Long-Range Hydration Effect of Lipid Membrane Studied by Terahertz Time-Domain Spectroscopy

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The hydration state of biomolecules is believed to affect their self-assembly. The hydration state of phospholipid bilayers is studied precisely by terahertz spectroscopy, by which water perturbed by a lipid membrane is detected sensitively from the observation of the relaxation dynamics of water molecules in the subpicosecond time scale. Combined with x-ray observation of the lamellar structure of the lipid, a long-range hydration effect on up to 4–5 layers of water is confirmed. Most water molecules in the lamellae fall into the hydration water, and condensation of them is also indicated.

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As the essential solvent for all living cells, the physical properties of water have been investigated widely for quite a long time. Especially, the characteristic properties of hydration water at the surface of biomolecules, different from those of bulk water, have been predicted to have a significant role on biological functions, since biomolecules are highly condensed in a cell in the mesoscale (nanometer-to-micron) range [1,2].

Phospholipid, which forms a bilayer in water, is one of the most universal biomolecules in a cell as the main component of the biomembrane. The hydration state of the lipid bilayer is believed to regulate the biochemical reactions on the membrane in cooperation with proteins. Furthermore, for the self-assembly of the lipid, hydration water is essential not only for hydrophobic interactions but also for bilayer interactions [3]. Thus, to understand the properties of hydration water in the vicinity of phospholipid bilayers at the molecular level, NMR and inelastic neutron scattering [4–6] have been employed from the viewpoint of dynamics of water molecules. While the rotational relaxation time of a bulk water molecule ranges up to a time scale of 10^{-13} s, the measured time scale of these experimental methods is $\tau \sim 10^{-9}$–10^{-11} s [6]. Therefore, only a single layer of strongly bound water molecules (5–6 water molecules per lipid) on the surface of a phospholipid bilayer has been observed. Actually, however, it is considered that there are still many waters which are weakly perturbed and slightly slowed, since water molecules at different distances from the lipid surface are perturbed to different extents. Such a long-range hydration effect of lipid membranes has been predicted by molecular dynamics simulations [7,8], which is also predicted for a protein or DNA [9,10].

To investigate the hydration effect of lipid bilayers in detail including the weakly perturbed water, it is essential to observe the dynamics of water molecules in an ultrafast time scale ($\tau \sim 10^{-13}$ s) by experiments, and slight change of water dynamics should be detected. Although there was no efficient experimental method until fairly recently, two types of experiments have been proposed to detect the ultrafast dynamics of water very recently: terahertz time-domain spectroscopy (THz TDS) [11–14] and pump-probe spectroscopy [11,15]. Both spectroscopies measure the ultrafast dynamics, although these dynamics cannot be compared to each other directly due to the different selection rule of the dynamical modes [11]. Though Zhao *et al.* applied the vibrational pump-probe spectroscopy to a lipid-water system, what we know so far remains that at least 16 water molecules per lipid at the surface do not behave as bulk water [15].

In the present study, we apply THz TDS for precise clarification of the hydration state of a lipid bilayer. Since the faster part of the rotational relaxation dynamics of water molecules lies in the THz frequency range (1 THz corresponds to $\tau = 1.6 \times 10^{-13}$ s $= 0.16$ ps) [16,17], we can evaluate the hydration state in the ultrafast time scale from the change in the complex dielectric constants in the THz region. Furthermore, by combining the THz TDS results with the structural information of multilamellar structures of the lipid observed by small-angle x-ray scattering (SAXS), we precisely show a long-range hydration water layer between the lipid membranes.

