Polyethylene glycol as a hydration agent in oriented membrane bilayer samples

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ABSTRACT Techniques such as NMR, ESR, fluorescence depolarization, and neutron scattering are commonly used to investigate the physical properties of membranes. Oriented membrane bilayer systems (single crystals) are often employed in these investigations. It is important to know and be able to control the level of hydration in these samples. In particular, one must have confidence that a sample is in fact "fully hydrated" and remains so during the course of the experiment. Full hydration is difficult to obtain by hydrating oriented samples using water-saturated vapor. An alternative method for hydrating oriented samples is to surround the oriented sample by a polymer solution. Higher hydration levels are achieved using this method. Three nuclear magnetic resonance studies using headgroup deuterated 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) were done to compare the hydration level of oriented headgroup samples surrounded by a polymer/water solution and fully hydrated multibilayer dispersions. Transition temperatures, quadrupolar splittings (at 50°C) and spin-lattice relaxation times (at 50°C) were measured. The simple tests of the transition temperature and quadrupolar splitting to determine full hydration, as my results show, are not sufficient. In this paper I demonstrate that more fully hydrated samples can easily be achieved by surrounding the oriented sample with a 5 wt % polyethylene glycol/water solution than by hydrating in water saturated vapor.

INTRODUCTION

Under biological conditions full hydration, defined as no change in physical properties with the addition of water, is generally ensured. Consequently, it is important to study model membranes in a fully hydrated environment. Membranes under physiological conditions are anisotropic fluids and many studies use this characteristic of membranes to investigate membrane physical properties. Single crystals are the ideal samples for anisotropic investigations, and for phospholipids this requires orienting the lipids between glass plates.

Oriented bilayer samples have been used for nuclear magnetic resonance (NMR) studies with ^{2}H (1, 2), ^{13}C (3), and ^{31}P (4, 5) nuclei. ^{2}H NMR relaxation measurements are powerful techniques for investigation of the dynamics of molecules (6), and one of the best ways to investigate the motions is through the anisotropy of the relaxation, i.e., the dependence of the relaxation on the orientation of the bilayer normal in the static magnetic field (7). Oriented membrane bilayer samples have been used to probe lipid motions fast (8–10) and slow (11) on the NMR timescale. In addition, electron spin resonance (12, 13), fluorescence depolarization (14), x-ray diffraction (15), and neutron scattering (16) are all techniques that have used oriented bilayer samples.

In oriented membrane bilayer samples the amount of water on the lipid bilayer is strictly controlled by the osmotic stress. The water in the multibilayers is brought to thermodynamic equilibrium with a neighboring known water phase. There are three ways of achieving this equilibrium: the multibilayer is subjected to a polymer solution whose large solutes cannot penetrate between the bilayers; or the multibilayer is physically squeezed under pressure in a chamber with a semi-permeable membrane to allow exchange with a water reservoir; or the multilayer is brought to equilibrium with a vapor of known relative humidity (17). These techniques have all been used, along with others, to investigate hydration forces between phospholipid bilayers (17). Rand and Parsegian (17) give the water content between bilayers in excess water for several lipid species. The weight percent of water varies from 46% for DPPC at 50°C to 21% for 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) at 30°C. In the same paper Rand and Parsegian state that lipids exposed to a water vapor of 100% humidity will not take up as much water as will the same sample in contact with liquid water. They give the example for a phosphatidylcholine multibilayer which will absorb 45–55 wt % water from the liquid, but only 30 wt % from a water-saturated vapor. Charged phospholipids are even worse. The other problem with using vapor pressure to control hydration is that large changes in the osmotic stress occur due to small changes in relative humidity resulting from tiny temperature fluctuations (17).

Polymers have long been used to regulate the amount of water in lipid systems in order to measure interbilayer forces (18, 19). If the bilayers are equilibrated with an external solution of a polymer such as dextran or polyethylene glycol, where the polymer molecules are too large to enter the lattice structure, water can be added or removed from the bilayers. Both the polymer and the lipid compete for water and a chemical equilibrium is established; thus, by increasing the concentration of the polymer in the water one can decrease the amount of water on the bilayer.

