Observation of buried water molecules in phospholipid membranes by surface sum-frequency generation spectroscopy

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We investigate the structure and orientation of water molecules at the water-lipid interface, using vibrational sum-frequency generation in conjunction with a maximum entropy phase retrieval method. We find that interfacial water molecules have an orientation opposite to that predicted by electrostatics and thus are likely localized between the lipid headgroup and its apolar alkyl chain. This type of water molecule is observed for phospholipids but not for structurally simpler surfactants. © *2009 American Institute of Physics*. [doi:10.1063/1.3257600]

Biological membranes are the barrier between the interior and the exterior of the cell, and support many proteins involved in important cell functions. It is becoming increasingly clear that water plays a key role in the structure, stability, function, and dynamics of biological membranes.[1] Despite the importance of water, many questions remain regarding its structure near cell membranes; in particular, the relationship of water structure and lipid headgroup chemistry and charge. Changes in lipid headgroup structure are expected to alter the interfacial water structure, but these effects, while important for a molecular understanding of membrane phase, are challenging to observe directly. We demonstrate here that the interfacial ordering of water depends intricately on details of the lipid headgroup. Our results strongly suggest the existence of "buried" water molecules located within phospholipid headgroups, but absent in structurally simpler surfactants.

We employ vibrational sum-frequency generation (VSFG) spectroscopy[2–5] to probe membrane-bound water. Owing to its unique selection rules, VSFG is able to probe the outermost few molecular layers at the surface, where the local symmetry is broken, making it ideal to investigate water molecules near lipid headgroups. VSFG can provide information about both the structure and the orientation of the interfacial water molecules by probing the water OH stretch modes, the frequency of which strongly depends on the water hydrogen bond strength, and therefore on the local environment. The water orientation determines the sign of the nonlinear optical susceptibility $\chi^{(2)}$, which governs the VSFG process.[3,4] This sign of $\chi^{(2)}$ can be determined by phase-resolved measurements (the interference of a VSFG signal from a sample and a well-characterized reference in the laboratory)[6] or retrieved through application of a numerical maximum entropy phase retrieval algorithm (MEM) from a single VSFG spectrum of a sample containing multiple interfering vibrational modes.[4,7] Using the latter approach, we here compare the water orientation and structure at different phospholipid and simple surfactant monolayers.

The VSFG setup has been described in detail elsewhere.[8] Briefly, the visible beam (VIS) (800 nm, ~30 μJ/pulse, spectral bandwidth of 25 cm^{-1}) is overlapped at the sample position with ~5 μJ/pulse, 150 fs infrared (IR) beam. The IR wavelength is continuously tuned from 3570 to 5110 nm, such that both the O–D and the C–H stretch vibrational region are covered. Both beams are focused down to a ~100 μm beamwaist. The incident angles of VIS and IR are, respectively, 35° and 40°, both defined relative to the surface normal. The SFG light generated is detected using a monochromator connected to a charge coupled device camera. All spectra shown here are collected under *s*-polarized SFG, *s*-polarized VIS, and *p*-polarized IR conditions. Spectra obtained are normalized to a reference signal from a z-cut quartz plate.

The lipid monolayer preparation procedure is described in detail elsewhere.[4,8] Here, lipid monolayers of lauric acid (LA) (anionic), octadecyl trimethyl ammonium bromide (OTAB) (cationic), 1,2-Myristoyl-*sn*-glycerol-3-phosphoserine (DMPS) (anionic), *L*-1,2-Dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) (zwitterionic), *L*-1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphoethanolamine (DPPE) (zwitterionic), and *L*-1,2-Dipamitoyl-3-trimethylammonium-propane (DPTAP) (cationic) were formed on phosphate buffer subphase (*p*H=7) containing D_2O (Cambridge Isotope Laboratories, 99.93% purity) at a surface pressure of ~20 mN/m. The corresponding area per molecule for OTAB, LA, DPTAP, DMPS, DPPC, and DPPE, are 60, 54, 57, 41, 51, and 41 $Å^2$, respectively. The surfactants and the lipids were purchased from Sigma-Aldrich and Avanti Polar Lipids, respectively. The concentration of ions in the water subphase is very low (<100 μM for all systems reported here), and counterions therefore do not significantly affect the structure or the orientation of the water molecules at the surfactant-water and lipid-water interface. The reason for this is that entropic effects, causing counterions to reside in the bulk, dominate at such low concentrations. We have shown previously[9] that for ions to affect the interfacial water structure, concentrations in the milliMach range are required.

