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Structure of polymerizable lipid bilayers: water profile of a diacetylenic lipid bilayer using elastic neutron scattering

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Elastic neutron scattering experiments have been used to study the hydration of multibilayers of 1,2-bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$). Previously published FTIR spectroscopic data had suggested, based on shifts in the carbonyl (C=O) stretch frequencies, that the phosphocholine headgroup in these polymerizable lipid bilayers was much less hydrated than that of saturated phosphatidylcholines. Our results demonstrate that the $DC_{8,9}PC$ headgroup is at least as well hydrated as that of dipalmitoylphosphatidylcholine (DPPC), a saturated lipid, under the same conditions.

I. Introduction

Meridional (001) X-ray diffraction data from stacked membrane multibilayers yields information concerning the projection of the electron density function onto an axis perpendicular to the membrane stacking direction [1]. Elastic neutron scattering yields analogous information, with the important distinction that scattering depends not on the electron density of the constituent atoms but on their neutron scattering cross section. Because the neutron scattering lengths of hydrogen and its isotope deuterium differ considerably from one another ($-0.38 \cdot 10^{-12}$ cm and $0.65 \cdot 10^{-12}$ cm, respectively), the magnitude of the scattered wave from a sample containing hydrogen will differ from an equivalent sample containing deuterium. By carrying out parallel experiments with specimens hydrated with H_2O and D_2O, neutron scattering becomes a powerful tool for directly determining (by difference) the locations of water in water-containing lattices [2].

1,2-Bis(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphocholine ($DC_{8,9}PC$) is a phosphatidylcholine (PC) that contains a polymerizable diacetylene group in each 23 carbon acyl chain (Eqn. 1).

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It has been observed that these lipids as well as others in the $DC_{m,n}PC$ series form extended hollow tubular structures with a diameter of about 0.5 μm, lengths as great as hundreds of μm, and walls composed of two to ten bilayers [3]. The tubules may be formed by either of two techniques: a thermal preparation in which tubules are formed by slowly cooling large unilamellar vesicles (LUVs) from above the hydrocarbon chain melting temperature, T_m [3], or precipitation from an organic solvent [4].

Investigators in this laboratory have studied the structures of a number of polymerizable lipid bilayers and have reported the electron density profile structures of three diacetylenic lipid bilayer systems using X-ray diffraction (Ref. 5, and Rhodes, D.G., et al., unpublished data). X-ray diffraction data from $DC_{8,9}PC$ show that the bilayer (lamellar) unit cell spacing (d) is small, comparable to d for saturated PCs having hydrocarbon chains 5–7 methylene carbons shorter. Based on a molecular model derived from the X-ray diffraction data, it has been suggested that the acyl chains are tilted to accomodate the additional space required for the longer acyl chains [5]. Because of the resolution of the experiment, no details regarding the conformation of the polar phosphate headgroup could be determined from these X-ray experiments.

Rudolph and Burke recently reported Fourier-transform infrared (FTIR) spectroscopy data on $DC_{8,9}PC$ and its saturated analog DTPC in various phases [6]. The authors report that the C=O stretching band for liquid crystalline phase $DC_{8,9}PC$ is composed of four bands at 1739, 1733, 1736, and 1718 cm^{-1}, with that at 1733 cm^{-1} having the greatest intensity. They suggest

that these bands may represent distinct rotational isomers, implying a more highly ordered headgroup than in bilayers of saturated PCs. During cooling of $DC_{8,9}PC$ to form gel-phase tubules the C=O stretching frequency decreased, with the 1717 cm^{-1} band becoming dominant at 0°C. This decrease in the C=O stretching frequency could be interpreted as indicating differences in packing or conformation. In summarizing the data, the authors conclude that the decrease in stretching frequency was the result of a change in the dielectric environment of C=O, and this change was an indication of 'significantly reduced hydration'. Although this interpretation is feasible, the exact origin of the observed shifts appeared uncertain. One might expect PC headgroups in $DC_{8,9}PC$ bilayers to have a conformation distinct from that of saturated PCs, and might further expect this change to result in hydration differences. By directly measuring the hydration of $DC_{8,9}PC$ bilayers using elastic neutron scattering, we have attempted to determine which of these factors (hydration or conformation) is responsible for the observed FTIR spectral shifts.

II. Methods

$DC_{8,9}PC$ was synthesized at the Naval Research Laboratory. Tubules were formed at room temperature using the solvent precipitation method of Georger et al. [4]. Tubule formation was verified by examining a small amount of the solution under an optical microscope. Although the solvent precipitation method of tubule formation varied from the thermal method used by Rudolph and Burke, the tubules themselves and the bilayer structures composing them are indistinguishable by a variety of diffraction methods (Blechner, S.L., unpublished data).