The level of hydration of the bilayer, as is well known, is important. The phase diagram for DPPC/water was

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established in the early 1980's (20). More recently, specifically deuterated phosphatidylcholine headgroups have been used to investigate the conformation of the headgroup in bilayer membranes (21) and the headgroup mobility (22) as a function of hydration. These studies were all done on multibilayer dispersions.

The phase diagram for the DPPC/water system has been determined from differential scanning calorimetry (20, 23, 24). The phase transition from gel to liquid crystalline is constant above ≈25 wt % water, as discussed in (20). Below this point the phase transition temperature increases with decreasing water content. Between ≈2.5 and 17 wt % water there is no ripple phase; instead, there is a coexistence phase of gel and liquid crystalline. The transition temperature varies between 43°C for a fully hydrated sample to 75°C for a 5 wt % water sample. The main transition temperature is a good method for determining the hydration level for low water content samples.

Bechinger and Seelig recently published a paper on the conformational changes of the phosphatidylcholine headgroup due to membrane dehydration (21). The quadrupolar splitting of the two deuterated methylene segments (α and β) of the headgroup of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was measured in multibilayer dispersions as a function of hydration in the range 10–40 wt % water. A distinct change in the alignment of the phosphatidylcholine headgroup was reported, for as the hydration decreased, the N^+ end of the phosphatidylcholine head group dipole moved closer to the surface of the bilayer. The quadrupolar splitting of the α deuterons decreased non-linearly and for the β position the splitting increased non-linearly, with increasing hydration up to ≈28 wt % where the splittings leveled off and then remained constant. Bechinger and Seelig do not specify the minimum amount of water needed for a fully hydrated sample. Based on their observations, one cannot distinguish, by quadrupolar splitting measurements, hydration levels above 28 wt % and thus from the perspective of the splitting 28 wt % is "fully hydrated."

Spin-lattice relaxation measurements have been done to study the dependence of phospholipid headgroup mobility on hydration by Ulrich et al., (22). They studied multibilayer dispersions of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) deuterated at the choline methyl group. They found that the effective rate of rotation increased almost linearly with the addition of water to a limiting hydration of 34 wt %, beyond which the motional correlation function remained constant. Spin lattice relaxation times varied between 20 and 65 ms for a variation between 10 and 70 wt % water. The sample was considered "fully hydrated" at 34 wt % water. This will be the most stringent test for full hydration used in this paper, because the transition temperature measurement cannot distinguish between hydrations above 25 wt % and the quadrupolar splitting does not change above 28 wt %. In the paper by Ulrich et al. (22), the experiments were performed at 30°C, which ensured the bilayers were in the liquid crystalline phase for DOPC since the transition temperature for a fully hydrated sample is −22°C. They comment that the transition temperatures were "somewhat higher for the partially hydrated samples," but gave no temperatures.

From the results discussed above it is clear that the hydration of the bilayer is an important parameter to consider. It is important to maintain a constant and well characterized hydration level for investigations into the orientations and motions of phospholipids. For powder samples it is straightforward to control the hydration, but for oriented samples it is not. The hydration of oriented samples generally takes place in a humid atmosphere, the time to hydrate varying from 4 to 72 hours, depending on the protocol of a given laboratory (9–11). As mentioned previously, this does not produce a fully hydrated sample.

I have compared the transition temperatures, the quadrupolar splittings at 50°C and spin-lattice relaxation times at 50°C for fully hydrated multibilayer dispersions and oriented samples surrounded by a 5 wt % polyethylene glycol (PEG) solution. DPPC specifically deuterated at the α position of the methylene segment in the headgroup (αDPPC-d_2) and DPPC specifically deuterated at the β position in the headgroup (βDPPC-d_2) were studied. An oriented sample of βDPPC-d_2 with a 50 wt % PEG solution, i.e., a partially hydrated sample, was investigated for comparison purposes.

