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Hydration and Hydrogen Bonding of Carbonyls in Dimyristoyl-Phosphatidylcholine Bilayer

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Abstract: We combine two-color ultrafast infrared spectroscopy and molecular dynamics simulation to investigate the hydration of carbonyl moieties in a dimyristoyl−phosphatidylcholine bilayer. Excitation with femtosecond infrared pulses of the OD stretching mode of heavy water produces a time dependent change of the absorption band of the phospholipid carbonyl groups. This intermolecular vibrational coupling affects the entire C═O band, thus suggesting that the optical inhomogeneity of the infrared response of carbonyl in phospholipid membranes cannot be attributed to the variance in hydration. Both the experimental and the theoretical results demonstrate that sn-1 carbonyl has a higher propensity to form hydrogen bonds with water in comparison to sn-2. The time-resolved experiment allows following the evolution of the system from a nonequilibrium localization of energy in the OD stretching mode to a thermally equilibrated condition and provides the characteristic time constants of the process. The approach opens a new opportunity for investigation of intermolecular structural relations in complex systems, like membranes, polymers, proteins, and glasses.

Introduction

The physical and chemical characteristics of the polar interfacial region in lipid molecules play a governing role in functional activation of the surface in biological membranes. Subtle modifications at the level of polar headgroups may result in alterations of collective functionality in such systems.[1−10] However, the knowledge on a molecular basis of the "fine mechanics" of structure and dynamics in the polar region is rather limited. This is mostly due to the difficulties of obtaining relevant experimental data. Even though X-ray and neutron diffraction techniques are informative about structure and arrangement of the hydrocarbon tails of membranes, they are of little help in respect to structural moieties at the surface.[11−15] Furthermore, the extensive line-broadening prevents obtaining structural information in nuclear magnetic resonance (NMR) studies of phospholipid membranes.[16−18] In this respect, optical (infrared) spectroscopy represents a unique experimental avenue to access information about the microscopic structural layouts in the interface of phospholipid bilayers.

Specifically, linear-infrared (IR) spectroscopy of phosphate and carbonyl moieties has been widely employed to investigate the orientation properties of molecular moieties in the polar compartment of phospholipid bilayers.[9,19−24] From the theoreti-

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cal standpoint, a number of molecular dynamics (MD) simulations accompanied this experimental activity, anticipating some information on the molecular organization and intermolecular interactions within the polar interface.[25−32]

Recently, a number of reports on IR third-order polarization response from carbonyl moiety in phospholipid membranes demonstrated that two-dimensional infrared (2D-IR) ultrafast spectroscopy may serve as a helpful tool to account for intermolecular relations in disordered environments.[33−35] The underlying motive of 2D-IR spectroscopy is rather similar to that of two-dimensional NMR (2D-NMR). In the case of 2D-NMR the information about intra- and intermolecular relations is extracted from a set of off-diagonal cross-peaks due to the coupling among the nuclear spins. Analogously, in 2D-IR spectroscopy information about structure and intermolecular interactions may be extracted from a set of cross-peaks, which are due to the vibrational coupling between the moieties under study. At variance with NMR techniques, nonlinear-IR spectroscopy of phospholipid membranes does not suffer from the limitations due to line broadening, and possesses the great advantage of the unparalleled time resolution.

Since the early spectroscopic studies, we know that the IR response of carbonyl groups in a phospholipid membrane demonstrates a complex band profile, which was attributed to inhomogeneous broadening and interpreted as the superposition of two or three subbands.[19−23] No really convincing explanation has been given as to the origin of the underlying substructure, though it is often considered to be due to the difference in hydration of the carbonyl groups.[21] The ambiguity in the interpretation of the IR response indicates that a productive discussion on spectroscopic data requires a clear and adequate view on the nature of the vibrational states under study: degree of delocalization of the excitation, orientation of the transition dipole moments, anharmonicity. In this respect, nonlinear-IR spectroscopy represents a helpful tool for probing those properties.[33−37] On one side, it allows to disentangle the homogeneous and inhomogeneous contributions to the band profile; on the other side, it provides the femtosecond time resolution to access the dynamical properties of the system.

