Water Interacting with interfaces, ions and itself

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8 Distribution of water molecules in lipid membranes

We study the structure and dynamics of water molecules embedded in 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) model membranes using polarization resolved, ultrafast pump-probe spectroscopy. We investigate the effect of the local environment of the membrane on the vibrational relaxation properties of water at various hydration levels (∼2-12 water molecules per lipid). We find that the water molecules that are hydrogen-bonded to different sites of the membrane exhibit different vibrational lifetimes. With increasing hydration level we observe a relative increase of the longer-lived, bulk-like water with respect to the shorter-lived lipid-bonded water molecules. We investigate the anisotropy decays of water molecules for different isotopic composition (D$_2$O/H$_2$O) of the hydrating water. For D$_2$O/H$_2$O ratios ≥0.1 the anisotropy decay of the OD vibration is dominated by vibrational energy transfer (VRET). The rate of VRET gives information on the distribution of water molecules at the membrane surfaces. We find this distribution to be very inhomogeneous.

8.1 Introduction

The smallest building blocks of living organisms are called cells. These building blocks are defined by a membrane that separates their content from the “outside” world. Membranes, in spite of being composed of only two layers of molecules (what makes them very flexible), are very tough, capable of withstanding high tension and compression from internal (cytoskeleton) and external (extracellular matrix) forces acting on them. The rigidity of these bio-membranes helps to maintain the integrity of cells while their flexibility allows cells to change their shape. Membranes regulate transport in and out of the cell, cell adhesion and motion, and signalling to other cells. An important role in the structuring of the membrane and its (bio)activity is played by the adherent water layers [89]. It has been shown that the structure and dynamics of these water layers strongly differ from bulk liquid water [16, 17, 20, 52, 118, 148, 161, 163, 164, 174, 177]. It has also been suggested that these water layers play an important role in the transport properties of the membrane. Water confined by lipid membranes is
not only present in the intercellular space between two neighboring cells, but also in the endocellular space where tightly packed hydrated membranes form the thylakoid (involved in photosynthesis) and the Golgi apparatus (crucial in the generation and distribution of molecules within the cell). Tightly packed hydrated membranes can also be found in biological transient states during vesicle fusion, endo- and exocytosis [90].

Water-membrane interactions and the structure of hydrated membranes were studied extensively using NMR [16, 162, 168, 174], X-ray and neutron scattering, infrared spectroscopy [16, 17, 118, 161, 163, 164, 177], MD simulations [14, 15, 54, 99, 176], CARS [30], SFG [20, 52, 148, 174] and dielectric spectroscopy [77, 155]. Thanks to recent developments in nonlinear infrared spectroscopy the study of ultrafast water-membrane interactions has now become possible [161, 163, 164, 177].

A common conclusion from the past work is that water near a lipid membrane behaves differently from water in the bulk. Water molecules at membrane interfaces are much less mobile than in bulk, due to the strong hydrogen-bonds formed with the lipid headgroups. Very little is still known about the structure and dynamics of the water layers in close contact to biological membranes, mainly because of the large variation of membranes structure, composition and activity. Moreover, there are only very few experimental techniques that can specifically probe the water molecules near the membrane surface.

To study the interactions between biological water, the membrane and other biomolecules embedded in the membrane, researchers usually turn to membrane model systems like liposomes, micelles or lamellar membrane stacks. Liposomes allow the study of water-membrane interactions under water-rich conditions, whereas membrane stacks form a well defined model system for studying the properties of water confined in between two membranes at rather water-poor conditions (water-poor in biological sense).

We have studied the vibrational properties of biological water using polarization resolved ultrafast pump-probe spectroscopy. This technique allows us to probe water molecules directly via their vibrational resonances, and not via the response of a nearby chromophore. We investigated the vibrational dynamics and localization of water molecules embedded in a membrane model composed of a stack of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) bilayers. We have varied the hydration level and isotopic composition of the hydrating water. We probe the OD vibrations and their mutual coupling by measuring the rate of the vibrational resonant energy transfer (VRET). As a result, we obtain highly specific information on the structure, distribution and dynamics of water molecules at the membrane interface.

8.2 Experiment

The infrared laser pulses needed for the experiments are generated via optical frequency conversion processes that are pumped by the output of a Ti:sapphire regeneratively amplified laser system (Spectra-Physics Hurricane). This system generates pulses at a wavelength of 800 nm with a pulse energy of $\sim 900 \, \mu J$ at
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A repetition rate of 1 kHz. The 800 nm beam is split into two parts. The first part is used as a seed for a white-light based OPA (Spectra Physics). After the OPA the idler is frequency doubled in a second BBO crystal. The doubled idler (1000 nm) is used as a seed in a parametric amplification process in a potassium niobate crystal (KNB), pumped with the remaining part of the 800 nm pulses, analogously to the infrared light generation scheme described in section 3.2. In the experiment we used pulses centered at a frequency of $\sim 2500 \text{ cm}^{-1}$ ($\sim 4 \mu\text{m}$) and a pulse energy of $\sim 6 \mu\text{J}$. The IR pump-probe setup is described in detail in section 3.3.

8.2.1 Sample preparation

We obtained 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) from Avanti Polar Lipids and used it without further purification. DOPC is a zwitterionic lipid, with an overall electrically neutral headgroup, carrying positive and negative charges on different atoms. Heavy and normal water (D$_2$O (99.9% D) and H$_2$O) are obtained from Sigma Aldrich. The dry lipids were dissolved in a methanol/chloroform mixture (1:3 volumetric ratio) at a concentration of 50 g/L. By adding methanol we achieved a more homogenous spreading of the solution on the CaF$_2$ substrate (due to its hydrophilic character). To prepare solid supported oriented lipid multibilayers we used a technique adapted from previous reports [4, 59, 65, 159, 160]. In this procedure we deposit a small amount of the solution (5 - 15 $\mu$L) on the CaF$_2$ window and wait for a few minutes until the solvent evaporates and the sample becomes solid like. We repeat this process until the desired thickness of the sample is achieved. The sample is then placed in a home-built sample holder. The holder consists of two connected metal cylinders, each holding one CaF$_2$ window. The sample holder is sealed from the environment in order to keep the sample at the desired hydration level. One of the cylinders is equipped with two gas ports (inlet/outlet) that allow us to purge the sample with air of the desired humidity. First we dry the sample by purging nitrogen gas through the sample holder. The time needed to dry the stack of multibilayers depends on the sample thickness and varies from 1h - 5h. To ensure the removal of all the solvent traces and water we equilibrate the samples overnight in the nitrogen buffer gas.

The sample is subsequently hydrated with the use of a home-built hydration system. The hydration system consists of a reservoir with water (D$_2$O/H$_2$O mixture), two precise electronically controlled valves, a PID controller and an electronic hygrometer (range: 10-95% RH, precision: 3% RH). The input of the humidity controller is a constant flow of nitrogen gas. The flow is split into two branches and the flow in each branch is controlled with a valve. One of the two nitrogen parts flows through a water reservoir and creates air saturated with water vapor. The two flows are again united and a hygrometer measures the relative humidity of the outgoing mixed air. Based on the hygrometer readings the two valves are adjusted via an active feedback loop to obtain the desired relative humidity (RH). The hydration device allows for generating air with relative humidities in the range of 15-95% RH with a precision of 3% RH.

