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Re-analysis of Magic Angle Spinning Nuclear Magnetic Resonance Determination of Interlamellar Waters in Lipid Bilayer Dispersions

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ABSTRACT A recent method to obtain the number of water molecules of hydration of multilamellar lipid vesicles using magic angle spinning nuclear magnetic resonance has been re-examined. The previous interpretation divided the water into bulk and interlamellar water and ignored water in defects (lakes) that are intrinsic to multilamellar lipid vesicles; the result was inconsistent with x-ray results for the lipid DOPC. The new interpretation takes advantage of the reduction of lake water with increased spinning and it uses osmotic pressure measurements to determine the loss of interlamellar water. The new result for DOPC from magic angle spinning is consistent with x-ray results.

INTRODUCTION

Recently, a new method has been introduced to study hydration of lipid bilayers that uses nuclear magnetic resonance (NMR) magic angle spinning (MAS) (Zhou et al., 1999). The first published results for dioleoylphosphatidylcholine (DOPC) bilayers were interpreted to give the number n_w of interlamellar water molecules per lipid molecule to be at least 37.5 in the L_α phase. This result is significantly larger than the value $n_w = 32.5 \pm 0.8$ obtained by Tristram-Nagle et al. (1998) using x-ray methods. This new MAS result would mean that the interfacial area/lipid is at least $A = 77$ Å^2, rather than the previously obtained $A = 72$ Å^2 (Tristram-Nagle et al., 1998; Rand and Parsegian, 1989), and the corresponding 7–10% differences would be obtained for the various bilayer thicknesses that are useful for discussing protein–lipid interactions. Because these quantitative NMR measurements took proper account of water spin-relaxation times and possible intensity contributions from spinning sidebands, an alternative explanation was sought for the discrepancy with the x-ray results. In this article, we offer a different interpretation of the new MAS method that obtains good agreement with the x-ray results.

Figure 1 shows the MAS sample cell and a rough schematic of the arrangement of multilamellar vesicles (MLVs), which, for simplicity, are represented as having spherical shapes. The central physical insight of Zhou et al. (1999) was that MAS is equivalent to placing the MLVs in a centrifuge cell. The MAS experiments are done in D_2O, which is about 10% denser than DOPC bilayers so that the excess D_2O centrifuges to the periphery of the spinning cell and the MLVs are pushed to the central part. (One caveat that may be mentioned is that the NMR signal comes from H_2O, which is a minor impurity in D_2O in the MAS experiment, and this raises the question whether the distribution of H_2O in the cell is the same as that of D_2O. It is the same, because the centrifugal energy difference of H_2O molecules in different parts of the MAS cell is negligible compared to thermal energy k_BT. In contrast, the centrifugal energy difference of MLVs, or even single bilayers, compared to D_2O, is much larger than k_BT because bilayers are so massive.)

The first interpretation of the MAS results (Zhou et al., 1999) made the classic assumption that all the water in the system is either interlamellar water neatly situated between well-stacked bilayers or is bulk water in the excess water phase. This assumption has plagued the lipid field for many years. It is the assumption made in the gravimetric (so-called Luzzati) method (Rand and Parsegian, 1989). This assumption has been widely recognized as incorrect for multilamellar vesicles near full hydration (McIntosh and Simon, 1986; Klose et al., 1988; Tristram-Nagle et al., 1993; Nagle et al., 1996; Kodama et al., 1997; Koenig et al., 1997). One now recognizes that there is a third kind of water that fills the spaces between MLVs and any other defect regions where the bilayers are not neatly stacked in well-oriented arrays. This water is indicated in Fig. 1 by the regions marked *L*, which may be thought of as "lake water." It is essentially bulk water, but confined to smaller regions rather than the larger, truly excess, water regions that may be called "oceans." Ocean water is shown near the periphery of the MAS cell in Fig. 1 and is marked *O*. In x-ray studies, lake water does not contribute to the measured d-space in the MLVs, but, as previously emphasized (Tristram-Nagle et al., 1993; Nagle et al., 1996), it contributes to the total weight, and this results in consistent overestimates of n_w using gravimetric data.