A sedimentation procedure described previously [5] was used to form multibilayers of flattened tubules, but because the samples required for the neutron scattering apparatus were twice the radius of those used in the X-ray diffraction experiments, four times the amount of sample per multilayer was used. Samples sedimented onto aluminum foil were mounted on flat glass (1×3 cm) and placed in sealed aluminum canisters. The relative humidity and mole fraction D_2O ($X(D_2O)$) inside the canister were regulated by the presence of small plastic cups of saturated salt solution in defined ratios of D_2O/H_2O, selected to fix the relative humidity at 98%, 84%, and 66%. Saturated salt solutions were made with reagent grade chemicals and D_2O/H_2O ratios (v/v) of 1.0, 0.75, 0.50, and 0.25. (The mole ratio was within 0.5% of the volume ratio). Canisters were placed in a constant temperature chamber at 15°C to equilibrate for at least 6 h before each experiment. At 15°C the samples were in the lipid gel phase, well below the transition temperature. The shifted C=O stretch frequency in the FTIR results of Rudolph and Burke appeared at 10°C, well below the phase transition temperature. All samples were unpolymerized and did not appear to be affected by exposure to the neutron beam.

Neutron scattering data were collected at the Brookhaven National Laboratories (BNL) High Flux Beam Reactor (HFBR) H3A Beamline. The beamline consists of a collimator, a pair of defining slits to trim the divergent beam, a θ–2θ diffractometer, and an 18×18 cm^2 thermal neutron area detector (256×192 channels) [7]. λ_{max} and $\Delta\lambda_{max}$ were experimentally determined to be 2.71 Å and 4.7%, respectively, at measured neutron flux of $4.5 \cdot 10^{+5}$ $n \cdot cm^{-2} \cdot s^{-1}$. The multilayers were centered on the ω axis of the θ–2θ diffractometer. Helium paths contained within thin aluminum windows were used to reduce air scattering. Five orders of Bragg diffraction for the 98% relative humidity data, and three orders for the 84% relative humidity data were easily observed without moving the detector from its initial value of 2θ, so data were collected by scanning ω only. For each sample condition (relative humidity, $X(D_2O)$), individual area detector patterns were collected until $1 \cdot 10^6$ counts were observed (approx. 20 min) by a monitor used to measure the flux of the incident beam at intervals of 0.2° in sample angle ω, through $\omega = 5.5°$. 100% D_2O data were collected at relative humidities of 98%, 84%, and 66%, the greatest scattering signal to noise ratio coming from multilayers at 98% relative humidity. Data were collected for samples at 98% and 84% relative humidity with D_2O/H_2O ratios of 1.0, 0.75, 0.50, and 0.25.

III. Results

The two-dimensional data arrays corresponding to each increment of ω were integrated by defining a strip in the meridional direction wide enough to capture the entire arc of each of the three to five orders. Data in this strip were summed in the perpendicular direction to create a one-dimensional (1-D) profile representing the total intensity as a function of scattering angle (2θ) for each value of ω. These 1-D files for each ω value were then summed to create a composite 1-D file, which was corrected for background noise using two exponential curves. A representative composite 1-D diffraction pattern (r.h. = 98%, $X(D_2O) = 1$) and the background correction used are shown in Fig. 1. The validity of the two exponential backgrounds was verified by collecting data from a blank aluminum foil mounted on flat glass, which gave the same intensities as the background correction.

To correct for the different rates at which the reciprocal lattice points sweep through Ewald's sphere of reflection, a Lorentz s-correction was applied to each diffraction order. Since the sample thickness was non-

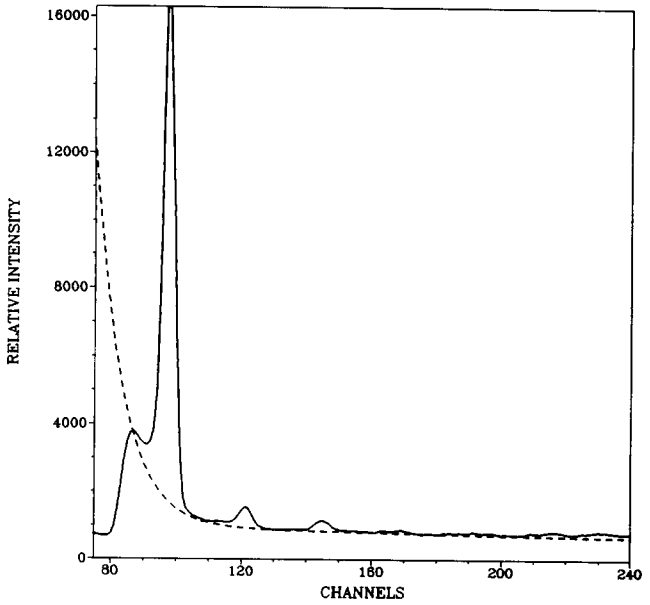

Fig. 1. One-dimensional neutron diffraction pattern of $DC_{8,9}PC$ at 98% relative humidity and $X(D_2O) = 1.0$. The dashed line is the two-exponential background correction.