Here, we report on experimental studies of the structural and dynamical aspects of the water−carbonyl interaction in samples of hydrated dimyristoyl−phosphatidylcholine (DMPC) membrane by means of two-color time-resolved nonlinear-IR measurements. In particular, we investigate the interactions of the sn-1 and sn-2 carbonyls (see Figure 1) with the stretching vibration of deuterium oxide, used as an aqueous medium for membrane fragments of this phospholipid. To separate the resonances of the two carbonyls we synthesized DMPC molecule, where carbonyl group in the sn-2 position was ^{13}C isotopically labeled.

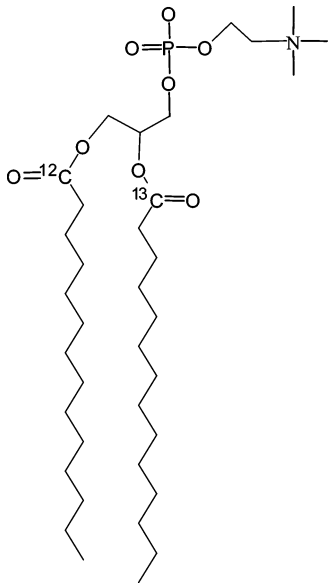

Figure 1. Molecular structure of the dimyristoyl-phosphatidylcholine. $^{12}C{=}O$ and $^{13}C{=}O$ are the sn-1 and sn-2 carbonyl groups, respectively.

The idea of the two-color experiment is to excite with a short IR pulse (the pump) the deuterated water stretching vibration around 2500 cm^{-1}, and to probe with a second pulse centered at about 1700 cm^{-1} the perturbation that the excitation produces on the absorption band of the carbonyl. Specifically, we measure the perturbation by comparing the probe spectrum with that of a reference beam which does not pass through the excited area (by the pump) of the sample. The ratio between probe and reference spectra provides the differential spectrum illustrating the modifications induced on the carbonyl absorption band by the excitation of the OD vibration. The appearance of spectral modifications is a clear indication that a coupling exists between the two groups, primarily related to the presence of hydrogen bonding.

We achieve the time dependence by varying the time delay between pump and probe pulses. The changes in the differential spectrum along with the time delay have direct relation to the mechanism of interaction between the excited (water OD stretching) and probed (DMPC carbonyl stretching) vibrations. If the excitation frequency is tuned through the water absorption band, the corresponding differential spectra of the probe allow reconstructing a two-dimensional spectrum (in frequency space), analogous to those obtained in two-color 2D-NMR.[38,39] The formulation of a reliable picture for the actual molecular system requires the support of a consistent model, which can be

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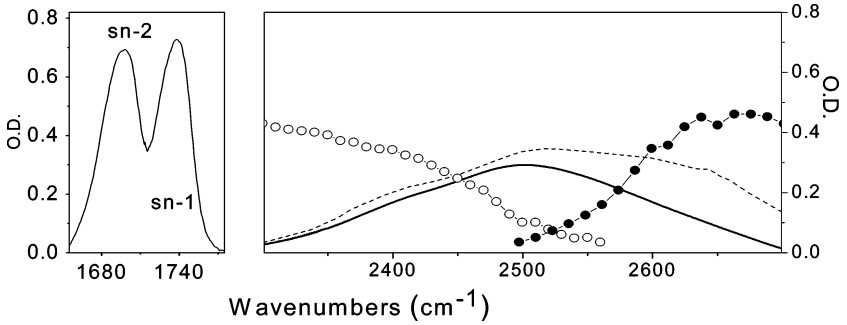

Figure 2. Left panel: linear-IR spectrum of the hydrated DMPC membrane fragments in the spectral region of the sn-1 and sn-2 carbonyl resonances. Right panel: linear-IR spectrum of hydrated membrane fragments (solid line) and of a neat D_2O sample (dashed line) in the spectral region of the OD stretching mode. The open-circle and the solid-circle lines represent the spectra of the pump pulse for "red" and "blue" excitation, respectively.

appropriately constructed on the basis of a full-atom MD simulation. In parallel with the experiments, we have then conducted an extensive simulation of the DMPC hydrated membrane, focusing our attention on the distribution of the C═O···D−O interactions in the bilayer and on the relative structural arrangement.