The MAS NMR approach to study MLV hydration sees two water populations in slow exchange (Zhou et al., 1999), but this observation does not require that only two types of water are present. In fact, much of the lake water will contribute to the interlamellar water signal as a simple estimate shows. Spherical MLVs that are of an order of 10

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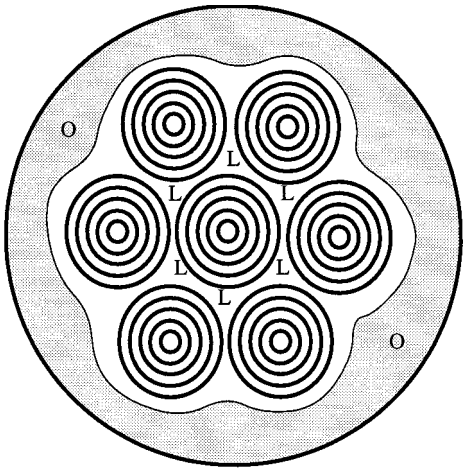

FIGURE 1 Rough schematic illustrating seven MLVs in a MAS cell spinning about the central axis, which is perpendicular to the page. The bilayer repeat is $D = 63$ Å and typical radii are 10 μm for the MLVs and 1.2 mm for the MAS cell, so there are many more bilayers per MLV than depicted and many more MLVs. Lake water is indicated by *L*, bulk (ocean) water by *O*, and interlamellar water (n_w per lipid molecule) is between bilayers in each MLV. The NMR MAS method sees only the shaded water as bulk water.

μm in diameter have lakes with linear dimensions of order 1–4 μm. Water in these lakes diffuses a distance of 5 μm on the NMR time scale (6 ms) and, therefore, samples the lipid headgroup environment and has the same chemical shifts as interlamellar water. It may also be noted that much of the true interlamellar water will also diffuse into the lake regions. The effective coefficient of diffusion of water through L_α phase bilayers is about $2 \cdot 10^{-9}$ cm^2/s, so the root mean square radial diffusion distance within MLVs is about 500 Å. These considerations suggest that the MAS method blurs the distinction between interlamellar and lake water. Even some of the ocean water near the MLVs will be seen by NMR as interlamellar water. (This could be called continental shelf water, but it must be recognized that this is an operational term, not a purely locational one, because, due to the vagaries of diffusion, some of this water will not have interacted with the MLVs on the NMR time scale and some will have done.) However, for truly excess water located more than 5 μm from MLVs, the environment is that of pure water on the NMR time scale.

If the simple picture of packed spherical MLVs shown in Fig. 1 were true, then one could estimate that the ratio of lake water to interlamellar water is about 0.6. However, MLVs generally have more complex shapes than simple spheres. It is more likely that lake regions will be smaller than shown because of deformation of the MLVs, especially when under centrifugal force. It is therefore not feasible to use geometrical models to try to account for this complicated effect.

The other effect of centrifugation in a MAS cell, pointed out by Zhou et al. (1999), is to remove interlamellar water from within the MLVs. Since this reduces n_w below that for fully hydrated MLVs, Zhou et al. (1999) used their lowest spinning frequency $\omega = 3$ kHz to estimate n_w. (Incidentally, extrapolating their data to $\omega = 0$ gives an even larger n_w that is $\geq$40 for DOPC.) However, these values are clearly only upper bounds to the true interlamellar n_w because of the inclusion of lake water n_l per lipid.

Nevertheless, the basic MAS measurement can be valuable because spinning at high frequency reduces the amount of lake water n_l. To implement this idea, we show in this article that the loss of interlamellar water Δn_w can be separately measured using x-ray data taken under osmotic pressure (Tristram-Nagle et al., 1998) together with a simple geometric conversion of MAS centrifugal force into osmotic pressure. When Δn_w is added to the MAS data, the sum decreases with increasing ω, indicating that lake water is indeed decreasing, consistent with the above picture. Extrapolating to high spinning frequency then enables estimation of n_w that is consistent with x-ray results.

DETERMINATION OF INTERLAMELLAR WATER LOSS

The centrifugal force on a D_2O molecule is $F(r) = (\Delta m)\omega^2 r$ where $\omega = 2\pi\nu$ is the spinning angular frequency, r is the distance from the spinning axis, and Δm is the mass of D_2O minus the mass of an equivalent volume of MLVs ($\Delta m = m_{D_2O} - \rho_{MLV} V_{D_2O}$). It is more fundamental to work with the centrifugal energy $E(r)$,

$$E(r) = -\int F(r)\mathrm{d}r = (\Delta m)\omega^2[r_w^2 - r^2]/2, \qquad (1)$$

where the choice of zero energy has been set to $r = r_w$, which is defined to be the radius that separates the MLV region from bulk water. We note that r_w decreases as ω increases because more water is moved from the MLV region to the bulk region and we now proceed to calculate r_w. Since the MAS chamber is a sphere (radius $r_c \approx 1.2$ mm), the volume of the MLV region is

$$v_{MLV} = (4\pi r_c^3/3)(1 - [1 - (r_w/r_c)^2]^{3/2}). \qquad (2)$$

We first divide v_{MLV} by the volume of the MAS cell and then consider the molecular partitioning of lipid and water, where n_o is defined to be the number of ocean water molecules per lipid given by the MAS data, $n_w + n_l$ is the number of water molecules per lipid in the MLV region (including both interlamellar water n_w and lake water n_l) and is given by the MAS measurement (where total water $n_w + n_l + n_o$ was fixed at 40 in the MAS experiment), $V_L = 1303$ Å^3 is the measured molecular volume of DOPC (Tristram-Nagle et al., 1998), and $V_w = 30.3$ Å^3 is the room temperature volume of the D_2O molecule. This yields

$$[1 - (r_w/r_c)^2]^{3/2} = n_o V_w/[V_L + (n_w + n_l)V_w + n_o V_w]. \qquad (3)$$

