Dynamics of water interacting with biomolecules

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8  STRUCTURE AND DYNAMICS OF WATER IN TRIGLYCERIDE OILS

It is commonly known that a small amount of water can be present in triglyceride oil, but a molecular picture of the structure and dynamics of these water molecules is lacking. In this chapter, we study the hydrogen-bond configuration and dynamics of water in triacetin, tributyryl and trioctanoin. We identify water molecules with a single strong hydrogen bond to the triglyceride, waters with two weaker hydrogen bonds to the triglycerides, and water clusters. These species do not interconvert on the 20 ps timescale of the experiment. The water molecules with two weaker hydrogen bonds to the triglyceride correspond to a single, specific hydrogen-bond configuration; these molecules likely bridge the carbonyl groups of adjacent triglyceride molecules, which can have a large influence on the properties of triglyceride oils.
8.1 INTRODUCTION

Water is known to actively influence the structure and functioning of biological molecules like proteins, DNA, and phospholipid membranes. However, the influence of water has been hardly studied for a major class of biomolecules: the triglycerides. Triglycerides are responsible for energy supply and storage needed for metabolic functions and are widely used in daily life as foods, pharmaceuticals, cosmetics and raw material for biodiesel.

Due to the hydrophobic nature of triglycerides, water is only soluble up to small amounts, but is nonetheless expected to have considerable influence on properties like the disorder of fatty acid chains, fluidity and molecular packing, in analogy with the effect of small amounts of water on phospholipids. The presence of water in triglycerides can also be very important for crystallization of oil soluble bioactive molecules and the types of crystals that are formed. The solubility of water in triglycerides can be critically important for stability of water-in-oil emulsions as well, as the diffusion of water can cause instabilities due to Ostwald ripening. Additionally, water decreases the long-term stability of triglyceride oils by promoting hydrolysis and auto-oxidation reactions.

In this chapter we study the hydrogen-bond configuration and dynamics of water in triglyceride oils, using linear infrared and 2DIR spectroscopy of the water hydroxyl stretch vibrations. We studied three saturated triglycerides that are widely used in industry and have different fatty acid chain lengths: triacetin, tributyrin, and trioctanoin (figure 8.1). By varying the fatty acid chain length, we effectively tune the hydrophobicity of the oil.

8.2 EXPERIMENTAL

SAMPLE PREPARATION Triacetin, tributyrin and trioctanoin are purchased from Sigma-Aldrich (purity >99%), and carefully dried. Even though the triglyceride oils are mostly hydrophobic, they do absorb small amounts of water (~0.5 ml/l) under atmospheric conditions. This water is removed using 4 Å molecular sieves (Sigma-Aldrich). After drying, solutions are prepared by adding controlled amounts of water (H₂O, D₂O, or 1:9 D₂O:H₂O) to the triglycerides. The ratio of 1:9 D₂O:H₂O is chosen as a compromise between having...
mostly HDO molecules (instead of D₂O), and still maintaining a sufficient concentration of OD oscillators and signal-to-noise to record 2DIR spectra. To ensure a homogeneous distribution of water in the oil phase, the solutions are left to equilibrate for at least five days. A long equilibration time is especially important for trioctanoin, in which water diffusion is very slow.

**Spectroscopy** We measure linear infrared spectra using a FTIR spectrometer (Bruker Vertex 80v). Each spectrum is background-corrected with an equivalent concentration of H₂O in triglyceride (for solutions with D₂O and 1:9 D₂O:H₂O) or with D₂O in triglyceride (for solutions with H₂O). This correction is more accurate than subtracting the spectrum of pure triglyceride only, since it takes into account the replacement of triglyceride volume by water.

We measure 2DIR spectra using the dual-color setup described in section 3.3. The pump and probe pulses are centered at $\sim$2640 cm⁻¹, in resonance with the OD stretch vibration of HDO molecules in triglyceride oil.