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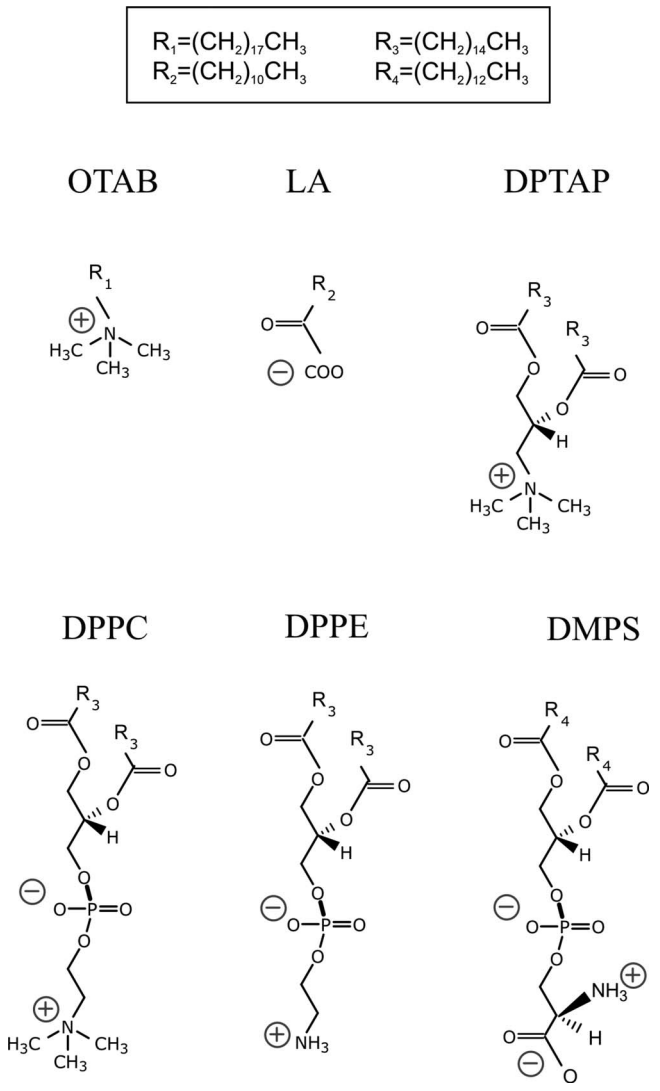

FIG. 1. The chemical structures of relatively simple surfactants OTAB, LA, and DPTAP (upper row), and more complex phospholipids DPPC, DPPE, and DMPS (lower row).