We next note that Δm increases with increasing ω because the average density of the MLV region,

$$\rho_{MLV} = \frac{m_L + (n_w + n_l)m_w}{V_L + (n_w + n_l)V_w}, \qquad (4)$$

decreases as the denser D_2O is moved to the ocean region. MAS measurements yield an overall average for $n_w + n_l$, but not the r dependence. However, the variation in Δm is only a few percent, so the average value will be used for all r. When $\omega = 0$, $\Delta m = 1.02$ g/mole D_2O and this increases to about 1.12 g/mole at the highest spinning frequencies.

The centrifugal energy $E(r)$ is equivalent to an osmotic energy,

$$E(r) = P(r)V_w, \tag{5}$$

where $P(r)$ is the osmotic pressure at radius r. Standard x-ray D-spacing measurements on MLVs under osmotic pressure (Rand and Parsegian, 1989) obtain the loss in interlamellar water Δn_w using

$$\Delta n_w = [D(0) - D(P)]A/2V_w, \tag{6}$$

where $D(P)$ is the D-spacing under osmotic pressure P and A is the area per lipid in the bilayer. Technically, one should allow for A to be a function of P, but, for the largest osmotic pressures applied at the center of the MAS cell (less than 7 atmospheres for 10 kHz) and using the measured area compressibility $K_A = 188$ dyn/cm (Tristram-Nagle et al., 1998), the decrease in A is only about 1% and will be neglected. This is equivalent to neglecting the change in bilayer thickness. The $D(P)$ data that were used to calculate Δn_w in Eq. 6 were obtained by Tristram-Nagle et al. (1998). Figure 8 of that paper shows $D'_W(P)$; to convert to $D(P)$ just add D'_B.

Conventionally, fits to x-ray data obtain $P(D)$ in terms of the conventional forms for interbilayer interactions, such as hydration repulsion, van der Waals attraction, and repulsive fluctuation pressures. The fit used in this article used the steric definition of water space thickness (McIntosh and Simon, 1993), where $D'_w = D - 45.3$ Å for DOPC (Tristram-Nagle et al., 1998). Using this convention, the exponentially decaying hydration force has $\lambda = 2.22$ Å and prefactor $P_h = 5.5 \cdot 10^8$ erg/cm^3, the exponentially decaying fluctuation pressure has $\lambda_{fl} = 5.8$ Å and prefactor $P_{fl} = 5 \cdot 10^6$ erg/cm^3, and the van der Waals attraction has Hamaker parameter $H = 4.7 \cdot 10^{-14}$ erg (Tristram-Nagle et al., 1998). (Other combinations of the parameter values fit the $P(D)$ data equally well; this ambiguity makes no difference in this application.) Then, $P(D)$ was inverted numerically to obtain $D(r)$ at each radius r in the MAS experiment. The total water loss Δn_w was then obtained by integrating over all radii r using Eq. 6 where the variable P is replaced by r using Eqs. 5 and 1,

$$\Delta n_w = v_{MLV}^{-1} \int_0^{r_w} \Delta n_w(r) 4\pi r [r_c^2 - r^2]^{1/2}\, dr. \tag{7}$$

RESULTS AND DISCUSSION

Figure 2 shows results for the loss of interlamellar water Δn_w as a function of spinning frequency squared ν^2, which is proportional to spinning energy and osmotic pressure. Figure 2 also shows the MAS result (Zhou et al., 1999) for bulk (ocean) water n_o. The increase in ocean water exceeds the loss of interlamellar water because lake water is also squeezed out of the MLV region into the ocean. In other words, ocean water is increasing and interlamellar water is decreasing, but the latter decrease is not as large as the former increase because lake water is also decreasing and this also feeds the increase in the ocean water. This effect is also quantitated in Fig. 2, which shows the amount of lake water, $n_l = 40 - n_o - n_w(0) + \Delta n_w$, where 40 is the gravimetric total number of water molecules in the MAS experiment and $n_w(0) = 32.5$ is the x-ray value for unstressed samples corresponding to $\nu = 0$.

