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Interfacial Water Structure Associated with Phospholipid Membranes Studied by Phase-Sensitive Vibrational Sum Frequency Generation Spectroscopy

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Abstract: Phase-sensitive vibrational sum frequency generation is employed to investigate the water structure at phospholipid/water interfaces. Interfacial water molecules are oriented preferentially by the electrostatic potential imposed by the phospholipids and have, on average, their dipole pointing toward the phospholipid tails for all phospholipids studied, dipalmitoyl phosphocholine (DPPC), dipalmitoyl phosphoethanolamine (DPPE), dipalmitoyl phosphate (DPPA), dipalmitoyl phosphoglycerol (DPPG), and dipalmitoyl phospho-L-serine (DPPS). Zwitterionic DPPC and DPPE reveal weaker water orienting capability relative to net negative DPPA, DPPG, and DPPS. Binding of calcium cations to the lipid phosphate group reduces ordering of the water molecules.

Introduction

Biological membranes are self-assembled bilayers of biomolecules such as lipids, proteins, and carbohydrates.[1] Among such complicated structures, phospholipids are the major constituents of the membrane structural backbone,[2] and the phospholipid composition greatly affects membrane functions and properties. The origin of the unique ability of a phospholipid to form self-assemblies arises from the amphiphilic nature of its structure and its interaction with the polar water environment. Phospholipids have similar two-part structures, the hydrophobic tail groups and the hydrophilic headgroup. The tail groups are the alkyl chains of varying lengths that congregate together in the central portion of the bilayer, allowing these hydrophobic entities to escape from the surrounding polar environment. The headgroup of a phospholipid is a negatively charged phosphate linked to a diverse set of functional groups that are immersed in the aqueous medium and thought to be well hydrated. Therefore, it is recognized that water molecules in the region of biomembranes play an important role in the biomembrane structure and function.[3−6]

Different experimental techniques have been employed to study phospholipids and associated water. To simplify the complicated natural biomembrane composition, model membrane bilayers or monolayers containing one type of phospholipid or a mixture of different types are often used. Previous NMR spectroscopic studies revealed that the ordering and dynamics of water near phospholipid head groups are changed upon lipid hydration.[7,8] X-ray and neutron scattering techniques have elucidated the positions and distances between phospholipid bilayers and provide information on membrane-membrane interactions and the hydration forces.[9,10] Infrared (IR) vibrational spectroscopy is a method sensitive to characteristic chemical bond strength and interactions and is used extensively to study the hydration sites of lipids and the hydrogen bonding network of water affected by the lipid head groups.[11−14] It has been found that the water molecules that reside near the head groups are hydrogen donors and form strong hydrogen bonds with the lipid phosphate and carbonyl groups. Concurrently, the electrostatic potential due to the charges on the headgroup leads to ordering of water dipoles and results in stronger hydrogen bonds between neighboring water molecules. In addition to experimental studies, molecular dynamics (MD) simulations provide more insight on the structure and physical properties of the phospholipid/water interface.[4,6,15−19] For example, water molecules are suggested to form stronger hydrogen bonds with the lipid phosphate groups than the carbonyl groups and the most

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(1) Zubay, G. *Biochemistry*, 2nd ed.; Macmillan: New York, 1988; p 154.
(2) Singer, S. J.; Nicolson, G. L. *Science* **1972**, *175*, 720.
(3) Rand, R. P.; Parsegian, V. A. *Biochim. Biophys. Acta* **1989**, *988*, 351.
(4) Gawrisch, K.; Ruston, D.; Zimmerberg, J.; Parsegian, V. A.; Rand, R. P.; Fuller, N. *Biophys. J.* **1992**, *61*, 1213.
(5) Milhaud, J. *Biochim. Biophys. Acta-Biomembr.* **2004**, *1663*, 19.
(6) Berkowitz, M. L.; Bostick, D. L.; Pandit, S. *Chem. Rev.* **2006**, *106*, 1527.
(7) Volke, F.; Eisenblatter, S.; Galle, J.; Klose, G. *Chem. Phys. Lipids* **1994**, *70*, 121.
(8) Konig, S.; Sackmann, E.; Richter, D.; Zorn, R.; Carlile, C.; Bayerl, T. M. *J. Chem. Phys.* **1994**, *100*, 3307.
(9) Fitter, J.; Lechner, R. E.; Dencher, N. A. *J. Phys. Chem. B* **1999**, *103*, 8036.
(10) Nagle, J. F.; Tristram-Nagle, S. *Biochim. Biophys. Acta-Rev. Biomembr.* **2000**, *1469*, 159.
(11) Hubner, W.; Blume, A. *Chem. Phys. Lipids* **1998**, *96*, 99.
(12) Pohle, W.; Selle, C.; Fritzsche, H.; Binder, H. *Biospectroscopy* **1998**, *4*, 267.
(13) Binder, H. *Appl. Spectrosc. Rev.* **2003**, *38*, 15.
(14) Binder, H. *Eur. Biophys. J. Biophys. Lett.* **2007**, *36*, 265.
(15) Alper, H. E.; Bassolinoklimas, D.; Stouch, T. R. *J. Chem. Phys.* **1993**, *99*, 5547.
(16) Marrink, S. J.; Berkowitz, M.; Berendsen, H. J. C. *Langmuir* **1993**, *9*, 3122.
(17) Zhou, F.; Schulten, K. *J. Phys. Chem.* **1995**, *99*, 2194.
(18) Jedlovszky, P.; Mezei, M. *J. Phys. Chem. B* **2001**, *105*, 3614.
(19) Lopez, C. F.; Nielsen, S. O.; Klein, M. L.; Moore, P. B. *J. Phys. Chem. B* **2004**, *108*, 6603.

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probable sites to accept a hydrogen bond are the double bonded oxygens connected to the phosphorus atom.[19] Moreover, at the phospholipid/water interface, water is thought to be perturbed up to 1 nm away by the dipole potential of the headgroup.[16] Despite ongoing research, direct probing of water molecules at the biomembrane interface has been relatively rare.