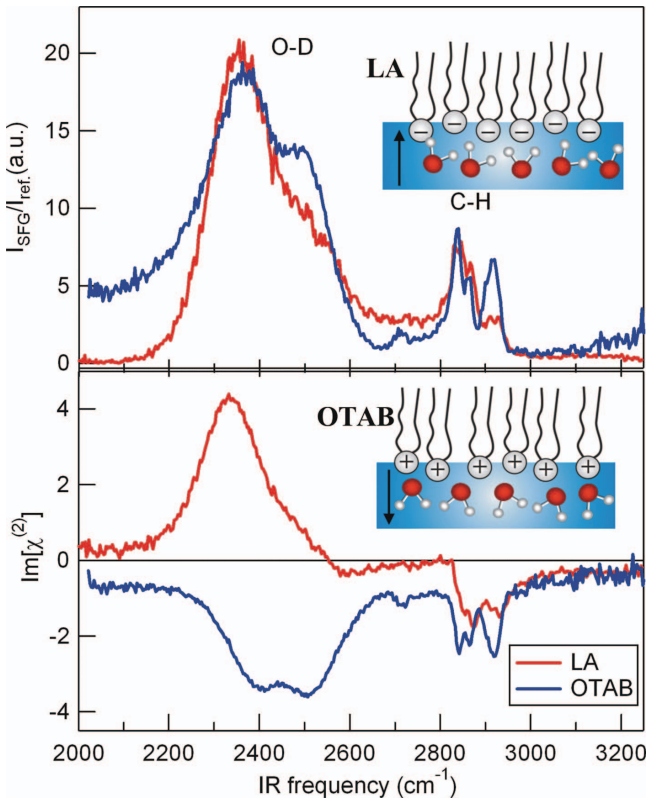

FIG. 2. Top panel: The $|\chi^{(2)}|^2$ spectra for LA (red) and OTAB (blue), respectively. Bottom panel: The corresponding imaginary part of the nonlinear susceptibility Im[$\chi^{(2)}$] obtained from the MEM analysis, as described in the supplementary material. Insets: Schematic representation of water orientation at surfactant-water interface. The arrow indicates the orientation of the electric field below the headgroup region.

The chemical structure of the surfactants and lipids used in this study are shown in Fig. 1 in approximate order of increasing structural complexity. SFG spectra for the simple surfactants LA (anionic) and OTAB (cationic) monolayers self-assembled on D_2O are displayed in Fig. 2. For both surfactants, the charge of the headgroup gives rise to an enhanced SFG signal when compared to neutral interfaces (e.g., the water-air interface,[3,10] see supporting information) due to (i) alignment of water molecules induced by the surfactant charge in the double layer region and (ii) possible contributions from third-order nonlinear optical effects due to the presence of a static field.[2,11] The latter contribution is relatively small, as evidenced by the relatively small nonresonant signal. The sharp peaks observed at the blue side of the spectra originate from the C–H stretch vibrations of the lipid chains: The CH_3 symmetric stretch, Fermi resonance, and asymmetric stretch at 2875, 2945, and 2960 cm^{-1}.[4,12]

Our MEM is used to infer the sign of the water contribution to $\chi^{(2)}$, which contains information about the orientation.[4] This is most easily observed from the imaginary part of $\chi^{(2)}$, Im[$\chi^{(2)}$], shown for both surfactants in the lower panel of Fig. 2. The obtained amplitudes of the CH_3 symmetric stretch (CH_3-*ss*) are negative for both surfactants, corresponding in each case to an orientation of the CH_3 symmetric stretch dipole moment away from the interface. This result is both in good agreement with our expectations—the surfactant tail orientation should be relatively insensitive to headgroup charge—and with literature results.[13] In the O–D stretch region, on the other hand, the values of Im[$\chi^{(2)}$] change sign with headgroup charge, corresponding to an inversion of the interfacial water orientation, as has been reported previously for simple, charged surfactants.[13] For the anionic LA monolayers, the water orients with their positively charged hydrogen atoms toward the surface, while for the cationic OTAB monolayers the orientation is opposite, as expected from simple electrostatics (see cartoons in Fig. 2). We note here that for both spectral regions and both surfactants, our phase retrieval analysis results in precisely the same Im[$\chi^{(2)}$] spectra as direct phase-resolved VSFG measurements,[13] lending further credence to this analysis technique.

Figure 3 displays the VSFG spectra for four lipids: Anionic DMPS, zwitterionic DPPC, zwitterionic DPPE, and cationic DPTAP. The corresponding Im[$\chi^{(2)}$] spectra indicate that the water molecules for all four cases are oriented as for the cationic OTAB, although both the surface charge and molecular structures are very different. Absent these data one might expect that the electric field created by the charged

