BIOPHYSICS

Saturation of charge-induced water alignment at model membrane surfaces

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The electrical charge of biological membranes and thus the resulting alignment of water molecules in response to this charge are important factors affecting membrane rigidity, transport, and reactivity. We tune the surface charge density by varying lipid composition and investigate the charge-induced alignment of water molecules using surface-specific vibrational spectroscopy and molecular dynamics simulations. At low charge densities, the alignment of water increases proportionally to the charge. However, already at moderate, physiologically relevant charge densities, water alignment starts to saturate despite the increase in the nominal surface charge. The saturation occurs in both the Stern layer, directly at the surface, and in the diffuse layer, yet for distinctly different reasons. Our results show that the soft nature of the lipid interface allows for a marked reduction of the surface potential at high surface charge density via both interfacial molecular rearrangement and permeation of monovalent ions into the interface.

INTRODUCTION

Biological membranes consist mainly of neutral, zwitterionic, and negatively charged lipids, which self-assemble into bilayers in the presence of water. Hence, biological membranes carry an effective charge with nominal densities varying from −0.002 to −0.3 C/m² (1). The resulting electrical surface potential of cell membranes is a key for biomolecular interactions at membranes (1), in addition to other membrane properties such as membrane stiffness (2) and intermembrane interactions (3). Therefore, the surface potential of charged lipid membranes in contact with water has been intensively studied (4–6), and mean-field models have been commonly used to account for the decay of the electrical potential into the aqueous environment (7).

At low surface charge densities, and correspondingly low surface poten- tials, the Gouy–Chapman model based on the linearized Poisson- Boltzmann distribution and some refined versions thereof are used to describe the diffuse distribution of counterions in the solution (8). In addition to this diffuse layer, at higher surface charge densities, condensa- tion of counterions to the membrane interface—as described by the-ories such as those of Stern or Manning—leads to a steep decay of the surface potential within a subnanometer distance from the interface (9–12). Above the critical charge at which counterion condensation takes place, no further change in the potential is expected in the diffuse layer, whereas in the Stern layer, the potential is expected to keep increasing both because of an increase in the charge density and because of a possible decrease in the dielectric constant. Beyond considerations based only on the electrostatic interactions, more advanced theories can also take specific ion effects into account (13–16). Even so, all these models assume mean-field approaches, not considering the molecular nature of the ions and the lipids (17). Also, the solvent, water, which through alignment of its permanent electrical dipoles contributes signif- icantly to charge screening, is treated as a continuum described by its linear response to an electric field. Yet, especially at high charge densities, the nonideal behavior of both counterions and water is expected (18) to substantially alter both the ion distribution and the water properties, which have been concluded to be responsible for hydration repulsion—even for neutral lipids (19, 20). For charged lipids, the different water structure may also lead to water having a reduced efficiency to screen charges (21). Because lipid interfaces in living organisms are very rich in biomacromolecules, such reduced screening can tremendously affect the solvation-free energy of molecules [including proteins (22)]. Thus, chemical equilibria, such as conformational transitions, ion association, acid dissociation constants, etc. (23), can be different near the lipid interface as compared to the bulk. Hence, insights into the precise water structure and potential at the membrane interface is crucial for understanding processes in any living cell, like for example, protein folding (24, 25). Also, for transport of ions or metabolites across the membrane, the barrier that needs to be overcome is determined not only by the hydrophobic membrane core but also by the aqueous interface (26–29). Despite its relevance, direct experimental information on the molecular structure at charged interfaces is limited (30, 31).

RESULTS

Here, we probe the molecular structure at membrane interfaces with varying charge using sum-frequency generation (SFG) spectroscopy (32–35). In an SFG spectroscopy experiment, a femtosecond broadband infrared (IR) pulse and a narrowband visible pulse are overlapped at the surface in space and time. The generated signal is spectrally dispersed in a spectrometer and detected with a charge-coupled device (CCD) cam- era. An SFG signal can only be generated in noncentrosymmetric media and is enhanced by vibrational resonances. This makes SFG molecularly specific and sensitive to the number of aligned interfacial molecules and their degree of alignment. We probe the alignment of interfacial water by recording the O–D stretch vibration of heavy water (D₂O) using SFG. To systematically vary the surface charge density of a lipid monolayer in contact with water, we use mixtures of positively charged DPTAP (1,2-dipalmitoyl-3-trimethylammonium-propane chloride salt) and negatively charged DPPG [1,2-dipalmitoyl-sn-glycero-3- phospho-(1’-rac-glycerol) sodium salt] lipids (Fig. 1A). The SFG results, along with molecular dynamics (MD) simulations, provide molecular-level information on the structure of the electrical double layer next to the membrane and, in particular, reveal a molecular re-structuring of the Stern layer at high membrane charge densities.