Vibrational sum frequency generation (VSFG), an interface-specific spectroscopy, is well suited as a probe to directly study water orientation at the phospholipid/water interface, as is shown here. VSFG spectroscopy is a second-order nonlinear optical spectroscopy that requires lack of inversion symmetry. This unique selection rule enables VSFG spectroscopy to probe the topmost few layers at an interface where the inversion symmetry is naturally broken.[20] To drive a VSFG process, two pulsed laser beams, one visible together with an infrared, are used. The VSFG signal is enhanced when the infrared frequency matches the vibrational frequency of an interfacial species, hence giving characteristic molecular information. Moreover, the VSFG response is sensitive to orientation and ordering of surface species, making it an ideal technique for direct probing of the biomembrane/water interface. Application of VSFG has already been well demonstrated on the neat air/water interface[21−30] and phospholipid/water interfaces.[31−35] For VSFG spectra of the neat water surface, the dangling OH bond of surface water molecules with the other OH bond hydrogen bonded to other interfacial water molecules is observed at 3700 cm^{-1}. In addition, hydrogen bonded OH modes (from 3000 to 3600 cm^{-1}) are clearly seen from the neat water surface spectra, although the detailed spectral assignments relating to specific water structures have not yet been fully agreed upon.[23,24,27−29] When the water surface is covered with a phospholipid monolayer, dangling OH bonds decrease significantly but still persist at the surface with a red-shifted frequency.[36] However, the VSFG signal in the hydrogen bonded region increases by many times in the presence of various kinds of phospholipids, providing evidence of an ordering of water molecules by the phospholipid head groups.[32]

Although much information about the lipid/water interface has been revealed by VSFG spectroscopy, the important question, namely the average orientation of water molecules at the lipid/water interface, is hardly answered. Whether the net interfacial water dipole is pointing toward the lipid tails or the bulk water interior determines the sign of the complex second-order nonlinear susceptibility, $\chi^{(2)}$, of surface modes. A conventional VSFG intensity spectrum is proportional to the absolute square of the nonlinear susceptibility, $|\chi^{(2)}|^2$; hence it does not distinguish the net water dipole direction. Recently, a phase-sensitive VSFG (PS-VSFG) method based on interference of the sample response with an additional signal of phase reference has been developed by Shen et al., which enables absolute determination of the sign of the second-order nonlinear susceptibilities of the water surface.[27,37,38] The PS-VSFG technique provides the imaginary part of the complex nonlinear susceptibility, Im $\chi^{(2)}$. PS-VSFG data clearly reveal that while the dangling OH bonds of a neat water surface are pointing out into the air side of the interface, the OH bonds that contribute to the hydrogen bonded OH stretching region of the VSFG spectrum are made up of both OH bonds pointing away from and toward the bulk aqueous phase. A brief description of PS-VSFG is shown in eqs 1−3.

$$I_{\mathrm{SFG}} \propto |\chi^{(2)}|^2 I_{\mathrm{vis}} I_{\mathrm{IR}} \propto |\chi^{(2)}_{\mathrm{NR}} + \sum_{\nu} \chi^{(2)}_{\nu}|^2 I_{\mathrm{vis}} I_{\mathrm{IR}} \tag{1}$$

$$\chi^{(2)}_{\nu} = \frac{A_{\nu}}{\omega_{\mathrm{IR}} - \omega_{\nu} + i\Gamma_{\nu}} \tag{2}$$

$$\mathrm{Im}\,\chi^{(2)} = -\sum_{\nu} \frac{A_{\nu}\Gamma_{\nu}}{(\omega_{\mathrm{IR}} - \omega_{\nu})^2 + \Gamma_{\nu}^2} \tag{3}$$

I_{SFG}, I_{vis} and I_{IR} are the intensities of output SFG, incident visible and IR pulsed laser beams. $\chi_{\mathrm{NR}}{}^{(2)}$, $\chi_{\nu}{}^{(2)}$ and $\chi^{(2)}$ are the nonresonant, resonant and total second-order nonlinear susceptibilities, respectively. The resonant enhancement occurs when the frequency of the incident IR is resonant with a vibrational mode of a molecule. A_{ν} is the transition moment strength, ω_{IR} is the incident infrared frequency, ω_{ν} is the frequency of the vibrational transition, and Γ_{ν} is the line width of the vibrational transition. The Im $\chi^{(2)}$ contains information of the sign of the second-order nonlinear susceptibility.

Neat water PS-VSFG spectra, similar to Shen et al., were reported by Tahara and co-workers using a broadband SFG system.[30] In addition to direct experiment determination of phase, Bonn et al. reported a phase retrieval algorithm applied to a conventional VSFG spectrum containing interfered modes from the phospholipid/water interface.[39] From the phase VSFG spectra deduced from the phase retrieval algorithm, they unexpectedly identified, albeit incorrectly identified as experimentally shown here, that water molecules orient with their hydrogens pointing *down* toward the bulk at a negatively charged phospholipid monolayer interface.[40] To explain this behavior which is contrary with the electrostatic interactions between phospholipid head groups and water, they suggested that the water molecules probed are above the headgroup, hence with their hydrogens still pointing to the negatively charged headgroup. However, this result is counterintuitive and is also not in agreement with the more recent MD simulation of VSFG