### 8.3 Results

#### 8.3.1 Linear spectra

![Figure 8.2](image)

**Figure 8.2.** Linear absorption spectra of the OD stretch vibrations of 1:9 D₂O:H₂O in triacetin (A), tributyrin (B), and trioctanoin (C), for different water concentrations. The spectra are corrected for the response of H₂O in triglyceride at the same water concentration. The integrated infrared absorption (bottom row) can be related to the water concentration by an empirical second order polynomial fit (solid lines). The water concentration at which the fit reaches the integrated OD stretch absorption of a saturated solution (dotted lines), indicated with arrows, is then an estimate for the water concentration of a saturated solution.
Figure 8.2 presents the linear absorption spectra of isotopically diluted water in the different triglycerides as a function of the water concentration. The OD stretch absorption increases gradually with increasing water concentration, until the saturation limit is reached and adding more water does not increase the OD stretch intensity further. This allows us to determine the water solubility, which is $57\pm2$ ml/l, $6.8\pm1$ ml/l and $3.4\pm1$ ml/l for triacetin, tributyrin, and trioctanoin respectively, corresponding to molar water to lipid ratios of 1:1.7, 1:9, and 1:11. The low water to lipid ratio for triglycerides with longer fatty acid chains directly reflects the lower solubility of water in these triglyceride oils.

Figure 8.3 presents the infrared absorption spectra of saturated solutions of water in the different triglycerides, for different water isotope compositions. All spectra are background-corrected and normalized to their peak intensity. For isotopically diluted water (fig. 8.3A), the infrared absorption spectrum reports on the hydrogen-bond strength, without the influence of intra- and intermolecular coupling of the hydroxyl stretch vibrations. We find we can decompose the spectra into three Gaussian bands; a broad band at lower frequencies ($\sim2600$ cm$^{-1}$), a narrower band at intermediate frequencies ($\sim2640$ cm$^{-1}$) and a small shoulder around 2715 cm$^{-1}$. The small shoulder corresponds to non-hydrogen-bonded OD groups$^{188}$.

With increasing fatty acid chain length, the broad band at low frequencies decreases in amplitude and shifts to higher frequencies by about 45 cm$^{-1}$, the narrow band at intermediate frequencies blueshifts by 13 cm$^{-1}$, and the amplitude of the small shoulder corresponding to non-hydrogen-bonded OD groups increases. This shows that the distribution of hydrogen-bond strengths shifts towards weaker hydrogen bonds for water in triglycerides with longer fatty acid chains. In addition the different spectral bands become narrower, which indicates that the distribution of hydrogen-bond strengths becomes more narrow.

Replacing isotopically diluted water by pure H$_2$O or D$_2$O (fig. 8.3B,C) leads to a splitting of the band at intermediate frequencies. The resulting two bands are assigned to delocalized symmetric and antisymmetric stretching modes that result from the intramolecular coupling of the hydroxyl stretch vibrations of H$_2$O and D$_2$O. The broad band at low frequencies does not show this behavior, which indicates that for these water molecules the two stretch vibrations do not or weakly interact, probably as result of a strong difference in hydrogen bond strength. Hence, the low frequency band likely arises from water molecules with one OH/OD group strongly hydrogen-bonded to the lipid and the other OH/OD group being free (giving rise to the small shoulder at 2715 cm$^{-1}$). The intermediate frequency band corresponds to water molecules that have both hydroxyl groups weakly hydrogen bonded to the lipid, or more specifically, to the C=O groups, considering that the frequency of this intermediate band matches very well with hydroxyl groups hydrogen-bonded to the C=O of acetone molecules$^{75,188}$.