We recorded linear spectra to monitor the dehydration and hydration pro-
cesses. In figure 8.1 we show the absorption of the OD stretch vibration at different hydration times. In the bottom of figure 8.1 the integrated area of the OD stretch absorption band is shown as a function of the hydration time. The sample is fully equilibrated after about 60 min.

After the hydration process the sample is put in between two CaF$_2$ windows separated with a teflon spacer thereby sealing the sample from the environment. In order to obtain sufficient optical density of the OD stretch mode we varied the spacer thickness in a range from 10 to 150 $\mu$m, depending on the hydration level and the isotopic composition of the hydrating water.

A properly oriented sample consists of a stack of planar bilayers with their normal parallel to the normal of the CaF$_2$ substrate. A sample with such a geometry can be compared to an uniaxial crystal [130]. It was shown by X-ray diffraction experiments that such a sample consists of planar lipid bilayers separated by layers of water [65, 91, 92]. In order to perform polarization resolved experiments we have to ensure that the sample does not change the polarization of the transmitted light.

We produced aligned monodomain DOPC multibilayers by means of a thermal and mechanical sample treatment reported previously [4, 59, 130, 159, 160]. Once the sample is hydrated and sealed between the CaF$_2$, we move the two
windows with respect to each other while gently applying pressure on the sample. The flow of the material resulting from the shear enables the sample to adapt to the energetically favorable planar form. Samples thicker than 50 µm could not be aligned with the mechanical treatment only. These samples were heated up to 40 °C - 100 °C (depending on the thickness and the hydration level), and are thus brought to a more fluid state. This implies that the viscosity of the sample decreases and the diffusivity increases causing, together with the applied shear stress, the multi-domain character to anneal away.

Following the hydration and alignment procedure described above we have placed every sample in between two orthogonally oriented polarizers and checked its transmission under orthoscopic white light illumination with a Nikon Eclipse Ti inverted microscope connected to a Photometrics Coolsnap HQ2 digital CCD camera.

Well aligned, monodomain lipid multilayers appear black when viewed between two crossed polarizers under an orthoscopic white light. In figure 8.2 we show the lipid multibilayer sample before (left) and after (right) the mechanical/thermal treatment. After the pump-probe measurements we re-examined each sample. We did not observe any changes in the linear absorption spectrum or the alignment of the samples.

The final hydration of the samples was checked by comparing their linear absorption spectrum with the absorption spectrum of a reference sample. The reference sample was a diluted, homogenous solution of DOPC in chloroform. We prepared 6 reference samples, each with addition of a precise amount of D$_2$O, such that we obtained a specific water/lipid molecular ratios (1:1, 1:2 ,1:4, 1:6, 1:8 and 1:10). We then measured linear absorption spectra of the reference samples and compared the relative magnitudes (by integrating the absorption band) of the absorption of the OD stretch band of water and the CH$_2$ stretch band of the lipid residing at ~2857 cm$^{-1}$. Figure 8.3 shows that the calculated OD/CH$_2$ band ratio (blue circles) for the reference samples correlates very well with the linear increase of the amount of water molecules per lipid. For each sample used in the experiment we calculated the OD/CH$_2$ absorption band ratio and compared to the reference ‘ruler’ (red squares and blue circles in figure 8.3).
The hydration numbers we obtained are in excellent agreement with a previous report [65] (see table I).

In our experiments we have studied lipid multibilayers at different hydration levels (25%, 50%, 75% and 100% RH - which correspond to \(x = 2.3, 3.5, 6.4\) and 11.5 water molecules per lipid respectively). For each hydration level we varied the isotopic composition of water from 10%, through 25%, 50% to 100% D\(_2\)O in H\(_2\)O. We further refer to the isotopic composition of water as the fraction of the OD oscillators in the system \(f_D\) (\(f_D=0.1, 0.25, 0.5\) and 1, respectively). Table I summarizes the hydration level - \(x\) and the isotopic composition of water - \(f_D\) of all the samples under study.

<table>
<thead>
<tr>
<th>RH [%]</th>
<th>(x) (\frac{\text{nw}}{\text{L}})</th>
<th>(f_D=0.1)</th>
<th>(0.25)</th>
<th>(0.5)</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>2.3 (2.5)*</td>
<td>0.5</td>
<td>1.1</td>
<td>2.3</td>
<td>4.6</td>
</tr>
<tr>
<td>50</td>
<td>3.5 (3.7)*</td>
<td>0.7</td>
<td>1.7</td>
<td>3.5</td>
<td>7</td>
</tr>
<tr>
<td>75</td>
<td>6.4 (6.2)*</td>
<td>1.3</td>
<td>3.2</td>
<td>6.4</td>
<td>12.8</td>
</tr>
<tr>
<td>95</td>
<td>11.5 (10.2)*</td>
<td>2.3</td>
<td>4.6</td>
<td>11.5</td>
<td>23</td>
</tr>
</tbody>
</table>
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8.3 Results and discussion

8.3.1 Linear spectra

![Linear absorption spectra of the OD stretch vibration of water molecules embedded in the lipid multibilayers.](image)

Figure 8.4. Linear absorption spectra of the OD stretch vibration of water molecules embedded in the lipid multibilayers. The top panel shows the absorption spectra for the two extreme hydration cases of \( x = 2.3 \) and \( x = 11.5 \) hydrated with the lowest \( \text{D}_2\text{O}/\text{H}_2\text{O} \) isotope mixture \( (f_D = 0.1) \). The dashed line represents the bulk spectrum of 4% \( \text{D}_2\text{O} \) in \( \text{H}_2\text{O} \). The bottom panel shows the linear absorption spectra of the OD stretch mode of samples hydrated at \( x = 11.5 \) and different isotopic compositions of water. The dashed line represents a spectrum of pure \( \text{D}_2\text{O} \).

In figure 8.4 we show linear absorption spectra of the OD-stretch vibration of water molecules embedded in the lipid multibilayers. The top panel shows that with increasing hydration level the spectrum shifts towards higher frequency. No significant changes in the spectral shape are observed, except for the vanishing of a weak shoulder at the high frequency side \( (\sim 2650 \text{ cm}^{-1}) \) with increasing hydration level. The bottom panel shows that upon increasing the \( \text{D}_2\text{O} \) content the absorption spectrum broadens and becomes more asymmetric.

For all hydration levels and isotopic compositions, the OD stretch absorption spectrum is redshifted with respect to that of bulk water (indicated by the dashed line in figure 8.4). This observation agrees with previous experimental observations and indicates that at all hydration levels a significant part of the water molecules form stronger hydrogen bonds with the lipids than with other water molecules \([16, 155, 177]\). The blueshift of the OD stretch vibration with increasing hydration has been quantitatively reproduced by recent molecular dynamics simulations by Gruenbaum et al. \([58]\). These simulations also showed
that the hydrogen bond between a water molecule and a phosphate moiety is stronger than that between a water and a carbonyl group. The latter hydrogen bond may still be somewhat stronger than that between water molecules [14].