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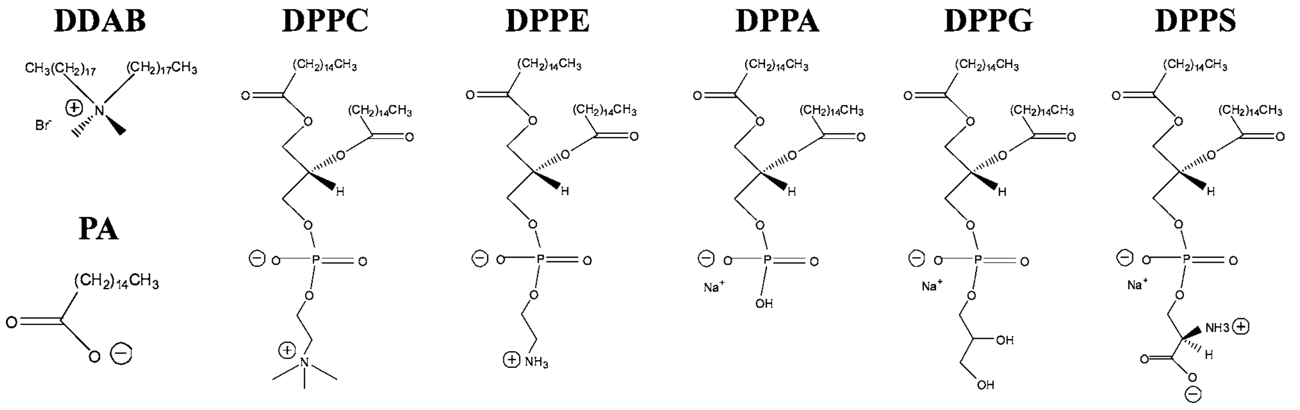
(20) Lambert, A. G.; Davies, P. B.; Neivandt, D. J. *Appl. Spectrosc. Rev.* **2005**, *40*, 103.
(21) Du, Q.; Superfine, R.; Freysz, E.; Shen, Y. R. *Phys. Rev. Lett.* **1993**, *70*, 2313.
(22) Gragson, D. E.; Richmond, G. L. *J. Phys. Chem. B* **1998**, *102*, 3847.
(23) Richmond, G. L. *Chem. Rev.* **2002**, *102*, 2693.
(24) Shen, Y. R.; Ostroverkhov, V. *Chem. Rev.* **2006**, *106*, 1140.
(25) Gopalakrishnan, S.; Liu, D. F.; Allen, H. C.; Kuo, M.; Shultz, M. J. *Chem. Rev.* **2006**, *106*, 1155.
(26) Gan, W.; Wu, D.; Zhang, Z.; Feng, R. R.; Wang, H. F. *J. Chem. Phys.* **2006**, *124*, 114705.
(27) Ji, N.; Ostroverkhov, V.; Tian, C. S.; Shen, Y. R. *Phys. Rev. Lett.* **2008**, *100*, 096102.
(28) Sovago, M.; Campen, R. K.; Wurpel, G. W. H.; Muller, M.; Bakker, H. J.; Bonn, M. *Phys. Rev. Lett.* **2008**, *100*, 173901.
(29) Fan, Y. B.; Chen, X.; Yang, L. J.; Cremer, P. S.; Gao, Y. Q. *J. Phys. Chem. B* **2009**, *113*, 11672.
(30) Nihonyanagi, S.; Yamaguchi, S.; Tahara, T. *J. Chem. Phys.* **2009**, *130*, 204704.
(31) Kim, J.; Kim, G.; Cremer, P. S. *Langmuir* **2001**, *17*, 7255.
(32) Watry, M. R.; Tarbuck, T. L.; Richmond, G. L. *J. Phys. Chem. B* **2003**, *107*, 512.
(33) Chen, X. Y.; Clarke, M. L.; Wang, J.; Chen, Z. *Int. J. Mod. Phys. B* **2005**, *19*, 691.
(34) Ma, G.; Allen, H. C. *Langmuir* **2006**, *22*, 5341.
(35) Sovago, M.; Wurpel, G. W. H.; Smits, M.; Muller, M.; Bonn, M. *J. Am. Chem. Soc.* **2007**, *129*, 11079.
(36) Ma, G.; Chen, X. K.; Allen, H. C. *J. Am. Chem. Soc.* **2007**, *129*, 14053.
(37) Ostroverkhov, V.; Waychunas, G. A.; Shen, Y. R. *Phys. Rev. Lett.* **2005**, *94*, 046102.
(38) Tian, C. S.; Shen, Y. R. *J. Am. Chem. Soc.* **2009**, *131*, 2790.
(39) Sovago, M.; Vartiainen, E.; Bonn, M. *J. Phys. Chem. C* **2009**, *113*, 6100.
(40) Sovago, M.; Vartiainen, E.; Bonn, M. *J. Chem. Phys.* **2009**, *131*, 161107.

Scheme 1. Molecular Structures of the Studied Fatty Acid, Surfactant, and Phospholipids



from the phospholipid/water interface, which suggests that water dipoles (dipole vector pointing toward the positive charge) are pointing *up* toward the (negatively charged) phospholipids.[41]

To reveal the water orientation at the phospholipid/water interface, we report here a direct PS-VSFG study of water structure associated with a series of phospholipid monolayers at the air/water interface. The five phospholipids that are studied here have differing headgroup structures and charge as shown in Scheme 1, which expectedly affect the nearby water structure. At physiological relevant neutral pH, dipalmitoyl phosphate acid (DPPA), dipalmitoyl phosphatidylglycerol (DPPG), and dipalmitoyl phosphatidylserine (DPPS) have net negatively charged head groups, while dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylethanolamine (DPPE) have zwitterionic head groups. We also examined water orientation in the case of simpler molecules, palmitic acid (PA, negatively charged after deprotonation), and dimethyldioctadecylammonium bromide (DDAB, positively charged) as a check for our phase determination.

Experimental Section

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphate sodium salt (DPPA), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol sodium salt (DPPG), 1,2-dipalmitoyl-*sn*-glycero-3-phospho-L-serine sodium salt (DPPS), dimethyldioctadecylammonium bromide (DDAB), and palmitic acid (PA) were purchased from Avanti Polar Lipids (Alabaster, AL). Spectrophotometric grade chloroform and methanol were purchased from Fisher Scientific and used as a mixed solvent for spreading of the phospholipids. The concentration of phospholipid stock solutions was ~1 mM. Deionized water (not purged of CO_2) with a resistivity of 18.2 $M\Omega\cdot cm$ and a measured pH of 5.5 was from a Barnstead Nanopure system.

Methods. Equilibrium Spreading of Phospholipids Monolayers. Phospholipids monolayers were overspread (~10 μL) on neat water in Petri-dishes (5 cm diameter). After spreading, 10 min was allowed for solvent evaporation and monolayer stabilization to equilibrium spreading pressure before PS-VSFG measurements. Under this condition, monolayers were equilibrated in the liquid condensed (LC) phase, which corresponds to a highly ordered monolayer on the water surface. When C16 PA was spread over pH 13 water, deprotonation and solvation of PA occurred, which resulted in decreasing VSFG signal with time.