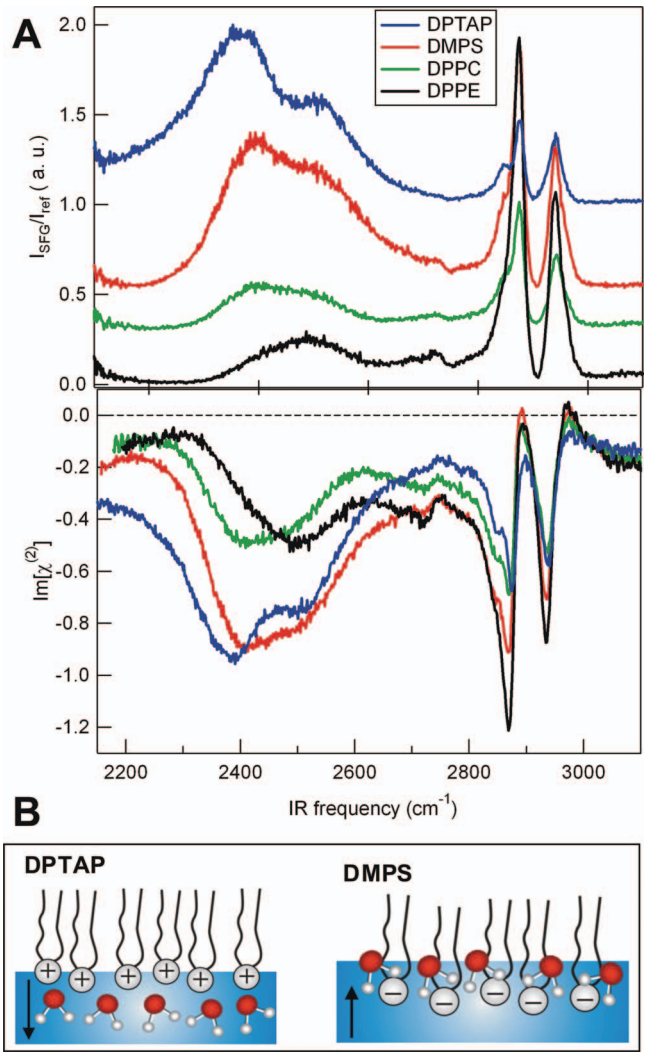

FIG. 3. (a) Top panel: The $|\chi^{(2)}|^2$ spectra for DPTAP (blue), DMPS (red), DPPC (green), and DPPE (black), respectively. Data are offset for clarity. Bottom panel: The corresponding imaginary part of the nonlinear susceptibility $\mathrm{Im}[\chi^{(2)}]$ obtained from the MEM analysis. (b) Lower panel: Schematic representation of water orientation at lipid-water interface, showing only the water molecules probed by VSFG. The arrow indicates the orientation of the electric field below the headgroup region.

interface would orient the water molecules in opposite direction when going from DPTAP (cationic) to DMPS (anionic) monolayers, as occurs when going from the cationic OTAB to the anionic LA. For cationic DPTAP monolayers, the water molecules are oriented as for cationic OTAB monolayers, in both cases as expected from electrostatics. At the negatively charged DMPS monolayer, on the other hand, water molecules orient in an *opposite* fashion to that expected from electrostatics. Furthermore, comparison of the extracted $\mathrm{Im}[\chi^{(2)}]$ spectra of interfacial water near DMPS with that near the zwitterionic phospholipids DPPE and DPPC highlights that for all these phospholipids, which differ in headgroup structure and charge, water orientation is the same. These observations can be explained as follows. DPTAP is not a phopholipid and has a headgroup structure that is both simpler than DMPS, DPPE, and DPPC and similar in complexity to the surfactants LA and OTAB (see Fig. 1). For LA, OTAB, and DPTAP, our data can be rationalized if we are detecting the water molecules situated beneath the headgroup: Those that terminate the bulk.