8.3.2 Vibrational energy relaxation

Figure 8.5 (left column) shows transient spectra (open symbols) at different delay times after the excitation of the OD stretch vibration for three different water isotope compositions. The most prominent changes are observed in the frequency range of the \( \nu = 0 \rightarrow 1 \) transition (\( \sim 2500 \text{ cm}^{-1} \)) representing the pump-induced depopulation of the OD stretch vibrational ground state. Below 2420 cm\(^{-1}\) the signal changes sign and we observe the high-frequency wing of the induced \( \nu = 1 \rightarrow 2 \) absorption. For the samples hydrated with pure D\(_2\)O the \( \nu = 1 \rightarrow 2 \) transition frequency is shifted towards lower frequency, and thus only the \( 0 \rightarrow 1 \) bleaching signal is observed within the experimental spectral window.

With increasing delay time the transient signals decay and the residual bleach slightly shifts towards lower frequencies. Simultaneously, at high frequencies (\( > 2570 \text{ cm}^{-1} \)) an induced absorption arises. This spectral shape is a signature of an increase in sample temperature. An increase in sample temperature leads to a blueshift of the fundamental absorption spectrum. As a result we observe a negative signal at the red side of the spectrum (\( < 2550 \text{ cm}^{-1} \)) and a positive signal at the blue side of the spectrum (\( > 2550 \text{ cm}^{-1} \)). The increase in temperature is the result of the vibrational energy relaxation and the subsequent excitation of low frequency librational and translational modes. For the samples at higher hydrations and with higher D\(_2\)O content, the thermal difference signal becomes stronger and comparable with the magnitude of the bleaching signal. After \( \sim 10 \text{ ps} \) the spectra do not change anymore.

Figure 8.6 shows the delay dependent isotropic signal recorded at a single probe frequency of 2500 cm\(^{-1}\). The top panel shows the delay traces for the two extreme hydration levels (\( x = 2.3 \) (squares) and \( x = 11.5 \) (circles)) and the lowest isotopic ratio of \( f_D = 0.1 \). At \( \sim 4 \text{ ps} \) we observe a clear deviation from mono-exponential behavior that is indicated with the dashed line. We also find that the relaxation rate is increasing significantly with increasing hydration level.

The data for \( f_D = 0.25 \) (not shown) resembles the data for \( f_D = 0.1 \). For \( f_D = 0.5 \) and \( f_D = 1 \) at \( x = 2.3 \) the transient signal decays in a similar non-exponential way (black squares), but the difference between the two rates is larger than for \( f_D = 0.1 \). At early delays (\( < 1 \text{ ps} \)), the signal decays much faster for \( f_D = 1 \) than for \( f_D = 0.1 \), but at later delays it seems to decay slower than for the \( f_D = 0.1 \) case. For higher hydrations (blue circles), due to very fast vibrational relaxation, the signal is, already at early delays, dominated by the thermal signal. Another interesting observation is that the heating signal for the samples hydrated with \( f_D = 1 \) decays at late delays (after \( \sim 10 \text{ ps} \)), suggesting a two step thermalization process.

The experimental observations for the samples with a low isotope content of \( f_D = 0.1 \) and 0.25, and all hydrations are in very good agreement with the results of recent work by Zhao et al. [177]. Based on our experimental observations and
Figure 8.5. Transient spectra of the OD stretch vibration as a function of delay time between excitation and probe pulses for the samples hydrated at $x = 6.4$ with $f_D = 0.1$ (top), $f_D = 0.5$ (middle) and $f_D = 1$ (bottom). The solid lines in the left column result from a fit to the kinetic model. The middle column shows the spectra that are extracted from the fit to the kinetic model. The right column presents a schematic representation of the kinetic models used to describe the data hydrated with water of different isotopic composition. The straight and wavy arrows indicate excitation and relaxation pathway of different water species, respectively.

This previous report, we describe our data for $f_D = 0.1$ and 0.25 with a model that involves two species of water molecules decaying independently with different rate constants (see the top schematic in figure 8.5).

The faster decaying component we attribute to water molecules being strongly hydrogen-bonded to the phosphate group of the lipids. The slower component we attribute to water molecules bonded to the carbonyl moieties and to other water molecules. The extracted relaxation rates from the fit are summarized in figure 8.7, panels A2 (open symbols). For both isotopic mixtures we find that the relaxation time constant $T_1$ decreases with increasing hydration. We find that the slower species (open circles) decays with $T_1 \approx 6$ ps at $x = 2.3$. Its lifetime decreases to $T_1 \approx 3$ ps at $x = 11.5$. The short lived species (open
Figure 8.6. Isotropic signals recorded at 2500 cm$^{-1}$ as a function of the delay between the pump and probe pulses. Each panel shows the delay traces for two extreme hydration levels ($x=2.3$ and $x=11.5$). The solid (red) lines result from a fit to the kinetic model described in the text. The dashed lines result from a mono-exponential function and act as guides to the eye.

squares) relaxes with a $T_1 = \sim 2.1$ ps at $x = 2.3$ and $T_1 = \sim 1$ ps at $x = 11.5$. Our findings are in excellent agreement with recent sum-frequency generation experiments on the vibrational dynamics of water molecules at water-lipid interfaces, which show that lipid-bound water molecules relax approximately two times faster than those bound to other water molecules [20]. In figure 8.5 (middle column) we show the extracted spectra corresponding to the two species. The water molecules exhibiting the shorter lifetime absorb at lower frequencies ($\sim 2470$ cm$^{-1}$), whereas the longer lived species absorb at higher frequencies ($\sim 2500$ cm$^{-1}$)

For $f_D = 0.5$ the isotropic signal decays faster than for the samples with lower $f_D$. Especially at short delay times after excitation we observe a very fast (sub-picosecond) decaying component. We find that the data can not be satisfactorily described with the model involving just two water species. The presence of an additional component in the delay curves most likely arises from a different population distribution of the different isotopes of water with respect to the samples with $f_D=0.1$ and 0.25. For this isotopic mixture statistically 50% of water molecules are HDO, 25% are D$_2$O and another 25% are H$_2$O. About 33% of the isotropic signal thus comes from the OD stretch vibration of D$_2$O molecules, for which the vibrational lifetime is expected to be short, due to efficient intramolecular coupling. For bulk D$_2$O we found a vibrational lifetime
T₁ of 400 fs (see chapter 4). We implement this contribution into the model as an additional third species decaying independently from the other two. We use this model to fit the data for \( f_D = 0.5 \) and the hydration levels of \( x = 2.3, 3.5 \) and 6.4.

For the fully hydrated sample (\( x=11.5 \)) we observe a change in the magnitude of the isotropic signal at late delays (>10 ps). The spectral shape of the thermal signal remains the same, only the amplitude decreases. This indicates a two-step thermalization process. In order to account for this late-delay dynamics, we have adjusted the kinetic model by adding an additional heat state. This additional heated state reflects the fact that upon vibrational relaxation water molecules do not reach the thermal equilibrium immediately but first reach a local hot state that is followed by cooling and full thermalization of the sample.

