Estimation of Differently Bound Water Molecules for the Gel Phase of Dimyristoylphosphatidylethanolamine-Water System as Studied by DSC and ^2H -NMR Spectroscopy

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Abstract. A measurement of ^2H spin-lattice relaxation time, T_1 , for D_2O was performed with a high resolution liquid NMR apparatus for two samples of dimyristoylphosphatidylethanolamine (DMPE)- D_2O system in a full hydration at varying temperatures of -20 , -10 , and 5°C , and both components and compositions of differently bound freezable water molecules were estimated from a best-fitted curve to experimental inversion recovery data. A choice of the best-fitted curve was based on a distribution of weighted residuals for the experimental data. A single component was found for a temperature of -20°C . At 5°C , where all the freezable water exists in the liquid state, three components were observed to be characterized by T_1 values of approximately 20, 100, and 200 ms, respectively. By comparing compositions of these individual components with those obtained in our previous DSC study, it was revealed that the first and secondary components are members of freezable interlamellar water and the last one is comparable to bulk water.

Key words: bulk water, ^2H -NMR spectroscopy, DMPE-water system, freezable interlamellar water, ice-melting DSC curve, spin-lattice relaxation time, water distribution diagram

1. Introduction

Studies of the interaction of lipid and water molecules have been performed with many techniques such as X-ray diffraction [1–4], NMR spectroscopy [5–14], and differential scanning calorimetry (DSC) [3, 15–23].

In our previous DSC studies [3, 15–23], the numbers of differently bound water molecules in phospholipid-water systems were estimated from a deconvolution analysis of the ice-melting DSC curves. In this connection, for a system of dimyristoylphosphatidylethanolamine (DMPE)-water system in a full hydration, the total number of nonfreezable and freezable interlamellar water molecules ($6\text{ H}_2\text{O}$ per molecule of DMPE) has been reported to agree with that estimated by X-ray dif-

fraction method based on a strip model for electron density profiles [3]. On the other hand, estimation of the number of water molecules in different bonding modes by a NMR technique has been almost performed from the quadrupole splitting of ^2H nucleus for D_2O in lipid- D_2O systems [5, 7, 8, 10, 11, 13, 14]. However, there are some studies on an alternate NMR technique based on ^2H spin-lattice relaxation time (T_1), but in these previous studies, differently bound water molecules have been reported to be detected in one component characterized by an average T_1 value [6, 9, 12].

In our previous DSC study for the DMPE- H_2O system [3], to estimate the ice-melting enthalpy for bulk water as accurately as possible, the melting DSC curves for freezable interlamellar water were deconvoluted into three components. Focusing on the three components for the freezable interlamellar water, in the present study, ^2H T_1 measurement based on a $180^\circ - \tau - 90^\circ$ pulse sequence was performed with a high resolution liquid NMR apparatus for two DMPE- D_2O samples in a full hydration at varying temperatures and in this experiment, a short interval was taken between interpulse delay times, τ , resulting in experimental inversion recovery data of approximately 100 points, which is quite different from the T_1 measurements previously reported by other workers [6, 9, 12]. Components and their compositions estimated from the present T_1 measurements were compared with the results of DSC.

2. Materials and Methods

2.1. SAMPLE PREPARATION

1,2-Dimyristoyl-3-*sn*-phosphatidylethanolamine (DMPE) was purchased from Sigma Co. (St. Louis, MO) and used without further purification as thin-layer chromatography of this lipid showed a single spot [24]. The DMPE (approximately 200 mg) in a 5-mm NMR sample tube was dehydrated under high vacuum (10^{-4} Pa) at room temperature for 3 days until no mass loss was detected by electroanalysis (Cahn Electrobalance) [3]. Using 1.8 wt% of water which the DMPE contains (which was obtained by the electroanalysis), the weight of dehydrated DMPE was determined. A desired amount of D_2O (99.97%D, Euriso-Top) was added to the dehydrated DMPE in the sample tube with a microsyringe in a dry box filled with dry N_2 gas and the tube was then sealed off. By reference to a water distribution diagram for the DMPE- H_2O system previously reported by us [3, 19], two samples in a full hydration were prepared at the D_2O /DMPE molar ratios, N_w , of 10.0 and 16.6. The two samples were thermally annealed with a temperature-controlled water bath by repeating thermal cycling (scanning rate: $0.2^\circ\text{C}/\text{min}$) at temperatures above and below the gel-to-liquid crystal phase transition until no change in the shape of ^2H -NMR spectrum was observed at 5°C . The annealing was continued up to several days to attain homogeneous mixing of the samples.