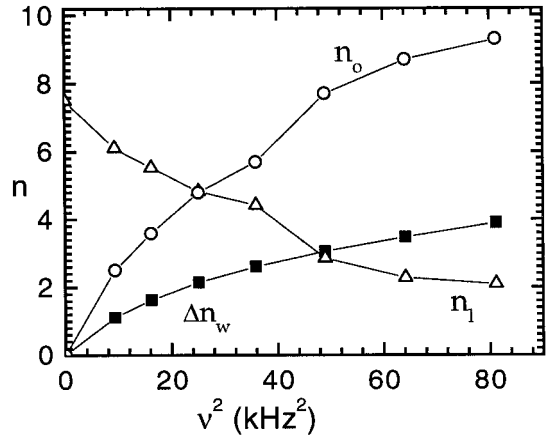

FIGURE 2 Ocean water n_o (*open circles*), loss of interlamellar water Δn_w (*solid squares*), and lake water n_l (*open triangles*) as a function of the square of the spinning frequency $(\omega/2\pi)^2$ (in kHz^2).

Figure 3 shows the MAS result for nonocean water, n_{wl}, which is called interlamellar water in (Zhou et al., 1999), but which, in this interpretation, is the sum of lake and interlamellar water, $n_{wl} = n_l + n_w$. As spinning frequency increases, $n_{wl}(\nu)$ decreases because both lake water and

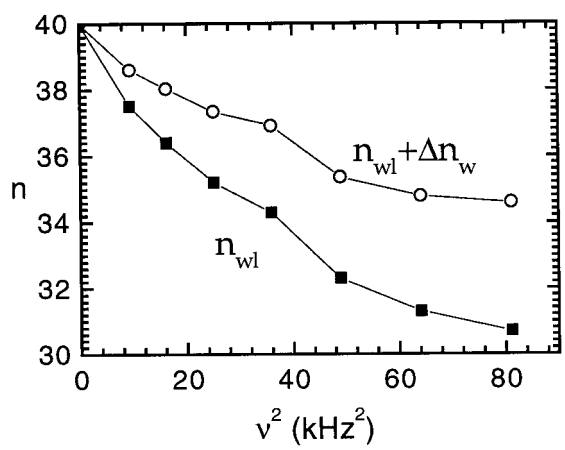

FIGURE 3 MAS result for nonocean water $n_{wl} = n_w + n_l$ (*solid squares*) and result for $n_{wl} + \Delta n_w$ (*open circles*) as a function of the square of the spinning frequency $(\omega/2\pi)^2$ (in kHz^2).

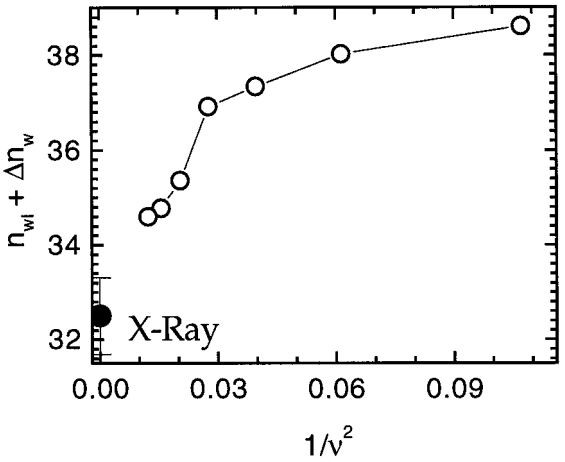

FIGURE 4 Extrapolation to high spinning frequency of $n_{wl} + \Delta n_w$ as a function of the inverse square of the spinning frequency $(2\pi/\omega)^2$. X-ray result is the solid circle.

interlamellar water are squeezed out of the MLV region. Using the result from the previous section, we can add back the loss of interlamellar water Δn_w at each ν. This gives $n_l(\nu) + (n_w(\nu) + \Delta n_w(\nu)) = n_l(\nu) + n_w(0)$, which is shown as $n_{wl} + \Delta n_w$ in Fig. 3. This latter curve should approach the unstressed, zero frequency, value $n_w(0)$ for interlamellar water if all the lake water is squeezed out at high ν. Indeed, $n_{wl} + \Delta n_w$ does decrease monotonically as shown in Fig. 3, although it is difficult to extrapolate its value for large ν from that figure. For this purpose it is more convenient to plot $n_{wl} + \Delta n_w$ versus ν^{-2}, as shown in Fig. 4. If one chooses to take the curvature of the three data at highest ν seriously, one would extrapolate to a value a bit higher than the x-ray result. This would be consistent with the possibility that there might be lake water that is highly resistant to centrifugation; one prime candidate would be lake water that is between MLVs that are near the bulk ocean because the centrifugal energy is very small at this location. However, given the obvious noise in the MAS data, it is at least as reasonable to conclude that extrapolation is consistent with the x-ray result $n_w(0) = 32.5 \pm 0.8$ (Tristram-Nagle et al., 1998).

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