The relaxation rates and populations of each of the three water species are shown in figure 8.7, panels B1 and B2. The band with an intermediate lifetime (open squares) shows a decreasing \( T_1 \approx 2 \) ps for \( x = 2.3 \) to \( T_1 \approx 0.8 \) ps for \( x = 11.5 \). Similarly the long-lived species (open circles) lifetimes decreases from \( T_1 \approx 4 \) ps for \( x = 2.3 \) to \( T_1 \approx 2 \) ps for \( x = 11.5 \). The lifetime of the most short lived species (open triangles) decreases from \( T_1 \approx 3 \) ps for \( x = 2.3 \) to \( T_1 \approx 0.8 \) ps for \( x = 11.5 \). Hence, at the highest hydration this lifetime is similar to the vibrational lifetime of the OD stretch vibration of D₂O water molecules in the bulk. The cooling time for the sample with the highest hydration \( x=11.5 \) was found to be \( \approx 8 \) ps (not shown). The extracted spectra are shown in figure 8.5, panel B.

For samples hydrated with pure D₂O (\( f_D = 1 \)) the water molecules binding to different sites at the bilayer interface show the same relaxation rate. We did not find any spectral signatures or variation in the vibrational lifetime that could help in distinguishing D₂O molecules forming hydrogen-bonds of different strengths (with different lipid moieties). We thus model the data with a consecutive model (a variation of that used previously for bulk water [128, 134, 135]). The excited state relaxes to an intermediate state. This step reflects the adaptation of the hydrogen bonds due to the sudden release of the vibrational energy and the excitation of lower frequency modes. This process is followed by a thermalization of the system. Similarly to the samples hydrated with \( f_D=0.5 \) we observe a further change of the thermal signal at later delays, after the vibrational relaxation from the OD stretch mode is complete. In this process, the energy is further redistributed out of the local, heated water cluster and the system cools down and equilibrates. We thus model our data with a 4 step consecutive relaxation model. This model describes the experimental data very well at all hydration levels. Panels A1 and A2 in figure 8.8 show the extracted populations and lifetimes of all the consecutive states.

**Discussion I**

The largest variation in the vibrational lifetime with hydration (\( x \)) is observed for membranes hydrated with a low isotopic mixture of D₂O in H₂O (\( f_D=0.1 \)). In such a dilute case the OD vibration of the HOD molecule is decoupled from other high frequency modes thus allowing the direct probing of the effect of the local binding site on its vibrational lifetime. We clearly observe two distinct
relaxation rates at all hydration levels. Figure 8.7, panel A2, shows the change of the two relaxation rates with hydration (open symbols). At the lowest hydration ($x=2.3$) the longer of the two lifetimes is more than 3 times longer, whereas the shorter one is about 2 times longer than the lifetime observed in bulk HDO:H$_2$O. Both lifetimes decrease when increasing hydration and reach a final value of $\sim$3 ps and $\sim$1.7 ps for hydrations $x > 6.4$. The extracted spectra are shown in figure 8.5, panel A. The blueshifted spectrum, absorbing at $\sim$2500 cm$^{-1}$ is associated with the longer vibrational lifetime, and the redshifted one with the shorter lifetime. We find that the positions of the two bands do not change with hydration. Only their relative amplitudes vary. The spectral position of the two bands confirms the notion that the strongly hydrogen bonded water molecules (presumably bonded to the phosphate group) have a shorter lifetime, most likely due to efficient coupling of the OD stretch vibration ($\sim$2500 cm$^{-1}$) with the stronger hydrogen bond and the vibrational modes of the phosphate group (1200 - 1300 cm$^{-1}$). The spectrally blueshifted spectrum reflects water molecules hydrogen bonded to carbonyl groups and/or other water molecules.

Assuming that the different water species have similar absorption cross sections, we directly obtain information about the dependence of the population of the two species on hydration level from the fit to the kinetic model. We find that with increasing hydration level the relative amount of redshifted, short-lived species decreases with respect to the long-lived species (see figure 8.7, panel A1). The phosphate group can be hydrated by at most 4 water molecules [55]. It is thus understandable that once the phosphate groups are saturated with water molecules, a further increase of the hydration will only add water molecules hydrogen-bonded to carbonyl groups or to other water molecules.

The hydration dependent population dynamics of the two species are in excellent, quantitative agreement with the recent molecular dynamics simulations by Gruenbaum et al. [58]. The population dynamics extracted from their simulations are marked with asterisks in figure 8.7 (A1). Gruenbaum et al. also calculated the isotropic signal decay using vibrational lifetimes of each of the water species as fit parameters. Their best-fit vibrational lifetimes are in very good agreement with those observed in our experiments (see figure 8.7 A2, marked with asterisks). Qualitatively our findings agree well with those reported for hydrated DLPC multilayers by Zhao et al. [177].

The population dynamics of the three species present in the samples hydrated with $f_D=0.5$ are shown in figure 8.7, panel B1. As for the lower isotope ratios, the amount of strongly bonded water molecules (solid squares) decreases relatively to the whole excited population with increasing hydration. The population of the additional, very short lived species increases with hydration. This may arise due to two effects. First, at higher hydrations more HDO molecules will be hydrogen-bonded to D$_2$O molecules. These HDO molecules may couple well to modes of the D$_2$O molecule and thus show a very fast relaxation. Another reason may be that at low hydration the D$_2$O molecules that are hydrogen-bonded to the lipids have a longer $T_1$ lifetime. With increasing hydration the relative amount of the lipid-bonded D$_2$O molecules will decrease, thus increasing the population of D$_2$O molecules that are hydrogen-bonded to water (HDO/D$_2$O/H$_2$O).
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Figure 8.7. Summary of the extracted lifetimes and populations of different water species embedded in the DOPC multibilayers for different isotopic content of hydrating water (A-B). Left column (A1-B1) shows the extracted populations as a function of the hydration level. The populations of each species were normalized to the sum of all the populations at that specific hydration. Right column (A2-B2) shows the dependence of the vibrational lifetime on the hydration level. The dashed lines are guides to the eyes.

For the highest hydration level we have introduced an additional thermal equilibration state in the relaxation process to account for the spectral changes at late delays. From the fit we find that the thermal equilibration time amounts to $\sim 8$ ps (not shown) and involves an amplitude decrease of the thermal spectrum (red to black spectrum in figure 8.5).

The isotropic data for the samples hydrated with pure D$_2$O ($f_D=1$) is fitted with a four state consecutive relaxation model. We do not find any spectral or dynamical features that would allow us to disentangle water molecules forming hydrogen bonds with the phosphate groups, carbonyl groups or with other water molecules. In figure 8.8 we show the extracted lifetimes. The excited state lifetime amounts to $T_1 = \sim 0.4$ p and does not change with hydration. No variation is also observed for the second state lifetime that represents the adaptation of the hydrogen bonds to the fast relaxation of the excited state. Interestingly, the cooling rate, decreases strongly with increasing hydration, from $T_\star = \sim 2.3$ ps for $x=2.3$ to $T_\star = \sim 6.3$ ps for $x=11.5$. The thermal equilibration between water clusters and lipids takes place via lipid-bonded water molecules. At low hydra-
Figure 8.8. Summary of the extracted lifetimes and populations of the water species embedded in between the DOPC multibilayers for samples hydrated with pure D$_2$O ($f_D=1$). The left panel shows the extracted populations as a function of the hydration. The populations of each state were normalized to the sum of all populations. The right panel shows the dependence of the vibrational lifetime on the hydration level. The dashed lines are guides to the eyes.