2.2. ^2H -NMR SPECTROSCOPY

(A) *Measurement of ^2H -NMR spectra and ^2H spin-lattice relaxation times (T_1)*

^2H -NMR spectra and ^2H -NMR spin-lattice relaxation times for D_2O in the DMPE- D_2O samples were measured with a Bruker ARX 400 MHz spectrometer (i.e., a liquid NMR apparatus of a maximal sweep width 125 KHz) using a broad band probe at a resonance frequency of 61.4 MHz. The NMR spectra were recorded with a 90° pulse, and the relaxation times were measured by an inversion recovery pulse sequence, $180^\circ - \tau - 90^\circ$. Considering that the 90° and the 180° pulse are important determinants in the accuracy of an estimated T_1 value, pulse widths (i.e., periods of pulse irradiation) characteristic of these respective pulses were determined as accurately as possible. Since the 180° pulse width was hard to determine directly from the NMR signal, the 90° pulse width giving the highest signal intensity was searched for by changing a pulse width at short intervals of $0.2\ \mu\text{s}$ and the 180° pulse width was then estimated by 2 times the 90° pulse width. The 90° pulse width thus obtained depended on the studied samples and temperatures and changed from 14.2 to $15.6\ \mu\text{s}$. An interpulse delay time of the pulse sequence, τ , was varied up to 1500 ms in the longest case (depending on $5T_1$) and the NMR signals were recorded for varying delay times. In particular, the delay times below 20 ms were changed at short intervals of 1 ms, so that τ values selected for each T_1 measurement were as many as 50–100 (i.e., indicating that the inversion recovery data of 50–100 were used for estimations of the T_1 value). Additional spectrometer parameters used for data acquisition after irradiation of the 90° pulse were as follows: sweep width 50 kHz, acquisition time 1.5 s ($>5T_1$) giving a dwell time $10\ \mu\text{s}$ (a resolution 0.6 Hz), pre scan delay (dead time) $45\ \mu\text{s}$. The number of recycle scans used to obtain a good S/N ratio was between 100 and 200.

(B) *Temperature calibration*

Sample temperatures monitored by a Bruker VT-2000 thermal controller using liquid N_2 were calibrated with a chemical shift, $\Delta\nu$, between the OH proton and the CH_3 protons of methanol and a calibrated sheath chromel-alumel thermocouple (Omega Engineering Inc.). The thermocouple was inserted directly into an open sample tube containing methanol placed in a sample holder of a Bruker NMR apparatus and a temperature of the methanol sample was changed stepwise from 210 to 290 K at intervals of 5 K according to the Bruker thermal controller. For each change in the temperature, both the chemical shift of methanol and the electromotive force of thermocouple were measured after ensuring thermal equilibrium of the sample. A least-squares fit for a plot of $\Delta\nu$ against the temperature read by the thermocouple gave a straight line over all the temperatures studied (i.e., written as $T/\text{K} = 477.5 - 0.285 [\Delta\nu]$). This equation was in good agreement with Van Geet's equation for 220–280 K, $T/\text{K} = 478.6 - 0.286 [\Delta\nu]$, scaled to 400 MHz [25]. Applying the equation obtained by us, the temperatures displayed by the

thermal controller were corrected for the temperature differences estimated from the chemical shifts.

For temperatures above 0 °C, the sample temperatures were kept with an estimated accuracy of at least ± 0.2 °C over a period of several hours studied, but the accuracy lowered gradually to approximately ± 2 °C at -60 °C with decreasing a temperature.

(C) *Analysis of inversion recovery data*

For a system of multiple components, a spin-lattice relaxation time of component i , T_1^i , is given by the equation

$$Mz^i = Mzo^i - 2Mzo^i \exp(-\tau / T_1^i) \quad (1)$$

where Mz^i and Mzo^i is magnetizations of component i at $t = \tau$ and $t (= \tau) = 0$, respectively. The magnetization for the whole system of multiple components is given by the sum of their individual magnetizations and is written as

$$\sum_i Mz^i = \sum_i Mzo^i - 2 \sum_i Mzo^i \exp(-\tau / T_1^i) \quad (2)$$

where $\sum_i Mz^i$ and $\sum_i Mzo^i$ are experimentally obtained by the intensities of inversion recovery signals observed at $t = \tau$ and $t (= \tau) = 0$, respectively. Using the designations, $Mz(\text{obs})$ and $Mzo(\text{obs})$, for these respective magnetizations, Equation (2) is replaced by

$$Mz(\text{obs}) = Mzo(\text{obs}) - 2 \sum_i Mzo^i \exp(-\tau / T_1^i) \quad (3)$$

