5.50 Å and b = 7.97 Å and contain 1 lipid molecule (Blaurock & McIntosh, 1986), dimensions similar to those of our hydrocarbon chain lattice.

SUMMARY

We have for the first time established the existence of a 2D molecular lattice in the subgel phase of DPPC on the basis of new X-ray diffraction studies on oriented multibilayers, excess water liposomes, and other diffraction data reported in the literature. Although molecular ordering of a lipid has been shown before, this is the first system which shows molecular ordering leading to the formation of a 2D molecular lattice containing 2 lipid molecules. For this molecular lattice, there exist six different molecular arrangements. Three of these belong to the $p1$ plane group while the rest belong to $p2$. The molecular lattices are found to be positionally correlated across the bilayer.

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