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PERSPECTIVE

No Ice-Like Water at Aqueous Biological Interfaces

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Abstract The surface vibrational spectrum of water at biological interfaces is often interpreted as having 'ice-like' and 'liquid-like' components. Here we show that the vibrational spectrum of water at both water–lipid and water–protein interfaces greatly simplifies upon H/D isotopic dilution, which is inconsistent with the presence of 'ice-like' structures. The changes in the spectra as a function of isotope content can be explained by intramolecular coupling between bend and stretch vibrations of the water molecules.

Biological membranes define the external boundaries of cells and various cell organelles. The main constituents of these membranes are amphiphilic (phospho-)lipid molecules that self-assemble into bilayers. Apart from compartmentalizing cellular material and providing barriers, biological membranes also regulate the molecular and ionic transport in and out the cells and cell organelles. The functionality of membranes originates from a variety of membrane-bound and embedded proteins, such as ion-channels and receptor proteins. In recent years, it is becoming increasingly clear that membrane functionality relies on an intricate interplay between different lipids, proteins and water molecules. Regarding the role of water, it has become apparent that the interaction with water co-determines the molecular and supra-molecular organization of lipids and proteins within the membrane. Membrane hydration drives the self-assembly of the bilayers, and studies of partially hydrated bilayers by X-ray scattering, neutron scattering and calorimetry indicated that the fluidity of the lipid phase—an essential parameter for membrane function—varies strongly with the degree of hydration [1]. Inversely, NMR experiments have shown that the lipid head groups have a strong influence on the local water structure [2–4].

In principle, vibrational spectroscopy allows one to probe water molecular properties directly through variations in the OH stretch frequency and linewidth, as the OH stretch vibration is a sensitive reporter to the local hydrogen-bonding environment of water molecules: the O–H stretch vibrational mode is known to vary strongly as a function of hydrogen-bond strength [5]. However, using infrared absorption or spontaneous Raman spectroscopy, it is extremely challenging to distinguish the few monolayers of interfacial, lipid-bound water from the generally much more abundant bulk water.

The study of bio-interfaces using vibrational spectroscopy has therefore benefited greatly from the increasingly widespread availability of the surface-specific vibrational spectroscopic technique of infrared–visible sum-frequency generation (SFG). SFG spectroscopy is a second-order nonlinear optical technique in which a visible and an infrared field are overlapped at an interface and the resulting emission at the sum of their frequencies is measured. This emission increases strongly when the infrared frequency is resonant with a vibration of surface molecules and is generally restricted to the 1–2 molecular layers closest to the interface [6], and increases strongly when the

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infrared frequency is resonant with a vibration of molecules within these molecular layers. Hence, for water underneath biomolecular (e.g. lipid or protein) monolayers, SFG spectroscopy provides the vibrational spectrum of only the lipid- or protein-bound water. Here we show, using SFG spectroscopy of (isotopically diluted) water underneath lipid and protein monolayers, that the water structure at these biological interfaces does not exhibit 'ice-like' and 'liquid-like' characteristics, as has been claimed previously [7–14].

Our SFG setup has been described in detail elsewhere [15]. Briefly, a regeneratively amplified Ti:sapphire system (Legend, Coherent Inc, USA) produces 100 fs pulses centered at 800 nm with a bandwidth of 12 nm. Out of the total output, 1 mJ/pulse is used for the generation of tunable mid-IR pulses using a commercial optical parametric amplifier and difference frequency generation unit (TOPAS, Light Conversion, Lithuania). Another 0.5 mJ/pulse is spectrally narrowed using an etalon resulting in a bandwidth of ~ 15 cm^{-1}. The IR and spectrally narrowed 800 nm beams are passed through half wave plates and polarizers and incident on the sample in a reflection geometry at angles of 40° and 35° with respect to the surface normal (IR and visible, respectively). The reflected SFG signal is focused into a spectrograph (Acton Instruments, USA) and dispersed onto an electron multiplied Charge Coupled Device (emCCD) camera (Newton, Andor, USA). The spectra reported in this study were collected under ssp polarization condition (s polarized SF, s polarized visible, p polarized IR), at room temperature. To correct for the spectral dependence of the input IR and visible pulses, the measured spectra were divided by the non-resonant spectra collected from a z-cut quartz plate. The spectra shown here were recorded for D_2O rather than H_2O, since our OPA/DFG set up generates substantially more IR in the 2,200–2,600 cm^{-1} frequency range than in the 3,100–3,600 cm^{-1} frequency interval.