Results and Discussion

The left panel of Figure 2 shows the linear-IR absorption of the carbonyl stretching in hydrated DMPC membrane fragments. The low and the high-frequency resonances corresponding to the sn-2 (^{13}C) and sn-1 carbonyl moieties, respectively, are well separated in the spectrum. In the right panel of the figure, we show the optical density of the OD stretching mode of the membrane sample (solid line). For comparison, we also show the OD stretching spectrum obtained from a neat D_2O sample (dashed line). The OD stretching band in the membrane coincides with that of neat water only in its low frequency part, whereas the blue side of the band is much weaker. It is generally accepted that the stretching modes in water are dominated by inhomogeneous broadening.[40−45]

The low frequency part of the band is ascribed to strongly hydrogen bonded water molecules, while the high frequency part is due to molecules which are only weakly bonded. Thus, we expect that a considerable fraction of D_2O molecules in the sample are involved in hydrogen bonding with the phospholipid moieties of the polar interface.

We base the present study on a comparison of the time-resolved response of the two carbonyls with the probe pulse frequency centered at 1700 cm^{-1}, whereas the pump pulse is tuned either to the low-frequency side ("red" OD excitation) or to the high-frequency side ("blue" OD excitation) of the OD stretching frequency (open and solid circle lines in the right panel of Figure 2). In Figure 3 (panels A and B), we show the two series of time-dependent differential spectra, which demonstrate the changes of the carbonyl absorption under the two excitation conditions. In Figure 4 we report the kinetic traces of the signal of the sn-1 and sn-2 carbonyls detected at 1740 and 1705 cm^{-1}, respectively, under red and blue OD excitation. As already noticed, the appearance of the differential spectrum is the manifestation of the coupling between the OD and the C═O vibrations. Experiments performed with parallel and perpendicular pump−probe polarization geometries (data not shown) demonstrate that the carbonyl response is rather isotropic already at very short delay times. This rules out the possibility that the signal is due to a direct OD to C═O energy transfer.

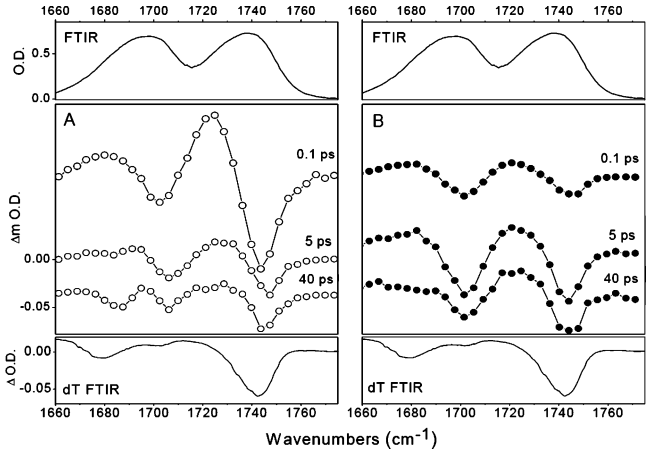

Figure 3. Time-resolved response of sn-1 and sn-2 carbonyls at indicated delay times under red (panel A) and blue (panel B) OD excitation in comparison with the FTIR spectrum (upper panels) and with the difference between FTIR spectra recorded at 30 °C and at 6 °C (dT FTIR in lower panels).