Phase-Sensitive Vibrational Sum Frequency Generation (PS-VSFG). Our PS-VSFG system is similar to the design reported by Nihonyanagi et al.,[30] which is based on heterodyne detection of broad bandwidth signals and Fourier transform analysis (Supporting Information Figure S1). Our broad bandwidth VSFG system[34,42,43] was redesigned for this application. A titanium/sapphire oscillator (Spectra-Physics, Tsunami) with an optimal center wavelength at 792 nm and a sub-50 fs pulse width seeds two 1 kHz regenerative amplifiers (Spectra-Physics, Spitfire, femtosecond and picosecond versions) that are pumped by a solid state Nd/YLF laser (Spectra-Physics, Evolution 30) at 527 nm. The resulting laser beams from the two respective regenerative amplifiers are ~85 fs pulses at 792 nm (22 nm bandwidth, > 1 W) and 2 ps pulses at 792 nm (17 cm^{-1} bandwidth, > 500 mW, used as the visible beam). For the tunable broadband infrared generation in an optical parametric amplifier (Light Conversion, TOPAS), the broadband femtosecond laser pulses (~ 85 fs, > 1 W) are used to generate amplified parametric waves (signal and idler) via a BBO (beta barium borate) crystal using three general steps: superfluorescence generation, preamplification, and power amplification of the signal beam. The amplified signal and idler beams are then used to create an infrared beam via an $AgGaS_2$ crystal in a nonlinear difference-frequency generation system (Light Conversion, NDFG connected to the TOPAS). A 300 μJ visible beam (s-polarized, 792 nm) and a 10 μJ infrared beam (p-polarized, OH stretch region) were spatially and temporally overlapped at incident angles of 50° and 60° on sample stage 1 (for samples and z-cut quartz), respectively. The visible, infrared, and generated sum frequency (s-polarized) beams reflected from sample stage 1 were refocused by a gold concave mirror (f = 100 mm) onto a GaAs (Lambda Precision Optics) surface on sample stage 2 to generate another sum frequency beam (local oscillator, LO). The sum frequency beam from sample stage 1 was delayed ~2.5 ps by passing through an ~1 mm thick silica plate positioned before the gold concave mirror. The time difference between the sum frequency beams from two sample stages introduced an interference fringe in the frequency domain. This interferogram was stretched in a monochromator (Acton Research, SpectraPro SP-500 monochromator with a 1200 g/mm grating blazed at 750 nm) and then detected by a liquid-nitrogen cooled charge-coupled device (CCD) (Roper Scientific, 1340 × 400 pixel array, LN400EB back illuminated CCD). The height of the sample surface which is critical for accurate phase determination is also checked by the image on the CCD. Spectra are averaged over two consecutive runs of 5 min integration times. Final spectra are processed from the raw interferograms through Fourier transformation. The data processing procedure is available in the Supporting Information.

Results and Discussion

It is noteworthy to interpret the VSFG results from a neat water surface before discussing the phospholipid/water inter-

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(41) Nagata, Y.; Mukamel, S. *J. Am. Chem. Soc.* **2010**, *132*, 6434.

(42) Hommel, E. L.; Allen, H. C. *Anal. Sci.* **2001**, *17*, 137.

(43) Tang, C. Y.; Allen, H. C. *J. Phys. Chem. A* **2009**, *113*, 7383.

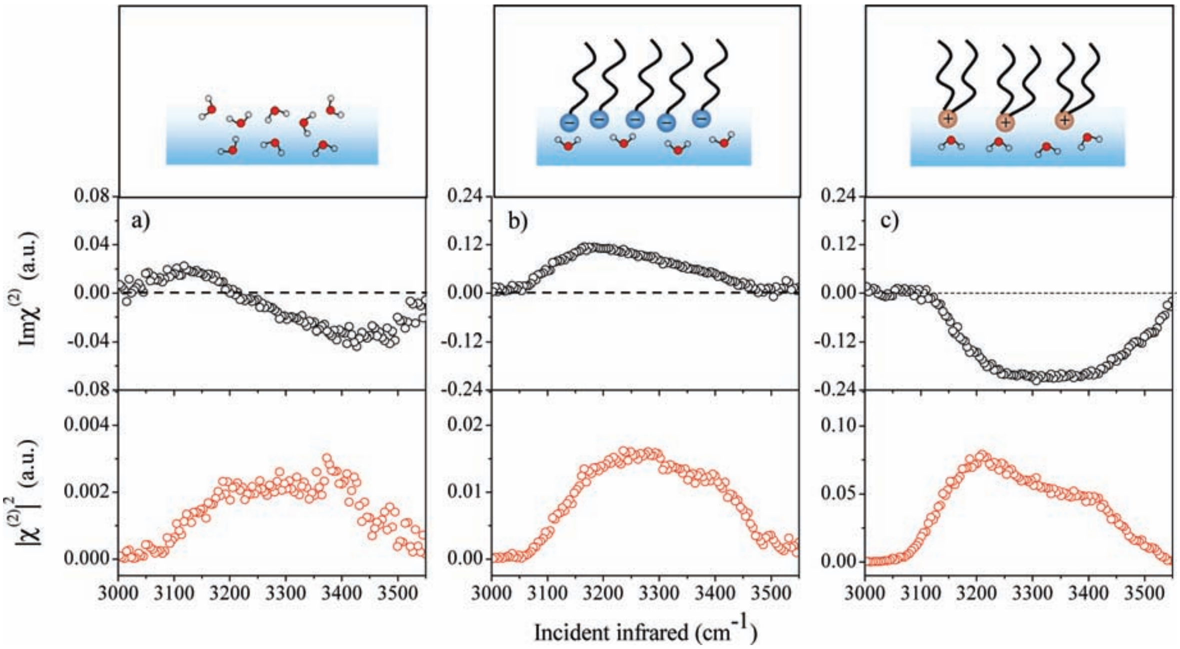

Figure 1. Im $\chi^{(2)}$ VSFG spectra (middle panel) and conventional VSFG spectra (bottom panel) of (a) water, with (b) PA on pH = 13 water and (c) DDAB. Illustrations of each interface are shown in top panel.