Lipid monolayers of 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP, Avanti Polar Lipids) were prepared at a surface pressure of 25 ± 3 mN/m at room temperature (22 ± 1°C) in a home-made Teflon trough. The surface pressure was measured with a tensiometer using the Wilhelmy plate method. The water subphase consists of pure D_2O (Cambridge Isotope Laboratories, Inc., 99.93% purity, used without further purification) and isotopic mixtures with H_2O (Millipore, 18 MΩ cm). Monolayers of lysozyme (Sigma-Aldrich) were prepared by dissolving lysozyme (1 mg/ml) in a 10 mM sodium phosphate D_2O buffer (pH = 7). Typically 3 h were waited for the surface protein monolayer(s) to reach an equilibrium state. In this state, the surface pressure amounted to 18 ± 2 mN/m. We performed SFG measurements while rotating the trough to reduce accumulated heating effects due to the repeated surface excitation. Experiments at isotope ratios D/(D + H) = 1 and 0.33 are reported here.

The measured spectra for pure D_2O in contact with lipids and proteins show a dominant double-peak structure for both the water–lipid and the water–protein interface, as illustrated in Fig. 1a and b. The peaks appearing at 3,200 and 3,400 cm^{-1} (2,350 and 2,500 cm^{-1} for D_2O) for water at bio-interfaces have traditionally been assigned (see, e.g. Refs. [7–14]) to 'ice-like' and 'liquid-like' interfacial

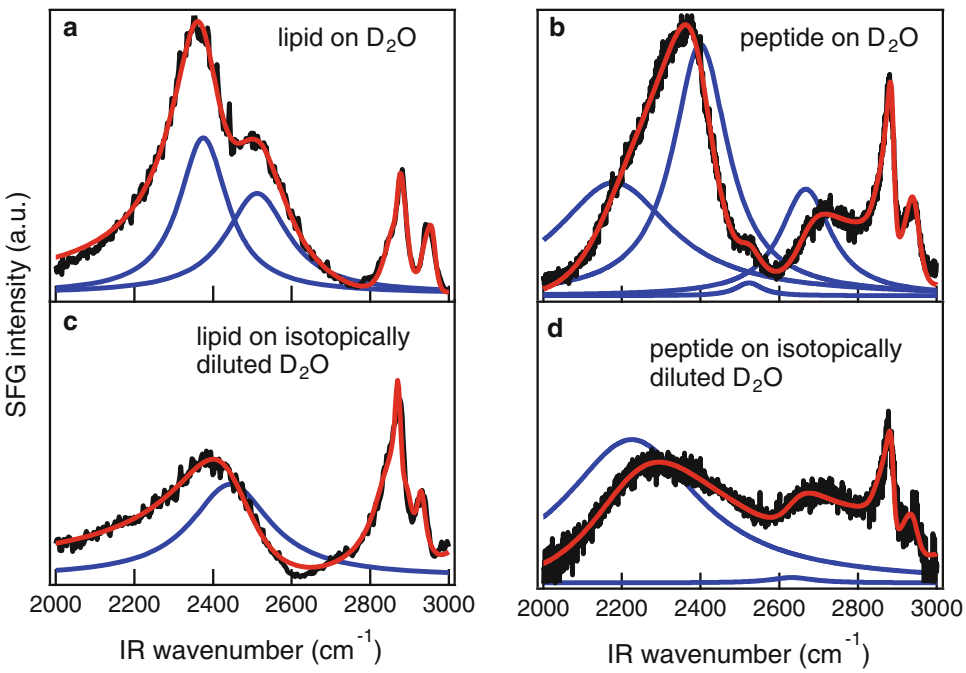

Fig. 1 SFG spectra in the O–D (2,100–2,750 cm^{-1}) and C–H (2,800–3,000 cm^{-1}) stretch region of heavy water D_2O (**a**, **b**; *top*) and isotopically diluted HDO (**c**, **d**; *bottom*) underneath a lipid (*left*) and protein (*right*) monolayer. In both cases several peaks appearing in the hydrogen-bonded region for the pure D_2O spectrum collapse onto one peak upon isotopic dilution. For pure D_2O the different peaks have traditionally been ascribed to different ('ice-like' or 'liquid-like') types of interfacial water. We show here that that interpretation is not correct. The *red lines* illustrate fits to the spectra, and the *thin blue lines* represent the different contributing fit components of the O–D stretch vibrations

water, as a result of the similarity between the frequencies observed and those observed in the infrared spectra of bulk ice and liquid water, respectively [6, 16]. We show here—for water interacting both with lipids and proteins—that this assignment is incorrect.