negligible (approx. 20 μm), a correction for absorption was applied. The values used for the absorption correction were estimated using various calculations found in the literature [8]. The calculated linear absorption coefficient was 0.19 cm for samples at $X(D_2O) = 1.00$, and slightly less for samples at lower mole ratios of D_2O.

Scattering data for five orders were collected for each D_2O/H_2O ratio for samples at 98% relative humidity. Samples at 84% relative humidity yielded only three integratable orders. Both diffraction patterns included a large first order, which decreased as $X(D_2O)$ decreased. The value of d was 63 Å for the 98% r.h. patterns, and 61 Å for the 84% r.h. patterns, which is consistent with the values obtained by X-ray diffraction. One expects the unit cell spacing to decrease as the water content decreases. The corrected integrated intensity values ($I_c(s)$) are listed in Table I.

IV. Analysis

The deuterium exchange method of Worcester [2] was used to discern at what D_2O content a phase change for a particular diffraction order occurred. For centrosymmetric structures, a plot of the square root of scattered intensity (for a particular diffraction order) versus $X(D_2O)$ should yield a straight line that crosses the line of zero intensity upon a change in phase. The non-linearity of these fits was used to obtain some measure of the error involved in the collection and measurement of the various diffraction orders. The three orders of $DC_{8,9}$ PC at 94% relative humidity are shown in the plots of Fig. 2. It is clear from the plot of the second order that a change in phase occurs at $X(D_2O)$

TABLE I

Corrected integrated neutron diffraction intensities of multilayers of $DC_{8,9}PC$ at a given relative humidity in the presence of various ratios of D_2O/H_2O

Diffraction order	Relative diffraction intensity ($I_c(s)$), D_2O/H_2O ratio			
	1.0	0.75	0.50	0.25
98% r.h. ($d = 63$)				
1	5097	3157	1505	492
2	391	149	19	101
3	288	186	171	170
4	131	132	147	287
5	131	161	33	56
84% r.h. ($d = 61$)				
1	2045	1563	925	373
2	134	63	–	97
3	220	187	115	55

$= 0.50$. It is also clear that each of the $X(D_2O) = 1.00$ points is slightly underestimated. If these points are removed from a linear regression analysis, (as drawn in Fig. 2) the residuals increase for the first, second, and third orders from 0.991 to 0.999, from 0.982 to 0.998, and from 0.980 to 0.999, respectively. Therefore, the difference profiles used for comparative analysis at 84% relative humidity were calculated from (75–50)%D_2O and (75–25)%D_2O. This method [2] addresses only phase changes, not absolute phase assignments and therefore led to a set of phase families for each diffraction pattern. A swelling analysis [1] from the limited number

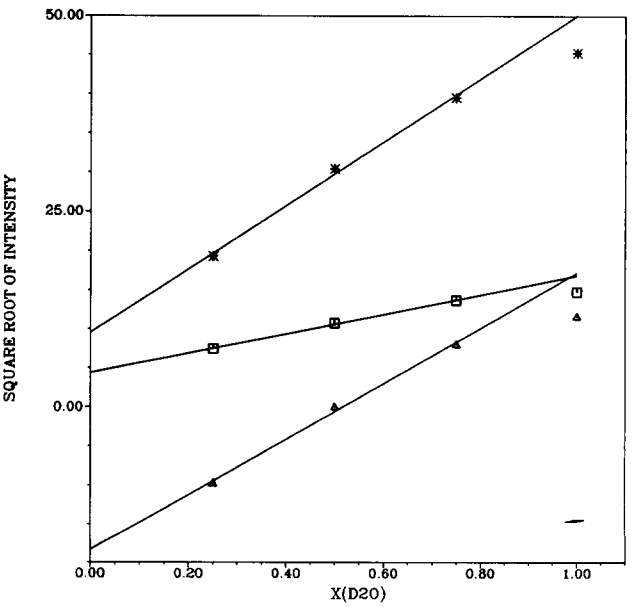

Fig. 2. A plot of the square root of neutron diffraction intensity versus $X(D_2O)$ for $DC_{8,9}PC$ at 84% r.h. The second order changes phase at $X(D_2O) = 0.25$. (*, first order; △, second order; □, third order.) The best-fit lines do not include the points at $X(D_2O) = 1.0$.