Figure 1B shows SFG intensity spectra for different DPTAP/DPPG ratios. Unless otherwise indicated, the experiments were performed for lipid monolayers on a D₂O subphase, at an average lipid surface area per
molecule of 54 Å². To retain constant ionic strength for the various lipid mixtures, we performed the experiments with 10 mM NaCl in the aqueous subphase, unless otherwise indicated. Experiments performed with 10 mM solutions of LiCl, KCl, and CsCl revealed identical behavior, within the sensitivity of the experiment (see fig. S1). The spectra in Fig. 1B show broad signatures from 2200 to 2700 cm⁻¹ and sharp peaks around 2900 cm⁻¹. The broad 2200 to 2700 cm⁻¹ signatures originate from the O-D stretch vibrations of the D₂O molecules, whereas the sharp 2900 cm⁻¹ peaks arise from the C-H stretch vibrations of the alkyl chain of the lipid molecules. The different frequencies of the C-H vibrations of the DPPG and DPTAP monolayers indicate the different order of the lipid molecules at the surface. In general, the observed intensities of the C-H vibrations are characteristic of highly ordered lipid layers as evident from the relatively low intensity of the CH2 symmetric stretch of the lipid molecules. The different frequencies of the C-H vibrations of the DPPG and DPTAP monolayers indicate the different order of the lipid molecules at the surface. In general, the observed intensities of the C-H vibrations are characteristic of highly ordered lipid layers as evident from the relatively low intensity of the CH2 symmetric stretch band that appears as a shoulder at ~2860 cm⁻¹ over the entire range of lipid compositions (see also table S1) (36). Despite the fact that there may be a variety of local structures, oppositely charged lipids homogeneously mix without forming segregated domains on larger length scales (37). Moreover, our signal is an average over a large area compared to the molecular structure. We thus assume that the nominal charge of the membrane surface is simply given by the sum of positive and negative charges carried by the lipids. As is apparent from Fig. 1B, the water response does not simply scale with the surface charge: The O-D signal for the only slightly negatively charged 40:60 mixture is quite similar to the signal for the pure DPPG system, whereas the 50:50 mixture yields a signal that is considerably smaller. To quantify the variation of the O-D stretch spectral features for different DPTAP/DPPG ratios, the SFG intensity \( I_{\text{eff}}(2) \) spectra are fitted as a function of the IR frequency using the following expression based on the Lorentzian model:

\[
\chi_{\text{eff}}^{(2)}(\omega) = A_\text{eff} \delta + \sum_i (A_i (\omega - \omega_i + i \Gamma_i)),
\]