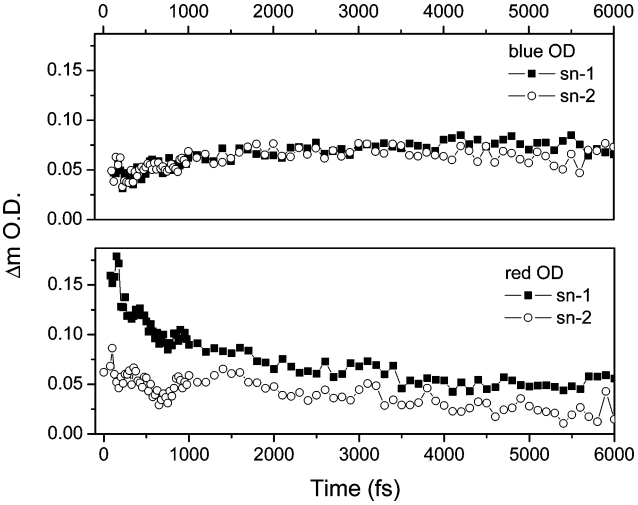

Figure 4. Kinetic traces of the signals of the sn-1 and sn-2 carbonyl groups under blue (upper panel) and red (lower panel) OD excitation.

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Furthermore, the practically instantaneous appearance of clear spectral features (spectra at 0.1 ps delay time in panels A and B of Figure 3) excludes that the effect is due to the heating of the sample, which would occur on a longer time scale.[46,47] Instead, we ascribe the signal to a time-dependent spectral shift of carbonyl resonances, due to the direct anharmonic coupling of the involved OD and C═O stretching vibrations. Excitation of the OD stretching mode instantaneously perturbs (flattens) the potential energy surface of the carbonyl moieties interacting with water. As a result, the resonant frequencies of the involved C═O groups experience a low energy frequency shift. The thermalization of the excitation initially localized in the OD modes is definitely a slower process, leading to the equipartition of the excess energy among all the degrees of freedom of the system. In other words, at short time delay the experiment brings the signature of non equilibrium energy distribution, whereas at later times the measured signal is due to equilibrium heat contributions. The sequence of spectra reported in Figure 3 (panels A and B) show (i) clear differences for red and blue OD excitation, and a (ii) not equal behavior of the spectral features corresponding to the sn-1 and sn-2 carbonyls. These observations represent the main findings of our experimental work.

It is a consequence of the hydrophilic nature of the polar groups at the bilayer surfaces, in contrast to the hydrophobic character of the inner part of the membrane, that the degree of hydration decreases rapidly from the outside to the inside of the bilayer. NMR and neutron scattering experiments demonstrate that water permeates the polar region and even hydrophobic compartment of phospholipid membrane.[48,49] Previous theoretical studies confirm this picture, showing that the phosphate group is the most hydrated, and that a lower, but still noticeable number of water molecules are bonded to the carbonyl groups.[25] Our MD simulation provides a quantitative description of the distribution of the water molecules in the membrane, and of the structure and distribution of the hydrogen bonds. Specifically, the amount of water molecules surrounding the sn-1 and sn-2 carbonyls within a distance of 5 Å from carbonyl's oxygen is practically the same, with a ratio 1.1 in favor of the sn-1 carbonyl. However, the distribution of water molecules within this distance range is significantly different for the two moieties. To show this difference, in Figure 5 we report the angle(α)−distance(*d*) distribution functions for the interactions of the sn-1 and sn-2 carbonyl groups with their first neighbor water molecule. The angle α is that formed by the O···D direction (O is the carbonyl oxygen) and the direction of the D−O covalent bond of water. A graphical representation of α and *d* is reported in Figure 5. The well-defined peaks observed at O···D distances lower than 2 Å correspond to C═O···D−O hydrogen bonds with quasi-collinear geometry (α > 140°). The peak heights (better seen in panels C and D of Figure 5) show that the sn-1 carbonyls have higher propensity to form hydrogen bonds to water than the sn-2 ones. In particular, we count about 52% of sn-1 and 39% of sn-2 carbonyls involved in optimal hydrogen bonding (*d* < 2.5 Å and α > 140°). At larger distances (*d* > 3 Å), there are instead more first neighbor water molecules around the sn-2 carbonyl comparing to sn-1 group. Moreover, we note that the distribution of the angle α becomes more symmetrically peaked around 90° with increasing *d* beyond 2.5 Å. This suggests that such water molecules are almost isotropically distributed with respect to the carbonyl group, implying a progressive loss of the water−carbonyl coupling with the distance.