DPPC, DPPE, and DMPS, the three phospholipids, have a more complex head group structure. Previous studies of these and other phospholipids have demonstrated that water penetrates the headgroup region up to the lipid chains, i.e., above the phosphate group, thus suggesting interfacial water at phospholipid monolayers may have a variety of possible hydrogen bonding environments.[14,15] However, our results suggest that for each phospholipid interfacial water is oriented the same: With its O–H groups pointing toward bulk water. The similarity of the VSFG response for these different types of phospholipids can be most simply explained if, in each case, we are sensitive to water above the phosphate group, oriented such that its dipole points toward bulk water[16] [see Fig. 3(b)]. The water density in this region is low compared to the bulk, but only by a factor of ~2,[14] so that water molecules can still hydrogen bond with neighboring water molecules and/or with the lipid headgroup. These water molecules will give rise to a broad vibrational response typical of hydrogen-bonded water, as each lipid is hydrated by 10–20 water molecules.[17] These buried water molecules are present through all range of surface pressures, as independent measurements show that the signal in the O–D stretch region does not change significantly as the lipid monolayer is compressed (data not shown).

Our interpretation, that water in contact with the net neutral, zwitterionic lipids DPPC and DPPE is oriented in the same fashion as water in contact with the anionic DMPS (Fig. 3) and that this water is situated above the phosphate group, can be rationalized from reference to the structure of each lipid. As Fig. 1 makes clear, in the region from the phosphate group to the hydrophobic tail the structure of all the phospholipids (i.e., DPPC, DPPE, and DMPS) is the same, thus suggesting, in agreement with our observations, water situated in this region should behave similarly for PC, PE, and PS. Note, that while this is the simplest way to interpret these results, our data are also consistent with a scenario in which water is oriented with its O–H groups pointing toward the bulk but is positioned just below the choline group (in the case of DPPC) and the amine group (in the case of DPPE and DMPS). In this scenario interfacial water molecules likely experience both different hydrogen bond donors (i.e., the choline and amine group) as well as possibly different hydrogen bond acceptors (i.e., the deprotonated carboxyl group and other water molecules) with changing lipid type. These interactions produce, in each case, water that is similarly oriented with the ones situated above the phosphate region. Such a scenario is possible, and it cannot be excluded based on the current data set, but is not likely to contribute greatly to our signal, considering that the headgroup charges are all oriented in the plane of the interface.[18]

As discussed above, our findings strongly suggest that for the phospholipid monolayers buried water molecules exist. VSFG is very sensitive to this type of water; more sensitive, it appears, than to the water underneath the headgroup region, which is oriented oppositely to the buried.[16] One might expect a larger contribution to the measured VSFG

spectral amplitudes from water near the bulk, as the water density below the headgroup is large compared to the water density above.[19] However, the charge-induced alignment of water molecules is strongly dependent on the local relative dielectric permittivity ε_r.[20] It has been shown that the dielectric permittivity has very large values in the region below the lipid headgroup, reaching several hundreds, compared to the value of 80 for bulk water.[20] This high local dielectric response is caused by the dipoles associated with the headgroup charges, and results in efficient screening of these charges, giving rise to less oriented water molecules below the headgroup. Above the headgroup, in the region of the alkyl chains, the dielectric function is low ($\varepsilon_r=3$),[20] and water will be strongly oriented.

Although the details of the spectral shape depend on the MEM analysis (see supporting information[21]), and Fermi resonance further complicates the SFG spectrum,[10] we can use the retrieved $\mathrm{Im}[\chi^{(2)}]$ spectra to determine the strength of the H-bond of the water molecules near the different lipid monolayers. Within the variation in the $\mathrm{Im}[\chi^{(2)}]$ spectra shown in Fig. 3, it is evident that the first moment of the O–D intensity distribution reveals that the H-bond strength increases in the order PE-PC-PS-TAP. This observation confirms the intuition that water molecules situated below the lipid headgroup where the water density is large (as for DPTAP) have stronger H-bonds than the buried water situated above the headgroup where the water density decreases (as it is the case for the phospholipids). The trend is also consistent with weaker hydration of PE lipids when compared to PC and PS lipids.[17] Furthermore, from the width of the two prominent peaks in the O–D stretch region, we can determine the heterogeneity in the interfacial water structure. The retrieved $\mathrm{Im}[\chi^{(2)}]$ spectra show broader peaks for the charged lipids when compared to the zwitterionic phospholipids. This is an indication that the water structure is more heterogeneous in the vicinity of the charged headgroups. An explanation for this observation may lie in the fact that the field acts to orient the dipole of the water molecules (bisecting the two O–H bonds), possibly giving rise to suboptimal orientation of some of the individual O–H bonds with respect to their H-bonding partners. Such a competition would result in a more heterogeneous water structure.

