Determination of DMPC hydration in the L_α and $L_{\beta'}$ phases by ^2H solid state NMR of D_2O

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Abstract The number of water molecules bound to the dimyristoylphosphatidylcholine (DMPC) interface was investigated both in the fluid (L_α) and gel ($L_{\beta'}$) phases by solid state deuterium NMR of D_2O. We determined that each DMPC molecule binds 9.7 ± 0.5 and less than 4.3 ± 0.5 D_2O in the fluid and gel phases respectively. These results are accounted for by considering the number of DMPC binding sites as well as the molecular organization in each phase.

Key words: Dimyristoylphosphatidylcholine; Solid state ^2H NMR; Bound water; Gel phase; Fluid phase

1. Introduction

Aqueous dispersions of dimyristoylphosphatidylcholine (DMPC) can exist under at least three lamellar phases: in the gel phase, $L_{\beta'}$, phospholipids are tilted as in the 'rippled' phase $P_{\beta'}$, which differs from the latter by a wavy interface. On the other hand, in the fluid phase L_α, molecules are statistically perpendicular to the lamellar plane. These lipid dispersions are thermotropic and lyotropic, i.e. phase transitions can be obtained by either temperature or hydration changes, respectively. For instance, at 20°C, removal of a portion of the interbilayer water converts the DMPC rippled phase into a gel phase [1–3]. This property indicates that each phase not only differs in molecular organization but also in its interfacial hydration. To study phospholipid hydration, several techniques have been considered in the last few decades. Infrared spectroscopy was used to determine lipid binding sites [4–6], the use of fluorescent molecules led to information on interface hydration [7,8] and differential scanning calorimetry (DSC) allowed quantification of bound water at the transition temperature [9]. Unfortunately, all these methods only provide a qualitative approach of hydration. In rare cases where a quantitative study is possible, as by DSC, one is restricted to the state close to a main phase transition. When attempting to quantify hydration as a function of temperature or phase nature, the most suitable technique appears to be deuterium NMR of D_2O [10–16]. To our knowledge the number of water molecules bound per DMPC in both gel and fluid phases is still unknown; hence we have determined this using NMR and DSC techniques. Experiments were performed on a dilution line, i.e. for several D_2O-to-DMPC molar ratios, R_i, and for temperatures far on each side of the gel-to-fluid phase transition. In doing so one is sure that spectra on a dilution line are always representative of the same phase ($L_{\beta'}$ or L_α). Such a phase transition determination is compulsory and was accomplished by DSC prior to the NMR experiments.

2. Materials and methods

2.1. Chemicals and sample preparation

DMPC was purchased from Interchim (Montluçon, France) while heavy water (D_2O) was obtained from CEA (Saclay, France). The purity of DMPC was checked by thin layer chromatography prior to and after completion of experiments, no degradation was detected.

DMPC powder was further dried by pumping under high vacuum for 12 h and transferred into 10 mm diameter glass tubes. Then, the appropriate amount of heavy water (D_2O) was added and the tubes sealed. To ensure a good homogeneity of the dispersion, samples were then heated to 45°C for 30 min, vortexed and cooled in liquid nitrogen. This cycle was repeated several times. For NMR experiments, samples were further transferred into a 5 mm diameter glass tube which was sealed with parafilm. To prevent the samples from being hydrated during transfers, preparations were made in a glove bag under dry nitrogen atmosphere.

2.2. Differential scanning calorimetry

Experiments were carried out on a Perkin-Elmer 7. 50 mg of dispersions of DMPC in D_2O were weighed. Samples were placed in a sealed pan to prevent dehydration during the thermotropic analysis. Thermograms were recorded at a rate of 3°C/min. To ensure that thermal equilibrium was reached, three thermograms on increasing and decreasing temperature were recorded for each sample. Measurements were performed on the last thermogram.

2.3. Nuclear magnetic resonance

Solid state ^2H NMR experiments were performed at 30.7 MHz using a Bruker MSL 200 spectrometer. ^2H NMR spectra were obtained on resonance using the quadrupolar echo pulse sequence with a 8-pulse Cyclops sequence [17]. Acquisition parameters were 50 kHz spectral window, 90° pulse duration of 10 μs, interpulse delay of 60 μs and recycle time of 2 s. Quadrature detection was used. Samples were allowed to equilibrate for at least 30 min at a given temperature before signal acquisition. Temperature was regulated to ±1°C.