MATERIALS AND METHODS

Materials

DPPC-d_2 specifically deuterated in each of the α and β positions of the headgroup, was a gift from Dr. Michel Roux at CEN-Saclay. The powder samples were prepared by dissolving 50 mg of lipid in excess chloroform. They were then dried under N_2 and placed under vacuum (10^{-2} mm Hg) for 15 hours. The dried lipid was suspended in 600 μL of ^{2}H-depleted water and submitted to three freezing (liquid nitrogen) and thawing (50°C) cycles. The sample was then transferred to a 10 mm (o.d.) NMR tube.

The oriented samples were prepared as follows. A 20 mg amount of DPPC was dissolved in 200 μL of chloroform (0.1 g lipid per mL of chloroform). The mixture was then spread onto approximately 25 glass plates (1.6 cm × 0.7 cm) 1 μL at a time until all the mixture was used. Following this, the plates were placed under vacuum at 50°C for 24 hours to evaporate off the chloroform. They were subsequently hydrated at 50°C in a humid atmosphere of ^{2}H-depleted water for 72 hours. The glass plates were stacked in a 10 mm (o.d.) NMR tube. Approximately 500 μL of a PEG-water solution was then inserted into the tube and left for 24 hours to equilibrate. Polyethylene glycol with a molecular weight of 8,000 was used for these experiments. For an x wt % sample $x(0.01)$ g of PEG was dissolved in $(1 - x(0.01))$ mL of ^{2}H-depleted water and heated to insure that the PEG fully dissolved

(most important for the large wt % samples). For notation purposes, an x wt % PEG/water solution will be denoted x-PEG.

NMR methods

Deuterium NMR experiments were performed in a field of 7.2 T on a home-built NMR spectrometer (25, 26) using a 90° pulsewidth of 4 μs and a dwell time of 5 μs. The signal was detected in quadrature with phase cycling for all pulse sequences (25, 27). Constant temperatures were maintained using a Bruker Model BV-T1000 temperature controller.

The orientation of the oriented sample in the magnetic field was controlled by a home-built goniometer, accurate to ± 0.5°.

Spectra were measured using a quadrupolar-echo pulse sequence (90°_y–τ–90°_x–t) with a τ value of 50 μs and a repetition time between pulse trains of 150 ms. Transition temperatures were determined from the spectra to within 1°. The transition temperature was observed in the powder by a reduction in the first moment and a sharpening of the resonance lines, while in the oriented samples only the sharpening of the resonance lines was used. The quadrupolar splittings were measured at 50°C.

Zeeman energy spin-lattice relaxation, T_{1z}, was measured using an inversion-recovery pulse sequence (180°_y–τ_1–90°_y–τ_2–90°_x–t), modified to have a decaying signal intensity with increasing τ_1. The modification involved subtracting the inverted spectrum from the quadrupolar spectrum (90°_y–τ_2–90°_x–t). Ten different τ_1 values between 1 and 90 ms were used; τ_2 remained constant at 50 μs. The temperature was maintained at 50°C for the quadrupolar splitting and relaxation time measurements.

Numerical methods

For the powder samples, single exponential relaxation rates were determined from the initial decay of the echo associated with the second and third pulses as a function of τ_1 (28). A five point average over the peak was used. For the oriented samples the spectra were used; the relaxation time for each side of the spectrum was evaluated individually and then averaged. The relaxation time was determined by fitting the area under the spectral peak to a single decaying exponential using a least-squares fitting routine. Five different orientations of the bilayer normal in the static magnetic field (angle θ) were measured. To compare with the powder relaxation times, which have contributions from all orientations, an average over the five angles needed to be done. Since the angles were not at equal intervals of cos θ, an equally weighted average of T_{1z}s could not be performed; alternatively, the average was determined by weighting each $1/T_{1z}$ by sin θ.

RESULTS

Deuterium NMR spectra were measured to compare fully hydrated powder samples with oriented samples surrounded by a PEG/water solution. The results for the quadrupolar splittings, the transition temperatures, and the spin-lattice relaxation times, T_{1z}, for each sample are summarized in Table 1.