where \( A_i \), \( \omega_i \), and \( \Gamma_i \) denote the area, half width at half maximum, and vibrational frequency of mode \( i \), respectively, whereas \( A_\text{eff} \) and \( \delta \) denote the amplitude and phase of the nonresonant contribution. \( \chi_{\text{eff}}^{(2)}(\omega) \) contains both "pure" \( \chi^{(2)} \) terms and possible contributions from \( \chi^{(3)} \) processes. It has previously been shown that interference effects between those contributions may distort the experimental SFG spectrum depending on the interfacial charge density and relative contribution of \( \chi^{(2)} \) and \( \chi^{(3)} \) terms (38). These interference effects, however, do not significantly affect the behavior of the water signal with varying surface charge density as shown in fig. S2. Because the change in total spectral area is the quantity of interest in the present work, we do not take these interferences into account (38). The fits are shown in Fig. 1B. The sign of \( A_i \) for the broad signatures, attributed to the O-D stretch vibrations, is opposite for DPTAP and DPPG (35) as a result of the different orientation of the water near the positively and negatively charged lipids. The different orientation of water is also the origin of a frequency shift of the SFG signals of the O-D stretch vibrations (see Fig. 1B). The sum of \( A_i \) of the O-D stretch vibrations obtained from the fit is a measure of the degree of alignment of the interfacial water molecules (39). The fitting parameter \( A_i \) represents the area of the Lorentzians of the resonances and describes the area of the imaginary part of the \( \chi^{(2)} \) spectrum. The data, summarized in Fig. 1C, reveal that the SFG spectral area linearly varies at very low nominal charge densities, while it starts to saturate at charge densities as low as ±0.05 C/m². At approximately ±0.1 C/m², corresponding to the ~60:40 and 40:60 mixtures, the SFG signal fully plateaus. Details on the exact peak positions and assignments to molecular vibrations can be found in the Supplementary Materials. The SFG spectra were recorded with \( s \) -polarized SFG, \( s \)-polarized Vis, and \( p \)-polarized IR light, that is, in \( ssp \) geometry, where \( s \) - and \( p \)-polarized light is perpendicular and parallel to the plane of incidence, respectively. Note that data collected in \( ppp \) and \( pss \) geometry reveal an identical dependence on the surface charge density (see figs. S3 and S4), which implies that the SFG intensity variations primarily reflect variations in the interfacial population of aligned molecules.

The observed saturation effect indicates that the degree of water alignment is not simply proportional to the surface charge. To exclude the possibility that the observed saturation of water alignment is caused only by this specific pair of lipids, we performed the same experiments on lipid mixtures with different headgroups (fig. S5A). We also observe the saturation for mixtures of DOTAP [1,2-dioleoyl-3-trimethylammonium-propane (chloride salt)] with DOPG [1,2-dioleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (sodium salt)], which exist in the liquid crystalline phase while DPTAP and DPPG exist in the gel state at room temperature (fig. S5B). Saturation of water alignment is also observed, when changing the lipid density by compression in a Langmuir trough (fig. SSC) and performing the experiment for different lipid coverages (fig. S6 combined with section S1 and fig. S7). These results show that the phase behavior and islands formation are not relevant to the observed (spatially averaged) effect. The effect is also observed for D₂O diluted with 75% of H₂O (fig. SSD), providing evidence that possible charge-induced changes in the vibrational coupling (40) do not affect the observed saturation upon variation of the surface charge. The observed saturation of the water SFG signal at approximately ±0.1 C/m² coincides with surface charge densities for which counterion...
condensation has been predicted to set in (10, 41). To investigate the occurrence of counterion condensation, we directly probed the presence of counterions at the interface using SFG. To this end, we performed SFG experiments with NaSCN in the subphase by directly probing the SCN$^-$ counterion through its vibrational mode at around 2080 cm$^{-1}$ (42, 43). In these experiments, we can observe the SFG response of the counterions and the oriented D$_2$O molecules simultaneously. Figure 2A shows the SFG spectra of D$_2$O solutions containing 10 µM NaSCN for different amounts of DPTAP on the surface. SCN$^-$ ions screen the charge of the positively charged DPTAP more efficiently than Cl$^-$ ions, and therefore, a lower concentration suffices to see counterion condensation (44). The spectra are again fitted with a Lorentzian model, varying only the amplitudes of the resonances. The SCN$^-$ signals are fitted with two peaks because it has previously been reported that two differently oriented SCN$^-$ species appear at a lipid monolayer (42). The peak positions and widths together with their assignment to the corresponding molecular vibrations are listed in the Supplementary Materials.

