THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

Cite This: J. Phys. Chem. B 2019, 123, 1085−1089

pubs.acs.org/JPCB

Hydration and Orientation of Carbonyl Groups in Oppositely Charged Lipid Monolayers on Water

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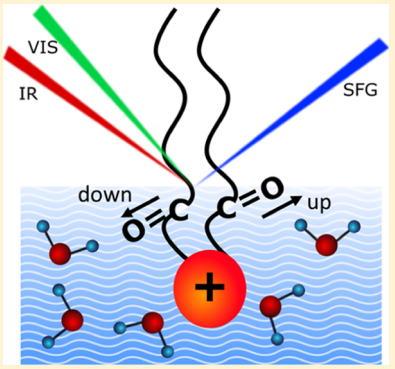
ABSTRACT: The carbonyl groups of glycerolipid monolayers on water play an important role in the formation of the interfacial hydrogen bond network, which in turn influences the interactions of lipids with, for example, metabolites. As the frequency of the carbonyl absorption band strongly depends on the hydration state of the lipid headgroups, the carbonyl band is a sensitive reporter of changes in the headgroup environment. Here, we use phase-resolved sum frequency generation spectroscopy to obtain information about the orientation and hydration of the carbonyl groups in lipid monolayers. We find that there are two distinct carbonyl moieties in the lipid monolayers, oppositely oriented relative to the surface plane, that experience substantially different hydrogen-bonding environments.



INTRODUCTION

In glycerolipids, the headgroups are connected to the lipid tails via a glycerol moiety.[1] The carbonyl groups of those ester linkages not only terminate the interfacial hydrogen bond network of water,[2] but also seem to dominate the signal in some surface potential measurement methods.[3] The ester carbonyl groups present in lipid molecules have a strong absorption around 1700−1750 cm^{-1}. The exact frequency of the absorption band is strongly influenced by the packing of the lipid acyl chains and headgroups as well as the hydration state of the headgroups.[4] It is thus a very useful band for characterization of changes in the environment and hydrogen bonding of lipid molecules and for identifying interactions with ligands.

There have been multiple studies using IR spectroscopy to investigate the absorption band of the carbonyl stretch vibration[5−9] as well as some sum frequency generation (SFG) studies.[10,11] The carbonyl signal has been reported to have an asymmetric appearance, which has been assigned to the existence of at least two overlapping bands. These bands have been predominantly assigned to hydrogen-bonded and non-hydrogen-bonded carbonyl groups. However, there are also some reports suggesting that the two signals originate from the two different carbonyl groups within one lipid molecule that experience a slightly different chemical environment.[6]

Thus, the position and strength of the carbonyl band provide useful information about the hydration state of the lipid molecules in contact with water. The hydration changes upon changing the lipid density; as the lipid layer becomes more densely packed, the headgroups become less hydrated which results in a shift of the carbonyl vibration to higher wavenumbers.[12] However, little is known so far about the absolute orientation of the lipid carbonyl groups in the monolayer, despite IR studies that characterize the relative orientation of carbonyls in lipid layers.[8,13] The orientation of the molecular moieties might well influence their ability to form hydrogen bonds to adjacent water molecules and thus the hydration state of the lipid molecules. The absolute orientation is also highly relevant for the contribution of the CO groups to the surface potential.[3] Therefore, investigating the orientation of the lipid carbonyl groups in the lipid monolayer is expected to yield valuable insights into the structure and properties of these biologically relevant interfacial systems. The absolute orientation of molecular moieties at the interface can be elucidated using phase-resolved SFG spectroscopy.

Here, we present intensity and phase-resolved SFG spectra of the lipid−water interface, using the positively charged lipid DPTAP and the negatively charged lipid DPPG as model systems for positively and negatively charged lipid layers, in the carbonyl vibration region at different lipid coverages. DPTAP was chosen for its relatively high surface tension at relatively low coverage. Interestingly, we observe two carbonyl signals with opposite sign. Furthermore, there are significant changes in the carbonyl signals upon changing the lipid coverage.