Five DPPC samples were used: two powder samples; one with the α position of the headgroup deuterated, the other the β position, and three oriented samples; a 5-PEG solution surrounding αDPPC-d_2 and two samples of βDPPC-d_2, one surrounded by 5-PEG and the other by 50-PEG.

The transition temperatures were measured for four of the five samples. Fig. 1 demonstrates how the spectra changes as βDPPC-d_2 undergoes a phase transition from the ripple to liquid crystalline phase; both the multibilayer dispersion and the 5-PEG oriented samples are shown. For the powder samples and the 5-PEG βDPPC-d_2 oriented sample, the transition temperatures all agree to within experimental error, indicating we have at least 25 wt % water for this 5-PEG sample. This is the least stringent test for a fully hydrated sample. The transition temperature increased for the 50-PEG sample, as expected from the phase diagram. Based on the phase diagram in reference 20, the hydration level is ≈20 wt % water.

TABLE 1 **Quadrupolar splittings, transition temperatures, and spin-lattice relaxation times for headgroup deuterated DPPC**

Sample	$\Delta\nu$	T_m	Ave T_{1z}
	kHz	*°C*	*ms*
α-Powder	6.15	43	26.9 ± 0.7
α-Oriented 5-PEG	6.2	NA	27.3 ± 0.5
β-Powder	4.95	43	31.7 ± 0.4
β-Oriented 5-PEG	4.95	43	30.5 ± 0.5
β-Oriented 50-PEG	4.7	46	24.2 ± 0.6

$\Delta\nu$ and Ave T_{1z} were measured at 50°C.

The quadrupolar splittings for the αDPPC-d_2 and βDPPC-d_2 powder samples were 6.15 and 4.95 kHz, respectively. In the oriented samples with the 5-PEG solution, the quadrupolar splittings were 6.2 kHz for the α position and 4.95 kHz for the β position, thus indicating at least 28 wt % water in the sample. The splitting decreased to 4.7 kHz for the 50-PEG sample as expected, since hydration decreases with increasing weight percent of PEG and, according to Bechinger and Seelig, the splitting of the β position decreases with decreasing hydration. Fig. 2 shows two spectra for βDPPC-d_2 samples oriented at 0° with respect to the magnetic field (maximum splitting). The dotted line is a spectrum of βDPPC-d_2 prepared in the method outlined in the previous section, with the exception of the addition of a PEG solution. The sample was simply left to hydrate for an additional 24 hours after stacking. As one can see, the splitting is less than it should be for this orientation. A 5-PEG solution was then added to the same sample, which was left to equilibrate for 24 hours prior to recording the solid line spectrum shown in the Fig. 1. The sample was stable over a period of a week, with no degradation in the orientation or change in splitting.

The spin-lattice relaxation times were measured in all five samples. For the powder samples the T_{1z}, as determined from the initial slope of the decay, represents the true T_{1z} and not one that has been reduced appreciably by the effects of diffusion of the DPPC molecules around the liposomes (28). Therefore, the relaxation times of the oriented samples, in which diffusion has no effect, can be compared with the powder. The relaxation times