In the present study, the unknown parameters T_1^i and Mzo^i (giving a fraction of component i) for the individual components were estimated by fitting Equation (3) to the inversion recovery data. The fitting to the experimental data was performed by using a computer program (ORIGIN, Microcal Software, Inc.) for multiple exponential decay curve analysis based upon a weighting nonlinear least squares procedure, and the best-fitted curve was finally determined from a distribution curve for a plot of weighted residual, r , given by Equation (4) against τ [26, 27].

$$r(\tau) = (1/y(\tau))^{1/2} (y_{\text{theor}}(\tau) - y(\tau)) \quad (4)$$

where $y(\tau)$ is an experimental data, and $y_{\text{theor}}(\tau)$ is a theoretical data obtained from a curve fitted to Equation (3).

Figure 1. Water distribution diagram for the gel phase of the DMPE-H₂O system. The cumulative number of nonfreezable interlamellar, freezable interlamellar, and bulk water molecules (per molecule of DMPE) is plotted as a function of the H₂O/DMPE molar ratio (N_w). N_w values of the DMPE-D₂O samples used for the present ²H NMR study are shown by dashed lines in this figure.

3. Results and Discussion

3.1. ESTIMATION OF THE NUMBERS OF DIFFERENTLY BOUND FREEZABLE INTERLAMELLAR WATER MOLECULES

In our previous calorimetric studies [3, 15–23], the number of water molecules in different bonding modes for lipid bilayer systems was estimated from a deconvolution analysis for the ice-melting DSC curves of varying water contents and was used to construct a water distribution diagram. The resultant water distribution diagram for the gel phase of DMPE-H₂O system previously reported by us [3, 19] is shown in Figure 1, where the cumulative number of nonfreezable interlamellar, freezable interlamellar, and bulk water molecules per molecule of DMPE is plotted against the H₂O/DMPE molar ratio (N_w). N_w values (10.0 and 16.6) of

Figure 2. Deconvolution analysis of ice-melting DSC curves for the gel phase of the DMPE-H₂O system at N_w values of (A) 10.0 and (B) 16.6. The deconvoluted curves, I, II, III, and IV, and their sum (the best-fitted curve) are shown by dotted lines and the experimental DSC curves by solid lines. ^2H T_1 measurements in the present study were carried out at temperatures shown by dashed lines in this figure.

the DMPE-D₂O samples used in the present NMR study are the same as those indicated by dashed lines in the diagram (Figure 1). As shown in the diagram, in a lipid-water system, freezable water exists in two types, freezable interlamellar and bulk water, for which the ice-melting DSC curve is observed. In Figure 2, the ice-melting DSC curves of the DMPE-H₂O samples are compared at N_w values of 10.0 and 16.6, thus, indicated in the diagram. The ice-melting curves (a solid line) are observed to be composed of at least two components, broad (a wide temperature range below 0 °C) and sharp (a narrow temperature range below 0 °C), derived from the freezable interlamellar and bulk water, respectively. Since the two components overlap at their basis, a deconvolution analysis was performed by using a computer program for multiple Gaussian curve analysis and by fitting both the half-height width and the midpoint temperature characterizing each deconvoluted curve to all the ice-melting curves of varying water contents (thus, the two factors are unchangeable throughout all the deconvolutions for varying water contents). The deconvoluted curves and their sum (i.e., the best-fitted curve) are shown by dotted lines in Figure 2. The deconvoluted curves I, II, and III are for the freezable interlamellar water, and the deconvoluted curve IV is for the bulk water. In Table 1, the ice-melting enthalpies (per mole of DMPE) of the individual deconvoluted curves, $\Delta H(I)$, $\Delta H(II)$, $\Delta H(III)$, and $\Delta H(IV)$, are shown, together with the numbers of differently bound water molecules (per molecule of DMPE), $N(I)$, $N(II)$, $N(III)$, and $N(IV)$. Thus, the $N(I)$, $N(II)$, and $N(III)$ values, the sum of which gives the total number of the freezable interlamellar water molecules, were calculated by using their average ice-melting enthalpy, 5.368 kJ/mol H₂O*, obtained in our previous paper [19], i.e., from $\Delta H(I)/5.368$, $\Delta H(II)/5.368$, and $\Delta H(III)/5.368$, respectively. The $N(IV)$ value giving the number of bulk water molecules was calculated from $\Delta H(IV)/6.01$ by applying the known molar ice-melting enthalpy for free water, 6.01 kJ/mol H₂O.