EXPERIMENTAL SECTION

Sample Preparation. 1,2-Dipalmitoyl-3-trimethylammonium-propane (chloride salt) (DPTAP) and 1,2-dipalmitoyl-

Received: December 21, 2018
Revised: January 7, 2019
Published: January 8, 2019

DOI: 10.1021/acs.jpcb.8b12297
J. Phys. Chem. B 2019, 123, 1085−1089

sn-glycero-3-phospho-(1′-*rac*-glycerol) (sodium salt) (DPPG) obtained from Avanti Polar Lipids were dissolved in a chloroform (Fischer Scientific, stabilized with amylene, >99%): methanol (VWR Chemicals, 99.8%) mixture (9:1) at a concentration of 4.3×10^{-4} mol/L. Sodium chloride (Riedel-de Haen AG, 99.8%) was baked in an oven for a couple of hours at 650 °C and immediately dissolved in D_2O (Carl Roth GmbH, 99.8%) at a concentration of 10 mM after cooling down. The D_2O solution was put into a trough, and a controlled amount of lipid solution was added using a click syringe. After preparation of the lipid monolayer, the system was left to equilibrate for 3 min. During that time and the measurement, the setup was flushed with N_2 to remove water vapor.

SFG Measurements. The SFG measurements were performed on a setup using a Ti:sapphire regenerative amplifier (Spitfire Ace, Spectra-Physics, centered at 800 nm, ~40 fs pulse duration, 5 mJ pulse energy, 1 kHz repetition rate). A part of the output was used to generate the broadband IR pulse in an optical parametric amplifier (Light Conversion TOPAS-C) with a noncollinear DFG stage. The other part of the output was directed through a Fabry−Perot etalon (SLS Optics Ltd.) to generate a narrow band VIS pulse with a bandwidth of ~25 cm^{-1}. The IR and VIS pulses were overlapped in space and time in a 10 μm thick *y*-cut quartz plate to generate the local oscillator (LO). The transmitted IR and VIS pulses, together with the generated LO, were focused and overlapped on the sample surface at angles of incidence of roughly 47° (VIS) and 62° (IR). The LO was delayed as compared to the VIS and IR pulses by directing it through a 2 mm thick fused silica plate. All spectra shown in this publication were recorded in ssp polarization and referenced to *z*-cut quartz. To ensure that the height and tilt of the sample surface and the *z*-cut quartz reference is the same, we used a height sensor and a HeNe laser. The data were analyzed using a previously described method.[14] Briefly, the spectra were inverse Fourier-transformed, filtered, and Fourier-transformed back into the frequency domain. Finally, the interferogram of the sample was normalized by the quartz reference interferogram. Like in the OH stretch region, pure D_2O spectra were acquired and used as a phase reference because D_2O does not have a vibrational resonance in the used frequency window. A 40° phase correction was applied to all DPTAP spectra, and a 20° correction was applied to the DPPG spectrum. The necessary phase correction is strongly dependent on the positioning of the quartz reference, which is done as accurately as possible, and the height change of the water surface during the measurement due to evaporation. Thus, there are slight variations in the phase correction for different experiments. There is an additional peak in the DPPG spectrum shown in Figure 4 at around 1660 cm^{-1}, which is an artifact from the experiment. It is also present, albeit less obvious, in the DPTAP spectra (Figure 1). This is due to the significantly higher nonresonant signal in the DPTAP spectra.

RESULTS AND DISCUSSION

Figure 1a shows the molecular structure of the positively charged glycerolipid DPTAP. Intensity SFG spectra of DPTAP on D_2O as well as Im $\chi^{(2)}$ spectra of the same system in the carbonyl stretch vibration region are shown in Figure 1b,c. We added 10 mM NaCl to the D_2O subphase to keep the ionic strength constant upon changing the lipid coverage. The Im $\chi^{(2)}$ spectra in panel c clearly show that there are two carbonyl

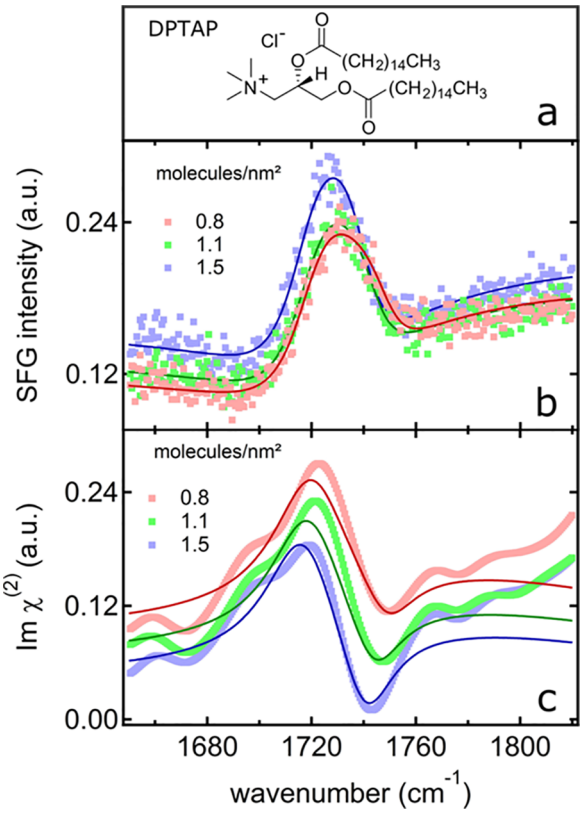


Figure 1. (a) Chemical structure of DPTAP, (b) intensity and (c) Im $\chi^{(2)}$ spectra of the DPTAP−D_2O interface at lipid coverages of 0.8, 1.1, and 1.5 molecules/nm^2. The dots in the panels b and c represent the experimentally acquired spectra and the solid lines the fits.