The band positions resulting from the Gaussian fit are summarized in figure 8.4 for the different triglycerides and isotopic compositions. For H$_2$O, the relative separation between the delocalized symmetric and antisymmetric stretching
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![Linear absorption spectra](image)

**Figure 8.3.** Linear absorption spectra of saturated solutions of water in triacetin, tributyrin and trioctanoin. A: 1:9 D$_2$O:H$_2$O, B: D$_2$O, C: H$_2$O. The spectra are corrected for the response of H$_2$O in triglyceride (A,B) or D$_2$O in triglyceride (C), and normalized on peak intensity. The dotted lines represent a Gaussian fit to the spectra.

![Center frequencies](image)

**Figure 8.4.** Center frequencies of the Gaussian bands that constitute the spectra (fig. 8.3) of saturated solutions of water in triacetin (red), tributyrin (green) and trioctanoin (blue). A: 1:9 D$_2$O:H$_2$O, B: D$_2$O, C: H$_2$O. Triangles: broad band (FWHM 90-220 cm$^{-1}$), circles: narrow band (FWHM 50-65 cm$^{-1}$), squares: small shoulder (FWHM 30 cm$^{-1}$).
modes is slightly smaller than the separation observed for D$_2$O, in accordance with their gas phase frequencies$^{189,190}$. The spectra for D$_2$O and H$_2$O show a small peak around 2385/3240 cm$^{-1}$ as well. The frequency of this peak is approximately twice the bend frequency, and follows the shift of the bend vibration with increasing fatty acid chain length, as can be seen in figure 8.5 (note this is in opposite direction of the OH stretch vibration). Hence, we assign this peak to the overtone of the bend vibration.

Besides a major contribution from OD stretch vibrations that are strongly hydrogen-bonded to the oil, the broad band at lower frequencies probably contains a contribution from small water clusters. Water molecules hydrogen-bonded to other water molecules give rise to a broad band centered at 2500 cm$^{-1}$. To determine the contribution of water clusters, we return to the linear spectra of isotopically diluted water in the triglycerides as a function of water concentration. These spectra were presented in fig. 8.2, but for clarity we normalize them on peak intensity and present them in fig. 8.6 as well. It can be seen that the shape of the spectrum changes considerably with water concentration for solutions of water in triacetin. A Gaussian fit (see fig. 8.7) shows that with increasing water concentration, the broad band at low frequencies increases in relative amplitude and shifts from 2620 cm$^{-1}$ to 2572 cm$^{-1}$, becoming more similar to the OD stretch absorption spectrum of bulk water. This observation indicates that this band contains a significant contribution of water clusters that increases with increasing water concentration. For tributyrin and trioctanoin we do not observe significant changes in spectral shape with increasing water concentration, so for these triglycerides the contribution of water clusters appears to be negligible, even at the solubility limit. Hence, for these triglycerides we can consider all water molecules to be isolated.

For low concentrations of isotopically diluted water (<4 ml/l), the OD stretch spectra of the three triglycerides are identical, so at low water concentrations the hydrogen-bond strengths and the relative contributions of each water species are the same. Hence, the differences between the water spectra

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**Figure 8.5.** Linear absorption spectra of saturated solutions of H$_2$O in triacetin, tributyrin and trioctanoin in the water bend region (A) and at approximately twice the frequency (B). The spectra are corrected for the response of D$_2$O in triglyceride and normalized on peak intensity between 1580 and 1680 cm$^{-1}$. The dotted lines indicate peak positions.
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**Figure 8.6.** Linear absorption spectra of 1:9 D$_2$O:H$_2$O in triacetin (A), tributyrin (B), and trioctanoin (C), for different water concentrations. The spectra are corrected for the response of H$_2$O in triglyceride at the same water concentration, and normalized on peak intensity. The dotted lines represent a Gaussian fit to the spectrum.

![Spectra](image)

**Figure 8.7.** Center frequencies and areas of the Gaussian bands that constitute the spectra of water in triacetin at different water concentrations (fig. 8.6A). Triangles: broad band (FWHM 90-160 cm$^{-1}$), circles: narrow band (FWHM 50-65 cm$^{-1}$), squares: small shoulder (FWHM $\sim$30 cm$^{-1}$).

