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Three Distinct Water Structures at a Zwitterionic Lipid/Water Interface Revealed by Heterodyne-Detected Vibrational Sum Frequency Generation

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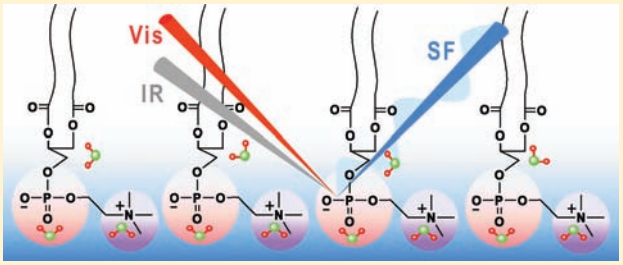
Supporting Information

ABSTRACT: Lipid/water interfaces and associated interfacial water are vital for various biochemical reactions, but the molecular-level understanding of their property is very limited. We investigated the water structure at a zwitterionic lipid, phosphatidylcholine, monolayer/water interface using heterodyne-detected vibrational sum frequency generation spectroscopy. Isotopically diluted water was utilized in the experiments to minimize the effect of intra/intermolecular couplings. It was found that the OH stretch band in the Im$\chi^{(2)}$ spectrum of the phosphatidylcholine/water interface exhibits a characteristic double-peaked feature. To interpret this peculiar spectrum of the zwitterionic lipid/water interface, Im$\chi^{(2)}$ spectra of a zwitterionic surfactant/water interface and mixed lipid/water interfaces were measured. The Im$\chi^{(2)}$ spectrum of the zwitterionic surfactant/water interface clearly shows both positive and negative bands in the OH stretch region, revealing that multiple water structures exist at the interface. At the mixed lipid/water interfaces, while gradually varying the fraction of the anionic and cationic lipids, we observed a drastic change in the Im$\chi^{(2)}$ spectra in which spectral features similar to those of the anionic, zwitterionic, and cationic lipid/water interfaces appeared successively. These observations demonstrate that, when the positive and negative charges coexist at the interface, the H-down-oriented water structure and H-up-oriented water structure appear in the vicinity of the respective charged sites. In addition, it was found that a positive Im$\chi^{(2)}$ appears around 3600 cm^{-1} for all the monolayer interfaces examined, indicating weakly interacting water species existing in the hydrophobic region of the monolayer at the interface. On the basis of these results, we concluded that the characteristic Im$\chi^{(2)}$ spectrum of the zwitterionic lipid/water interface arises from three different types of water existing at the interface: (1) the water associated with the negatively charged phosphate, which is strongly H-bonded and has a net H-up orientation, (2) the water around the positively charged choline, which forms weaker H-bonds and has a net H-down orientation, and (3) the water weakly interacting with the hydrophobic region of the lipid, which has a net H-up orientation.



INTRODUCTION

Biological membranes are semipermeable barriers that separate a cell or cellular organelles (nucleus, mitochondria, etc.) from the surroundings. This separation is essential to maintain the environment inside the organelles and cell for their proper functioning. The function of membranes and membrane proteins is crucially dependent on the aqueous environment at the inner and outer surfaces of membranes. It is because, for example, the membrane−water interaction affects membrane electrostatics that regulates vital processes, such as the signal transduction as well as transport of ions, drugs, and biomolecules across the membrane. Therefore, the elucidation of the molecular-level structure and physicochemical properties of water at the membrane/water interface is very important.

Membranes are complex assemblies of lipids, proteins, carbohydrates, and cholesterols so that direct probing of the real membrane/water interface is difficult. Thus, model systems, such as the lipid multibilayer or lipid monolayer on the water surface, have been extensively studied to understand the physicochemical properties of the membrane/water interfaces. The zwitterionic lipid is a major constituent of biological membranes and pulmonary surfactants. The properties of water at zwitterionic lipid interfaces have been investigated by various types of spectroscopy such as NMR,[1−4] IR,[5−11] and terahertz (THz) spectroscopy[12,13] using a lipid multibilayer made up of about micrometer-thick films of hydrated membrane fragments. These studies on lipid multibilayer systems indicated that the properties of water at zwitterionic lipid/water interfaces is significantly different from that of the bulk water. For example, steady-state IR studies showed that the water associated with the phosphate in phosphatidylcholine is strongly hydrogen-bonded (H-bonded).[6,8,13] Peculiar biexponential dynamics was observed for the vibrational and orientational relaxation of the water by time-resolved IR spectroscopy, and it was argued that this arises from two different water environments existing at the interface of the lipid multibilayer.[6,8,9] In addition, the dielectric relaxation

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of water in the lipid multibilayer was measured and suggested multiple water species existing in the different environments in the lipid multibilayer.[13] All these experimental studies which were performed for the lipid multibilayer with varying water content suggested inhomogeneous water structures at the zwitterionic lipid interfaces. However, the microscopic picture of the water structures at the interface has not fully been obtained yet.

Interestingly, the theoretical study based on molecular dynamics (MD) simulations of a fully hydrated lipid bilayer indicated that water forms different local structures around the positive site and negative site in the headgroup of the zwitterionic lipid.[14−18] In the work of Damodaran[17] and Klein,[14] for example, the atomic density of the water oxygen exhibits a well-defined peak within ∼5 Å from the phosphate phosphorus as well as from the choline nitrogen of phosphatidylcholine. Furthermore, it was suggested that the water molecules in these local hydration structures have different orientations.[16,18] This picture of the local water structures at the zwitterionic lipid/water interface seems to be believed in the theoretical community, but there has been no direct experimental evidence that supports the existence of such multiple local hydration structures. This is partly because the "non-interface-selective" spectroscopic methods, such as NMR, IR, and THz spectroscopy, cannot selectively probe the interface of a fully hydrated zwitterionic multibilayer lipid system.