Figure 1c and d shows the effect of isotopic dilution on the SFG spectra of water at the water–lipid (DPTAP) and water–protein (lysozyme) interface, respectively. In both cases the rather complex, multi-peaked response in the O–D stretch region (2,100–2,800 cm^{-1}) shown in Fig. 1a and b greatly simplifies. The results of fits to the data using the well-known expression for the recorded SFG intensity [8] are shown as red solid lines in Fig. 1. The fit parameters reflecting the simplification of the SFG spectra upon isotopic dilution are shown in Tables 1 and 2 for the water–lipid and water–protein interfaces, respectively.

This observation cannot be reconciled with the presence of two distinct sub-ensembles of strongly ('ice-like') and weakly ('liquid-like') hydrogen-bonded interfacial water [17–21]. If the two peaks are due to two different types of OD groups, i.e. if for D_2O strongly and weakly hydrogen-bonded OD groups exist, these should also be present for HDO, meaning that for HDO the same spectrum should be observed (but with lower intensity owing to the lower density of OD groups). The dramatic change in spectral response, besides the expected decrease in intensity, going from D_2O to HDO directly shows that such distinct hydrogen-bonded sub-ensembles do not exist. A plausible and consistent explanation for the observations is that the two peaks originate from an intramolecular coupling between the overtone of the bending mode and the fundamental of the stretch mode, as will be explained in the following.

Table 1 Fit results for lipid (DPTAP) on D_2O

	Pure D_2O	Isotopically diluted D_2O
Amplitude	195	246
Frequency (cm^{-1})	2,375	2,445
Width (cm^{-1})	150	246
Amplitude	200	
Frequency (cm^{-1})	2,512	
Width (cm^{-1})	193	

Table 2 Fit results for peptide on D_2O

	Pure D_2O	Isotopically diluted D_2O
Amplitude (cm^{-1})	2,029	2,664
Frequency (cm^{-1})	2,181	2,225
Width	354	413
Amplitude (cm^{-1})	−1,446	1,667
Frequency (cm^{-1})	2,400	2,632
Width	169	135
Amplitude (cm^{-1})	−157	
Frequency (cm^{-1})	2,523	
Width	80	
Amplitude (cm^{-1})	870	
Frequency (cm^{-1})	2,667	
Width	156	

For D_2O and H_2O, the frequency of the bending mode amounts to 1,210 and 1,650 cm^{-1}, respectively. Hence the frequency of the overtone (0→2) transition of the bending mode closely resembles that of the stretch fundamental. This degeneracy gives rise to a strong anharmonic interaction between the two modes, as shown schematically in Fig. 2a. This interaction between the bend overtone and the stretch fundamental leads to new coupled vibrational resonances for which the absorption cross-section possesses a minimum at the frequency of the overtone of the bending mode, as illustrated in Fig. 2b: the resonance between the overtone of the bend mode (a dark state) and the stretch mode causes the appearance of a transmission window ('Evans window') at the overtone frequency in the spectrum of the coupled modes. This explains the double-peaked feature in the SFG spectra of aqueous interfaces in general, and those of biological interfaces in particular. It also explains why, as can be observed in Table 1, generally the HOD resonance is broader than that of the two individual resonances present for D_2O: the full density of