In preference to ²H-NMR experiments, DSC of a DMPE-D₂O sample was performed at N_w 10.0 under the same condition as that previously reported by us [3]. Compared with the ice-melting curve of the DMPE-H₂O sample shown in Figure 2A, the melting curve of the DMPE-D₂O sample is the same in shape, but it is different in temperature, thus, its overall temperature is shifted by approximately 3 °C to a high temperature side.

3.2. ESTIMATION OF COMPONENT AND COMPOSITION OF DIFFERENTLY BOUND WATER MOLECULES BY ²H-NMR MEASUREMENT

Figure 3 shows a variation of ²H-NMR spectra of the DMPE-D₂O sample at N_w 10.0 with an increase in temperature. The sample in the NMR apparatus was cooled from a temperature of the liquid crystal phase to -60 °C and then kept at the temperature for at least 1 h before beginning the ²H-NMR measurement. The

* The value was obtained from a slope of linear line for a plot of the sum of $\Delta H(I)$, $\Delta H(II)$, and $\Delta H(III)$ against N_w .

Figure 3. (A) A series of typical ^2H -NMR spectra of the DMPE- D_2O sample at N_w 10.0 ranging in temperature from -60 to 30 °C. (B) ^2H -NMR spectra are compared for temperatures of -60 to -20 °C in an enlarged scale.

sample temperature was changed at intervals of 5 °C, and the ^2H -NMR spectra were measured after ensuring thermal equilibrium of the sample for each change in the temperature. In Figure 3, the typical spectra (A) and the enlarged scale spectra (B) are compared, respectively.

As shown more clearly in Figure 3B, the spectrum appears for the first time around -30 °C (curve d) although it extends over a wide frequency range of +5 kHz to -5 kHz, and the temperature (~ -30 °C) is comparable to the onset temperature of ice-melting DSC curves shown in Figure 2. The broad spectrum gradually grows up to -20 °C simultaneously with a sharpening in the shape (curves e and f), after which a doublet splitting spectrum is clearly observed as shown in curves d and e of Figure 3A.

Furthermore, as shown in curve e (at 5 °C), the splitting spectrum is found to be superimposed on a sharp single spectrum. However, such a superposition becomes unclearer with an increase in temperature (curves f, g, and h).

By comparing Figure 3B with Figure 2, it is noticeable that the temperature (-30 °C), at which the spectrum shows up itself, is the same as that, at which the ice obtained from the freezable interlamellar water begins to melt. Accordingly, it is understandable that the NMR spectra shown in Figure 3 are derived from water (D_2O) molecules existing in the liquid state, thus, the freezable interlamellar and bulk water. So, the NMR spectrum derived from the nonfreezable interlamellar

water is undetectable because the sweep width of the present NMR apparatus is limited in a narrow range of 120 kHz in a maximum. On this basis, the splitting spectra observed in the present study are assigned to the freezable interlamellar water molecules which are located under the influence of electrostatic gradient induced by the headgroups of DMPE present as dipolar zwitterions (thus, the quadrupole interaction). In addition, the sharp spectrum observed at 5 °C is considered to be derived from the bulk water molecules existing outside the bilayers, and this water molecules are exchanged with the freezable interlamellar water molecules so that a gradual broadening of the spectra (in other words, a decrease in the splitting) with increasing a temperature occurs above 5 °C.

The present NMR spectra were found to arise from two types of the freezable water. However, more detailed estimation for the number of freezable interlamellar water molecules has been made by the DSC study, as shown in Table I. From this viewpoint, measurements of ^2H spin-lattice relaxation time (T_1) were performed for two DMPE- D_2O samples at N_w 16.6, in addition to N_w 10.0, at desired temperatures shown by dashed lines in Figure 2, and both components and compositions (fractions) of differently bound freezable water molecules were estimated by applying Equation (3) for a multiple component system.