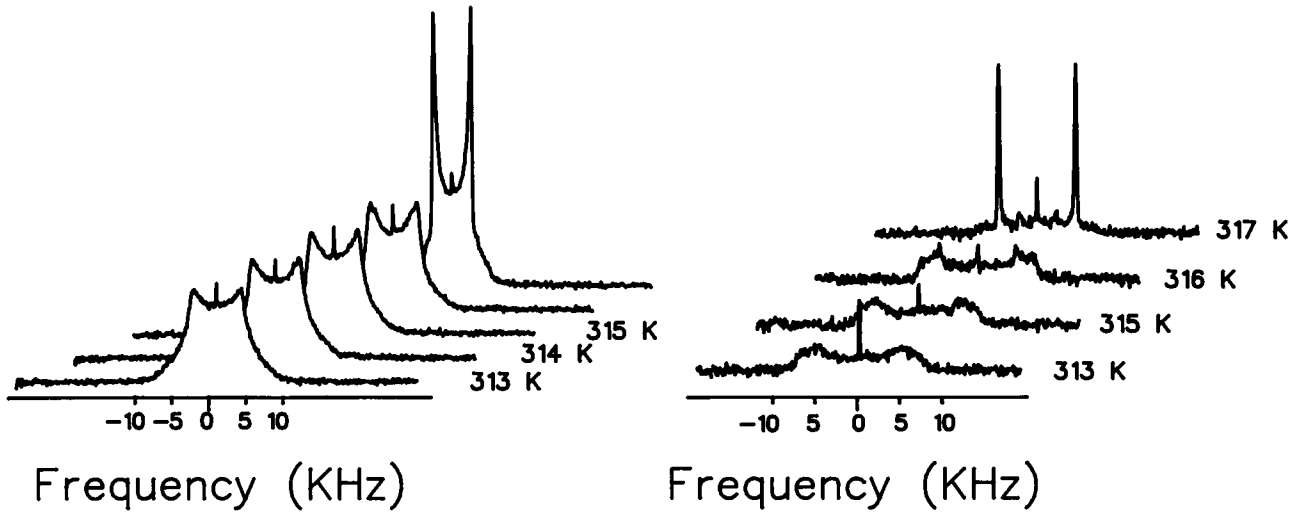

FIGURE 1 Transition temperatures for βDPPC-d_2: powder (*left*) and oriented with 5-PEG (*right*) (0° orientation with respect to magnetic field).

are listed in Table 1 with the errors representing only the error in the fits. The spin-lattice relaxation times for both powder and oriented samples are not significantly different, indicating we have a hydrated sample which, according to Ulrich et al., is at least 34 wt % water. The 50-PEG sample showed a decrease in the relaxation time from 31 to 24 ms. It should be noted that all relaxation times were measured at the same absolute temperature