The lipid/water interfaces in a fully hydrated condition can be studied with a Langmuir monolayer of lipids prepared at the water surface by interface-selective vibrational sum frequency generation (VSFG) spectroscopy.[19−22] Nevertheless, conventional homodyne VSFG has not provided any evidence for the different local hydration structures suggested by the theoretical studies. Very recently, heterodyning of electronic[23] and vibrational[24−27] sum frequency generation has been realized, which opens a new way to obtain molecular-level information of water interfaces. In particular, heterodyne-detected vibrational sum frequency generation (HD-VSFG) provided the imaginary part of the vibrationally resonant $\chi^{(2)}$ ($\mathrm{Im}\chi^{(2)}$; $\chi^{(2)}$ is the second-order nonlinear susceptibility) of interfacial molecules, which can be directly compared to the infrared absorption spectra of the molecule that corresponds to $\mathrm{Im}\chi^{(1)}$. Moreover, the sign of the $\mathrm{Im}\chi^{(2)}$ signal contains direct information about the up/down orientation of molecules at an interface. HD-VSFG enables us to measure the "true" spectral responses and to obtain proper understanding of molecules at the interface, which are often missed in conventional VSFG measurements.[28] Actually, we recently studied the charged (anionic and cationic) lipid/water interfaces with HD-VSFG spectroscopy and clarified that the interfacial water is preferentially oriented according to the direction of the electric field created by the charge of the lipid headgroup.[29] Our work, along with a similar experiment by the Allen group,[30] has corrected wrong arguments that were made on the basis of the analysis of conventional homodyne VSFG data.[31] Because the $\mathrm{Im}\chi^{(2)}$ spectra are much more informative than the $|\chi^{(2)}|^2$ spectra obtained by conventional homodyne VSFG, it is highly desirable to apply HD-VSFG spectroscopy to the zwitterionic lipid/water interface to elucidate the heterogeneous water structures at the interface.

In this paper, we report on our HD-VSFG study of a zwitterionic phosphatidylcholine lipid/water interface, which is a prototypical model of a fully hydrated neutral membrane that has both positive and negative charges in the headgroup. The high sensitivity of HD-VSFG spectroscopy allows us to carry out experiments for the interface of isotopically diluted water. This minimizes the spectral broadening due to intra/intermolecular coupling and enables us to obtain vibrational spectra that give straightforward information about the water structure at the interface. The present work unveils that there are three different kinds of water at the zwitterionic lipid/water interface.

EXPERIMENTAL SECTION

Materials. The lipids and surfactant used in the present study are shown in Figure 1. The lipids (zwitterionic, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC); cationic, 1,2-dipalmitoyl-3-(trimethylammonium)propane (DPTAP); anionic, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG)) were purchased as lyophilized powders from Avanti Polar Lipids. The zwitterionic surfactant *N*,*N*-dimethyldodecylamine *N*-oxide (DDAO) (≥99%) was purchased from Sigma-Aldrich and was used as received. Chloroform (99.7%, GC grade) was purchased from Kanto Chemical Co. and was used as obtained. Milli-Q water (18.2 MΩ cm resistivity) was used for all measurements as H_2O. D_2O (NMR grade, 99.9%) was purchased from Wako. To prepare a Langmuir monolayer of the lipids at the water surface, stock solutions ($\sim 5 \times 10^{-6}$ mol dm^{-3}) were prepared by dissolving a few milligrams of lipids in chloroform or in chloroform/methanol (10:1, v/v) and were spread on the water surface (pH ≈ 6) in a Petri dish (3 cm diameter). A soluble zwitterionic surfactant (DDAO) solution (1×10^{-4} mol dm^{-3}) spontaneously forms a Gibbs monolayer on the aqueous surface.

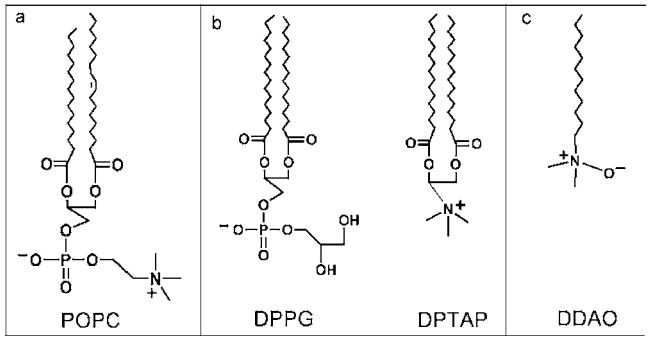

Figure 1. Chemical structures of different lipids: (a) zwitterionic POPC, (b) negatively charged DPPG and positively charged DPTAP, and (c) zwitterionic surfactant DDAO. The counterions of the charged lipids (Na^+ for DPPG and Cl^- for DPTAP) are not shown for simplicity.

Setup of HD-VSFG. The HD-VSFG setup has been described previously.[27] Briefly, a part of the output from a Ti:sapphire regenerative amplifier (Spectra Physics, SpitfireProXP, average power ∼3.5 W, repetition rate 1 kHz, pulse width ∼100 fs, center wavelength 795 nm) was used as the input of a commercial optical parametric amplifier and a difference frequency generator (Spectra Physics, TOPAS C & DFG1) to generate broad-band IR (ω_2, center wavelength 2900 nm, bandwidth ∼400 cm^{-1}, pulse energy 16 μJ). The other part of the amplifier output passed through a narrow band-pass filter (CVI, center wavelength 795 nm, bandwidth 1.5 nm (24 cm^{-1})) and was used as the visible (ω_1) pulse for the sum frequency generation. The ω_1 and ω_2 beams were spatially and temporally overlapped on the sample surface with incident angles of 48° and 61.5°, respectively, to generate the sum frequency at $\omega_1 + \omega_2$ (SF1). The reflected ω_1 and ω_2 beams from the sample surface were refocused on a GaAs(110) surface by a spherical concave mirror to generate the sum frequency once more (SF2), which acted as the local

oscillator in the heterodyne detection. The SF1 was delayed with respect to the SF2 by passing through a 2 mm thick silica plate located between the sample and the concave mirror. Finally, the SF1 and SF2 pulses were introduced into a polychromator and detected by a charge-coupled device (CCD). In the polychromator, the two SF pulses were dispersed and temporally stretched and hence interfered with generation of an interference fringe in the frequency domain. The frequency domain spectrum was inversely Fourier transformed, and the heterodyned signal was extracted using a suitable filter function in the time domain. Then the extracted heterodyned component was Fourier transformed back to the frequency domain. Similarly, the frequency-domain heterodyne spectrum from a *z*-cut quartz crystal was recorded as a reference to calibrate the intensity and phase of the spectrum of the sample. With this procedure, we obtained the corrected imaginary (Im) and real (Re) $\chi^{(2)}$ spectra of the sample. To obtain reliable Im$\chi^{(2)}$ spectra of the lipid/water interfaces, we took special care of the phase error during the measurements (see the Supporting Information). The SF, visible, and IR beams were s-, s-, and p-polarized (ssp polarization), respectively, in the present work.