signals with opposite sign at 1720 and 1750 cm^{-1}. It has previously been reported that the C═O moieties are oriented at a very large angle to the surface normal.[8,13] We thus expect that both C═O populations are positioned almost parallel to the water surface, albeit being oriented in opposite directions. A positive (negative) signal indicates that the C═O dipole is pointing toward air (water). Thus, the carbonyl groups contributing to the low-frequency signal at 1720 cm^{-1} are oriented with their oxygen atoms pointing toward the water subphase. This orientation toward water enables the formation of hydrogen bonds to adjacent water molecules. As hydrogen bonding lowers the frequency of the carbonyl stretch vibration, we attribute the signal at 1720 cm^{-1} to hydrogen-bonded carbonyl groups, which is consistent with previous IR studies.[4,7,15] The opposite sign of the signal at 1750 cm^{-1} shows that there are also carbonyl moieties that are oriented with their oxygen atoms pointing up toward the air. The fact that they are oriented in this direction makes hydrogen bonding to water molecules underneath unlikely. The frequency of this signal is accordingly significantly higher, which indicates that it originates from carbonyl groups that do not participate in hydrogen bonding.[15] A schematic of the orientation of the two carbonyl groups on a lipid is shown in Figure 2. Note that the two oppositely oriented carbonyl groups do not necessarily have to reside on the same molecule. One molecule might have two carbonyl groups with both oxygen atoms pointing down while another molecule might have two carbonyl groups with both oxygen atoms pointing up. Experimentally, we cannot distinguish between the two

DOI: 10.1021/acs.jpcb.8b12297
J. Phys. Chem. B 2019, 123, 1085−1089

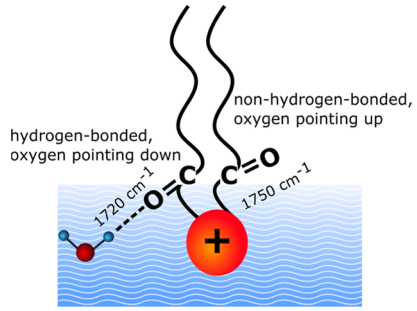


Figure 2. Schematic of a lipid molecule on a water surface. One carbonyl group is oriented with the oxygen atom pointing toward the water and is hydrogen-bonded to a water molecule. The other carbonyl group is oriented with the oxygen atom pointing toward the air. The two different types of carbonyl group are depicted here as part of one molecule. The two carbonyl groups could also originate from separate lipid molecules.

scenarios of the two opposite orientations appearing within one molecule or in separated molecules.

Upon comparing the spectra of the DPTAP−water interface at different coverages, one sees that the high-frequency signal increases in amplitude upon increasing the lipid coverage. Furthermore, the frequency of both carbonyl signals shifts to lower wavenumbers upon increasing the lipid coverage. To quantify those changes, the intensity and Im $\chi^{(2)}$ spectra are fitted with a model using Lorentzian lineshapes: $\chi^{(2)}_{\text{eff}} = A_0\, e^{i\varphi} + \sum_n (A_n/(\omega_{\text{IR}} - \omega_n + i\Gamma_n))$, where A_0 and φ represent the area and phase of the nonresonant contribution and A_n, ω_n, and Γ_n are the area, frequency, and half-width at half-maximum of the resonances. The data are fitted using a nonresonant phase and amplitude and three vibrational resonances. The vibrational resonances at 1720 and 1750 cm^{-1} with a width of 35 and 27 cm^{-1}, respectively, are attributed to the carbonyl stretch vibration. A third resonance at 1800 cm^{-1} with a width of 200 cm^{-1} is required to adequately describe the data. The nonresonant phase decreases from −0.22 to −0.15 and −0.09 upon increasing the lipid coverage, while the nonresonant amplitude increases slightly from −0.38 to −0.39 and −0.42. While the change in the nonresonant amplitude is responsible for the offset between the intensity SFG spectra at different lipid coverages shown in Figure 1b, the change in the nonresonant phase induces the offset in the imaginary spectra shown in Figure 1c. The corresponding intensity and Im $\chi^{(2)}$ spectra are fitted using the same parameters.