faces. Despite unambiguous assignment of 3700 cm^{-1} to the dangling OH stretch, also referred to as the free OH, the interpretation of VSFG spectra of the hydrogen bonded OH stretch region (3000 to 3600 cm^{-1}, Figure 1a, bottom panel) has been controversial for a long time.[23−25,27−29] The intensity spectrum of neat water exhibits two characteristic bands centered at ∼3200 and ∼3400 cm^{-1}, which are commonly referred to as "ice-like" and "liquid-like" water structures.[44] Without knowing the sign of the nonlinear susceptibility (χ), interpretations based on fitting of conventional VSFG spectra of the air/water interface are not unique; hence, this leads to different structural interpretations.

The Im $\chi^{(2)}$ spectra provide not only the resonant information of modes but also the sign (orientation) of each mode. Our Im $\chi^{(2)}$ spectrum of neat water in the hydrogen bonded stretching region is similar with the result reported by Shen and coworkers[27] (Figure 1a, middle panel). It is suggested by Tian et al. that the VSFG signal from the neat water surface is largely due to the top two layers of water molecules and the sublayers are mainly disordered and give little contribution.[45] From previous work[27] in the hydrogen bonded region, the negative region from 3450 to 3600 cm^{-1} has been assigned to donor-bonded OH stretches of 3-coordinate DDA and DAA water molecules in the topmost layer. (Here D and A denote donor and acceptor hydrogen bonds respectively, with which water molecules hydrogen bond to nearest neighbors.) The negative region from 3200 to 3450 cm^{-1} has been assigned to asymmetrically donor-bonded 4-coordinate DDAA molecules, while the positive region from 3000 to 3200 cm^{-1} was mainly attributed to symmetrically donor-bonded DDAA molecules.[27] The above assigned spectral regions overlap with each other, which leads to a crossover point around 3200 cm^{-1}. The negative sign in an Im $\chi^{(2)}$ spectrum corresponds to the OH stretches with a net orientation of the hydrogens pointing down toward the bulk and the positive sign corresponds to a net orientation with hydrogens pointing up.

The fatty acid, surfactant, and phospholipids that were studied here form self-assembled monolayers on water and were overspread to form a condensed phase monolayer prior to the PS-VSFG measurements. Palmitic acid and DDAB are amphiphilic molecules with relatively simple head groups, that is, a carboxylic group and ammonium group, respectively. Their monolayers on the water surface therefore serve as references for a negatively charged surface (PA) and a positively charged surface (DDAB). PA on pH 13 water has its carboxylic group in a deprotonated state based on a reported pK_a of ∼9 from previous studies of an arachidic acid monolayer.[43,46] The conventional VSFG spectra of a water surface with deprotonated PA and DDAB monolayers exhibit similar 3200 and 3400 cm^{-1} bands as that from the neat water surface as shown in the bottom panel of Figure 1, but with significantly higher intensities. Such high intensities clearly suggest ordering of water molecules by the strong surface electric field, which increases the probing depth of the VSFG.[25] However, the Im $\chi^{(2)}$ spectra shown in the middle panel of Figure 1 in the case of PA and DDAB are completely different. Unlike the coexistence of both negative and positive regions for neat water, broad continuous regions in either purely positive or purely negative phase are observed for PA and DDAB respectively. The negatively charged PA monolayer orients water molecules with hydrogens pointing up toward the air phase and the positively charged DDAB monolayer reveals the opposite water orientation with hydrogens pointing down.

The Im $\chi^{(2)}$ and conventional (power) VSFG spectra of the water surface with net negatively charged phospholipid monolayers DPPA, DPPG, and DPPS are shown in Figure 2. These conventional VSFG spectra are also consistent with published VSFG spectra for similarly charged phospholipids,[32] although the 3200 cm^{-1} intensities in our spectra are slightly lower

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(44) Tian, C. S.; Shen, Y. R. *Chem. Phys. Lett.* **2009**, *470*, 1.

(45) Tian, C. S.; Ji, N.; Waychunas, G. A.; Shen, Y. R. *J. Am. Chem. Soc.* **2008**, *130*, 13033.

(46) Le Calvez, E.; Blaudez, D.; Buffeteau, T.; Desbat, B. *Langmuir* **2001**, *17*, 670.

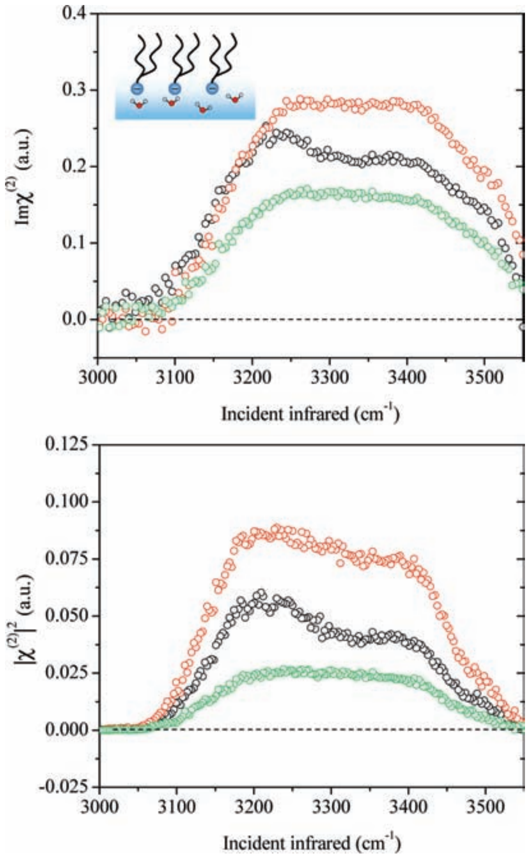

Figure 2. Im $\chi^{(2)}$ VSFG spectra (top) and conventional VSFG spectra (bottom) of water with net negatively charged phospholipid monolayers: DPPA (black), DPPG (red), and DPPS (green, lowest intensity spectrum).