HD-VSFG Measurements of the Monolayer. HD-VSFG measurements of the Langmuir monolayer of the lipids were performed at a temperature of 296 K and at a surface pressure of 25 ± 3 mN/m, which corresponds to the liquid condensed (LC) phase.[32] During the HD-VSFG measurements, the surface pressure was monitored with a commercial surface tension meter (Kibron, Inc., Helsinki, Finland). For the lipids studied, we measured the Im$\chi^{(2)}$ spectra at different surface pressures (18, 25, and 35 mN/m) and confirmed that the spectra are essentially the same within the LC phase (see the Supporting Information). For the zwitterionic surfactant DDAO, the Gibbs monolayer (soluble monolayer) formed at the surface of a 1×10^{-4} mol dm^{-3} aqueous solution was measured. It is known that the OH stretch region of the interfacial water spectra is heavily affected by inter- and/or intramolecular coupling.[33,34] Therefore, we mainly used isotopically diluted water (H_2O/D_2O = 1/4 (v/v), i.e., $H_2O/HOD/D_2O$ = 1/8/16) in the present study to eliminate the effect of the intra- and intermolecular coupling. Therefore, water means isotopically diluted water in this paper, if not mentioned. (We use the symbol HOD to denote the isotopically diluted water hereafter.) The concentration of the counterion in the charged and mixed lipid solution was $\leq 1 \times 10^{-6}$ mol dm^{-3}. Under such a low concentration, the counterion effect on the measured spectra is negligible.[29]

RESULTS AND DISCUSSION

Im$\chi^{(2)}$ Spectra of Water at Charged and Zwitterionic Lipid/Water Interfaces. HD-VSFG provides vibrationally resonant $\chi^{(2)}$ spectra of molecules at the interface. The Im$\chi^{(2)}$ spectrum can be directly compared to the infrared absorption spectrum in solution which corresponds to Im$\chi^{(1)}$ spectra. Furthermore, the vibrational band in the Im$\chi^{(2)}$ spectrum appears with a positive or negative sign, reflecting the polar orientation of the relevant vibration. Consequently, Im$\chi^{(2)}$ gives direct information about the absolute orientation of water at the interfaces. In this section, we first briefly describe the Im$\chi^{(2)}$ spectra of charged (cationic and anionic) lipid/water interfaces as a starting point, although they have already been reported.[27,29] Then we discuss the Im$\chi^{(2)}$ spectrum of the zwitterionic lipid/water interface, which is the main subject of the present study.

The inset of Figure 2 shows the Im$\chi^{(2)}$ spectra of the anionic DPPG and cationic DPTAP/water (HOD) interfaces in the CH and OH stretch regions. In these spectra, the CH stretch bands around 2900 cm^{-1} have a negative sign, whereas the OH stretch bands appear with different signs in the region of 3000–3600 cm^{-1}. The same sign of the CH stretch band implies that the terminal methyl groups of alkyl chains in the lipids are oriented in the same way for the cationic and anionic lipids (i.e., the methyl hydrogens pointing toward the air[27]). This is reasonable because the orientation of the terminal methyl is independent of the sign of the charge of the headgroup. In contrast, the opposite sign of the OH stretch bands demonstrates that the net orientation of the interfacial water is opposite for the anionic and cationic lipids. In the case of the OH stretch vibration of water, the sign of the vibrational band coincides with the net direction of the H ← O dipole moment of water at the interface. The positive sign of the OH stretch band at the anionic DPPG/water (HOD) interface represents a net H-up orientation of water, whereas the negative sign of the OH stretch band at the cationic DPTAP/water (HOD) interface demonstrates a net H-down orientation of water. The Im$\chi^{(2)}$ spectra of the charged lipid/water interfaces clearly show that the net orientation of the interfacial water is determined by the sign of the charge of the lipid headgroup. More specifically, the water orientation is governed by the direction of the static electronic field created by the charge of the headgroup existing at the interface.[29]

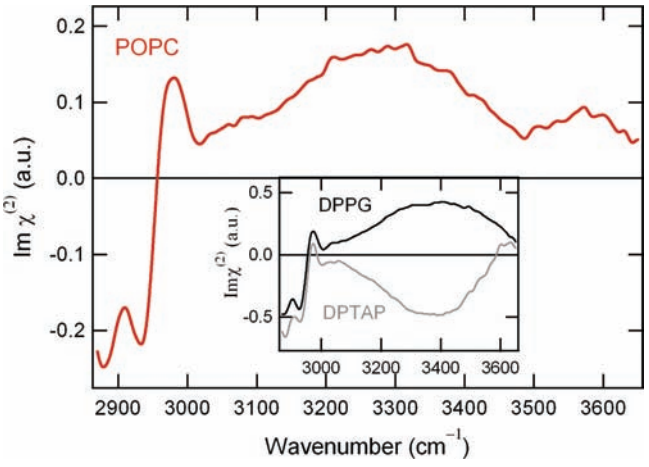

Figure 2. Im$\chi^{(2)}$ spectrum of the zwitterionic POPC/water (HOD) interface (red) in the OH and CH stretch regions. Inset: Im$\chi^{(2)}$ spectra of the anionic DPPG/water interface (black) and cationic DPTAP/water interface (gray). Isotopically diluted water ($H_2O/HOD/D_2O$ = 1/8/16) was used, and all the measurements were done with the ssp polarization combination (s, sum frequency polarization; s, visible polarization; p, IR polarization). The surface pressures of the lipid monolayers were maintained at 25 ± 3 mN/m during the measurements. The $|\chi^{(2)}|^2$ spectrum of the POPC/water interface is shown in the Supporting Information.