The sum of the absolute values of the fitting parameters $A_m$ of the two SCN$^-$ vibrational signals and that of the O-D stretch vibrations is plotted against the charge density of the DPTAP layer in Fig. 2B. The results show that the thiocyanate counterions can already be detected at low charge densities. When the water signal starts to level off at a charge density of ~0.15 C/m$^2$, the thiocyanate signal rises. This provides direct evidence that the plateauing of the water signal, as seen in Fig. 1B, is closely interlinked with the condensation of counterions. Further increasing the charge density even leads to a reduction of the water signal. Experiments, where the surface charge density is varied by mixing DPTAP with DPPG, show the same maximum in water signal at phospho-(1rac-glycerol) (sodium salt) mixtures were studied, because no force fields were available for DPTAP. We set the surface area of the lipid to 61 Å$^2$ and vary the concentration of DOTAP/POPG as follows: 0,100, 20,80, 40,60, 45,55, 50,50, 55,45, 60,40, 80,20, and 100%. We used a total of 80 lipids and 10,820 water molecules. To (electro-) neutralize the system, we added either Na$^+$ or Cl$^-$ ions. Details on the simulation can be found in Materials and Methods. Please note that we used a water slab with lipids only on one slide of the slab. The other side resembles the water-vacuum interface.

To quantify the water alignment, we determine the axial profile of the dipole moment density of water projected onto the surface normal $z$ (Fig. 3A). The origin point $z = 0$ nm is set at the position of the Gibbs dividing surface of the water-vacuum interface. The positive (negative) dipole moment reveals that the water is aligned with its dipole moment pointing up (down) toward the water-lipid interface (bulk). Moreover, the orientation of the water molecules changes upon varying the effective surface charge density using different mixtures of positively and negatively charged lipids. In line with the experimental data in fig. S8, the magnitudes of the net orientation obtained from the MD simulations differ between the positively charged lipid surface as compared to the interface with the same absolute negative surface charge (Fig. 3A). This asymmetry is most apparent within the Stern layer and can be related to different hydrogen bonding interactions between the lipid interface results from the balance of electrostatic attraction of the ions to the interface and the (effectively repulsive) entropic gain from the randomization of counterions (9). Counterion condensation can neutralize the excess surface charge, preventing the effective surface charge from increasing beyond a value equal to the critical surface charge density (10), which intuitively leads to saturation of the water orientation in the diffuse layer. Yet classical double-layer models predict that the formation of the surface charge-counterion layer, similar to a capacitor, results in a steady increase of the electric field and thereby the polarization within the Stern layer with the increase of the surface charge density (46). Therefore, on the basis of the response of dipolar water to the surface field, water in the Stern layer should be expected to exhibit enhanced alignment with increasing charge density. By contrast, our results on the O-D stretch vibration of water (Figs. 1B and 2B) show that the overall water alignment is insensitive to increasing surface charge density, and thus, water alignment saturation in both the Stern and the diffuse layer must occur.

To explore the origin of this saturation, we performed MD simulations at the lipid-water interface for different lipid mixtures. In the MD simulations, DOTAP/POPG [1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (sodium salt)] mixtures were studied, because no force fields were available for DPTAP. We set the surface area of the lipid to 61 Å$^2$ and vary the concentration of DOTAP/POPG as follows: 0,100, 20,80, 40,60, 45,55, 50,50, 55,45, 60,40, 80,20, and 100%. We used a total of 80 lipids and 10,820 water molecules. To (electro-) neutralize the system, we added either Na$^+$ or Cl$^-$ ions. Details on the simulation can be found in Materials and Methods. Please note that we used a water slab with lipids only on one slide of the slab. The other side resembles the water-vacuum interface.

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headgroups bearing different charge and water molecules. Specifically, with a negatively charged moiety, water interacts with a hydrogen bond of one of its O-H groups. By contrast, with the positively charged moiety, water interacts through its dipole (47). Hence, the alignment of O-H groups relative to the surface plane can be quite different, which explains the differences between the signal magnitudes for the positive and negative interfaces (see also the Supplementary Materials). To quantify the change in water alignment with lipid composition, the dipole moment integrated along the surface normal for \( z > 0.42 \text{ nm} \) is plotted as a function of charge density in Fig. 3B. In agreement with the experimental results, the integrated dipole moment follows a sigmoidal trend upon changing the surface charge density.