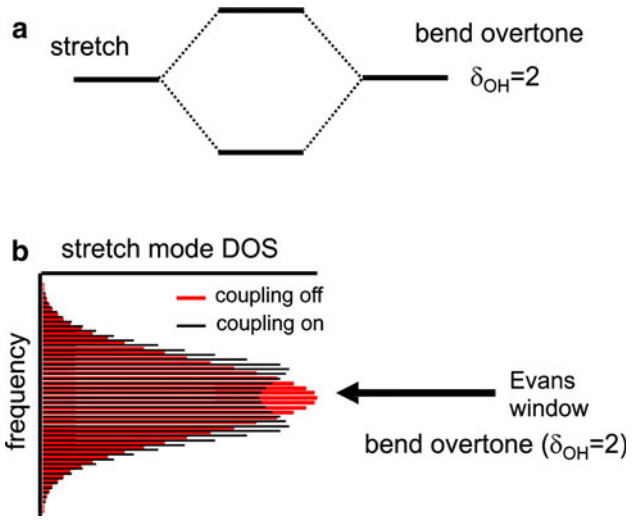

Fig. 2 **a** The anharmonic interaction between two degenerate vibrational levels, in this case the fundamental transition of the OH/OD stretching mode and the overtone of the OH/OD bending mode, causes a splitting of the energy levels into symmetric and asymmetric combination modes. **b** The broad, inhomogeneously broadened [22, 23] water O−D or O–H stretch mode density of vibrational states (DOS) couples strongly to the overtone of the bending mode. The bend overtone couples most strongly to those modes within the quasi-continuum of stretch modes that have the same frequency

vibrational states is now contained in the one resonance, rather than being split into two. We note that the simplification of the SFG spectra demonstrated here in the O−D stretch region upon the isotopic dilution of D_2O occurs in the equivalent way for the O–H stretch region upon the isotopic dilution of H_2O. Examples can be found in the Supplementary Information and Ref. [17].

For HDO, the bending mode is located at 1,450 cm^{-1}, whereas the O–D and O–H stretch vibration within the HDO molecule remain at $\sim$2,400 and $\sim$3,300 cm^{-1}. Since the mixing of the vibrational states is inversely proportional to the energy difference between the bend overtone and the stretching mode, for HDO the mixing is effectively 'switched off'. For an interface that is enriched in HDO, the two peaks therefore merge into one, as the mixing is switched off for HDO, at both the O−D and O–H stretch frequencies. Isotopic dilution experiments therefore provide a strict test of the 'ice-like'/'liquid-like' hypothesis vs. an hypothesis relying on vibrational mode coupling, and clearly show the 'ice-like'/'liquid-like' hypothesis to be false. We note that, while the intramolecular coupling between the bending overtone and the stretch mode provides a complete and sufficient description of the observed spectral changes upon isotopic dilution, effects resulting from intermolecular (dipole–dipole) coupling may also affect the spectra. The amount of intermolecular vibrational mixing (exciton formation) strongly depends on the vibrational frequencies of adjacent water molecules, and this mixing will also be reduced upon isotopic dilution.

In any case, it is evident that no distinct sub-ensembles of water at these biological interfaces exist. This does not mean, however, that all water molecules are experiencing the same type of environment. Rather, we expect there to be a continuous distribution of hydrogen bond strengths, both between water molecules themselves and between water molecules and lipids/proteins. Indeed, lifetime measurements of the O–H stretch vibration of interfacial water [24] and 2-dimensional surface sum-frequency generation spectroscopy [22, 23] have revealed a continuous heterogeneity of water molecules at the water–air interface and have evidenced the presence of an additional type of water at the water–lipid interface, presumably water that is strongly hydrogen-bonded to the lipid head group.

The simplified SFG spectra directly reflect the hydrogen bond strength of interfacial water at the interface and the possible distribution of hydrogen bond strengths of interfacial water. It is thus evident that isotopic dilution is an essential tool to identify different water species at biological aqueous interfaces [18]. In addition to isotope dilution, there are two additional SFG-based approaches that can shed additional light on the structure of water at biological interfaces. Firstly, there are phase-sensitive measurements, which are able to provide the real and imaginary parts of the interfacial vibrational response [25–29]. Such measurements provide information on the orientation of the water molecules and contain more details of the band shapes of the vibrational response. It has already been shown that phase-sensitive measurements in conjunction with isotopic dilution is particularly helpful in elucidating interfacial water structure [17]. In addition, the above-mentioned vibrational lifetime measurements (see e.g. Refs. [24, 30, 31]) and 2-dimensional surface sum-frequency generation spectroscopy [22, 23] hold great potential in elucidating the (in-)homogeneity of interfacial water hydrogen bonding strength.

In conclusion, we have shown that the two-peaked structure generally observed for SFG spectra recorded from aqueous biological interfaces is not the result of 'ice-like' and 'liquid-like' structures at these interfaces. Rather, the peak splitting can be explained from an intramolecular coupling between different vibrational modes. We show that isotopic dilution 'switches off' this coupling and results in a strong simplification of the SFG spectra.

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