The Im$\chi^{(2)}$ spectrum of the zwitterionic POPC/water (HOD) interface is shown with a red curve in Figure 2. The OH stretch band appears with a positive sign, which shows that interfacial water has a net H-up orientation at the interface although the zwitterionic POPC has no net charge on the headgroup.[30,35] This implies that the negatively charged phosphate group is more capable of orienting interfacial water than the positively charged choline group. This result is consistent with the charge of each site estimated by the CHARMM electrostatic model,[36] which suggested that the negative charge on the phosphate group (−1.2e) is larger than the positive charge on the choline group (+0.78e). However, compared to the simple OH band of the charged lipid/water interfaces, the OH stretch band of the zwitterionic lipid/water interface exhibits a very peculiar band shape. In a broad positive OH stretch band in the 3000–3650 cm^{-1} region, there is a clear dip around 3470 cm^{-1} which is completely absent in the OH

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stretch band of the anionic DPPG/water (HOD) and cationic DPTAP/water (HOD) interfaces. This dip is not due to the intramolecular coupling (e.g., Fermi resonance) of the interfacial water because we used isotopically diluted water ($H_2O/HOD/D_2O$ = 1/8/16) and hence the observed OH stretch band is predominantly due to the HOD species.

The characteristic OH stretch band at the POPC interface strongly indicates that the water structure at the zwitterionic lipid/water interface is significantly different from that at the charged lipid/water interfaces. For the charged lipids, the sign of the total charge of the headgroup determines the net orientation of interfacial water.[29] For the zwitterionic lipid, on the other hand, the headgroup is electrically neutral so that the electric field in the far field must be zero. Nevertheless, as the total sign of the OH stretch band indicates, the negative phosphate generates the local electric field that induces H-up orientation of water molecules in the vicinity. This means that the positive choline group can also generate the local electric field, which may create a different water structure in the vicinity. Because the $\mathrm{Im}\chi^{(2)}$ spectra are linear to the molecular response, the spectra are the cumulative sum of the multiple components, if they exist. We considered that the complex OH band shape of the zwitterionic POPC/water interface highly likely arises from the simultaneous presence of the different water structures around the negative and positive charges at the interface. To examine this idea, we carried out the experiments for a structurally simpler zwitterionic surfactant/water (HOD) interface as well as the mixed lipid monolayer/water (HOD) interfaces, which we describe in the following sections.

$\mathrm{Im}\chi^{(2)}$ Spectrum of Water at a Zwitterionic Surfactant/Water Interface. At the zwitterionic POPC/water interface, because of the larger negative charge on the phosphate group, the OH stretch band appears with a positive sign in total. This situation obscures the contribution of the water associated with the positively charged choline. DDAO is a surfactant that has a headgroup equivalent to trimethylamine *N*-oxide (Figure 1c), and ab initio molecular orbital (MO) calculations indicated that the excess charges on the anionic and cationic sites of DDAO are comparable (−0.65e on the oxygen and +0.65e on the trimethylamine).[37,38] Thus, we can expect that the effect of the negative and positive charges will appear with comparable magnitudes at the DDAO/water interface.

Figure 3 shows the $\mathrm{Im}\chi^{(2)}$ spectrum of the DDAO/water (HOD) interface measured by HD-VSFG. Like those of the lipids, the $\mathrm{Im}\chi^{(2)}$ spectrum of the DDAO/water (HOD) interface exhibits negative CH stretch bands due to the terminal methyl of the alkyl chain in the <3000 cm^{-1} region. Remarkably, the $\mathrm{Im}\chi^{(2)}$ spectrum shows both a positive signal (3000−3380 cm^{-1}) and a negative signal (3380−3600 cm^{-1}) in the OH stretch region, which clearly demonstrates the existence of different types of water having opposite orientations at the interface. Because DDAO has the positive and negative sites in the headgroup, it is natural to assign the positive OH band in the $\mathrm{Im}\chi^{(2)}$ signal to the net H-up-oriented water associated with the negatively charged oxygen (O^-) and the negative OH band to the net H-down-oriented water associated with the positively charged trialkylammonium ($R(Me)_2N^+$). The OH stretching frequency of the former is lower than that of the latter, which indicates that the water associated with the anionic group is more strongly H-bonded than that associated with the cationic group. This $\mathrm{Im}\chi^{(2)}$ spectrum of the DDAO/water (HOD) interface reveals that the distinct water species appear at the zwitterionic interface.

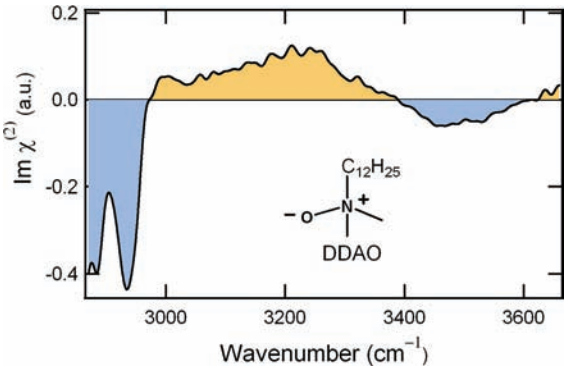

Figure 3. $\mathrm{Im}\chi^{(2)}$ spectrum of the zwitterionic surfactant DDAO/water (HOD) interface in the OH and CH stretch regions, measured with the ssp polarization combination. The bulk concentration of DDAO was 1×10^{-4} mol dm^{-3}, and isotopically diluted water was used.

We note that these distinct positive (~3000−3380 cm^{-1}) and negative (~3380−3600 cm^{-1}) bands were not clearly observed and the negative signal becomes predominant in the $\mathrm{Im}\chi^{(2)}$ spectrum of the DDAO/H_2O interface (see the Supporting Information). This difference is probably due to the intra- and intermolecular couplings of H_2O, and the distinct structures become evident only in the spectrum of the isotopically diluted water interface where the intra- and intermolecular couplings are suppressed.