Data treatment was performed on a Bruker Aspect 3000 and VAX/VMS 4000 computers. A Lorentzian broadening of 50 (for highly hydrated samples) to 100 Hz (for weakly hydrated samples) was applied to the free induction decays before Fourier transformation.

2.4. Theoretical background

In monophasic lamellar samples (i.e. without excess water), two types of water molecules may be distinguished: *trapped* and *bound* water [10,11,14–16]. Trapped water is incorporated between bilayers with movement characteristics similar to those of free water [11,15,16]. Bound water is hindered in its movement by the lipidic interface, partially oriented in that the OH bond direction is maintained to a limited degree by hydrogen bonding. Bound water molecules are exchanging between the different binding sites and with trapped water molecules. It is possible to quantify bound water molecules in a monophasic lamellar sample applying the two-site exchange theory to the ^2H NMR spectra of heavy water [18–21]. This determination results from variation of the D_2O quadrupolar splitting, $\Delta\nu_{obs}$ (Fig. 1), with sample hydration. If $\Delta\nu_B$ and $\Delta\nu_T$ are the quadrupolar splittings of heavy water bound to DMPC and trapped between DMPC bilayers, respectively, then in the case of fast exchange, only one quad-

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rupolar splitting is detected in the resulting ^2H NMR spectrum [20,22]:

$$\Delta\nu_{obs} = \frac{n_B}{n}\Delta\nu_B + \frac{n_T}{n}\Delta\nu_T = \frac{n_B}{n}\Delta\nu_B \quad (1)$$

where n_B and n_T are respectively the number of bound and trapped water molecules, n is the number of moles of water in the sample ($n = n_B + n_T$). $\Delta\nu_T$ is averaged to zero because trapped water exhibits the movement characteristics of free water [16]. Eq. 1 can also be written introducing the molar ratio of water to DMPC, R_i:

$$\Delta\nu_{obs} = \frac{N_D n_D}{n}\Delta\nu_D = \frac{1}{R_i}(n_D\Delta\nu_D) \quad (2)$$

where N_D is the number of moles of DMPC in the sample and n_D the number of bound water molecules per lipid. $\Delta\nu_D$ is the quadrupolar splitting of water molecules bound to DMPC molecules.

When $n > N_D n_D$, bound and trapped water are present, this is the *swelling* regime. In such situations, n_D is constant since hydration of the surface is completed, $n_D = n_D^*$. $\Delta\nu_D$ is likely to be constant since Ulrich and Watts [23] showed that the quadrupolar splittings given by deuterated methylenes in the phosphocholine headgroup are indeed constant in that regime, $\Delta\nu_D = \Delta\nu_D^*$. Consequently, Eq. 2 is transformed to:

$$\Delta\nu_{obs} = \frac{1}{R_i}(n_D^*\Delta\nu_D^*) \quad (3)$$

The dependence between $\Delta\nu_{obs}$ and $1/R_i$ should then be linear and intersect the origin [13] in that regime. In situations where swelling is not observed, i.e. when $n < N_D n_D$, the system is in the *hydration* regime. Eq. 2 is still valid but n no longer includes trapped water: n is then equal to $N_D n_D$ so that $\Delta\nu_{obs} = \Delta\nu_D$. As a consequence of the above, the variation of $\Delta\nu_{obs}$ as a function of $1/R_i$ makes it possible to determine $R_i^* = n^*/N_D = n_D^*$, the water-to-DMPC molar ratio observed when the system goes from the hydration regime to the swelling regime, i.e. the number of water molecules bound to each molecule of DMPC when hydration of the interface is achieved.

In the following, n_D^* values will be determined for DMPC membranes both in fluid and gel phases.