In Figure 4, $[M_{zo}(\text{obs}) - M_z(\text{obs})]/2M_{zo}(\text{obs})$ derived by Equation (3) is plotted in semilogarithmic form as a function of delay time, τ , and the best-fitted curves to experimental data are shown by solid lines. T_1 values of individual components and their compositions obtained from the best-fitted curves are summarized in Table II. The T_1 measurement was carried out at temperatures of -20, -10, and 5 °C for N_w 10.0, respectively, and at only a temperature of 5 °C for N_w 16.6. As shown in Figure 2A, at -20 °C (a), the freezable water existing in the liquid state is only one type characterized by the deconvoluted curve I and is two types characterized by the deconvoluted curves I and II at -10 °C (b). For 5 °C (c of Figure 2A and d of 2B), at which all the freezable water is present in the liquid, the deconvoluted curves I, II, III, and IV are observed for the two fully hydrated samples. In Figure 5, curves of $(M_{zo}^i - M_z^i)$ versus τ for component i (given by Equation (1)) estimated for each best-fitted curve are shown by dotted lines, thus, the sum of these individual curves gives the best-fitted curve shown by a solid line in this figure. The best-fitted curve in Figure 5A (at -20 °C) is a single exponential decay, i.e., indicative of one component designated I'. However, the best-fitted curves other than that of Figure 5A are shown to be composed of multiple components, i.e., two components of I' and II' for Figure 5B (at -10 °C) and three components of I', II', and III' for Figure 5C and 5D (at 5 °C). The choice of best-fitted curve was based on a distribution of weighted residual (r) given by Equation (4) plotted against delay time (τ). For an example, in Figure 6, the distribution of r values for experimental recovery data (measured at 5 °C for the sample of N_w 10.0) are compared for two (A) and three components (B). In Figure 6A for two components, the distribution is characterized by undulation and so one component was further added. The resultant distribution of three components is shown in Figure 6B, where r values distribute

Table 1. The numbers of water molecules (per molecule of DMPE), $N(I)$, $N(II)$, and $N(III)$; and their fractions, $F(I)$, $F(II)$, and $F(III)$, estimated from ice-melting enthalpies (per mole of DMPE), $\Delta H(I)$, $\Delta H(II)$, and $\Delta H(III)$, of deconvoluted curves, I, II, and III, for freezable interlamellar water, together with $N(IV)$ and $F(IV)$ estimated from $\Delta H(IV)$ of deconvoluted curve IV for bulk water, in the gel phase of the DMPE-H₂O system

N_w	Temperature (°C)	Component of deconvoluted curve	Ice-melting enthalpy kJ/mol DMPE	Number of water molecules per molecule of DMPE ¹	Fraction (%)
10.0	-20	I	$\Delta H(I)$ 3.29	$N(I)$ 0.6	$F(I)$ 100.0
	-10	I	$\Delta H(I)$ 5.64	$N(I)$ 1.0	$F(I)$ 54.1
		II	$\Delta H(II)$ 4.82	$N(II)$ 0.9	$F(II)$ 45.9
	5	I	$\Delta H(I)$ 5.74	$N(I)$ 1.1	$F(I)$ 13.6
		II	$\Delta H(II)$ 10.14	$N(II)$ 1.9	$F(II)$ 23.4
		III	$\Delta H(III)$ 5.74	$N(III)$ 1.1	$F(III)$ 13.6
		IV	$\Delta H(IV)$ 24.14	$N(IV)$ 4.0	$F(IV)$ 49.4
16.6	5	I	$\Delta H(I)$ 5.84	$N(I)$ 1.1	$F(I)$ 7.5
		II	$\Delta H(II)$ 10.63	$N(II)$ 2.0	$F(II)$ 13.6
		III	$\Delta H(III)$ 5.84	$N(III)$ 1.1	$F(III)$ 7.5
		IV	$\Delta H(IV)$ 63.15	$N(IV)$ 10.5	$F(IV)$ 71.4

¹ An average standard deviation for the numbers of water molecules is 0.2 [3].

² The total number of freezable interlamellar water molecules is given by $N(I) + N(II) + N(III)$.

³ Values of 37.0 and 20.9 are given by $F(II) + F(III)$ for N_w values of 10.0 and 16.6, respectively.

Figure 4. Plots of experimental inversion recovery data of ^2H T_1 measurement against delay time (τ) for the gel phase of the DMPE- D_2O samples at N_w values of 10.0 and 16.6. For N_w 10.0, the measurement was performed at three different temperatures of (A) -20 , (B) -10 , and (C) $5\text{ }^\circ\text{C}$, respectively, and was performed at a temperature of (D) $5\text{ }^\circ\text{C}$ for N_w 16.6. The best-fitted curve is shown by a solid line for each plot, and the plot for 100% D_2O (at $5\text{ }^\circ\text{C}$) is shown for comparison.