$\mathrm{Im}\chi^{(2)}$ Spectra of Water at Mixed Lipid/Water Interfaces. The $\mathrm{Im}\chi^{(2)}$ spectrum of the zwitterionic surfactant/water interface shows that the local charges in the headgroup can induce a distinct water structure around each site even if the net charge at the interface is zero. This strongly suggests that the distinct hydration structures are formed around the positive and negative sites also in the case of the zwitterionic lipid. To further confirm this argument, we carried out HD-VSFG experiments for the mixed lipid monolayer/water interfaces. In this experiment, we mixed positively charged DPTAP and negatively charged DPPG, which have headgroups similar to the positive or negative sites of the zwitterionic POPC (Figure 1). We formed a mixed lipid monolayer at the water surface and measured $\mathrm{Im}\chi^{(2)}$ spectra while changing the ratio of the anionic and cationic lipids. This experiment clarifies how the $\mathrm{Im}\chi^{(2)}$ spectrum changes as the interfacial charge gradually changes from positive to negative.

The $\mathrm{Im}\chi^{(2)}$ spectra of the mixed lipid/water interfaces are shown in Figure 4. The black line in Figure 4 (spectrum a) is the $\mathrm{Im}\chi^{(2)}$ spectrum of the mixed lipid/water interface measured at a molar ratio of DPPG/DPTAP = 0.3/1.0. This ratio means that the cationic lipid is predominant and the water surface is positively charged. As expected, the $\mathrm{Im}\chi^{(2)}$ spectrum has a negative sign in the OH stretch region (maximum ~3410 cm^{-1}), demonstrating a net H-down orientation of interfacial water. With an increase of the relative amount of the negative DPPG, the intensity of the negative band around 3410 cm^{-1} gradually decreases (spectra a−d). However, the OH band does not vanish completely, and both the positive and negative bands appear at DPPG/DPTAP = 0.5/1.0 (spectrum d) as in the case of the zwitterionic DDAO/water interface. This gradual spectral change (from spectrum a to spectrum d) strongly indicates that the H-up-oriented water gradually appears with an increase of the negatively charged group at the interface, and that H-up-oriented water and H-down-

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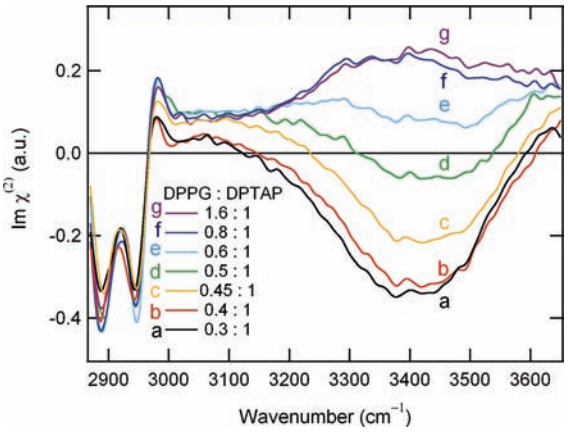

Figure 4. Im$\chi^{(2)}$ spectra of mixed lipid/water (HOD) interfaces with different molar ratios of the anionic (DPPG) and cationic (DPTAP) lipids. The molar ratio corresponding to each spectrum is shown in the figure. The measurements were carried out with ssp polarization, and isotopically diluted water was used. The surface pressures of the lipid monolayers were maintained at 25 ± 3 mN/m during the measurements.

oriented water coexist at the interface when the effective charge densities of the positive and negative sites are comparable (spectrum d).

With a further increase of the relative amount of DPPG, the Im$\chi^{(2)}$ spectrum exhibits a positive band with a dip around 3450 cm^{-1} (spectrum e; DPPG/DPTAP = 0.6/1.0). This spectrum is similar to the spectrum of the POPC/water interface shown in Figure 2. Obviously, the dip feature in spectrum e corresponds to the negative feature in spectrum d, implying that the H-down water associated with the cationic choline does not lose its identity although its response becomes weaker and it gets buried in the positive signal due to the H-up water generated by the negative phosphate. On a further increase of the relative amount of the anionic lipid, the spectral dip around 3450 cm^{-1} vanishes (spectra f and g), and the Im$\chi^{(2)}$ spectrum exhibits a single broad positive band. This spectrum resembles the Im$\chi^{(2)}$ spectrum of the negatively charged DPPG/water interface where the structure and orientation of the interfacial water are determined by the electric field created by the negative charge of the anionic phosphate group. It is noted that there is no significant change in the CH stretch region with a change of the relative molar ratios of DPPG and DPTAP. This assures that the drastic spectral evolution observed in the OH stretch region is solely due to the change in the interfacial water, which is caused by the change of the charge at the interface.

As already mentioned above, the observed spectral change in the OH stretch region is readily rationalized as the cumulative sum of the contribution of the positive and negative bands, which arise from the water around the negatively charged phosphate and positively charged choline groups, respectively. Furthermore, it is straightforward to assign the dip feature around 3450 cm^{-1} in spectrum e to the H-down-oriented water aligned around the positive choline. On the basis of the result obtained from the mixed lipid/water interface, we can now safely conclude that the characteristic spectral feature of the zwitterionic POPC/water interface arises from a positive band due to the H-up-oriented water associated with the phosphate and a negative band due to the H-down-oriented water around the choline in the headgroup of POPC.