Figure 3a illustrates the shift of both carbonyl vibrational resonances to lower frequency upon increasing the lipid coverage. This shift is more prominent in the high-frequency peak. One possible explanation for this shift is dipole−dipole interactions between the carbonyl groups. Upon increasing the amount of lipid on the surface, the molecules move closer together and the strength of these interactions increases. A strengthening of dipole−dipole interactions leads to a shift to lower wavenumbers.[16] Coulomb interactions between carbonyl groups in adjacent strands in parallel β-sheets have been proposed previously to result in a 27 cm^{-1} shift of the amide 1 band to lower frequencies.[17] The strength of this transition dipole coupling is strongly dependent on the distance between the carbonyl groups. β-Sheets on average consist of 6 strands that have an aggregate width of around 25 Å.[18] They are thus separated by roughly 4 Å. At an area per molecule of 54 $Å^2$ (1.85 molecules/nm^2), the average distance between carbonyl

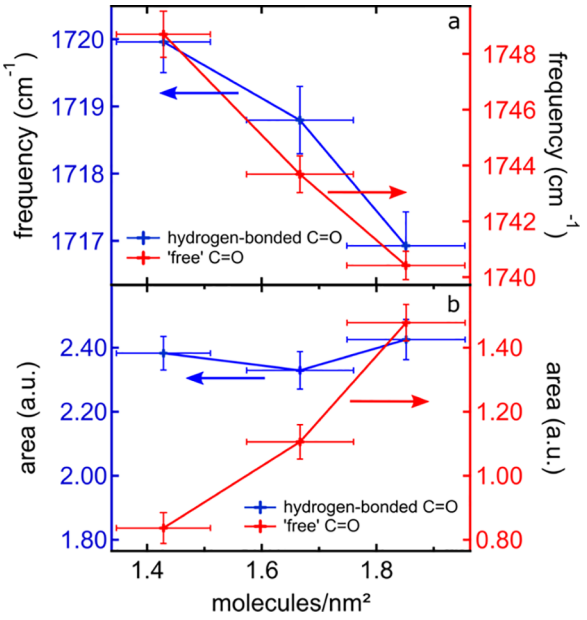


Figure 3. Peak frequencies (a) and absolute peak areas (b) of the two different DPTAP carbonyl signals as a function of the number density (molecules per nm^2). The error bars in *y*-direction represent the standard deviation from the respective fitting coefficients while the error bars in *x*-direction are an estimate of the error in amount of lipid added to the surface. The reported trends have been observed reproducibly.

groups is about 5 Å, because there are two carbonyl groups per lipid molecule. It is thus plausible that dipole−dipole coupling is responsible for the observed red shift with increasing density.

As mentioned above, the red shift is more pronounced in the high-frequency than in the low-frequency signal. This might be explained by the interconversion of hydrogen-bonded to "free" carbonyl population upon increasing the lipid coverage. This interconversion would give rise to the average distance between "free" carbonyl groups decreasing more quickly than expected from the increase in density, resulting in increased coupling and larger frequency shift. As it is known that water is squeezed out from the headgroup region upon increasing the lipid density of the monolayer,[19] it is likely that the relative number of hydrogen-bonded carbonyl groups decreases.

The absolute areas of the two carbonyl resonances obtained from the fits as a function of lipid coverage are shown in Figure 3b. Interestingly, the low-frequency signal remains constant while the high-frequency signal increases with increasing amount of lipid in the monolayer. Upon increasing the lipid coverage, the number of lipid molecules increases. As a result, a larger SFG signal is expected. The fact that the area of the low-frequency signal stays constant while the "free" carbonyl signal increases is thus consistent with the interpretation that there is interconversion from hydrogen-bonded to "free" carbonyl moieties. However, one has to keep in mind that the SFG signal intensity depends not only on the number of molecules but also on their orientation. It is therefore difficult to unambiguously interpret these changes in the data. The constant area of the low-frequency signal might also be explained by a change in the orientation of the hydrogen-bonded carbonyl groups that compensates an increase in the number of oscillators. The increase in the high-frequency signal might also result from a change in the orientation and not necessarily mainly from an increase in the number of

DOI: 10.1021/acs.jpcb.8b12297
J. Phys. Chem. B 2019, 123, 1085−1089

oscillators. Nevertheless, the observed frequency shifts combined with the changes in the peak areas seem to support our interpretation. A decrease in headgroup hydration upon increasing the lipid coverage has also been reported by Ma and Allen,[19] who investigated the phosphate vibration of the lipid headgroup.