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, Wako Pure Chemical Industries) was obtained as a neutral charged phospholipid in the powder form and used without further purification. The DMPC powder was dissolved in pure water (MilliQ) with different molar ratios: $R = [H_2O]/[\mathrm{DMPC}] = 190, 113, 78, 60, 35, 19$. The lipids formed multilamellar vesicles. For the preparation of a dried phospholipid film, the DMPC powder was dissolved in the mixture of chloroform and methanol (2:1 v/v). After the evaporation of the organic solvents, a dried phospholipid film (total thickness: 2–3 mm) remained in the sample cell. The relative humidity was about 50%, which corresponds

to $R = [H_2O]/[DMPC] \approx 4$ for the dried phospholipid film [18]. All samples were maintained at a constant temperature of $36 \pm 0.1\,^{\circ}C$ during THz TDS and SAXS measurements.

THz TDS measurements were performed by using the setup reported in the previous literature [13,14,19], where an Er-doped fiber laser (780 nm, 100 fs, 50 MHz) was used as a light source. With using a silicon Dove prism (refractive index: 3.4), the attenuated total reflection measurement was performed for the accurate determination of the complex dielectric function of the solutions. The penetration depth of the THz wave was about 20 μm. In these TDS measurements, the data have high reliability in the 0.5–2.6 THz region in our experiments.

SAXS measurements were performed at BL-15 A, Photon Factory, High Energy Accelerator Research Organization, Japan. The wavelength of the x ray used was 1.5 Å, and the ray were detected by using a CCD (C7300, Hamamatsu Photonics). The sample-to-detector distance was about 1 m, which was calibrated by using standard samples (lead stearate and silver behenate).

Figure 1 shows the repeat distance d of DMPC multilayers, obtained by SAXS, for each DMPC solution ($R = [H_2O]/[DMPC] = 190, 113, 78, 60, 35, 19$). The repeat distance is obtained from the position q of the Bragg peak ($d = 2\pi/q$) due to the regular stacking of bilayers in the lamellar phase. For $R > 35$, the lamellar repeat distances are almost constant at about 62 Å, since the repeat distance of fully hydrated DMPC bilayers is determined only by the balance between the attractive interaction (van der Waals interaction) and the repulsive interactions (steric ''Helfrich'' repulsion and hydration repulsion) [3]. Since the thickness of a DMPC bilayer is about 37 Å, the thickness of the water layer is about 25 Å [20]. Half of the layer (12.5 Å thick) corresponds to the water layer for a monolayer of the lipid. Surplus water molecules are excluded from the multilamellar vesicles and become bulk water. At $R = 35$, the repeat distance decreases slightly, which is consistent with previously reported results [21]. From the result, it is evaluated that about 35–40 water molecules per phospholipid molecule are confined between DMPC bilayers in the fully hydrated condition at 36 °C. For $R < 35$, the lipid bilayers are unable to sustain the fully hydrated condition, and the lamellar repeat distance decreases as the water ratio decreases. At $R = 0$, the repeat distance is expected to be the same as the bilayer thickness. The SAXS results indicate that about 35–40 water molecules per a lipid exist in the 12.5-Å-thick water layer at the fully hydrated condition. Even if it is possible that some kind of defect regions exist in the lipid solution and the number of water molecules in the lamellae is overestimated [20], a certain degree of water condensation in the lamellae compared with bulk water is indicated from the SAXS result: If there was no condensation, 25 water molecules were sufficient for filling the water phase (12.5 Å water layer thickness and the cross-sectional area of a lipid is 60 $Å^2$ [20], while the volume of a bulk water molecule is 30 $Å^3$).

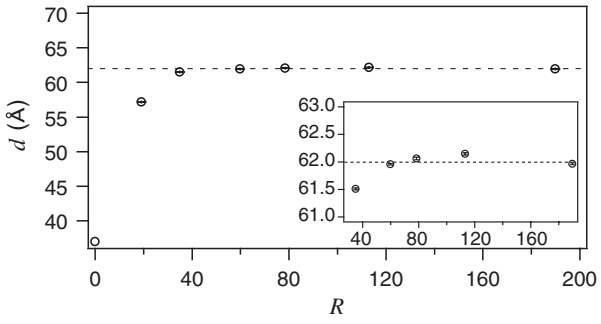

FIG. 1. Lamellar repeat distance for different concentrations of lipid ($R = [H_2O]/[DMPC]$), obtained by SAXS measurements. The inset shows a magnified plot at around $d = 62$ Å. Dashed lines indicate a constant repeat distance, at 62 Å.