It is worth noting that a spectral feature similar to that of the zwitterionic POPC/water interface is observed at a mixed lipid ratio of DPPG/DPTAP = 0.6/1.0, not at 1.0/1.0 that realizes electric neutrality. This may be due to the difference in the accessibility of the water molecule to the phosphate and choline sites at the two interfaces. At the zwitterionic POPC, the anionic phosphate and cationic choline groups are linked by covalent bonds, which give some restriction for the vertical position of the two groups at the interface. A simulation study suggested that the dipole vector from the phosphorus atom to the nitrogen atom in the headgroup of POPC is not parallel to the water surface, but is tilted by ~20°.[39] For the mixed lipids, on the other hand, the anionic and cationic groups are not covalently linked, and hence, their vertical positions are determined more freely. Thus, it is likely that the relative vertical positions of the anionic and cationic groups are different at the two interfaces. Because the water density sharply changes along the vertical direction,[40] a slight difference in the vertical position likely changes the number of water molecules that can be associated. In addition, even if the relative vertical positions of the anionic and cationic groups are the same at the two interfaces, the surface areas occupied by the phosphate and choline groups in POPC are presumably different from that in the mixed lipids even at the same surface pressure because the numbers of alkyl chains per charged group are different (i.e., two alkyl chains for one zwitterionic headgroup at the POPC interface whereas four alkyl chains in total for anionic and cationic groups at the mixed lipid interfaces). This difference in the surface area also seems to cause a difference in the number of water molecules that can be associated with the cationic and anionic groups at the two interfaces. We think that this difference in the accessibility of the water molecules to the phosphate and choline groups gives rise to the difference in the magnitude of the Im$\chi^{(2)}$ response of the H-up-oriented water and H-down-oriented water at the two interfaces and hence that the spectral feature resembling the zwitterionic POPC is realized for the mixed lipid at a DPPG/DPTAP ratio of 0.6/1.0, not at 1.0/1.0.

Evidence of Weakly Interacting Water at the Zwitterionic Lipid/Water Interface. On the high-frequency side of the OH stretch band (>~3550 cm^{-1}), the Im$\chi^{(2)}$ spectra of all of the lipid/water interfaces (i.e., the interfaces between water and the cationic, anionic, cationic/anionic mixtures, or zwitterionic lipid) show a positive signal (Figures 2 and 4). This positive signal is also observed at the zwitterionic surfactant/water interface (Figure 3). These positive signals are not due to experimental artifacts due to the phase error. We realized a high phase accuracy in the entire OH stretch region by a precise control of the optical path length during the measurements (see the Supporting Information), and the phase accuracy was checked by measuring the Im$\chi^{(2)}$ spectrum of the lipid/D_2O interfaces that shows zero Im$\chi^{(2)}$ signal in the OH stretch region. Thus, these positive Im$\chi^{(2)}$ signals in the high-frequency region manifest the existence of the third type of water at the interface. The high OH stretching frequency indicates its weakly interacting nature, and the positive sign of the signal implies its H-up orientation regardless of the sign of the charge and chemical structure of the headgroup of the lipid or surfactant.

To examine this high-frequency signal in more detail, we measured the Im$\chi^{(2)}$ spectrum of the POPC/H_2O (not HOD) interface, optimizing the frequency of the infrared ω_2 pulse to the high-frequency region of the OH stretch band. In this

experiment, we used H_2O, not isotopically diluted water, to realize a higher signal-to-noise ratio (S/N), because the effect of the intra/intermolecular coupling (e.g., Fermi resonance) is not significant in the spectral region around 3600 cm^{-1}. The obtained $Im\chi^{(2)}$ spectrum of the POPC/H_2O interface is compared to the spectrum of the air/H_2O interface in Figure 5.

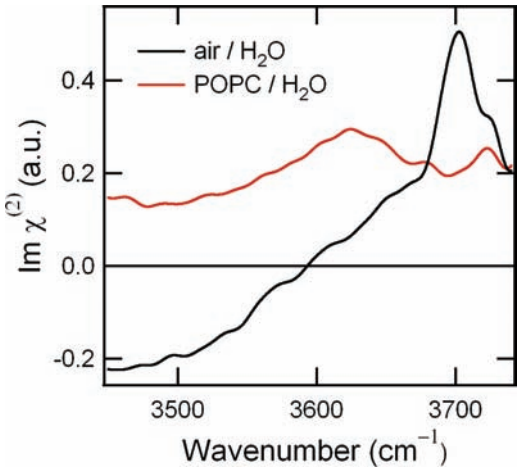

Figure 5. $Im\chi^{(2)}$ spectra of the zwitterionic lipid POPC/H_2O interface (red) and the air/H_2O interface (black) in the high-frequency region. The measurements were carried out with ssp polarization, and the surface pressure of the lipid monolayer was 25 ± 3 mN/m.

At the air/H_2O interface, the $Im\chi^{(2)}$ spectrum has a positive sign in the >3600 cm^{-1} region and exhibits a sharp peak at ~3700 cm^{-1} that is due to the "free OH" pointed toward the air.[27,41,42] Also at the POPC/H_2O interface, the $Im\chi^{(2)}$ spectrum exhibits a broad positive band peaked at ~3620 cm^{-1}. It has already been suggested by a number of experimental and theoretical studies that weakly interacting water exists in the hydrophobic region of the lipid.[6,13,35,43] In particular, a conventional homodyne VSFG study showed that the free OH band at the air/water interface shifted toward the lower frequency, keeping its identity, as the water surface was gradually covered by lipid molecules. Because this high-frequency OH band remained even when the water surface was fully covered by lipid, this band has been assigned to the weakly interacting OH of the water molecules existing in the hydrophobic region of the lipid/water interface.[43] Therefore, the positive $Im\chi^{(2)}$ signal observed around 3580 cm^{-1} (Figure 2) is safely attributed to the water molecules residing in the hydrophobic region of the lipid monolayer.

Fitting Analysis and Three Water Structures at the Zwitterionic Lipid/Water Interface. As described in the previous sections, the $Im\chi^{(2)}$ spectrum of the zwitterionic POPC/water interface reveals that there are three different types of water at the interface: (1) net H-up-oriented water associated with the negatively charged phosphate (we denote it with "W_P" hereafter), (2) net H-down water associated with the positively charged choline (W_C), and (3) net H-up-oriented water in the hydrophobic region of the lipid (W_H). These three kinds of water differ in the H-bond strength and net orientation, reflecting their location at the zwitterionic lipid/water interface. Because the observed OH stretch band of the $Im\chi^{(2)}$ spectrum is the sum of the $Im\chi^{(2)}$ responses of W_P, W_C, and W_H, we carried out a fitting analysis to obtain more quantitative information about these three water species. In this analysis, we assumed the Gaussian band shape for the $Im\chi^{(2)}$ response of the three water species at the zwitterionic lipid/water interface and made a fitting using the following functional form:

$$R(\bar{\nu}) = R_P(\bar{\nu}) + R_C(\bar{\nu}) + R_H(\bar{\nu})$$
$$= a_P \exp\left[-\frac{(\bar{\nu}-\bar{\nu}_P)^2}{2f_P^{\,2}}\right] + a_C \exp\left[-\frac{(\bar{\nu}-\bar{\nu}_C)^2}{2f_C^{\,2}}\right]$$
$$+ a_H \exp\left[-\frac{(\bar{\nu}-\bar{\nu}_H)^2}{2f_H^{\,2}}\right]$$

Here, a_i (i = P, C, H) denote the amplitude (or the weight factor) of corresponding components, and $\bar{\nu}_i$ and f_i represent the center frequency and band width of each Gaussian band, respectively. The best fit obtained is shown in Figure 6 with a green line, and the corresponding parameters are given in Table 1.