All the water molecules are bonded to the lipids, thus the (equilibration) redistribution of the vibrational energy between the low frequency modes of water and lipid molecules occurs relatively fast. At the highest hydration, however, the water forms nanopools, and these pools cool slower with increasing volume to surface ratio. The final, thermally equilibrated state is thus reached on a slower time scale than in the case of low hydration. The excited state spectral line shape (see figure 8.5) follows the strongly asymmetric shape of the linear absorption spectrum of D$_2$O shown in figure 8.4 (bottom panel). In addition the excited state spectrum is somewhat deformed which results most likely from a partial excitation of the inhomogeneous absorption band. In the experiment we used infrared pulses with a bandwidth of about $\sim$150 cm$^{-1}$ (FWHM) which is not sufficient to cover the whole OD stretch absorption spectrum of pure D$_2$O.

The two thermal states - local hot state and final thermally equilibrated state have similar spectral shapes since both result from a blueshift of the fundamental absorption spectrum. The final equilibrated state however has a smaller amplitude since the vibrational energy is redistributed over the whole system thus the blueshift of the absorption spectrum is smaller. A similar cooling process following vibrational relaxation has been observed for water confined in ionic micelles [37], for which the local thermalization among the water molecules is followed by the thermal equilibration between the water and the apolar solvent outside the micelle.

8.3.3 Anisotropic data

In figure 8.9, we present the time dependent anisotropy parameter $R(t)$ for
8.3 Distribution of water molecules in lipid membranes

Figure 8.9. Anisotropy parameter $R(t)$ as a function of the delay time between the pump and probe pulses, for all the studied samples. Each panel shows anisotropy decays for different isotopic compositions of hydrating water ($f_D$) at one particular hydration level ($x$). For comparison the dashed and dash-dotted lines represent the anisotropy decays for diluted mixture of HDO in H$_2$O ($\tau_r \sim 2.5$ ps) and for pure D$_2$O ($\tau_r \sim 400$ fs) [128]. The solid black lines result from the fit to the model described in the text.

all studied samples. Each panel shows the anisotropy decay for samples at a specific hydration ($x$) and the four studied isotopic mixtures ($f_D$) of H$_2$O and D$_2$O. The anisotropy time traces were constructed according to equation 2.33 with parallel ($\Delta \alpha_{\parallel}(t, \nu)$) and perpendicular ($\Delta \alpha_{\perp}(t, \nu)$) signals averaged over $\sim 30$ cm$^{-1}$ around the central frequency of 2500 cm$^{-1}$. Before constructing the anisotropy, the isotropic data were corrected for the heating effect, resulting from the dissipation of the vibrational energy. The correction involves subtraction of the heat signal from the isotropic data at all delay times. The time dynamics of the heat signal are assumed to follow the vibrational relaxation dynamics [134]. For the samples hydrated with $f_D = 0.5$ (highest hydration of $x = 11.5$) and pure D$_2$O (all hydrations) we find that the intermediate local hot state is anisotropic. The presence of such an anisotropic heat state can be explained as follows. After the fast relaxation of the excited state of the OD stretch mode of D$_2$O molecules (which is much faster than the relaxation of the OD stretch vibration of the HDO molecules, see figure 8.7 B2 and figure figure 8.8), the vibrational energy is redistributed over the lower frequency modes.
among water molecules in the direct surroundings of the excited molecule. Due to the limited mount of water, this energy remains localized within the cluster for a longer time, forming a local, transient hot spot. Hence, the heating effect primarily influences the response of the originally excited OD vibrators, thus retaining the anisotropy of the transient spectrum. We correct for this effect by subtracting the anisotropy value taken at a delay where the vibrational relaxation of the OD stretch excited state is completed and all the population is in the intermediate hot state (∼6 ps for \( f_D = 0.5 \) and \( x = 11.5 \); ∼4→2.5 ps for \( f_D = 1 \) and \( x = 2.3 \rightarrow 11.5 \)). We subtract this anisotropy value at all delays with a time evolution defined by the vibrational relaxation.

The anisotropy traces at all hydration levels are strongly non-exponential and are characterized by a fast initial decay (within the first ∼500 fs) followed by a much slower decay (>10 ps). After ∼5 ps, the anisotropy traces decay on such a slow timescale that in our experimental window we consider them reaching a constant end level. With increasing hydration we observe a moderate acceleration of the anisotropy decay for data hydrated with \( f_D = 0.1 \) (open red circles in each panel). The anisotropy decay changes from essentially non-decaying for \( x = 2.3 \) to slowly decaying on a ∼20 ps timescale for \( x = 11.5 \). We observe a similar trend for the anisotropy decays for the samples hydrated with \( f_D = 0.25 \) and 0.5. The anisotropy decays for the samples hydrated with pure D\(_2\)O \((f_D=1)\) show a very fast initial decay (within ∼1 ps) to an end level for all hydration levels.

The most significant changes in the anisotropy decay profile are observed when varying the isotopic composition of the hydrating water. Increasing the D\(_2\)O content leads to a strong speed up of the decay within the first ∼2 ps. At later delays the decay rate is comparable for different \( f_D \), except that the end level has decreased significantly with increasing D\(_2\)O content.

The dashed lines in figure 8.9 represents the anisotropy for a dilute, bulk D\(_2\)O/H\(_2\)O mixture [128]. It is clear that for all measured samples the anisotropy decays faster at early delays and slower at later delays than in the case of bulk water. The dash-dotted lines indicate the anisotropy decay for pure bulk D\(_2\)O [128]. With increasing D\(_2\)O content the anisotropy decays resemble more and more that of pure D\(_2\)O. Especially for \( x = 11.5 \) and \( f_D = 1 \) (black open triangles) the decay up to 500 fs resembles very much that of bulk D\(_2\)O. The discrepancy however occurs at later times at which the measured anisotropy does not decay to zero but reaches a non-zero end level.

The main part of the anisotropy decay may be caused by two effects (as discussed in sections 2.5.1 and 2.5.1): reorientation of the excited water molecules or vibrational resonant intermolecular energy transfer (VRET). For the samples hydrated at \( x = 2.3 \) with a dilute mixture of D\(_2\)O in H\(_2\)O \((f_D = 0.1)\), one can assume that the intermolecular dipole-dipole coupling, due to its \( 1/r^6 \) dependence, is very weak. Moreover, the lipids also act as a diluting medium, thus further increasing the distances between the OD oscillators. Thus the observed moderate acceleration of the anisotropy decay when increasing the hydration (∼150 ps → ∼25 ps), most likely results from an increase in the (partial) mobility of the water molecules. We have tested this hypothesis by measuring the anisotropy decay for a sample hydrated at \( x = 11.5 \) and a very dilute
mixture of 2% D$_2$O in H$_2$O ($f_D = 0.02$). It is clear that at these low isotope ratios the anisotropy decay is driven by the (limited) reorientation of water molecules rather than the vibrational resonant energy transfer. We observed that the anisotropy decay is identical to that of the sample hydrated at $x=11.5$ and $f_D=0.1$.