Figure 2 shows the complex dielectric constant of pure water, DMPC solution samples ($R = 190, 113, 78, 60, 35, 19$), and dried DMPC film ($R = 4$) in the terahertz region, obtained by THz TDS with attenuated total reflection measurement. Since the imaginary part of the dielectric constant of the dried DMPC film is almost zero in the THz region, no specific absorption by the DMPC molecule is expected in this region. This indicates that the complex dielectric constant of DMPC solutions comes from the relaxation or resonant dynamics of water molecules. The obtained dielectric constant for pure water includes slow relaxation (relaxation time $\tau \approx 8$ ps), fast relaxation ($\tau \approx 0.25$ ps), and an intermolecular stretching vibration mode (at about 5 THz) in the THz frequency range [16,17]. From the reduction of the dielectric constant of the DMPC solution from that of pure water, a decrease of the amount of bulk water by the hydration effect is evaluated, which becomes the hydration water [11–14].

Recently, a similar experiment on a partially hydrated lipid film has been reported [18]. In the study, the dielectric

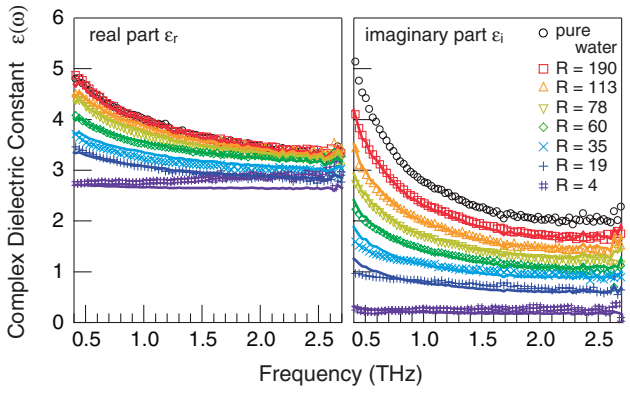

FIG. 2 (color). Complex dielectric constant of pure water, DMPC solutions ($R = 190, 113, 78, 60, 35, 19$), and DMPC film ($R = 4$), obtained by THz TDS measurements (0.4–2.7 THz). The left figure shows the real part of the dielectric constant, while the right one is the imaginary part. The solid lines show the fitting result by using Eq. (1) with fixing $r_h = 0.944$.

constants were fitted with the superposition of Debye functions for each relaxation mode of water, which is the usual analysis of pure water in the THz region [17]. However, in a solution with a high concentration of a solute, the local electric field due to the solute becomes predominant, and the superposition of Debye functions cannot be applied [22]. Furthermore, to determine the hydration state of lipid bilayers in water, we have to observe the hydration state in the fully hydrated condition, because lipid bilayers exhibit strange properties in the partially hydrated condition [23]. Actually, their estimation of the amount of hydration water seems so small compared to the previous studies [4] and is inconsistent with our results (see below). In our analysis of the fully hydrated lipid, we applied a revised model of the dielectric constant taking into consideration the local electric field [13]:

$$\begin{aligned}\epsilon_0[\epsilon(\omega)-1] &= \frac{(M_w - n_h M_l)\tilde{g}}{1-\tilde{h}\tilde{\alpha}_w^{\mathrm{vib}}(\omega)/r_w^3}\left[\tilde{\alpha}_w^{\mathrm{vib}}(\omega)+\frac{\tilde{\alpha}_w^{\mathrm{GHz}}(\omega)+\tilde{\alpha}_w^{\mathrm{THz}}(\omega)}{1-h\alpha_w/r_w^3}\right] \\ &+ \frac{n_h M_l \tilde{g}}{1-\tilde{h}\tilde{\alpha}_w^{\mathrm{vib}}(\omega)/r_h^3}\left[\tilde{\alpha}_w^{\mathrm{vib}}(\omega)+\frac{\tilde{\alpha}_w^{\mathrm{THz}}(\omega)}{1-h\alpha_w/r_h^3}\right] \\ &+ \frac{M_l\tilde{g}}{1-\tilde{h}\alpha_l/r_l^3}\alpha_l, \end{aligned} \quad (1)$$

where M_w and M_l are the number density of water molecules and lipid molecules in the solution, respectively, and are calculated as

$$M_w = \frac{3W_w}{4\pi[r_w^3 W_w + r_l^3 W_l - (r_w^3 - r_h^3)n_h W_l]}, \quad (2)$$

$$M_l = \frac{3W_l}{4\pi[r_w^3 W_w + r_l^3 W_l - (r_w^3 - r_h^3)n_h W_l]}. \quad (3)$$

W_w and W_l are the number of moles of water and lipid in the solution, respectively, and r_w, r_l, and r_h are radii of a bulk water molecule, a lipid molecule, and a hydration water molecule, respectively. r_w and r_l are obtained from the literature as 1.93 and 6.4 Å, respectively [20]. The polarizabilities [α_w, $\tilde{\alpha}_w^{\mathrm{vib}}(\omega)$, $\tilde{\alpha}_w^{\mathrm{THz}}(\omega)$, and $\tilde{\alpha}_w^{\mathrm{vib}}(\omega)$] and the parameters $\tilde{g}$, $\tilde{h}$, and h are determined from the complex dielectric constant of pure water or from observed $\epsilon(\omega)$ of lipid samples by using the method reported in Ref. [13]. Thus, the fitting parameters for the complex dielectric constant $\epsilon(\omega)$ are the number of hydration water molecule per lipid defined in the ultrafast time scale n_h, radius of the hydration water molecule r_h, and polarizability volume of the lipid α_l. The first, second, and third terms in Eq. (1) correspond to the dielectric polarization of the bulk water, hydration water, and lipid, respectively. This model assumes that hydration water loses the slow relaxation mode due to the considerable slowdown of its dynamics than that of bulk water by the hydration effect of the solute, which cannot be observed in the THz region [18,24].

Our SAXS results indicate a certain degree of water condensation in the lamellar phase, compared to the bulk water. The condensation of hydration water has been reported by other x-ray and neutron scattering results for a protein [25], and the density of water σ_w compared to bulk water has been estimated as 1.05–1.25. Our SAXS results exhibit that the lamellar repeat distances are constant for $R > 35$, which indicates that the hydration states of the lipid bilayers are also constant in this fully hydrated condition. Thus, to determine the degree of the condensation from the THz TDS, the measured dielectric constants of lipid solutions in the THz region were fitted by Eq. (1) for $R > 35$. By averaging the fitting result of r_h for $R > 35$, $r_h = 0.944$ is obtained, which corresponds to $\sigma_w = (r_w/r_h)^3 = 1.19$. Since this is consistent with reported condensation of hydration water, we fixed $r_h = 0.944$ for all samples to determine the hydration number of the lipid bilayer precisely. Furthermore, the polarizability volume of the lipid, α_l, was also determined from the free fitting by Eq. (1) for the lipid film sample ($R = 4$) as 86 Å^3. Thus, the hydration numbers for all concentration samples were obtained by the restricted fitting with remaining only one fitting parameter, n_h. The fitting results are indicated by solid lines in Fig. 2.