In conclusion, our results show that water molecules reverse their orientation when going from negative to positive surfactant-water interface. For phospholipid monolayers, we experimentally reveal the existence of buried water molecules, situated between the lipid headgroups and their alkyl chains that have a weaker H-bond network than that of bulk water. Those interfacial water molecules clearly do not simply terminate the bulk. Their presence illustrates, once again, the complexity of biological aqueous interfaces.

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[1] P. M. Wiggins, Microbiol. Rev. **54**, 432 (1990); B. Kasemo, Surf. Sci. **500**, 656 (2002).
[2] D. E. Gragson and G. L. Richmond, J. Am. Chem. Soc. **120**, 366 (1998).
[3] Q. Du, R. Superfine, E. Freysz, and Y. R. Shen, Phys. Rev. Lett. **70**, 2313 (1993).
[4] M. Sovago, E. Vartiainen, and M. Bonn, J. Phys. Chem. C **113**, 6100 (2009).
[5] O. Esenturk and R. A. Walker, J. Chem. Phys. **125**, 174701 (2006); M. M. Knock, G. R. Bell, E. K. Hill, H. J. Turner, and C. D. Bain, J. Phys. Chem. B **107**, 10801 (2003); S. Roke, J. Schins, M. Muller, and M. Bonn, Phys. Rev. Lett. **90**, 128101 (2003); G. Ma and H. C. Allen, Langmuir **22**, 5341 (2006).
[6] N. Ji, V. Ostroverkhov, C. S. Tian, and Y. R. Shen, Phys. Rev. Lett. **100**, 096102 (2008); V. Ostroverkhov, G. A. Waychunas, and Y. R. Shen, *ibid.* **94**, 046102 (2005).
[7] P. K. Yang and J. Y. Huang, J. Opt. Soc. Am. B **14**, 2443 (1997).
[8] M. Sovago, G. W. H. Wurpel, M. Smits, M. Muller, and M. Bonn, J. Am. Chem. Soc. **129**, 11079 (2007).
[9] G. W. H. Wurpel, M. Sovago, and M. Bonn, J. Am. Chem. Soc. **129**, 8420 (2007).
[10] M. Sovago, R. K. Campen, G. W. H. Wurpel, M. Muller, H. J. Bakker, and M. Bonn, Phys. Rev. Lett. **100**, 173901 (2008).
[11] P. L. Hayes, E. H. Chen, J. L. Achtyl, and F. M. Geiger, J. Phys. Chem. A **113**, 4269 (2009); X. L. Zhao, S. W. Ong, and K. B. Eisenthal, Chem. Phys. Lett. **202**, 513 (1993); S. W. Ong, X. L. Zhao, and K. B. Eisenthal, *ibid.* **191**, 327 (1992).
[12] P. Guyot-Sionnest, J. H. Hunt, and Y. R. Shen, Phys. Rev. Lett. **59**, 1597 (1987).
[13] S. Nihonyanagi, S. Yamaguchi, and T. Tahara, J. Chem. Phys. **130**, 204704 (2009); M. Oh-e, A. I. Lvovsky, X. Wei, and Y. R. Shen, *ibid.* **113**, 8827 (2000).
[14] F. Zhou and K. Schulten, J. Phys. Chem. **99**, 2194 (1995).
[15] V. V. Volkov, D. J. Palmer, and R. Righini, J. Phys. Chem. B **111**, 1377 (2007); P. Jedlovszky and M. Mezei, *ibid.* **105**, 3614 (2001); K. V. Damodaran and K. M. Merz, Langmuir **9**, 1179 (1993).
[16] G. A. Yeghiazaryan, A. H. Poghosyan, and A. A. Shahinyan, Physica A **362**, 197 (2006).
[17] T. J. McIntosh and S. A. Simon, Biochemistry **25**, 4948 (1986).
[18] H. Dominguez, A. M. Smondyrev, and M. L. Berkowitz, J. Phys. Chem. B **103**, 9582 (1999).
[19] R. A. Bockmann, A. Hac, T. Heimburg, and H. Grubmuller, Biophys. J. **85**, 1647 (2003).
[20] H. A. Stern and S. E. Feller, J. Chem. Phys. **118**, 3401 (2003).
[21] See EPAPS supplementary material at http://dx.doi.org/10.1063/1.3257600 for sum-frequency generation theory and maximum entropy method.