Figure 5. Comparison of exponential decay curves (dotted lines) for components I' , II' , and III' estimated from the respective best-fitted curves shown by A, B, C, and D in Figure 4. A, B, C, and D of this figure correspond to those for the best-fitted curves in Figure 4, respectively.

Table II. Components, I' and II', for freezable interlamellar water estimated from the best-fitted curve to experimental inversion recovery data; their ^2H spin-lattice relaxation times, $T_1(\text{I}')$ and $T_1(\text{II}')$; and their fractions, $F(\text{I}')$ and $F(\text{II}')$, together with $T_1(\text{III}')$ and $F(\text{III}')$ for component III' comparable to bulk water, in the gel phase of the DMPE- D_2O system at varying temperatures, -20 , -10 , and 5°C

N_w	Temperature ($^\circ\text{C}$)	Component estimated from best-fitted curve	T_1 / ms	Fraction (%)		
10.0	-20	I'	$T_1(\text{I}')$	4.8 ± 1.5	$F(\text{I}')$	99.7 ± 0.5
		II'	$T_1(\text{II}')$	31.8 ± 2.8	$F(\text{II}')$	20.2 ± 0.3
	-10	I'	$T_1(\text{I}')$	5.8 ± 1.2	$F(\text{I}')$	79.8 ± 0.5
		II'	$T_1(\text{II}')$	100.6 ± 3.4	$F(\text{II}')$	34.0 ± 0.3
	5	I'	$T_1(\text{I}')$	20.0 ± 1.3	$F(\text{I}')$	8.0 ± 0.5
		II'	$T_1(\text{II}')$	104.8 ± 5.0	$F(\text{II}')$	21.1 ± 0.3
16.6	5	III'	$T_1(\text{III}')$	199.5 ± 7.9	$F(\text{III}')$	70.9 ± 0.3

in a random mode, so that the fitted curve composed of three components was selected, as shown in Figure 5C.

Comparing Table II with Table I, obtained by the NMR and DSC studies, respectively, noticeable points are summarized as follows:

1. The present NMR study reveals an increase in the number of components for the freezable water with increasing a temperature, similarly to the result of DSC study.
2. For temperatures of -20 and -10°C , the two techniques give concordant results such as one component at -20°C and two components at -10°C , indicating that both components, I' and II', estimated from the T_1 measurements are members of the freezable interlamellar water.
3. However, for 5°C , the disagreement is observed between two techniques, i.e., three components of I', II', and III' for the NMR and four components of I, II, III, and IV for the DSC.

Focusing on the disagreement discussed above (3), the problem could be solved by a fraction, F , occupied by the individual components shown in Tables I and II. Thus, for two fully hydrated samples at N_w values of 10.0 and 16.6, a F value for component I', $F(\text{I}')$, obtained by the T_1 measurement is very close to a F value of component I, $F(\text{I})$, for the freezable interlamellar water obtained by the DSC. Additionally, a point to be noted is that $F(\text{II}')$ for component II' is nearly equal to the sum of $F(\text{II})$ and $F(\text{III})$ for the freezable interlamellar water and $F(\text{III}')$ for component III' characterized by the longest relaxation time (~ 200 ms) is also close to $F(\text{IV})$ for the bulk water. Presumably, fairly rapid exchanges occur between components II and III (detected by the DSC) present as the freezable interlamellar

Figure 6. Distributions of weighted residual (r) for experimental ^2H inversion recovery data of varying delay time (τ), calculated for two (A) and three components (B) by using Equation (4) in the text, respectively.

water and so the T_1 technique is unable to detect the two components individually, resulting in one component denoted as II'. T_1 value of approximately 200 ms for component III' comparable to the bulk water is shorter than that of 273.8 ms for free water obtained at 5 °C by us (Figure 4) [28, 29], indicating exchanges between the freezable interlamellar and bulk water. Finally, when a value of $F(\text{I}')/[F(\text{I}') + F(\text{II}')] for the freezable interlamellar water (obtained by the T_1 measurement) is$

compared between the two fully hydrated samples, 28.3% for N_w 10.0 is nearly equal to 27.5% for N_w 16.6 (therefore, $F(\text{II}')/[F(\text{I}') + F(\text{II}')] value is also nearly the same for both samples). This indicates that the amount of freezable interlamellar water, as well as its T_1 value, is equal for the two samples in a full hydration, similarly to the result of DSC, thus, the sum of $N(\text{I})$, $N(\text{II})$ and $N(\text{III})$ is the same for N_w values of 10.0 and 16.6 as shown in Table I.$

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