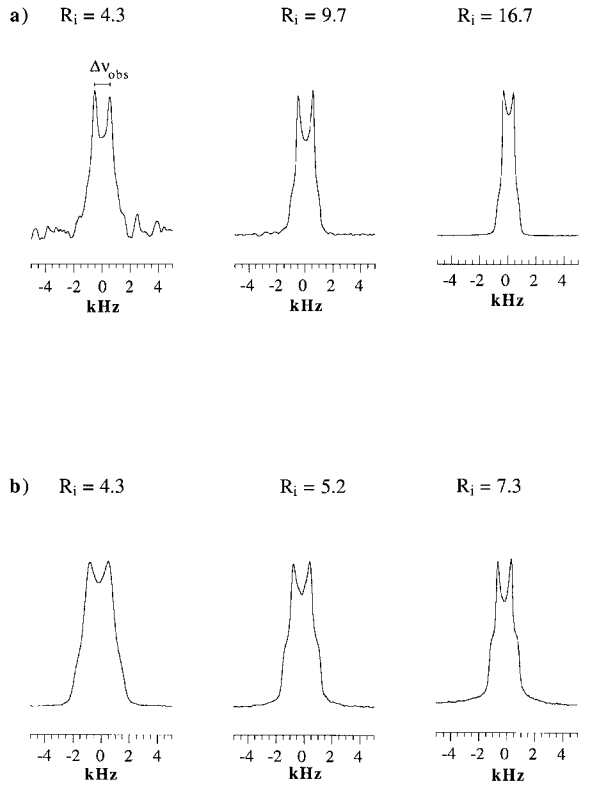

Fig. 1. Selected ^2H NMR spectra of DMPC-D_2O systems at 55°C (a) and 5°C (b). The amount of heavy water in samples is defined by R_i, the D_2O-to-DMPC molar ratio. The error of R_i is estimated to ±0.5. The quadrupolar splitting, $\Delta\nu_{obs}$, is the frequency range between the two spectrum maxima and is measured to ±25 Hz.

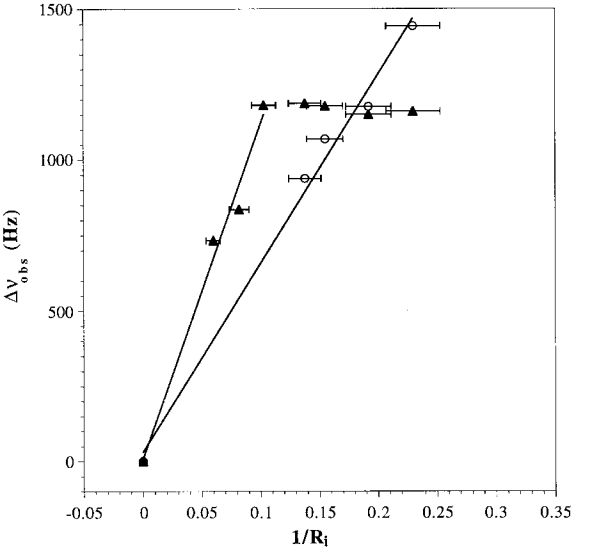

Fig. 2. Evolution of the quadrupolar splitting of D_2O with the DMPC-to-D_2O molar ratio, $1/R_i$, for DMPC-D_2O systems. (○) At 5°C: points are fitted by a straight line. (▲) At 55°C: points are fitted by a straight line for $R_i < 9.7$. The experimental error on quadrupolar splitting (±25 Hz) is included in the symbol. Horizontal bars represent the experimental error on the reciprocal of R_i.

3. Results

3.1. Transition temperatures of DMPC-D_2O mixtures versus hydration

As already mentioned, dispersions of DMPC in water experience gel-to-fluid transitions at temperatures which depend on the water content [1,9,15,24]. Because the determination of the number of bound water molecules in a given phase requires a study of samples with various R_i, a DSC analysis of the samples was first necessary to determine the correct temperature for which all of them are in the same phase. Table 1 gives the transition temperatures for DMPC-D_2O mixtures we worked on. For $R_i < 12.2$, dispersions of DMPC in heavy water experience a transition directly from the gel phase to the fluid phase which occurs at a hydration-dependent temperature: the less hydrated the sample, the greater the transition temperature, in agreement with the literature [1,24,25]. For $R_i > 12.2$, dispersions of DMPC are first organized in a ripple phase, $P_{\beta'}$, before forming the L_α phase. The latter phase appears at ca. 24°C according to Table 1. As a consequence, the temperature must be set higher than 40°C or lower than 15°C to be sure that all the samples are respectively in the fluid or gel phase.