The fast component in the anisotropy decays observed at early delay times results most likely from a wobbling in a cone motion (hinging rotation) of the OD oscillators. Even strongly bonded and confined water molecules will maintain some freedom and fluctuate thermally giving rise to a spread of the direction of the OD oscillator within a cone. Such molecular motions has been observed for water molecules hinging between DMA molecules [156], water in reverse micelles [38, 109], and more recently for water embedded in DLPC stacked bilayers [177]. The amplitude of the fast component is approximately two times bigger (anisotropy amplitude drop of $\sim 20\%$) than for bulk water ($\sim 10\%$), suggesting a larger angular cone spread. This may occur if the measured OD group of the water molecule is hydrogen-bonded to the lipid and the other OD group is free. The free OD may then rotate in propeller-like motion, “wobbling” the whole molecule around, thus increasing the angular spread of the hydrogen-bonded OD group. This picture is consistent with previous dielectric relaxation measurements on lipid multibilayers [155]. We observed, even at low hydration levels ($x<4$), a presence of a fast (nearly bulk-like) rotational component in the THz spectra.

With increasing concentration of OD oscillators the mutual distances get smaller and the resonant dipole-dipole interactions become important. For higher D$_2$O/H$_2$O ratios the decay of the anisotropy is mainly dictated by VRET, as observed for bulk water [128, 171]. VRET leads to a fast delocalization of the vibrational energy among randomly oriented water molecules. Thus VRET leads to a complete decay of the anisotropy to zero. The experimental data, however show that the anisotropy traces decay to a non-zero end level. This observation indicates that not all the water molecules participate in VRET which suggests that a fraction of the water molecules is located far enough from the other water molecules not to take part in the energy transfer. These isolated water molecules are likely hydrogen-bonded to the lipid and show very little or no reorientation just as the water molecules at $x=2.3$ and $f_D=0.1$.

In the case of pure D$_2$O very fast intramolecular energy transfer will occur. Such intramolecular energy transfer leads (quasi-instantaneously) to a decay of the anisotropy to a value of $\sim 0.12$ (energy transfer over an angle of $104^\circ$). This effect will contribute to the anisotropy decay for the samples hydrated with $f_D = 0.5$ and $f_D = 1$. Part of the initial decay observed for $f_D = 0.5$ (diamonds) and $f_D = 1$ (triangles) samples will thus result from this intramolecular energy transfer.

Based on the above observations we can construct a model to describe the experimental data. We consider all three contributions that may lead to the anisotropy decay:
- reorientation of the water molecules - $R_{\text{reor.}}(x; t)$,
- vibrational resonant energy transfer between the water molecules - $R_{\text{VRET}}(x, f_D; t)$,
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- intramolecular energy transfer for D$_2$O molecules - $R_{\text{IET}}(f_{D};t)$.

The time dependent anisotropy can be thus described with the following formula:

$$ R(x, f_{D}; t) = \left[ R_{\text{reor.}}(x; t) \cdot R_{\text{VRET}}(x, f_{D}; t) \cdot A(x) + B(x) \right] \cdot R_{\text{IET}}(f_{D}; t), \quad (8.1) $$

where, $A(x)$ is the fraction of the water molecules participating in VRET and $B(x)$ is the fraction of the isolated water molecules that do not show VRET or (fast) reorientation. These molecules can only show intramolecular energy transfer if they are D$_2$O molecules.

The intramolecular energy transfer term is given by:

$$ R_{\text{IET}}(f_{D}; t) = \left( \frac{0.3f_{D2O}(f_{D})e^{-k_{D2O}t} + (1 - f_{D2O}(f_{D}))e^{-k_{HDO}t}}{f_{D2O}(f_{D})e^{-k_{D2O}t} + (1 - f_{D2O}(f_{D}))e^{-k_{HDO}t}} \right), \quad (8.2) $$

where, $f_{D2O}$ is the fraction of the D$_2$O water molecules showing intramolecular energy transfer. The factor of 0.3 in the formula indicates a decay of the anisotropy from the initial value of $R(t) = 0.4$ to $R(t) = 0.12$ (30% of the initial value). The D$_2$O and HDO water molecules have different vibrational lifetimes which determine their “visibility” in the anisotropy. It is therefore necessary to normalize each of these contributions to their lifetimes, thus the exponential terms characterized by $k_{D2O}$ and $k_{HDO}$. In the global fit we use the vibrational lifetimes extracted from the fits to the isotropic data: the D$_2$O lifetime $1/k_{D2O}$=0.4 ps, and the hydration dependent HDO lifetime, as depicted in figure 8.7.

Reorientation of the water molecules is described with a biexponential decay (as proposed in previous reports [14, 58, 176, 177]):

$$ R_{\text{reor.}}(x; t) = c(x) e^{-k_{r1}t} + (1 - c(x)) e^{-k_{r2}t}, \quad (8.3) $$

where $c(x)$ and $(1-c(x))$ are the two fractions reorienting with $k_1$ and $k_2$ respectively.

Finally, the term describing the intermolecular energy transfer is given by:

$$ R_{\text{VRET}}(x, f_{D}; t) = \exp \left( -F(x, f_{D}; t) \right), \quad (8.4) $$

where

$$ F(x, f_{D}; t) = \frac{4}{3} \pi [2f_{D}C_{W}(x)]a^3 \exp \left( -\frac{k_{1}r_{0}^{6}t}{a^6} \right) - \frac{4}{3} \pi^{3/2} [2f_{D}C_{W}(x)] \sqrt{k_{1}r_{0}^{6}t} \ \text{erf} \left( \sqrt{\frac{k_{1}r_{0}^{6}t}{a^6}} \right), \quad (8.5) $$

where $[C_{W}(x)]$ is the concentration of water molecules involved in the VRET (expressed in $1/\text{Å}^3$), $k_1$ is the vibrational relaxation rate of the OD oscillator ($k_1 = 0.6$) and $r_0$ is the so-called Förster radius. Parameter $a$ is the minimal distance over which VRET can take place.

Here we modified the well known formula describing the resonant energy transfer in order to explicitly separate the two contributions: VRET and IET.
The derivation details can be found in appendix 8.5. In order to exclude the possibility for IET to contribute to the decay described by VRET term, we allow VRET to occur only between neighboring molecules and not between the two hydroxyl groups within the same molecule (see appendix 8.5). This is achieved by choosing the minimum value $a$ (over which energy transfer can take place) larger than the distance between the OD transition dipole moments within D$_2$O molecule. This way we account for the two energy transfer contributions to the anisotropy decay separately.

Equation 8.5 differs from the well known, so-called Förster formula used previously to describe VRET in bulk water [128, 171].

$$R_{\text{VRET}}(x; t) = \exp \left( \frac{-4\pi^{3/2}}{3} [C_W(x)] \sqrt{k_{r1} r_0^6 t} \right), \quad (8.6)$$

This equation describes the anisotropy decay in bulk water very well, but it does not distinguish the contributions from VRET and IET.

In the fitting procedure we first separately fit the reorientation term $R_{\text{reor}}$ to the data hydrated with $f_{D} = 0.1$ at all hydrations. We use the fitted values as constants in the global fit for all the data with increasing $f_{D}$. We find that our model is in excellent agreement with the experimental data. The result of the global fit is shown with solid, black lines in figure 8.9. The fit parameters are summarized in table II.