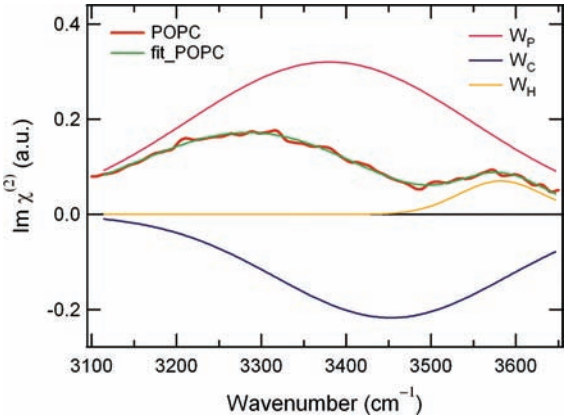

Figure 6. Fitting analysis of the $Im\chi^{(2)}$ spectrum of the zwitterionic lipid POPC/water (HOD) interface in the OH stretch region. The best fit (green) and its three Gaussian components are shown. The magenta, blue, and orange lines represent the components due to the water associated with phosphate (W_P), choline (W_C), and the hydrophobic region (W_H), respectively. The experimental $Im\chi^{(2)}$ spectrum is also shown with a red line.

Table 1. Parameters of the Best Fit of the $Im\chi^{(2)}$ Spectrum of the POPC/Water (HOD) Interface

component	amplitude (a_i)	center frequency ($\bar{\nu}_i$) in cm^{-1}	band width ($\sqrt{2}f_i$) in cm^{-1}
i = P	0.032	3380	238
i = C	−0.022	3452	192
i = H	0.007	3581	70

As seen in this figure, the characteristic feature of the OH stretch band is well reproduced as the sum of the three Gaussians. The largest component (the magenta line) is positive and peaked at ~3380 cm^{-1}, which represents the signal of net H-up-orientated water associated with phosphate groups (W_P). The peak of this band is approximately 30 cm^{-1} red-shifted compared to the maximum of the infrared absorption of bulk HOD (~3410 cm^{-1}). This red shift indicates the stronger H-bonding of the phosphate-associated water. At the anionic phosphate, the negatively charged (non-ester) oxygen atoms directly form H-bonds with water. Because they have higher negative charge density than the oxygen atom of water, the H-

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bond between the phosphate oxygen and water becomes stronger than that between water molecules. This result is consistent with the IR studies of the weakly hydrated phosphatidylcholine multilayer system.[6,8,13] The second largest component (the blue line) has a negative sign and is peaked at ~3450 cm^{-1}, which represents the signal of H-down-oriented water associated with choline groups (W_C). The peak of this component is approximately 40 cm^{-1} blue-shifted compared to the OH stretch band of bulk HOD, indicating that the H-bonding of the choline-associated water is weaker than that of W_P or even that of bulk water. This result is readily rationalized as follows. The positive charge at the nitrogen in the choline is surrounded by the three methyl groups, which makes the choline act like a hydrophobic cation in the H-bonding network. It is known that a hydrophobic solute acts as a breaker of the H-bond network in bulk water[44,45] and that it gives rise to a blue shift of the OH stretch band. Thus, it is natural that the OH stretch band of the water associated with the hydrophobic choline group appears at a higher frequency also at the interface. The third component (the orange line) exhibits an intensity maximum around ~3580 cm^{-1} with a positive sign. The small amplitude of this component suggests that this water species is minor compared with the other two components. This component is attributable to the weakly interacting water in the hydrophobic region of the lipid (W_H), as already discussed in the previous section.

The results of the fitting analysis clearly showed that the characteristic $Im\chi^{(2)}$ spectrum of the POPC/water interface is quantitatively rationalized with the three spectral components that correspond to the three distinct water structures having different net orientations and different H-bonding strengths. Although the existence of different types of water at the zwitterionic lipid/water interface has been suggested by several NMR and IR studies,[1,6,9,13] the present HD-VSFG study provides the first direct experimental evidence of the distinct local water structures around the phosphate and choline groups at the zwitterionic lipid/water interface.

Recently, Allen and co-workers[30] reported the $Im\chi^{(2)}$ spectra of a phosphatidylcholine lipid/H_2O interface, which were measured by HD-VSFG employing the optical configuration developed by us.[23,27] They observed a broad positive OH stretch band of the interfacial water, being consistent with the observation in our present study. However, they failed to notice the characteristic spectral feature that represents the distinct local water structures at the zwitterionic interface. We think that this discrepancy is for several reasons: First, to discuss a detailed spectral feature of the $Im\chi^{(2)}$ spectra, it is crucial to measure the spectra with a high phase accuracy. Judging from the description in their paper,[30] the phase error of their measurements seems substantially large, especially during the exposure of the CCD. Second, they measured spectra of the phosphatidylcholine/H_2O interface with H_2O as the liquid phase, not with the isotopically diluted water (HOD). As already mentioned, the OH stretch band of the H_2O interface is substantially broadened by intra/intermolecular coupling, which obscures the spectral features in the OH stretch region.[28,33,34] Third, they reported the $Im\chi^{(2)}$ spectra only in the range of 3000−3550 cm^{-1}. The OH stretch band of the weakly interacting water in the hydrophobic region of the lipid layer is outside the frequency region of their measurement.