Figure 3 shows the hydration number n_h defined in the time scale of 10^{-13} s for all samples, obtained by the fitting with fixing $r_h = 0.944$ and $\alpha_l = 86$ Å^3. This result shows the same tendency as the SAXS result (Fig. 1), which confirms our analysis for THz TDS, since with decreasing concentration of the lipid (increasing R) n_h increases for $R < 35$, while for $R > 35$ the hydration number is almost constant within errors, where the lamellar repeat distances of the DMPC multilayer are constant at full hydration condition. By averaging n_h for $R > 35$, the number of hydration water molecules on fully hydrated DMPC multilayers is found to be 28.1. This result indicates that over

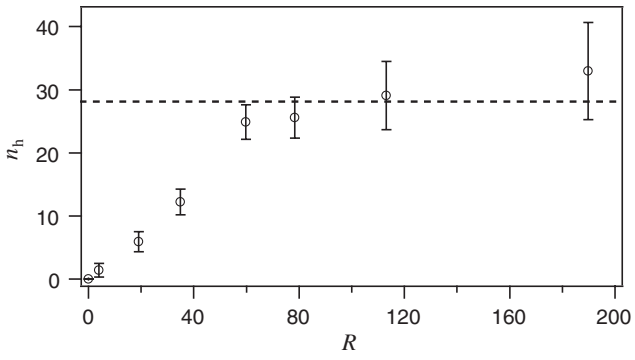

FIG. 3. Number of hydration water molecules per DMPC molecule, n_h, indicated in the 10^{-13} s scale. The results are obtained by using Eq. (1) with fixing $r_h = 0.944$ ($\sigma_w = 1.19$) and $\alpha_l = 86$ Å^3. The dashed line is at $n_h = 28.1$, i.e., the averaged value for the fully hydrated condition $R > 35$.

75% of the water molecules in the lamellar phase of fully hydrated DMPC are perturbed by the membrane. This hydration number corresponds to 4–5 layers of water molecules, and the corresponding correlation length of the hydration effect is roughly about 10 Å. On the other hand, for $R \leq 35$, the fraction of hydration water is smaller (about 35%). The sample condition, i.e., whether the system contains excess water or not, possibly causes this transitionlike behavior of the fraction, since the total free energy of the system determines the hydration state. The transitionlike behavior of the fraction is consistently comprehensive, when we consider almost the same total volume of the interlamellar water during $R = 35$–40 (Fig. 1) in spite of changing the number of the interlamellar water, because hydration water is found to be condensed.

The observed long-range hydration effect seems inconsistent with previous results of NMR or neutron scattering [4–6]. However, as mentioned in the introduction, our result does not contradict these previous results, since the hydration effect is a matter of a dynamical property and the definition of hydration water considerably depends on the observed time scales of water dynamics. In THz TDS, the faster part of the relaxation dynamics of water molecules is measured on the order of 10^{-13} s, with which slightly perturbed water molecules are all defined as hydration water, while in NMR or neutron scattering ($\tau \sim 10^{-9}$–10^{-11} s), sufficiently slowed water molecules have been only observed. On the other hand, our result of the long-range hydration effect is consistent with the previously reported results of the molecular dynamics simulation [8–10], since the time step in such a simulation is normally about 10^{-15} s and the typical observed time scale is 10^{-13}–10^{-12} s, which has affinity to our experiment with THz TDS. Furthermore, our result strongly confirms the observation of hydration water in the ultrafast time scale by pump-probe spectroscopy on lipid hydration [15] or other THz spectroscopy on the nanometer-scale water pool in reverse micelles [26].

In this study, by complementary use of THz TDS and SAXS, we clarified precisely the relation between the hydration state and the lamellar structure of hydrated phospholipid bilayers. This combination leads to a conclusion of the presence of the long-range hydration water layer on the lipid. This long-range hydration water includes slightly perturbed water molecules by lipids compared to bulk water. The correlation length of the hydration effect is about 10 Å from the lipid surface, and most of the water molecules in the lamellar phase are regarded as hydration water in the ultrafast time scale. The result also suggests the condensation of water molecules in the hydration layer.

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