![Band areas](image)

For the different oils, as shown in fig. 8.3, are mostly due to differences in water concentration. There are still subtle differences: at low water concentrations, the water bend vibration still shows a significant redshift with increasing fatty acid chain length ($\sim$ 6 cm$^{-1}$ from triacetin to trioctanoin). The redshift of the bend vibration is also responsible for small changes of the H$_2$O and D$_2$O hydroxyl stretch spectra with fatty acid chain length, due the coupling of the water bend and hydroxyl stretch vibrations for non-isotopically diluted water.
8.3.2 2DIR spectra

To investigate the dynamics of the different hydrogen-bonded water molecules and their possible interconversion, we measured time-resolved 2DIR spectra. We recorded spectra for water in triacetin and tributyrin. Unfortunately, for trioctanoin the water concentration was too low to measure 2DIR spectra with sufficient signal-to-noise.

Figure 8.8 presents the 2DIR spectra of saturated solutions of water in triacetin at 0.4 picoseconds after the excitation. The top row shows the isotropic (rotation-free) 2DIR spectra. For isotopically diluted water, the isotropic 2DIR spectrum shows a negative absorption feature along the diagonal, corresponding to the bleaching of the $0\rightarrow1$ vibrational transition, and a positive absorption feature corresponding to the $1\rightarrow2$ vibrational transition, which is redshifted along the probe axis by about 100 cm$^{-1}$ due to the anharmonicity of the OD stretch vibration. We find the spectrum of isotopically diluted water in triacetin at 0.4 ps to be very inhomogeneous: the spectrum shows an almost perfect correlation between excitation and detection frequency (nodal line slope of $\sim 1$). In comparison, for isotopically diluted bulk water this correlation is largely lost at 0.4 ps, as a result of rapid spectral diffusion$^{191}$. This indicates that the hydrogen-bond strength of water molecules confined in triglyceride oil is modulated on a much longer timescale than in bulk water, reflecting much slower water dynamics.

For pure D$_2$O in triacetin, we observe two negative features along the diagonal corresponding to the symmetric and antisymmetric OD stretch vibration (and corresponding peaks due to the $1\rightarrow2$ transition). In addition, off-diagonal features (cross-peaks) appear at all delay times due to the vibrational coupling between the symmetric and antisymmetric stretch vibrations. Note that the symmetric to antisymmetric cross-peak appears around $\omega_{pump,probe}=2580$, 2680 cm$^{-1}$, while the corresponding diagonal signal is centered at a lower frequency of $\sim 2550$ cm$^{-1}$. This lower frequency is the result of the contribution of OD stretch vibrations of singly hydrogen-bonded water molecules and water clusters; these vibrations absorb at lower frequencies compared to doubly hydrogen-bonded water molecules. For these water molecules, the hydrogen-bond configuration is asymmetric, and the OD vibrations do not form symmetric and antisymmetric vibrations. Hence, these water molecules only contribute to the diagonal signal and not to the cross-peaks of the symmetric and antisymmetric OD vibrations of the D$_2$O molecules with two weak hydrogen bonds of similar strength. The non-hydrogen-bonded OD stretch vibrations are not observed in the 2DIR spectra, because their cross section is very low and the diagonal 2DIR signal depends quadratically on the cross section.

The bottom row of figure 8.8 presents the polarization-difference 2DIR spectra. In the polarization-difference spectrum the diagonal signals are eliminated, and the cross-peaks become more visible, provided that the cross-peaks have an anisotropy value that differs from that of the diagonal peaks$^{60}$. The polarization-difference spectra reveal that the spectrum of isotopically diluted water contains weak cross-peaks as well. These have a similar position and shape as the cross-peaks measured for D$_2$O, but much lower intensity ($\sim 1/100$).
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Figure 8.8. 2DIR spectra of saturated solutions of 1:9 D$_2$O:H$_2$O (A) and D$_2$O (B) in triacetin, at 0.4 picoseconds after excitation. The top row shows the isotropic 2DIR spectrum (eq. 2.41, rotation free) and the bottom row the polarization-difference 2DIR spectrum (eq. 2.53, cross-peaks enhanced). The spectra are normalized on peak intensity.