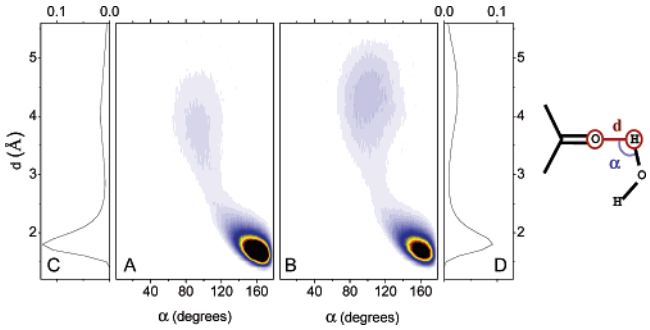

Figure 5. Angle(α)-distance(*d*) distribution functions g(α,d) for the C═O and O−H first neighboring pairs for the case of the sn-1 (panel A) and sn-2 (panel B) carbonyls according to the geometric definition represented at the right of the figure. Panels C and D are the first neighbor radial distribution functions g(d) for sn-1 and sn-2 cases, respectively.

We already noticed that the low-frequency part of the D_2O absorption band around 2500 cm^{-1} corresponds to the population of water molecules which are strongly hydrogen bonded to other water molecules and/or to the polar groups (including carbonyls) of DMPC. The observation that, for short time delay, the intensity of the signal measured upon red OD excitation is markedly higher than obtained under blue OD excitation (Figure 3, panels A and B) confirms the anticipated dominant role of the C═O···D−O hydrogen bonds in determining the vibrational coupling between water and carbonyl groups. The spectral profile and the time evolution of the two carbonyls' responses upon red and blue OD excitation can thus be interpreted on the basis of the difference of CO-water coupling. In this respect, it is important to consider the statistical distribution of water molecules around each carbonyl in the proximal distance range (up to 5 Å), as it results from our MD simulation. Despite the different degree of hydration of the two carbonyls at very short distance (see above), the distribution of water molecules "seen" by the two moieties within the distance range of 5 Å is very similar. 19% and 17% of the water molecules are involved in optimal hydrogen bonding to the sn-1 and sn-2 carbonyls, respectively. Such water would absorb on the red side of OD FTIR band and also would show most effective anharmonic coupling to the C═O stretching vibration. Therefore it largely contributes to the transient response measured in our experiment. About 3.9% of water proximal to sn-1 and 4.5% of water proximal to sn-2 carbonyls are truly solitary, i.e., not hydrogen bonded either to phospholipid moieties or to water. These water molecules would absorb in the blue side of the OD band. The rest of the water (∼77%) is effectively involved in hydrogen bonding to phosphate, to other carbonyls different from the tagged one, and/or to other water molecules. We expect such water molecules to absorb in the red side of the OD band, but not to contribute to the instantaneous modification of the absorption band of the C═O group, to which they are not

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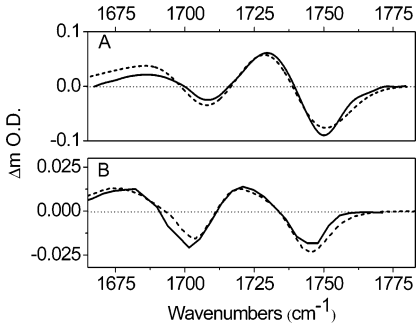

Figure 6. Solid lines represent the time dependent differential spectra at 0.1 ps delay time, with red (panel A) and blue (panel B) OD excitations. Dashed lines show the spectra obtained by subtracting a frequency downshifted FTIR response from the original one. In the case of the red OD excitation (panel A) the intensity of the band at ~1700 cm^{-1} has been lowered by 25% to take into account that sn-2 carbonyls are less hydrogen bonded.

directly hydrogen bonded. Excitation of their OD stretching mode would then contribute to the time-dependent response of the tagged C═O group via the slow thermalization processes mainly.