(three) of values of relative humidity helped to verify the correct phase assignment.

The structure factor was determined by calculating the square root of $I_c(s)$. Using the phases determined by D_2O/H_2O exchange, neutron scattering profiles were determined by calculating the Fourier transform of the structure factor. Phases for all orders except the second remained the same throughout the D_2O/H_2O exchange sequence (Fig. 2). The second order changed phase at $X(D_2O) = 0.25$ for samples at both 98% and 84% relative humidity. As mentioned above, the phasing method of deuterium exchange leads to a set of phase families for each diffraction pattern. Given data from only one $X(D_2O)$, phases could not be determined unambiguously. Therefore, a sequence of neutron scattering profiles were calculated with a change in phase in the second order. The profiles were compared as $X(D_2O)$ increased. Combinations of phases were disregarded when those combinations yielded neutron scattering profiles that did not vary in a consistent manner as D_2O was replaced by H_2O. Two realistic phase families were found in this way for each diffraction pattern. The neutron scattering profiles obtained by using these two families were related by an inversion and a shift of $d/2$. Both profiles resembled those expected for lipid bilayer structures, with large peaks on the edges of the unit cell and a minimum at the center (i.e., origin) of the unit cell. Water (difference) profiles were produced from the two phase families and used to identify the correct phase combination. The neutron scattering profiles used to calculate the difference profiles were constrained to have the same value at the origin where the least amount of water was to be expected. The difference profiles from the incorrect phase family indicated significant levels of water near the origin at the methyl terminus of opposing hydrocarbon chains.

V. Discussion

The two significant features in the neutron scattering profiles were a large peak at the edges of the unit cell (+32 and −32 in Fig. 3) corresponding to the water region near the bilayer phosphatidylcholine headgroup, and a minimum at the center of the unit cell (0 in Fig. 3) resulting from both the lack of water inside the bilayer and disorder where the hydrocarbon chains of opposing monolayers meet. As the deuterium content in the membrane was decreased, the neutron scattering in the water region outside the polar headgroup decreased, while the bilayer interior remained relatively unchanged. Difference profiles calculated from these scattering profiles were flat and featureless over the region corresponding to the hydrophobic bilayer interior, and had maxima at the outer cell edges, the magnitude of which increased with $X(D_2O)$.

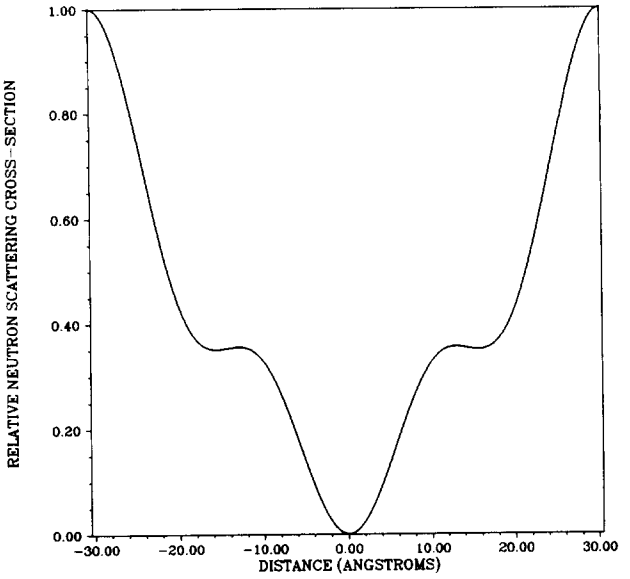

Fig. 3. Neutron scattering profile of $DC_{8,9}PC$ at 84% relative humidity and $X(D_2O) = 1.0$ calculated by performing a Fourier Transform on the square root of the integrated diffraction intensities.

The neutron scattering profiles of Worcester [9] from dipalmitoyl phosphatidylcholine (DPPC) bilayers at 84% relative humidity and 23°C were used as a basis for comparison with $DC_{8,9}PC$ profiles at 84% relative humidity and 15°C. Structure factors from DPPC at $X(D_2O) = 1.0$ and $X(D_2O) = 0.0$ (Worcester, D.L., unpublished data) were used to construct a comparative water profile at three order resolution. Both lipids were at temperatures well below their respective gel phase transition temperatures.