This indicates that these cross-peaks are due to vibrational coupling between the symmetric and antisymmetric stretch vibrations of the 1% of D$_2$O molecules that are left in the isotopically diluted mixture. At all time delays, only cross-peaks arising from the coupling between the symmetric and antisymmetric stretch vibrations are observed, both for water in triacetin and tributyrin. The absence of other cross-peaks indicates that the different hydrogen-bonded water species do not interconvert on the 20 picosecond timescale of the experiment.

To study the vibrational dynamics of the different hydrogen-bonded water species in more detail, we analyze slices of the isotropic 2DIR spectra at different excitation frequencies. Figure 8.9 presents these slices for isotopically diluted water in triacetin and tributyrin. For each excitation frequency, the vibrational response decays within several picoseconds, reflecting the decay of the excited vibrations back to the vibrational ground state. Based on the linear spectra, we expect at least two spectral components to contribute to the vibrational response; a component corresponding to the OD stretch vibrations of water molecules with a single strong hydrogen bond to the triglyceride, and a component corresponding to OD stretch vibrations of water molecules with two weaker hydrogen bonds to the triglyceride. We therefore analyze the spectra using a spectral decomposition model with two components that decay independently.
to a heated ground state (eq. 4.7). This state is included to account for the small residual signal due to heating of the sample. The vibrational lifetimes of each component, $T_{1,1}$ and $T_{1,2}$, are the primary fit parameters, while the spectral components $\sigma_1$ and $\sigma_2$ are calculated using a singular value decomposition. The spectrum of the heated ground state is taken directly from the data at 100 ps. The results of the least-squares fit are presented in figure 8.9 and describe the data well. In this analysis, we did not consider spectral diffusion effects, which are expected to lead to a slight broadening of the bands with time.

Figure 8.10 presents the spectral components. Spectral component 2, corresponding to OD vibrations of water molecules with two weak hydrogen bonds to the triglyceride, varies in amplitude, but hardly in shape, with the excitation frequency. This finding indicates that the absorption band of the water molecules with two weak hydrogen bonds is close to homogeneously broadened. In contrast, the spectral signature of component 1, corresponding to the OD vibrations of water molecules with a single strong hydrogen bond to the triglyc-
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eride, varies strongly with the excitation frequency. This indicates that the absorption band of the singly hydrogen-bonded water molecules is inhomogeneously broadened and represents a heterogeneous ensemble of water molecules with different hydrogen-bond strengths. This heterogeneity likely arises from the fact that water molecules are hydrogen-bonded to different groups of the triglyceride (carbonyl or ether oxygen), and from the presence of different local conformations of the glycerol backbone and fatty acid chains of the triglyceride surrounding the hydrogen-bonded water molecules. In the case of triacetin, there is an additional contribution of water clusters to the spectrum as well. This contribution manifests itself as a broadening and increase in amplitude of spectral component 1.

The vibrational lifetimes of the OD stretch vibration of water in triacetin are $2.0 \pm 0.6$ ps and $11 \pm 1$ ps, for component 1 and 2, respectively. For water in tributyrin the lifetimes are comparable: $2.3 \pm 0.7$ ps for component 1 and $17 \pm 3$ ps for component 2. The lifetime of component 2, corresponding to OD vibrations of doubly hydrogen-bonded water molecules, is quite long compared to the $1.8$ ps lifetime of the OD stretch vibration observed for HDO molecules in bulk isotopically diluted water\textsuperscript{74}. This longer lifetime points at a weaker anharmonic coupling of the excited OD vibration and low-frequency accepting modes of the triglyceride solvent, which can be explained from the weak hydrogen bonds. In this perspective, the lifetime of spectral component 1 is remarkably short, especially when it is excited at high frequencies (corresponding to weak hydrogen bonds).