Table II. Parameters extracted from the global fit.

<table>
<thead>
<tr>
<th>$x$</th>
<th>2.3</th>
<th>3.5</th>
<th>6.4</th>
<th>11.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A$</td>
<td>0.21</td>
<td>0.24</td>
<td>0.25</td>
<td>0.3</td>
</tr>
<tr>
<td>$B$</td>
<td>0.11</td>
<td>0.07</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>$c$</td>
<td>0.04</td>
<td>0.05</td>
<td>0.065</td>
<td>0.045</td>
</tr>
<tr>
<td>$1/k_{r1}$ [ps]</td>
<td>1</td>
<td>0.8</td>
<td>0.65</td>
<td>0.5</td>
</tr>
<tr>
<td>$1/k_{r2}$ [ps]</td>
<td>150</td>
<td>80</td>
<td>50</td>
<td>25</td>
</tr>
<tr>
<td>$C_W \left[ \frac{n}{\AA^3} \right]$</td>
<td>0.018</td>
<td>0.0225</td>
<td>0.028</td>
<td>0.027</td>
</tr>
<tr>
<td>$a$ [Å]</td>
<td></td>
<td>1.7±0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$r_0$ [Å]</td>
<td></td>
<td>2.4±0.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For each specific hydration level there will be a corresponding distribution profile of water molecules within the bilayer. Since the distribution does not change with the isotopic composition of the hydrating water, the distribution profile for each hydration level is determined by a single set of $A$, $B$ and $c$ fractions (see table II). To compensate for the uncertainty in the exact hydration level between the data sets hydrated with different $f_{D}$ we allowed the $A$, $B$ and $c$ fractions to vary within 10%. Thus the fractions are denoted as average values in table II. We have fitted the concentration of water molecules $C_W(x)$ that
participate in VRET provided that these molecules are all D$_2$O ($f_D$=1). For samples hydrated with isotopic mixtures the amount of water molecules showing VRET is given by: $C_{OD} = 2f_DC_W$ ($f_D$=0.1, 0.25 or 0.5). The value of $C_W$ was allowed to differ by $\sim 15\%$ from this value to account for the uncertainty in the exact hydration level.

**Discussion II**

In figure 8.10 (top panel) we show the fractions A (water molecules involved in VRET) and B (isolated water molecules). We find that at very low hydrations, approximately 40% of the water molecules are involved in VRET. This is surprising taking into account the hydration level of only $\sim 2$ water molecules per lipid. Due to the $r^{-6}$ dependence of the transfer rate these water molecules need to reside rather close to each other to exhibit the experimentally observed VRET. Our findings thus suggest that at low hydrations water molecules form clusters. With increasing hydration level the distribution of water molecules becomes more and more homogenous and at the hydration level of $\sim 12$ water molecules per lipid (which is the maximal amount of water molecules a DOPC molecule can coordinate [72]) nearly all water molecules are involved in VRET. One can imagine that at this hydration level water forms a 2-dimensional layer separating the two bilayers, whereas at lower hydrations this 2D water sheet is torn at places. “Touching” bilayers will expel water molecules at some places, “forcing” them to aggregate elsewhere. At the hydration level of $x = 12$ DOPC bilayers start to swell, the interbilayer distance increases and water is comfortably accommodated between the bilayers forming a continuous layer [65].

The bottom panel in figure 8.10 shows the extracted fractions of D$_2$O ($f_{D_2O}$) molecules exhibiting intramolecular energy transfer. The dashed line indicates the theoretical amount of D$_2$O water molecules in the sample. As expected the fraction of intramolecular energy transfer increases with increasing D$_2$O/H$_2$O isotope ratio. Interestingly we also observe that $f_D$=1 the amount of D$_2$O molecules showing intramolecular energy transfer increases with the hydration level. The intramolecular energy transfer in bulk water is extremely efficient because of the strong spectral overlap of the OD stretch vibrations. At low hydration levels, all water molecules are hydrogen bonded to the lipids, with which they from stronger bonds than among each other ($\text{HB}_{phosphate} > \text{HB}_{carbonyl} > \text{HB}_{water}$). For many of these D$_2$O molecules one of the hydroxyl groups will be hydrogen bonded to the lipid (phosphate or carbonyl group) and the other one will be bonded to other water molecules or will remain free. Due to this asymmetry, the two vibrations of the two hydroxyl groups will not be as strongly mixed as in bulk (red circles in figure 8.10, lower panel). For samples with higher hydration levels there are more and more molecules forming hydrogen bonds to other water molecules, thereby increasing the fraction of D$_2$O molecules for which the OD vibrations are in resonance, and thus show rapid IET.

From the fit of the anisotropy decays to the model we can determine the hydration level dependent specific concentration of OD oscillators $[C_{OD}(x) = 2f_DC_W(x)]$ involved in the resonant energy transfer. The extracted values are indicated in figure 8.11 with open circles. It is interesting to compare this con-
8.3 Distribution of water molecules in lipid membranes

Figure 8.10. Top panel: Fractions A and B as a function of the hydration level. Bottom panel: The fraction of D$_2$O molecules showing intramolecular energy transfer as a function of the isotopic composition at studied hydration levels. The dashed line indicates the fraction of D$_2$O molecules present in the system and overlaps well with the extracted D$_2$O fractions for $x=11.5$. The dotted lines represent quadratic fit at each hydration level (D$_2$O fraction $= f_D^x$). For lower hydration levels the extracted D$_2$O fractions for $f_D=1$ are smaller than the amount of D$_2$O molecules in the system indicating that not all the D$_2$O exhibit intramolecular energy transfer.

centration with the average concentration of OD oscillators, assuming a homogeneous distribution over the lipid layers. This concentration is given by:

$$N_{OD} = \frac{2f_D x}{V_L + V_W x},$$  \hspace{1cm} (8.7)

where $x$ is the hydration number, $V_L$ is the volume of a DOPC molecule and $V_W$ is a volume of water molecule. The volume of the DOPC molecule in the lamellar liquid crystalline phase amounts to $\sim$1300 Å$^3$ \cite{56, 169}. The volume of a water molecule is $\sim$30 Å$^3$. The concentration of OD oscillators based on this estimation is indicated with the dashed line in figure 8.11. Clearly at all hydrations, the concentration of the OD oscillators extracted from the fit to the observed VRET, is much higher than the concentration of water homogenously distributed within the bilayer. It is reasonable to assume that nearly all water molecules are located, as shown by numerous x-ray and neutron scattering experiments, in between the lipid bilayers and protrude the bilayers only up to the carbonyl moieties \cite{65, 159}. By using the volume of a lipid headgroup (estimated to be $\sim$1/3 of a volume of the whole lipid), instead of the
Figure 8.11. Concentration of OD groups $C_{OD}$ showing VRET as a function of the hydration level. The dashed line indicates the average OD concentration when the water molecules are homogeneously distributed over the complete volume of the lipid. The solid line indicates the average OD concentration when the water molecules are homogeneously distributed over the volume of the lipid headgroup. The dash-dotted lines indicate the OD concentration of bulk water for each isotope mixture.

volume of the whole lipid, we can thus calculate the average concentration of OD oscillators assuming the water molecules to be located only near the headgroup volume of the membrane. We indicate this concentration as a function of the hydration level with the solid lines in figure 8.11. We find that at low hydration levels this concentration is still lower than the concentration of OD oscillators extracted from the fit to the observed VRET. The two values become similar for maximum hydration of the membranes.