3.2. Determination of the number of water molecules bound to DMPC in the L_α phase

Fig. 1a shows selected ^2H NMR spectra of D_2O-DMPC systems at 55°C. For all spectra, only one quadrupolar splitting is observed and such a behavior is detected whatever R_i. Spectra are very similar in shape and width to those obtained

Table 1
Transition temperatures for DMPC-D_2O mixtures of different R_i, water-to-DMPC molar ratio, as determined by DSC

$R_i = n/N_D$	Transition temperature (°C)
4.3	40[a]
5.2	38[a]
6.5	36[a]
7.3	35[a]
9.7	28[a]
12.2	16[b], 26[c]
16.7	15[b], 24[c]

Accuracy: ±1°C.
[a]Transitions from the gel phase, $L_{\beta'}$, to the fluid phase, L_α.
[b] Transitions from the $L_{\beta'}$ phase to the rippled phase, $P_{\beta'}$.
[c] Transitions from the $P_{\beta'}$ phase to the L_α phase.

by Pope et al. [15] on the same system when performing temperature variations. The presence of a single splitting is indicative of a fast exchange, in the NMR time scale, between bound and trapped water (see Section 2.4), i.e. this means that the time constant is smaller than 1/1200 Hz (0.8 ms). As this feature is observed even for very low hydration, i.e. when saturation of the phospholipid interface has not yet been completed (see next paragraph), one can also say that exchange between the bound water sites is also fast in the NMR time scale. The quadrupolar splitting, $\Delta\nu_{obs}$, is plotted with the reciprocal of R_i in Fig. 2. Error bars on $1/R_i$ and quadrupolar splittings have been calculated by considering that R_i is defined at ±0.5 and $\Delta\nu_{obs}$ measured at ±25 Hz. This plot clearly indicates that a change in the quadrupolar splitting behavior occurs at $R_i = 9.7$. For $R_i < 9.7$, points are well fitted by a straight line which demonstrates that $\Delta\nu_D{}^*$ is independent of hydration in the swelling regime as was supposed in Section 2.4. The change occurring at $R_i = 9.7$ reflects the transition from the hydration to the swelling regime and indicates that a molecule of DMPC binds 9.7 ± 0.5 water molecules when in the fluid state. Below this hydration, the value of the quadrupolar splitting stays about constant at 1200 ± 25 Hz and represents the quadrupolar splitting given by water molecules bound to DMPC.

3.3. Number of water molecules bound to DMPC in the $L_{\beta'}$ phase

An equivalent study was done at 5°C where all the samples are in the gel state, $L_{\beta'}$, whatever the hydration (Table 1). Selected ^2H NMR spectra of DMPC-D_2O systems are plotted in Fig. 1b. As in the fluid phase, their shape is characteristic of axially symmetric powder patterns and only one quadrupolar splitting is observed indicating the onset of fast exchange between different kinds of water molecules. The $\Delta\nu_{obs}$ value increases with decreasing R_i. The variation of $\Delta\nu_{obs}$ with $1/R_i$ is reported in Fig. 2. All plotted points have been fitted to a straight line which means that all the studied samples are in their swelling regime. In order to observe a change in the slope of the plot, we would have needed to prepare samples with lower R_i. However, it is very difficult to make homogeneous samples at these low water concentrations. Therefore, only an estimate of the upper limit of the number of bound molecules to DMPC can be deduced from this graph: a DMPC molecule binds less than 4.3 ± 0.5 water molecules when in the gel state. Moreover, we can only conclude that $\Delta\nu_D{}^*$ is greater than 1400 ± 25 Hz.

4. Discussion

Results reported in the previous section show unambiguously that the number of water molecules bound per DMPC molecule as well as their quadrupolar splitting is dependent on the phase nature. To our knowledge, no study of the interfacial hydration of DMPC had been performed yet. However, DPPC (analog to DMPC with two additional carbons in both acyl chains) was investigated and shown to bind 10–11 water molecules in the fluid phase [26,27] which is very close to what we obtained for DMPC (9.7 ± 0.5). This suggests that hydration does not depend on chain length. This is not surprising since the literature indicates that hydration is related to the nature of the lipid headgroup and to the degree of unsaturation of its acyl chains, both parameters influencing the headgroup area. For instance, phosphatidylcholines (PC) bind one to two water molecules more than phosphatidylethanolamines (PE) in the fluid phase [8,11,27,28] and it is known that the headgroup area of PE is slightly smaller than that of PC [29]. Dioleoylphosphatidylcholine (unsaturated lipid chains) binds 20 water molecules in the fluid phase [9] whereas DMPC binds ca. 10 water molecules (our study). A comparison of their headgroup area shows that the former is larger than the latter: 82 Å^2 versus 62 Å^2 [29]. Therefore, as DMPC and DPPC have very comparable headgroup areas (62 Å^2 versus 59 Å^2), it is not surprising to find comparable $n_D{}^*$ values.