Five separate bases of comparison between the DPPC and $DC_{8,9}PC$ water profiles were made in order to discern whether the diacetylenic lipid was more or less hydrated than the standard model saturated PC system, DPPC. They were: the half-width at half-maximum of the maxima in the water profiles at $\pm d/2$; the distance from the center of the water region to the point where the water profile has decreased to 5% of the maximum; the relative amount of water at the phosphate position; the extent of the water peak inside the bilayer beyond the phosphate position to the position corresponding to 5% of the maximum; and the relative amount of water at the C=O region of the glycerol backbone.

All comparisons were based on the water profiles calculated at the same resolution from DPPC (100%–0%)D_2O, and the $DC_{8,9}PC$ (75%–50%)D_2O (Fig. 4). (Comparisons based on the difference profile calculated using (75%–25%)D_2O yielded similar results in all of the five criteria.) (1) The half-width at half-maximum for the DPPC water profile is 6.4 Å compared to 7.0 Å for the polymerizable lipid. (2) The distance from the maximum water position to 5% of the maximum is about 11.4 Å for DPPC whereas the analogous distance in the $DC_{8,9}PC$ bilayer is 12.7 Å. (3) The phosphorus

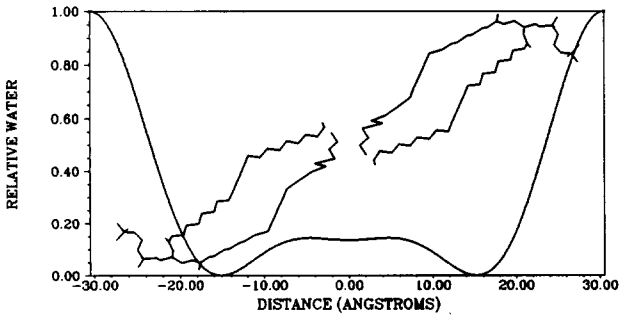

Fig. 4. Water (difference) profile of $DC_{8,9}PC$ at 84% relative humidity, calculated from the difference between neutron scattering profiles at $X(D_2O) = 0.75$ and $X(D_2O) = 0.50$ used for comparison with DPPC water profile. A model molecular conformation derived from the X-ray diffraction data of hydrated $DC_{8,9}PC$ bilayers has been included for reference (Ref. 5 and Blechner, S.L., unpublished data).

position for DPPC was estimated from X-ray diffraction of multilayers at 84% r.h. in our laboratory and other laboratories [10] to be at 22.3 Å from the bilayer center. At this position, the amount of water present is about 48% of the maximum amount of water in the interbilayer space. $DC_{8,9}PC$ bilayers appear to be more hydrated, approx. 56% of the maximum, at the phosphorus position, which is about 24 Å from the bilayer center. (4) For both DPPC and $DC_{8,9}PC$ bilayers, the water extends into the bilayer about 6 Å beyond the phosphate position. (5) The extra electron density of the glycerol backbone appears as a shoulder on the phosphate peak in moderately high resolution (better than 8 Å) electron density profiles, and was used to estimate the C=O position. At the C=O position for DPPC (approx. ± 17.6 Å from the bilayer center), the amount of water is equal to about 6% of the maximum. However, at the C=O position for $DC_{8,9}PC$ (approx. ± 21.0 Å from the bilayer center), there is about 26% of the maximum water present.

VI. Conclusion

From the forgoing analysis it seems clear that bilayers of $DC_{8,9}PC$ are not less hydrated than those of the saturated PC, DPPC. The data show that at the C=O region, $DC_{8,9}PC$ is clearly more hydrated than DPPC. In discussing their FTIR results, Rudolph and Burke suggested two possible explanations for the C=O stretch data, either the conformation of the $DC_{8,9}PC$ headgroup is very different from that of the saturated PC analog or the $DC_{8,9}PC$ headgroup is less hydrated. These authors gave much greater weight to the latter explanation, with the justification being the resemblance of the $DC_{8,9}PC$ spectrum to that of saturated PCs in anhydrous phases.

Our work has shown that the polar region in the bilayers of tubules formed by the solvent precipitation method is at least as well hydrated as that of the non-diacetylenic phosphatidylcholine, DPPC. It does not seem likely that the bilayer composition of the tubules formed by the thermal method (as in Rudolph and Burke's work) and the solvent method (this work) are significantly different based on spectroscopic data [4] or diffraction data (Blechner, S.L. et al., unpublished data). The FTIR results reported by Rudolph and Burke therefore indicate that the conformation of the $DC_{8,9}PC$ phosphate headgroup is quite distinct from that of normal phosphatidylcholines.

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