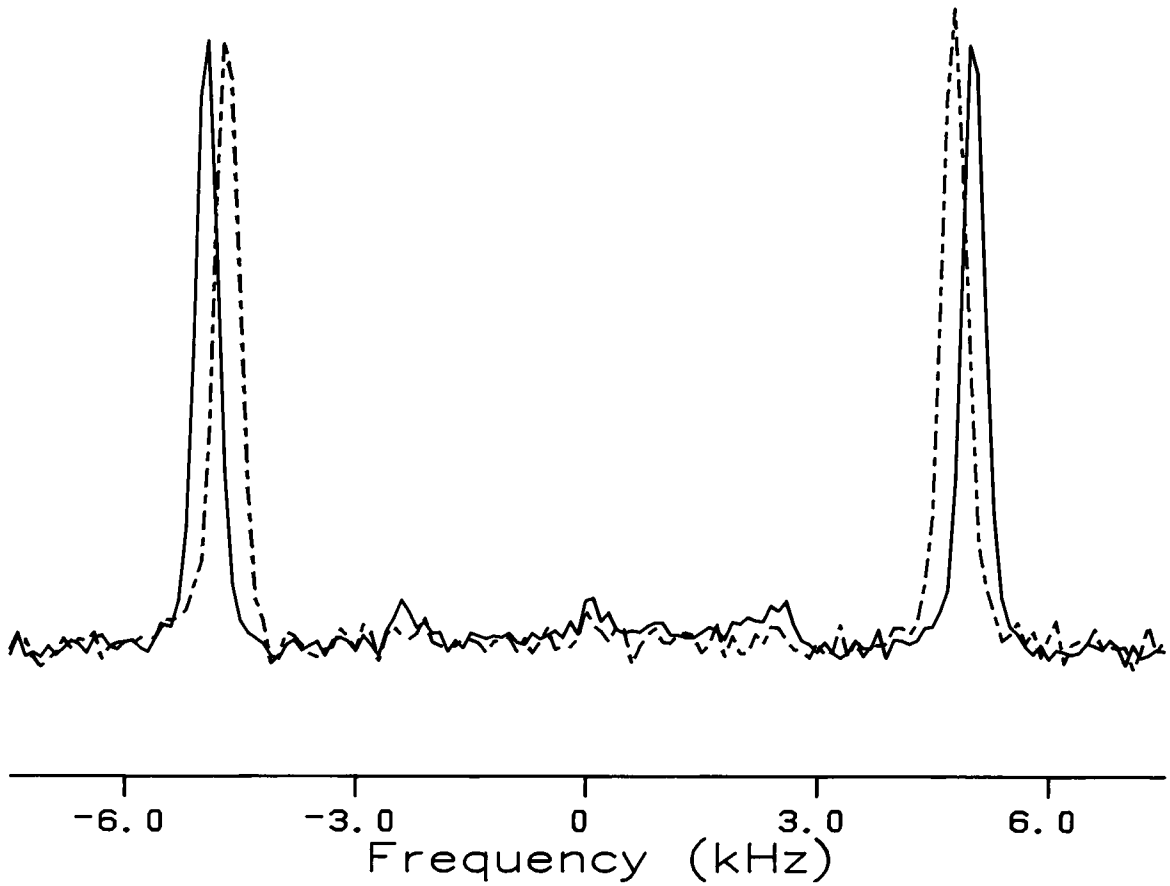

FIGURE 2 Spectra for βDPPC-d_2 with 5-PEG (*solid line*) and without PEG (*dashed line*). 0° orientation with respect to magnetic field.

of 50°C; however, the phase transition temperature differed by only two degrees in the 50-PEG sample. For an oriented sample of αDPPC-d_2 with no PEG solution, the relaxation times were considerably shorter (21 ms compared with 27 ms for the powder) and were not reproducible (due to the sample dehydrating over the course of the long experiment).

We also looked at surrounding the glass plates with bulk water, 1-PEG and 2-PEG solutions, but the orientation of the lipids was destroyed under such circumstances.

DISCUSSION

We have shown that an oriented sample surrounded by a 5-PEG solution is fully hydrated as determined by the transition temperature, the quadrupolar splitting and the average spin-lattice relaxation rate. Based on the spin-lattice results, the sample must be hydrated to at least 34 wt % water at 50°C. Our NMR criteria are not sensitive to the addition of any more water. The additional 12 wt % water, which Rand and Paresegian (17) report is present in a fully hydrated sample, does not affect the spin-lattice relaxation time. Therefore, from the perspective of the fast motions of the headgroup, the additional water plays no role and thus the sample can be considered fully hydrated at 34 wt % water. The additional water may simply cause the bilayers to separate more. The definition of fully hydrated depends on the measurement technique and what the technique is sensitive to. The question arises whether a 5-PEG solution hydrates or dehydrates the sample. For oriented samples this method hydrates the sample more than water saturated vapor does and is a more consistant and stable method for hydrating and maintaining hydration. Surrounding the sample with bulk water would be the best way to ensure full hydration, but unfortunately the orientation of the lipids cannot be maintained under these conditions.

Polyethylene glycol can also be used to dehydrate the sample, as was indicated with the 50-PEG sample. It is now clear that by surrounding these samples with a large wt % of PEG in water the hydration can be significantly reduced (Lees, I., unpublished results).

Using a PEG/water solution is a good technique for regulating the amount of water on lipid bilayers oriented on glass plates. A more hydrated sample can be made by simply surrounding the stack of glass plates with a 5 wt % PEG/water solution than can be achieved by hydration in a humid atmosphere alone. With 5 wt % PEG the sample is hydrated to at least 34 wt % water. The hydration remains constant over a long period of time even at high temperatures, which is difficult to maintain in samples without PEG.

I wish to thank Professor Evan Evans and Professor Peter Rand for useful suggestions on using a polymer to control hydration, Irene Lees for telling me about her unpublished results, and Professor Myer Bloom for helpful discussions.

This work was supported by the Natural Sciences and Engineering Research Council of Canada.

Received for publication 8 June 1992 and in final form 30 November 1992.

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