The concentration of OD oscillators showing VRET is quite independent of the hydration level. This means that the average distance between the OD groups does not decrease with increasing hydration level. The OD concentration only depends on the isotope composition. This suggests that the water molecules form small clusters. With increasing hydration level the number density of these clusters increases as expressed in the increase of the fraction $A$. The independence of $C_{OD}$ on the hydration level indicates that the composition and size of the clusters is very similar at all hydration levels.

The non-homogenous character of water distribution at the interface of lipid membranes is consistent with Monte Carlo simulations of the interactions between stacked lipid bilayers [54]. Gouliaev et al. demonstrated the presence of
strong local perturbations (ripples) in the membrane ordering, often leading to soft collisions between the bilayers. Especially at low hydrations these collisions will assist water molecules to cluster at places where there is space available between the bilayers and at the same time water will be “expelled” from places where the two bilayers touch.

The very slow reorientation time of water molecules at the membrane interface at low hydration and \( f_D = 0.1 \) is most likely dictated by the residence time of water molecules at the binding site, which then essentially reflects the hydrogen bond lifetime. Using molecular dynamics simulations, Bhide et al. determined the residence time of water molecules at different regions in the membrane: Region I (phosphate and carbonyl associated water molecules) - \( \sim 600 \) ps, region II (lipid headgroup) - \( \sim 40 \) ps [14]. We can thus assume that within the time we determine the anisotropy parameter the measured water molecules remain hydrogen-bonded. In region III (second hydration shell around lipid headgroup) the reorientation time is \( \sim 3 \) ps, however we do not reach hydration levels high enough for these water molecules to contribute significantly [14].

### 8.4 Conclusions

We used polarization-resolved, ultrafast pump-probe spectroscopy to study the vibrational energy relaxation and anisotropy decay of water molecules at a cell membrane model interface. The studied samples consisted of a stack of monodomain DOPC multibilayers. In the experiment we vary both the hydration level of the membrane and the isotopic composition of the hydrating water (\( \text{D}_2\text{O}/\text{H}_2\text{O} \) ratio).

We find that water molecules at the membrane’s interface experience very different local environments depending on which site of the lipid they are hydrogen-bonded to. Water molecules forming strong hydrogen-bonds with phosphate relax faster than water molecules that are hydrogen-bonded to carbonyl groups and other water molecules. For samples that are highly hydrated and/or contain a high fraction of \( \text{D}_2\text{O} \) (\( \geq 50\% \)), the vibrational relaxation is observed to be followed by a thermalization and cooling process.

We also performed polarization-resolved experiments to measure the anisotropy dynamics of the OD oscillators. By varying the isotopic composition of the hydrating water we find that the anisotropy decays are dominated by two contributions: intermolecular resonant (Förster) energy transfer and intramolecular energy transfer within \( \text{D}_2\text{O} \) molecules. There is also a fraction of water molecules for which there is no vibrational resonant energy transfer (VRET). These water molecules are apparently isolated. With increasing membrane hydration level the fraction of isolated water molecules decreases and at a hydration of approximately 12 water molecules per lipid, the water between the two bilayers forms a two-dimensional continuous sheet. Our findings demonstrate that in tightly packed membranes with a low hydration level the distribution of water molecules is highly inhomogeneous. Throughout the membrane interface there are regions poor in water and regions with nearly bulk-like water pools as schematically depicted in figure 8.12.
Figure 8.12. A cartoon showing the difference between the water distribution between the two lipid monolayers at low ($x \sim 2$) and high ($x \sim 10$) hydration levels.

8.5 Appendix: Anisotropy

In section 2.5.1 we showed how the Förster formula for resonant energy transfer is derived. We showed that the anisotropy decay due to this transfer is described by the excitation survival probability $\rho(t)$:

$$
\rho(t) = \left\{ \frac{4\pi}{V} \int_0^R \exp \left( -\frac{tr_0^6}{T_1r^6} \right) r^2 dr \right\}^N,
$$

(8.8)

This approach assumes a statistical distribution of molecules, thus includes all possible distances between them. Hence the integral is performed from $R=0$. In the case of water molecules it thus intrinsically takes into account an intramolecular energy transfer. To describe the anisotropy decays for water molecules at the lipid membranes interface we want to separate the contributions from vibrational resonant energy transfer (VRET) and intramolecular energy transfer (IET). We account for IET with equation 8.2. From the term describing solely VRET we need to exclude the possibility of energy transfer within the same molecule (over a distance of $\sim 1.55$ Å). This is achieved by performing the integration from a distance $R = a$, which should be on the order of an intermolecular distance ($\sim 2.2$ Å considering the intermolecular hydrogen-hydrogen distance).

The excitation survival probability $\rho(t)$ is thus given by:

$$
\rho(t) = \left\{ \frac{4\pi}{V} \int_a^R \exp \left( -\frac{tr_0^6}{T_1r^6} \right) r^2 dr \right\}^N,
$$

(8.9)

Performing the integration, we obtain:

$$
\rho(t) = \left\{ \frac{4\pi}{V} \left[ \frac{1}{3} R^3 \exp (-f(t)) - \frac{1}{3} a^3 \exp (-g(t)) + \frac{1}{3} \sqrt{\frac{\pi tr_0^6}{T_1}} \text{erf} \left( \sqrt{f(t)} \right) - \frac{1}{3} \sqrt{\frac{\pi tr_0^6}{T_1}} erf \left( \sqrt{g(t)} \right) \right] \right\}^N,
$$

(8.10)
Figure 8.13. Excitation survival probability function for various minimal energy transfer distances $a$. VRET becomes slower for larger intramolecular distances $a$. For $a \to 0$ the decay profile is given by equation 8.6

where

$$f(t) = \frac{t r_0^6}{T_1 R^6} \quad \text{and} \quad g(t) = \frac{t r_0^6}{T_1 a^6}$$

Following the steps shown in section 2.5.1 we obtain:

$$\rho(t) = \exp \left( -\frac{4\pi C_{OD} \alpha^3}{3} \exp(-g(t)) - j(t) \operatorname{erf}\left(\sqrt{g(t)}\right) \right), \quad (8.11)$$

where,

$$j(t) = \frac{4\pi^{3/2} C_{OD} r_0^3 \sqrt{t}}{3\sqrt{T_1}}. \quad (8.12)$$

Here $C_{OD}$ is expressed as the number of OD groups per unit volume ($1/\text{Å}^3$). This formula can be used to describe resonant energy transfer for any system with a non-zero minimal distance between the donors and acceptors.

From the above equation it follows that for $a \to 0$ the above formula converges to the well known form derived in section 2.5.1 (also see figure 8.5):

$$\rho(t) = \exp \left( \frac{4\pi^{3/2} C_{OD} r_0^3 \sqrt{t}}{3\sqrt{T_1}} \right). \quad (8.13)$$