Finally, we measure the anisotropic spectrum of the water molecules confined
in the triglycerides. The anisotropy is directly proportional to the second order rotational correlation function, and decays due to molecular reorientation and resonant (Förster) energy transfer. Figure 8.11 presents the anisotropy decay of the vibrational excitation for saturated solutions of water in triacetin and tributyrin, obtained by averaging over a frequency range of 20 cm\(^{-1}\) around the main diagonal peak of the 2DIR spectrum. For isotopically diluted water, the anisotropy decays only partially within the time window of the experiment. This indicates that the water molecules do not fully reorient on this timescale, but instead sample a limited angular space. We can describe the partial decay of the anisotropy using a single exponential with an offset: 

\[ R = R_1 e^{-t/\tau_r} + R_0. \]

This yields a reorientation time \(\tau_r\) of about 4 ps (\(\tau_r = 4.0 \pm 0.5\) ps for triacetin, and \(3.8 \pm 0.5\) ps for tributyrin) and an offset of 0.14 \(\pm 0.04\), which corresponds to reorientation within a cone angle of 46° (see eq. 2.51).

In bulk liquid water, water molecules fully reorient on a 2.5 picosecond timescale\(^{74}\) by a mechanism which involves the switching of hydrogen-bond partners\(^{13}\). The transition state for reorientation requires the approach of another water molecule. For water molecules confined in triglycerides the situation is very different, as there are no water molecules to switch to (or very few, in the case of water clusters). This lack of potential new hydrogen-bonded partners explains why the reorientation only occurs within a limited cone angle: without the possibility of forming a new hydrogen bond, the hydrogen bond to the triglyceride is maintained, which limits the angular space of the reorientation.

For D\(_2\)O, we only average the anisotropy decay over the diagonal peak that corresponds to the antisymmetric stretch vibration. By doing so, the effect of intramolecular coupling is eliminated: the antisymmetric stretch vibrations that transfer energy to the symmetric mode show up in another part of the spec-
trum (the cross-peak) and thus do not contribute to the anisotropy decay of the diagonal peak. However, intermolecular coupling due to coupling between antisymmetric vibrations located on neighboring water molecules, can still contribute to the anisotropy decay. It can be seen in fig. 8.11 that the anisotropy decay for D$_2$O in tributyrin is very similar to the decay for isotopically diluted water in the same triglyceride, while the anisotropy decay for D$_2$O in triacetin is clearly faster than its isotopically diluted counterpart. This is in line with our earlier observation that triacetin contains a significant amount of water clusters, that also contribute to the absorption at 2690 cm$^{-1}$. Within a water cluster, intermolecular energy transfer is possible, while in tributyrin all water molecules are isolated and intermolecular energy transfer does not happen.

Water clusters are likely only present in triacetin due to the higher concentration of hydrophilic groups versus hydrophobic groups for this triglyceride, as a result of the shorter hydrophobic fatty acid chain. This makes it more likely for a water molecule that is hydrogen-bonded to the hydrophilic carbonyl group of the triglyceride to be in the vicinity of a water molecule that is hydrogen-bonded to the carbonyl group of another triacetin. As a result, it may become favorable for other water molecules to accumulate in between and in the vicinity of these hydrogen-bonded water molecules, thus leading to the formation of water clusters.