To demonstrate the generality of the finding of different carbonyl groups that are differently oriented, we also measured SFG spectra of the negatively charged DPPG on D_2O in the carbonyl vibration region. Figure 4a shows the molecular structure of DPPG. The resulting intensity and Im $\chi^{(2)}$ spectra are shown in Figure 4b,c. The spectra are fitted with three carbonyl peaks at 1697, 1718, and 1743 cm^{-1}. A fourth resonance at 1800 cm^{-1} with a width of 200 cm^{-1} is used to fit the data. The nonresonant phase is −0.01, and nonresonant amplitude −0.02. Note that, even though the negative signal at 1743 cm^{-1} in Figure 4c is quite small compared to the experimental noise, we are confident that it is indeed a signal as it was observed in multiple measurements and a negative signal at that frequency is also needed to fit the intensity spectrum in Figure 4b. Fitting the spectrum with three carbonyl signals is consistent with previous IR studies for DPPG,[7] where Blume et al. attributed the two low-frequency peaks to differently hydrogen-bonded carbonyl groups due to the presence of glycerol OH in the lipid headgroup. The peak at 1697 cm^{-1} that we observe for the DPPG monolayer has a significantly lower frequency than the carbonyl signals of the DPTAP monolayer shown in Figure 1. This could indicate that hydrogen bonding to the carbonyl groups in the PG headgroup is stronger than in the TAP headgroup. However, one should realize that the coverage of the reported DPPG spectrum is higher than that of the DPTAP spectra. Unfortunately, we cannot measure at lower coverages as the sample is locally heated by the laser beams and the lipid molecules are displaced out of the measurement area at low surface pressure.[20]

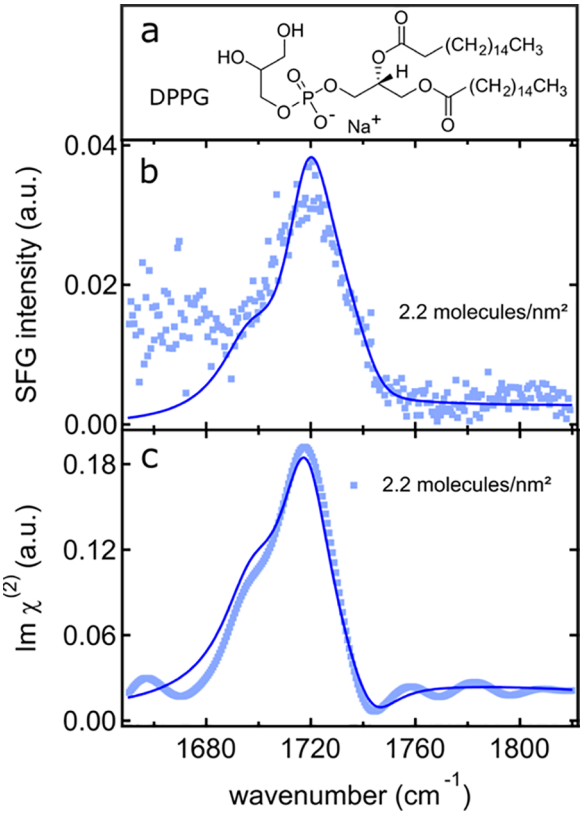


Figure 4. (a) Molecular structure of the negatively charged lipid DPPG, (b) intensity and (c) Im $\chi^{(2)}$ spectra of the DPPG–D_2O interface at a lipid coverage of 2.2 molecules/nm^2. The dotted lines in the panels b and c represent the experimentally acquired spectra and the solid lines the fits.

CONCLUSIONS

We have shown phase-resolved SFG spectra of the lipid carbonyl groups of DPTAP and DPPG monolayers on D_2O at different lipid coverages. Our results indicate that there are two differently oriented carbonyl groups in the monolayer. The low/high frequency of the positive/negative signal strongly suggests that the orientation affects the ability of the carbonyl groups to form hydrogen bonds. For DPTAP, the frequency of the resonance as well as the area of the signals changes substantially upon changing the lipid coverage. The frequency shift together with the change in the signal area indicates that there is an increase in the amount of "free" carbonyl groups upon increasing the lipid density. This is consistent with the previously reported change in hydration state that had been inferred from observed shifts in the phosphate signal from the lipid headgroup upon changing the lipid coverage.[19]

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Yuki Nagata and Christoph Bernhard for fruitful discussions. L.B.D. is a recipient of a position funded by the Deutsche Forschungsgemeinschaft through the Excellence Initiative by the Graduate School Materials Science in Mainz (GSC 266). This work is funded by a DFG grant (BA 5008/3).

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