The value of 10 bound water molecules can be accounted for by the number of binding sites of the DMPC molecule. According to infrared spectroscopic studies [4,5], the hydrogen binding sites of dipalmitoylphosphatidylcholine are composed of all the oxygens of the phosphate group and that of the *sn*-2 carbonyl group, which would be partially hydrated [4]. Therefore, the number of binding sites is equal to 11 (9 on PO_4^- and 2 in C=O). This value is in very good agreement with our finding, proving that in the L_α phase, all the binding sites are accessible and occupied. In the gel phase, DMPC binds less than 4.3 ± 0.5 water molecules, thus distinctly less than in the fluid phase. The main difference between these two phases is the orientation of phospholipids in the lamellae. In the fluid phase, they are statistically parallel to the bilayer normal while in the gel phase they are tilted by 30° [30,31]. This results in a decrease of the DMPC headgroup area in the gel phase (53 Å^2) compared to that of the fluid phase (62 Å^2) [29]. Moreover, phospholipid tilting in the $L_{\beta'}$ may hide some of the binding sites. The combination of these two effects certainly accounts for the drop of headgroup hydration with decreasing temperature.

All of the NMR spectra are axially symmetric even for the lowest temperature investigated. This indicates that water motion, even at low temperature, is fast enough for spectra to be axially symmetric. Moreover, quadrupolar splitting values are far from the theoretical value of motionless D_2O (220 kHz) [32]. This shows that water rotation about its C_2 axis is still fast even at 5°C and for weakly hydrated samples. $\Delta\nu_D{}^*$ depends on temperature: it is greater in the gel phase than in the fluid phase. The quadrupolar splitting of bound water is related to the dynamics of water. The lower value of $\Delta\nu_D{}^*$ in the case of lamellae in the L_α phase can partly be attributed to an increase in phospholipid motions on going to the fluid phase. Moreover, experiments carried out at 55°C show that $\Delta\nu_D{}^*$ is constant in the hydration regime which suggests that all the binding sites of DMPC are equivalent. Assuming that the four

oxygens of the phosphate group are motionally equivalent, it is however difficult to believe that they behave as that of the carbonyl group. Because the fraction of water bound to the carbonyl is much lower than that bound to the phosphate, its contribution to $\Delta\nu_D{}^*$ may be considered negligible. Hence, this leads to an essentially constant $\Delta\nu_D{}^*$ value in the fully hydrated regime.

At this stage of the discussion, it is important to compare our findings with the work of Volke et al. [16], who describe the behavior of water in such systems without distinguishing between bound and trapped water, which may appear contradictory to our approach. A close inspection of their model shows that it only accounts for the behavior of the interbilayer water that we call trapped water. However, the above authors recognize that there is a small fraction of water significantly influenced by the surface in slowing down its mobility. This is in total agreement with our model, simply we call that type of water the bound fraction. In our work we used NMR to determine the bound fraction whereas they used the same technique to describe the behavior of the interbilayer (trapped) water. Moreover, our experimental data show unambiguously that there are indeed two types of water, at least for fluid phase membranes, since there is a very clear break in the variation of the water quadrupolar splittings as a function of R_i (water content) as demonstrated by Fig. 1. This had never been reported before and is in full agreement with our model.

To conclude, we showed here that it is possible to quantify molecular hydration at a given temperature using deuterium NMR of heavy water. We found that a molecule of DMPC binds less than 4 D_2O and ca. 10 D_2O respectively in the gel and fluid phases. These results confirm that there is a tight relationship between hydration and the phase nature. Until now, phase transitions were mostly discussed in terms of chain order. Our findings indicate that interactions between headgroups and water must also be taken into account to explain phase formation. One interesting point for future work would be to study the evolution of hydration with temperature in a given phase and near the phase transition.

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