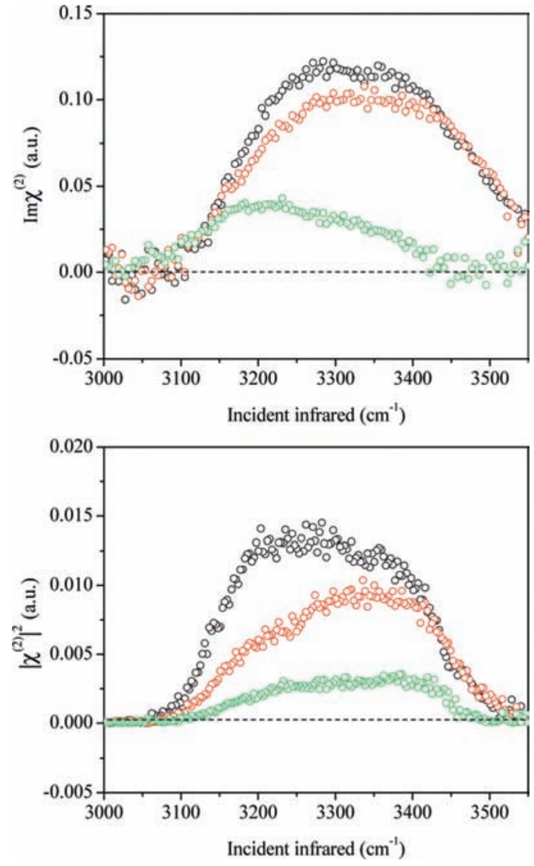

Figure 3. Im $\chi^{(2)}$ VSFG spectra (top) and conventional VSFG spectra (bottom) of water with net neutral zwitterionic monolayers: DPPC (black), DPPE (red) and DPPC on 0.4 M $CaCl_2$ (green, lowest intensity spectrum).

(Supporting Information). It has been shown in the early 1990s by Eisenthal and co-workers that the third-order susceptibility ($\chi^{(3)}$) mechanism can make a significant contribution to the detected nonlinear polarization.[47−49] According to a study on a charged monolayer/water surface,[48] the electric-field-induced second harmonic generation (EFISHG) from water molecules induced by the negative charge of an *n*-docosyl sulfonate monolayer shows a decrease in the SHG signal as compared to that from the neat air/water interface. This is in contrast with the much greater VSFG signal relative to the neat air/water interface for negatively charged phospholipids (Figure 2). In addition, the PS-VSFG studies on water at highly charged surfaces completed by Shen and co-workers were interpreted based on $\chi^{(2)}$ contribution.[24,50] Therefore, the VSFG intensity increase observed in our spectra for the negatively charged phospholipid monolayers are attributed to predominantly a $\chi^{(2)}$ mechanism.

Similar to PA at pH 13, all negatively charged phospholipids order the water molecules with hydrogens pointing up. This indicates that the electrostatic potential of the phospholipid head groups dictates the net interfacial water orientation despite the difference in functional groups and headgroup orientation. The above phospholipids together with their sodium counterion form an electric double layer (EDL) in the interfacial region. Previous MD simulations on DPPG[51] and dioleoylphosphatidylserine (DOPS)[52] bilayers suggested that sodium ions reside in the vicinity of the negative centers of phospholipid head groups. The order of magnitude higher VSFG intensity in the Im $\chi^{(2)}$ spectra of negatively charged phospholipids compared to that from the neat water surface suggests that water molecules below the negatively charged head groups are aligned preferentially with their hydrogens up toward the surface.

Phospholipids with zwitterionic (net neutral) head groups such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are most studied because of their common presence in biomembranes. The water structure associated with these types of net neutral head groups is particularly intriguing. The Im $\chi^{(2)}$ and conventional VSFG water spectra of DPPC and DPPE monolayers are shown in Figure 3. The positive sign for both Im $\chi^{(2)}$ spectra over the full hydrogen bonded OH stretching region indicates that interfacial water molecules orient on average with hydrogens up toward the surface similar to negatively charged monolayers, although the lower relative intensity suggests a weaker orientating effect compared to the net negative DPPA and DPPG phospholipids. Previous studies have also shown that interfacial water structure can be very different at neutral surfaces. For example, specific anions can be absorbed into the air/polymer/water interface and can cause ordering of interfacial

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(47) Ong, S. W.; Zhao, X. L.; Eisenthal, K. B. *Chem. Phys. Lett.* **1992**, *191*, 327.

(48) Zhao, X. L.; Ong, S. W.; Eisenthal, K. B. *Chem. Phys. Lett.* **1993**, *202*, 513.

(49) Geiger, F. M. *Annu. Rev. Phys. Chem.* **2009**, *60*, 61.

(50) Tian, C. S.; Shen, Y. R. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 15148.

(51) Pimthon, J.; Willumeit, R.; Lendlein, A.; Hofmann, D. *J. Mol. Struct.* **2009**, *921*, 38.

(52) Bhide, S. Y.; Berkowitz, M. L. *J. Chem. Phys.* **2005**, *123*, 224702.

water molecules.[53] Interfacial water molecules are also found to be accommodated in neutral 4″-*n*-octyl-4-*p*-cyanobiphenyl (8CB) Langmuir monolayers and give rise to a new band at ∼3520 cm^{-1}.[54]

Our results are in accordance with the recent MD simulations, which suggest that the charge on a PC headgroup viewed by the interfacial water molecules is negative despite its zwitterionic nature.[5,16−18] However, our Im $\chi^{(2)}$ spectra of the phospholipid/water interfaces are *opposite* with the calculated Im $\chi^{(2)}$ results from the phase retrieval algorithm furthered by Bonn et al., although it should be pointed out that our Im $\chi^{(2)}$ spectra of PA and DDAB are similar to the references they use, that is, lauric acid (LA, negatively charged) and octadecyl trimethyl ammonium bromide (OTAB, positively charged).[40] Bonn et al. attributed the negative OH stretch response in their retrieved Im $\chi^{(2)}$ spectra of the phospholipid/water interface to the water molecules sitting above the phospholipid head groups, hence the net orientation of these water OH bonds would point down to the negative charge of the head groups. Indeed there are water molecules above the headgroup because significant numbers of dangling OH bonds remain at the phospholipid/water interface even under full monolayer coverage.[36] However, the orientation of water in localized regions above as well as directly around the headgroup, the hydrating water molecules, are not discernible from the larger number of water molecules that are oriented by the electrostatic potential induced by a phospholipid monolayer.