8.4 Discussion

The spectra of water molecules confined in the triglyceride oils are similar to the spectra of water molecules confined in other solvents. Studies on mixtures of water and acetone$^{75,188}$, acetonitrile$^{192}$, DMSO$^{75,193}$, poly-ether$^{194}$ and dioxane$^{195}$ showed a strong narrowing and blueshift of the water hydroxyl stretch vibrations when the water concentration was decreased. For all these solvents, a decrease of the water fraction leads to a replacement of the interconnected water hydrogen-bond network by specific hydrogen-bond configurations involving water and hydrophilic groups of the solvent. Arguably the most closely related system is water in acetone clusters$^{188}$. Infrared spectra of the water OH stretch vibration showed that water molecules in acetone clusters form either a single strong hydrogen bond to an acetone carbonyl group, or two weaker hydrogen bonds to two acetone carbonyl groups, which is similar to what we currently observe for water molecules in the triglycerides. The main difference is that water in acetone clusters can more easily switch hydrogen-bond partners: on a 1.3 picosecond timescale, water molecules change between a single strong hydrogen bond and two weaker hydrogen bonds to the acetone carbonyl groups. Most likely, this difference is due to the much higher density of carbonyl groups in acetone, and due to the much higher mobility of the light acetone molecules compared to the bulky triglyceride molecules.

The 2DIR spectra showed that water molecules with two hydrogen bonds to the triglyceride constitute a single species, which indicates that these water molecules have a specific hydrogen-bond configuration. Considering the posi-
tioning of the carbonyl groups of the triglyceride, the most likely hydrogen-bond configuration is one where the water molecule bridges two adjacent triglyceride molecules. Below the melting point triglycerides adopt different polycrystalline phases that are thought to persist in the liquid\textsuperscript{22}. Different models have been proposed for the liquid ordering; a smectic phase with triglycerides in a chair-like conformation\textsuperscript{178}, a nematic phase with triglycerides in the same conformation but with twisted chains\textsuperscript{179} and a stacked-disk ordering where each disk consists of a single y-shaped triglyceride molecule\textsuperscript{180}. Each of these structures would allow bridging water molecules between carbonyl groups of adjacent triglyceride molecules, while intramolecular bridging is unlikely. As such, water is expected to have a large influence on the liquid triglyceride properties such as viscosity and molecular ordering during crystallization, which is of great importance for many biological and industrial systems.

8.5 Conclusions

We have investigated the hydrogen-bond structure and vibrational dynamics of water in triglyceride oils, using linear infrared and time-resolved 2DIR spectroscopy. From the linear spectra of the water hydroxyl stretch vibrations measured at different isotopic dilutions, we identify several water species: waters with a single strong hydrogen bond to the triglyceride, waters with two weaker hydrogen bonds to the triglycerides, and water clusters. The latter species is only present in triacetin, the triglyceride with the shortest fatty acid chains. For tributyrin and trioctanoin the amount of clusters is negligible, and all water molecules can considered to be isolated.

The 2DIR spectra of the OD stretch vibration of D\textsubscript{2}O in triacetin and tributyrin contains cross-peaks between the symmetric and antisymmetric stretch vibrations of D\textsubscript{2}O. The absence of other cross-peaks shows that the different water species do not interconvert on the 20 picosecond timescale of the experiment.

The vibrational lifetimes of the OD stretch vibration of HDO in triacetin are 2.0 ± 0.6 ps for HDO molecules with a single strong hydrogen bond, and 11 ± 1 ps for HDO molecules with two weak hydrogen bonds. For tributyrin the lifetimes are 2.3 ± 0.7 ps and 17 ± 3 ps, for the singly and doubly hydrogen-bonded water molecules, respectively. Within the experimental time window, the anisotropy of the vibrational response decays only partially; the decay corresponds to reorientation on a 4 picosecond timescale within a limited cone angle of 46°. The transient spectral response of water molecules with a single strong hydrogen bond to the triglyceride depends strongly on the excitation frequency, revealing the presence of different subspecies of singly-bound water molecules with different hydrogen-bond strengths. In contrast, the water molecules with two weaker hydrogen bonds to the triglyceride correspond to a single, specific hydrogen-bond configuration. Considering the structure of the triglycerides, these water molecules likely bridge adjacent triglyceride molecules. As such, water is expected to have a large influence on liquid triglyceride properties.