It is also worth mentioning a recent theoretical work of Nagata and Mukamel,[35] who simulated the $Im\chi^{(2)}$ spectra of water at a phosphatidylcholine lipid/H_2O interface. In agreement with the experiment, their simulated $Im\chi^{(2)}$ spectrum showed a positive sign over the OH stretch region. They analyzed their results to gain more insight and simulated the change of the $Im\chi^{(2)}$ response with variation of the depth at the interface. Then they concluded that there are three kinds of water at the interface: (i) the water in the hydrophobic glycerol region ($Im\chi^{(2)}$ maximum ~3590 cm^{-1}), (ii) water adjacent to the lipid headgroup region (maximum ~3470 cm^{-1}), and (iii) the near-bulk water (maximum ~3290 cm^{-1}) that is non-adjacent to the lipid headgroup. Their first type of water accords with our observation of weakly interacting interfacial water that gives rise to the positive OH stretch band at ~3580 cm^{-1} (W_H). However, the second and third types of water are different from the water structures we have identified in the present study. In fact, in their study, the water adjacent to the lipid headgroup region was treated as a whole and it only provided a gross spectral response of the interfacial water. In other words, their analysis did not distinguish the water associated with the phosphate and choline groups, which are simultaneously present at the headgroup region with opposite orientations. In fact, the responses of the water associated with the phosphate and choline groups cannot be separated if only the spectral evolution along the vertical direction of the interface is analyzed because the vertical positions of the phosphate and the choline groups are not well separated.

Figure 7 sketches the three distinct water structures existing at the zwitterionic phosphatidylcholine lipid/water interface,

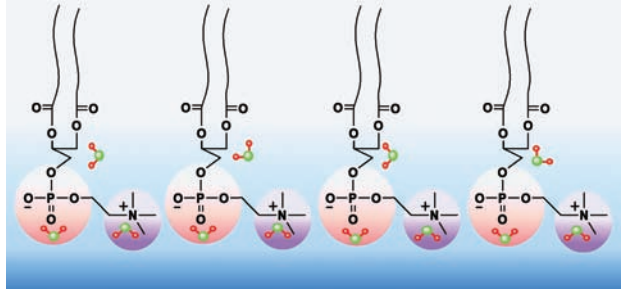

Figure 7. Schematic representation of the three distinct waters at the zwitterionic lipid/water interface.

which have been revealed by the present HD-VSFG study. The phosphate-associated water (W_P) is depicted by a red sphere around the phosphate group. The single water molecule in the sphere is a symbolic representation of net H-up-oriented water at the vicinity of the phosphate group. Similarly, the violet sphere around the choline represents the choline-associated water (W_C) having a net H-down orientation. The water in the hydrophobic region of the lipid represents the weakly interacting water with a H-up orientation (W_H) on average.

SUMMARY AND CONCLUSIONS

We investigated the water structure at a zwitterionic phosphatidylcholine monolayer/water interface using HD-VSFG spectroscopy with isotopically diluted water. We found that the OH stretch band in the $Im\chi^{(2)}$ spectrum of the phosphatidylcholine lipid/water interface exhibits a characteristic feature which is markedly different from that of the OH stretch bands of the charged lipid/water interfaces. To interpret this peculiar spectrum of the zwitterionic lipid/water interface, we measured $Im\chi^{(2)}$ spectra of a zwitterionic surfactant/water interface and mixed lipid/water interfaces. The $Im\chi^{(2)}$ spectrum

of the zwitterionic surfactant/water interface clearly shows both positive and negative bands in the OH stretch region, demonstrating that the water species having opposite orientations coexist at the interface, i.e., the strongly H-bonded water existing in the vicinity of the anionic moiety with net H-up orientation and the weakly H-bonded water existing in the vicinity of the cationic moiety with net H-down orientation. For the mixed lipid monolayer/water interfaces, while gradually varying the ratio of the anionic and the cationic lipids at the interface, we observed a gradual spectral change in which spectral features similar to those of the neat anionic, zwitterionic, and cationic lipid/water interfaces appear successively. This result also strongly indicates the presence of H-up-oriented and H-down-oriented water structures around the negative and positive charges at the lipid interfaces. In addition, all the interfaces of the lipids as well as the surfactant showed positive OH stretch bands around 3600 cm^{-1} regardless of the sign of the charge and the chemical structure of the headgroup. This indicates that there is weakly interacting water in the hydrophobic region in the lipid and surfactant monolayers. Consequently, we concluded that there are three different types of water species at the zwitterionic POPC/water interface: (1) the water associated with the negatively charged phosphate, which is strongly H-bonded and has a net H-up orientation, (2) the water around the positively charged choline, which is relatively weakly H-bonded and has a net H-down orientation, and (3) the water in the hydrophobic region of the lipid, which is very weakly interacting and has a net H-up orientation. The $Im\chi^{(2)}$ spectrum at the zwitterionic lipid/water interface can be interpreted as a cumulative sum of the signals arising from three distinct water species existing at the interface. Reflecting the difference in the strength of the H-bonding, these different water molecules exhibit substantially different OH stretch frequencies so that their $Im\chi^{(2)}$ signals do not completely cancel each other in spite of different signs and show the peculiar spectral feature. We note that such clear data can be obtained because of the linear nature of $Im\chi^{(2)}$ and that it is almost impossible to properly interpret the $|\chi^{(2)}|^2$ spectrum obtainable with traditional homodyne-detected VSFG (see the Supporting Information). The present HD-VSFG study has provided new insights into the orientation and structure of water at the zwitterionic phosphatidylcholine lipid/water interface, which are highly relevant to the elucidation of the properties and functions of membrane/water interfaces.

■ ASSOCIATED CONTENT

Supporting Information

(1) Raw and the filtered heterodyne spectra of the reference *z*-cut quartz, (2) comparison of $Im\chi^{(2)}$ spectra of DPPC/water (HOD) and POPC/water (HOD) interfaces, (3) $Im\chi^{(2)}$ spectra of DDAO/H_2O and DDAO/HOD interfaces, (4) $Im\chi^{(2)}$ spectra POPC/water (HOD) interface at different surface pressures and (5) complex $\chi^{(2)}$ and $|\chi^{(2)}|^2$ spectra of the POPC/water interface. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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