Theoretical simulations help to provide additional insight on the DPPC/water interfacial structure. Berkowitz suggested that water molecules are able to penetrate up to the carbonyl groups of the alkyl chains in the lipid tails.[6] The carbonyl, phosphate, and choline groups are at different positions if projected on the normal to the phospholipid layer with the carbonyl group on the tail ∼5 Å above the PC head.[5,16,55] The phosphorus atom to nitrogen atom (P−N) vector is nearly parallel to the surface plane, ∼10° away from the surface plane as reported by many others, with the phosphate slightly higher than the choline group, although the distribution probability is broad, over several tens of degrees.[56−58] The electrostatic potential due to the DPPC molecules can be arbitrarily set to continuously increase from zero to positive values as one goes from the tail group to the bulk water.[6] The contributions to this positive electrostatic potential are largely from the negatively charged phosphate, and then from the carbonyl groups.[59] As a result, on average, the interfacial water molecules orient with their dipole (hydrogens) pointing toward the surface (toward the DPPC tails) to compensate for the electrostatic potential. The orientation of interfacial water molecules can also be explained by the cumulative charge distribution of the DPPC layer that is experienced by the water molecules. Starting from the carbonyl groups on the tails to the bulk water, the simulated cumulative charge becomes more negative until it peaks at the position of the phosphate, and then gradually increases back to zero after the choline group.[18] This suggests that the water molecules between the phosphate and the choline groups experience the strongest orientational ordering. Additionally, the net orientation of water molecules below the carbonyl groups would always be with their hydrogens pointing up toward the DPPC tails, which is in agreement with our measured Im $\chi^{(2)}$ spectra.

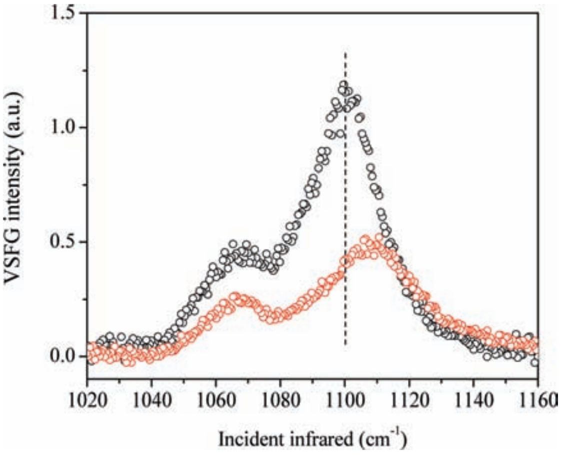

Figure 4. Conventional VSFG spectra of DPPC PO_2^- on water (black, highest intensity spectrum) and on 0.4 M $CaCl_2$ (red, lowest intensity spectrum) subphases.

The Im $\chi^{(2)}$ water spectra of negatively charged phospholipids clearly show higher amplitudes as compared with net neutral zwitterionic phospholipids. This might suggest that DPPC and DPPE mainly perturb water molecules between the tail carbonyls and the choline of the headgroup, while the negatively charged head groups could impact the water orientation over a longer range. Recent MD simulated VSFG spectra from the DMPC/water interface suggest as well that the main contribution to the VSFG spectra is the water dipoles located over several Ås in depth, corresponding to the PC headgroup region.[41]

To further elucidate the influence of zwitterionic head groups on the interfacial water structure, an Im $\chi^{(2)}$ water spectrum of DPPC on a $CaCl_2$ subphase was collected and is also shown in Figure 3. The amplitude of the Im $\chi^{(2)}$ water spectra decreases greatly on the 0.4 M $CaCl_2$ subphase as compared with that of the neat water subphase, although the sign of $\chi^{(2)}$ remains positive over the hydrogen bonded spectral region. (Note that the *y* axis scales can be directly compared between figures.) Cations are attracted to the carbonyl and phosphate groups of the phospholipids.[60] Owing to the greater negative charge carried by the phosphate group, it is reasonably conceived that Ca^{2+} ions would favor binding to the phosphate compared to the carbonyl group.

Conventional VSFG spectra of the DPPC phosphate group were obtained on neat water and on a 0.4 M $CaCl_2$ subphase as shown in Figure 4. Two peaks at ∼1070 and ∼1100 cm^{-1} are observed in these spectra. The former peak is assigned to the P−O ester stretch and the latter is assigned to the symmetric stretch of PO_2^-.[61] The frequency of the PO_2^- symmetric stretch is sensitive to the hydration state of the phosphate group and blue shifts upon dehydration.[34,62] A theoretical study of the PO_2^-

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(53) Chen, X.; Yang, T.; Kataoka, S.; Cremer, P. S. *J. Am. Chem. Soc.* **2007**, *129*, 12272.

(54) Zhang, Z.; Zheng, D. S.; Guo, Y.; Wang, H. F. *Phys. Chem. Chem. Phys.* **2009**, *11*, 991.

(55) Pandit, S. A.; Bostick, D.; Berkowitz, M. L. *Biophys. J.* **2003**, *84*, 3743.

(56) Gabdoulline, R. R.; Vanderkooi, G.; Zheng, C. *J. Phys. Chem.* **1996**, *100*, 15942.

(57) Dominguez, H.; Smondyrev, A. M.; Berkowitz, M. L. *J. Phys. Chem. B* **1999**, *103*, 9582.

(58) Mashl, R. J.; Scott, H. L.; Subramaniam, S.; Jakobsson, E. *Biophys. J.* **2001**, *81*, 3005.