The shape and kinetics of the transient spectra (Figures 3, and 4, respectively) are then consistent with the following picture. Blue OD excitation deposits energy in water molecules which are rather weakly bonded (or even not bonded at all) to the membrane polar groups. The resulting effect on the C═O spectral response is essentially indirect and not selective at all. The change of the optical density in the region of the carbonyls' absorption is small at early times, since the specific coupling of C═O oscillators to the blue OD modes is weak. As the delay time increases, the excitation energy redistributes among other water molecules and, more generally, among the other degrees of freedom of the system. The moderate increase of the signal observed in the first four picoseconds (upper panel in Figure 4) is the net result of this energy transfer to vibrational modes which are coupled to the probed carbonyl modes.

The situation is quite different when we excite the system on the red side of the OD stretching band. In this case the excited water molecules are strongly hydrogen bonded. We already noticed that the majority of those molecules are bonded to phosphate groups or to other water molecules, and only a relatively small fraction of them is actually bonded to C═O groups. However, since we are probing in the carbonyl spectral region, the signal (panel A of Figure 3) originates mainly from the contribution of C═O···D−O bonded pairs. The excitation of the OD stretching mode causes, due to anharmonic coupling, a red shift of the absorption spectrum of the carbonyl. This effect sets up instantaneously, and results in the large intensity of the signal near zero delay time. The signal corresponding to the sn-1 oscillator is larger than that of sn-2 (see Figure 4). This is consistent with the different ability of the two carbonyl groups to form hydrogen bonds resulting from the MD simulation (Figure 5).

It is quite instructive to compare the differential signal at 0.1 ps delay time in the panels A and B of Figure 3 with an FTIR difference spectrum obtained by subtracting a frequency downshifted (by 5−9 cm^{-1}) FTIR response from the original one as in Figure 2. Specifically, in panel A of Figure 6, we compare the differential signal at 0.1 ps delay time under red OD excitation with such FTIR difference spectrum, where we have decreased the intensity of the band at ~1700 cm^{-1} by 25%. This intensity decrease allows accounting the lower number of hydrogen bonds predicted for the sn-2 carbonyl by our MD simulation (see above). In panel B of Figure 6, we compare the differential signal at 0.1 ps delay time under blue OD excitation with the FTIR difference spectrum, calculated without any modification. In both cases, the agreement is rather good, supporting the hypothesis that the effect of the anharmonic coupling to the excited OD vibration (via hydrogen bond) is an instantaneous red shift of the C═O band. The entire carbonyl stretching band appears to be equally and simultaneously affected by the excitation of the OD vibration. This is in contrast with the interpretation that the IR line-shape of carbonyl in membranes is due to a superposition of different subbands, corresponding to moieties with different hydration. A different interpretation of the complex structure of the carbonyl band, which takes into account the delocalized nature of vibrational excitation appears more appropriate. We will discuss this point together with other aspects of the 2D-IR response of DMPC, in a forthcoming paper.

The intensity of the signal decreases with time delay, as the OD excitation energy is redistributed among other degrees of freedom (primarily to other water molecules), less efficiently coupled to C═O. The bottom panel of Figure 4 clearly indicates that the intensity of the sn-1 band has a fast initial decay that reaches a much slower regime after about 2 ps. The fast decay time (1.1 ± 0.2 ps) is consistent with the vibrational lifetime of the OD stretching mode measured for HOD in H_2O.[50] The signal corresponding to the sn-2 carbonyl demonstrates a similar decaying component but of lower amplitude. In this case, the fast decaying component apparently competes with a rising contribution, analogous to the one observed for the blue OD excitation. The limited time interval considered here (we detected the kinetics up to 40 ps; data not shown) does not allow an accurate determination of the slow time constant. However, we can estimate it to be in the range of 20−30 ps, and thus to assign it to the thermalization process of the excitation energy.