Erratum: "Observation of buried water molecules in phospholipid membranes by surface sum-frequency generation spectroscopy" [J. Chem. Phys. 131, 161107 (2009)]

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In our recent paper, we reported the counterintuitive orientation of water at charged lipid monolayer model membranes,[1] i.e., interfacial water molecules having an orientation opposite to that predicted by electrostatics. Since this type of water molecule was observed for phospholipids but not for structurally simpler surfactants, we concluded that most likely this water is localized between the lipid headgroup and its apolar alkyl chain-buried water. This conclusion was drawn from an analysis of Sum-Frequency Generation (SFG) spectra using the maximum entropy method (MEM),[2] which allows for phase retrieval from intensity spectra. Knowledge of the phase of the SFG signal is a prerequisite for obtaining insights into the absolute orientation of interfacial water from SFG spectra.

A recent paper by Nagata and Mukamel,[3] reporting an MD simulation of water in contact with lipid monolayer and the resulting calculated SFG spectra was at odds with our conclusions. Moreover, two recent experimental papers by the Tahara[4] and Allen[5] groups, who performed phase-resolved (heterodyne detected) SFG measurements on water in contact with very similar lipid monolayers, also showed that the orientation of interfacial water at the lipid interfaces is intuitive and for charged lipids it is the same as for charged surfactant/water interfaces.

As shown in Ref. 2 and the supporting information of Ref. 1, there are certain caveats and boundary conditions that have to be met for the successful application of the maximum entropy method to the analysis of SFG spectra (as opposed, for example, of using the same analysis tool to investigate coherent anti-Stokes Raman scattering spectra[6]). The recent reports in Refs. 3, 4, and 5 prompted us to reinvestigate the maximum entropy method procedure used in our analysis, as both the Mukamel, Tahara, and Allen results were consistent with a picture of water simply situated below the head group charge and oriented by the electrostatic interaction with that charge.

Our new analysis revealed the following two important points:

(1) There is an unexpected dependence of the retrieved phase on the order in which the data is analyzed—that is, whether it is analyzed in the order of increasing or decreasing frequency. If the original intensity spectrum I for N spectral points reads $I(i)$, $i = 1, \ldots, N$, then performing the analysis on the inverted function y defined as inversely, $y(1) = I(N)$, $y(2) = I(N-1), \ldots, y(N) = I(1)$, gives a different phase retrieval result: the results of the analysis with increasing or decreasing fre-