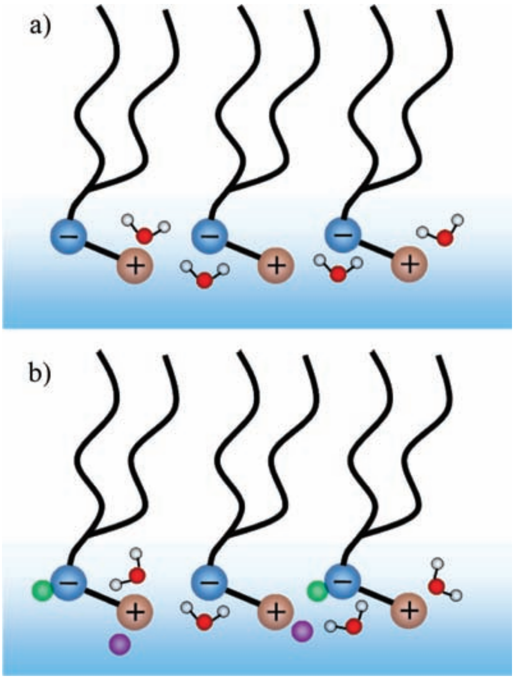
(59) Pandit, S. A.; Bostick, D.; Berkowitz, M. L. *J. Chem. Phys.* **2003**, *119*, 2199.

(60) Binder, H.; Zschornig, O. *Chem. Phys. Lipids* **2002**, *115*, 39.

(61) Casal, H. L.; Mantsch, H. H.; Paltauf, F.; Hauser, H. *Biochim. Biophys. Acta* **1987**, *919*, 275.

(62) Pohle, W.; Selle, C.; Fritzsche, H.; Bohl, M. *J. Mol. Struct.* **1997**, *408*, 273.

Scheme 2. Illustration of Water Orientation at the Interface of (a) DPPC on Neat Water and (b) DPPC on 0.4 M $CaCl_2$ Subphase[a]



[a] The phosphate and choline groups of DPPC are labeled negative and positive charge, respectively. Ca^{2+} ions which bind to the phosphate group and Cl^- ions are represented as green and purple spheres in (b). Water orientation becomes less ordered at the interface in the presence of Ca^{2+} ions.

moiety of methylphosphocholine showed that upon hydration there is a loss of electron density of the P═O bonds due to strong hyperconjugation with the water O−H antibonding orbital, resulting in weakened P═O bonds and a red shift.[63] In the presence of 0.4 M $CaCl_2$, the PO_2^- symmetric stretch blue shifts to ~1109 cm^{-1}, suggesting that calcium binding results in phosphate dehydration. The observed spectral blue shift of the PO_2^- peak is also consistent with the theoretical study on dimethyl phosphate in the presence of water and calcium, which showed that as the Ca^{2+} approaches the PO_2^- oxygens, and a blue shift of both the symmetric and asymmetric stretches of the PO_2^- was observed.[64]

Upon the basis of the spectra in Figures 3 and 4, it can be concluded that the decrease in the amplitude of the Im $\chi^{(2)}$ water spectrum of DPPC on the 0.4 M $CaCl_2$ subphase is due to the binding of Ca^{2+} to the phosphate group. In addition, it should be pointed out that the PO_2^- symmetric stretch frequency remains nearly unchanged at a 0.5 M NaCl subphase (data not shown) relative to that on neat water. This suggests that the electrostatic interaction between sodium ions and the phosphate is relatively weak and does not affect the hydration shell of the phosphate, while the interaction of the calcium ions results in direct binding where calcium replaces hydrating water molecules. As a consequence, the presence of Ca^{2+} shields part of the phosphate charge on the DPPC headgroup, resulting in a lessened impact on the ordering of the interfacial water molecules as illustrated in Scheme 2. At the Ca^{2+} concentration studied here, interfacial water molecules still experience a net negative charge from the DPPC molecules so that the Im $\chi^{(2)}$ water spectrum is still positive.

The strength of the water hydrogen bonding network under the influence of a phospholipid monolayer is also of great interest. It is generally thought that water hydrogen bonding strength correlates with the frequency of the OH stretch, namely higher OH stretch frequency equates to weaker hydrogen bonding strength. However, the intermolecular and intramolecular coupling of water O−H bonds causes a splitting of the OH stretch bands, which complicates spectral interpretation.[28] A very recent study of water near the highly charged CTAB/water interface reveals that the 3200 and 3400 cm^{-1} OH bands merge into one band as the water is gradually replaced by HOD.[65] This result suggests that decoupling of the O−H bonds is required for determination of the actual OH stretch frequency and the hydrogen bonding strength, although it is suggested by MD simulation that the structure of water molecules in and near the phospholiplid/water interface are heterogeneous and the organization corresponds to different moieties of the headgroup.[41]

Conclusions

In summary, direct PS-VSFG measurement of water structure associated with a series of phospholipid monolayers reveals that interfacial water molecules orient accordingly to the net phospholipid headgroup charges. Negatively charged head groups (DPPA, DPPG and DPPS) have greater impact on water ordering than neutral zwitterionic head groups (DPPC and DPPE). In the case of zwitterionic phospholipids, the negative charge on the phosphate group dominates the interfacial water orientation. Binding of Ca^{2+} to the phosphate group reduces the effective charge experienced by surrounding water molecules, resulting in less influence on water orientation.

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Supporting Information Available: Experimental configuration and analysis details of the PS-VSFG and IR energy profiles from VSFG spectra from z-cut quartz with GaAs are shown and discussed. This material is available free of charge via the Internet at http://pubs.acs.org.

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(63) Mrazkova, E.; Hobza, P.; Bohl, M.; Gauger, D. R.; Pohle, W. *J. Phys. Chem. B* **2005**, *109*, 15126.

(64) Petrov, A. S.; Funseth-Smotzer, J.; Pack, G. R. *Int. J. Quantum Chem.* **2005**, *102*, 645.

(65) Nihonyanagi, S.; Yamaguchi, S.; Tahara, T. *J. Am. Chem. Soc.* **2010**, *132*, 6867.