At the bottom of Figure 3, we show the difference spectrum obtained by subtracting the FTIR spectrum of the membrane measured at 6 °C from that recorded at 30 °C. It is unambiguous that the shape of the differential spectra (Figure 3, panels A and B) evolves toward the profile of the FTIR temperature difference spectrum. We can say that the transient differential spectra monitor the thermalization of the energy initially deposited in high-frequency molecular vibrations. Also, the visual inspection of the two series of spectra (see for instance, the feature observed at about 1680 cm^{-1} in the temperature difference FTIR spectrum, whose appearance is definitely delayed in the spectra in panel B) reveals that the thermalization is faster when the excitation takes place in the low-frequency tail of the water band. Energy exchange and relaxation are then more efficient in the presence of strong hydrogen bonding.

Conclusions

Time-resolved two-color pump−probe infrared spectroscopy provides a unique tool to monitor hydrogen bonding and hydration within the polar groups of phospholipid membranes. The method has analogies with the two-color 2D-NMR spectroscopy. The anharmonic coupling of different vibrations is

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responsible for the appearance of cross-peaks from which structural and dynamical information can be extracted with femtosecond time resolution. In particular, the experiments performed on DMPC membranes show that the absorption bands of the two carbonyls are affected throughout their entire width by the excitation of the OD stretching vibration of water. Under this respect, the interpretation of the C═O stretching FTIR band profile of DMPC membrane as due to the presence of substates corresponding to different degrees of hydration appears inadequate.

The changes in the carbonyl absorption band upon excitation of the water OD stretching set up instantaneously and are attributed to the anharmonic coupling of the vibrations. In particular, excitation of weakly hydrogen bonded water molecules ("blue" OD excitation) produces for sn-1 and sn-2 carbonyls quite similar and fairly weak spectral modifications that show only minor changes with increasing pump−probe time delay. Excitation of OD involved in stronger hydrogen bonds ("red" OD excitation) produces instead a rather intense time-dependent response, stronger for sn-1 carbonyl than for sn-2. This finding, in agreement with the results of the MD simulation, points to a higher propensity of carbonyls in sn-1 position to form hydrogen bonds with water molecules comparing to the sn-2 ones. For red OD excitation, the evolution of the C═O differential spectrum at early times is very fast, with a time decay constant of 1.1 ps. It represents the signature of the energy relaxation process from the initially excited OD vibrations to other degrees of freedom of the system. The fully thermalized time-dependent response is finally reached with a much slower process whose characteristic time is estimated in the range of 20−30 ps.

We have shown that the experimental approach based on two color pump−probe infrared technique is able to provide relevant information on hydrogen bonding and hydration in complex systems such as phospholipid membranes. We believe that it can be successfully extended to the investigation of similar problems in other large systems, like proteins, polymers, and glasses.

Experimental Section

The details regarding the synthesis isotopically substituted DMPC (Figure 1), the sample preparation, and the technical aspects of the ultrafast infrared spectrometer are given in the Supporting Information. Classical MD simulation of DMPC bilayer was performed on a sample consisting of 112 phospholipid molecules and of 3016 water molecules. The MD protocol was described earlier.[25−27] In the current run, besides introduction of surface tension to Nose extended ensemble,[27] we incorporated a hydrostatic pressure control,[51] where pressure is intended to be maintained equal in all three directions. This is proper for stable simulations in the case of anisotropic molecules in anisotropic simulation cell. After initial thermalization (10 ns), 2 ns long trajectories were produced. For the analysis we used the last 1 ns time segment, in which we sampled atomic coordinates every 0.1 ps.

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Supporting Information Available: Details regarding the synthesis isotopically substituted DMPC, the sample preparation, and the technical aspects of the ultrafast infrared spectrometer. This material is available free of charge via the Internet at http://pubs.acs.org.

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