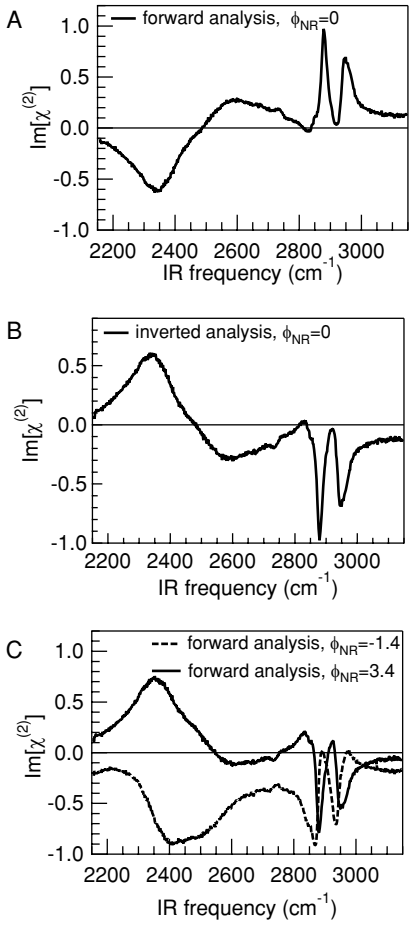

FIG. 1. The imaginary spectra obtained from MEM analyses of the SFG spectrum of a DMPS monolayer in the O–D and C–H stretch region. Panel A: "forward" analysis with increasing frequency input. Panel B: "reverse" analysis with increasing frequency input. Note that the uncorrected Im[$\chi^{(2)}$] spectra are identical when multiplied by a factor of –1. The upper trace requires a significant phase correction to meet the criteria for phase correction mentioned in the text. Panel C: the wrongly phase-corrected data ($\phi_{NR} = -1.4$), with a negative Im[$\chi^{(2)}$] signal in the O–D stretch region implies that water would be pointing "down." The correct phase-correction requires a larger correction ($\phi_{NR} = 3.4$) and gives results that are consistent with the heterodyne-detected SFG results:[4,5] the O–D groups are pointing "up."

quency are different by exactly a factor of π of the retrieved phase. This is equivalent to a multiplication of the retrieved imaginary part by –1, as illustrated in Figs. 1(a) and 1(b), which shows retrieved Im[$\chi^{(2)}$] spectra without any error phase correction ($\phi_{NR} = 0$). This dependence on the mode of analysis is typical of the fast-Fourier transform (FFT) involved in the analysis routine (and is present in all commercial FFT software we have checked), and was therefore also observed for the surfactants.

It means that the retrieved overall phase should be considered as the retrieved phase modulo π. This implies that *a priori* assumptions about the nonresonant phase, such as that the phase correction should be small owing to the absence of electronic—or other—resonances in the frequency regions of infrared, visible, and SFG fields should be made with extreme care. Analysis in both directions is possible but the nonresonant phase correction will be different by a factor of π.

(2) The caveats and criteria for correcting the raw phase retrieved from the MEM procedure to obtain the purely resonant phase, were mentioned in Ref. 2 and the supplementary information of Ref. 1. Briefly, the phase correction should meet the following criteria: first of all, for the systems studied here, the Im[$\chi^{(2)}$] should be negative in the C–H stretch region; second, Im[$\chi^{(2)}$] ≈ 0 in the regions without resonances; third, the C–H resonances must appear as (negative) peaks in the Im[$\chi^{(2)}$] at frequencies known with an accuracy of a few inverse centimeters, e.g., for CH_3 symmetric stretch the peak position is around 2875 cm^{-1}. If we apply these criteria, *without any a priori assumptions about the nonresonant phase (see point 1)*, we conclude that a phase correction close to π (3.4) should be applied to the forward-corrected data [see Fig. 1(c)], rather than the value of –1.4 we concluded in our original manuscript. The retrieved Im[$\chi^{(2)}$] spectrum appropriately corrected for the nonresonant phase now corresponds well to the results obtained using experimental approaches to determine the Im[$\chi^{(2)}$] spectrum and are consistent with an intuitive orientation of the interfacial water, with O–D groups pointing "up."

These results reiterate our previous conclusion[2] that MEM can be a very useful addition to the toolbox of SFG data analysis but that the nonresonant phase correction should be performed with great care.

[1]M. Sovago, E. Vartiainen, and M. Bonn, J. Chem. Phys. **131**, 161107 (2009).
[2]M. Sovago, E. Vartiainen, and M. Bonn, J. Phys. Chem. C **113**, 6100 (2009).
[3]Y. Nagata and S. Mukamel, J. Am. Chem. Soc. **132**, 6434 (2010).
[4]J. A. Mondal, S. Nihonyanagi, S. Yamaguchi, and T. Tahara, J. Am. Chem. Soc. **132**, 10656 (2010).
[5]X. Chen, W. Hua, Z. Huang, and H. C. Allen, J. Am. Chem. Soc. **132**, 11336 (2010).
[6]E. M. Vartiainen, H. A. Rinia, M. Muller, and M. Bonn, Opt. Express **14**, 3622 (2006).