Figure 3A highlights the different behavior for the regions of \( z > 5.8 \text{ nm} \) and \( z < 5.8 \text{ nm} \). At \( z < 5.8 \text{ nm} \), the total water dipole is almost independent of the surface charge density as soon as it exceeds \( \pm 0.05 \text{ C/m}^2 \) (60:40 or 40:60 lipid ratio), consistent with water in the diffuse layer experiencing a constant effective surface charge given by the combined charge of the lipid headgroups and the condensed counterions. The \( z > 5.8 \text{ nm} \) region, relevant to counterion condensation, reveals variations in the water organization also at higher surface charges. To specifically explore water alignment within these two regions, we separately integrate the dipole moment in the near-interfacial region (\( z > 5.8 \text{ nm} \), further referred to as the Stern layer) and the near-bulk region (\( 0.4 \text{ nm} \leq z \leq 5.8 \text{ nm} \), further referred to as the diffuse layer). We arbitrarily defined the Stern layer to have a thickness of \( \sim 1 \text{ nm} \) (45, 48). We thus define the diffuse layer to start at \( z = 5.8 \text{ nm} \), which is distanced \( \sim 1 \text{ nm} \) from the Gibbs dividing surface of the lipid water interface. The assumed thickness of the Stern layer does not change our results qualitatively. Figure 3B shows the results of this analysis, along with sigmoidal fits with parameters reported in Table 1. This analysis reveals that the contributions of the diffuse layer and the Stern layer are comparable. Water alignment in the Stern layer shows a broad sigmoidal trend, whereas the diffuse layer is characterized by a much sharper transition of the orientation of the water molecules. The counterions condense in the Stern layer, thereby effectively screening the excess charge on the lipid surface resulting in the saturation of water alignment in the diffuse layer. This saturation at low charge densities in the diffuse layer fits well to Manning’s calculation of the critical charge density at which charge condensation occurs (black line in Fig. 3B) (10). The water orientation in the Stern layer also saturates: With increasing charge density, the water alignment in the vicinity of the charged headgroups and the condensed counterions levels off as well. This contrasts common double-layer models (8, 46) for which one would expect the amount of oriented water in the double layer to increase continuously with increasing surface charge.

**DISCUSSION**

The observed saturation within the Stern layer can, in principle, originate from two distinctively different molecular mechanisms. As the charge density increases, the electric field in the Stern layer continuously increases. However, above a critical value, the water alignment reaches saturation (that is, the water dipole aligns parallel to the electric field). This saturation has been predicted (21) and experimentally inferred for mineral interfaces (49). Alternatively, at a certain point, water alignment saturates because the counterions approaching the lipid effectively screen the excess charge on the lipid headgroups.

Our simulation suggests that the experimentally and computationally observed saturation of the water orientation in the Stern layer at the lipid interface can be explained by the second mechanism, a charge density–dependent structure of the Stern layer. This restructuring of the Stern layer is shown in Fig. 4A. For increased charge density, the counterion approaches the headgroup more closely by several angstroms. As a result, for highly charged lipid surfaces, some water molecules are “squeezed out” of the Stern layer by the strong interaction between the lipid headgroup charge and the counterion. These counterions efficiently screen the lipid charges, and above some critical values of the charge density, water molecules, within the Stern layer, effectively experience the same electric field. The ionic fields (calculated from the integral of the charge distribution of all charged molecules, that is, lipids and counterions along the \( z \) axis) are virtually the same for the 80:20 mixture and the 100:0 mixtures, as shown in Fig. 4B. This saturation of the electric field shows that the soft nature of the lipid interface (as opposed to solid-water interfaces) allows for a nearly full compensation of the increased charge.
density above \( \sim 0.15 \text{ C/m}^2 \): The lipid-water interface is permeable to counterions, and penetration of counterions into the region of the charged headgroups can efficiently compensate the increased charge density. The penetration of counterions into the headgroup region is evident from the approach of the maxima of the N atom of the \(-\text{N(CH}_3\text{)}_3\) and the \(\text{Cl}^-\) distribution with increasing charge density in Fig. 4A. The lipids can rearrange to allow for this permeation (see the wider distribution of the N atoms for the 100:0 mixture as compared to the 80:20 mixture in Fig. 4A). We note that simulations with different background electrolyte concentrations qualitatively reveal similar behavior (see fig. S10).

Therefore, our results show that the saturation of water alignment upon increasing the lipid charge density stems from two distinct saturation mechanisms: Saturation in the diffuse layer is caused by counterion condensation, which keeps the effective surface potential in the diffusive layer constant in agreement with the Manning theory (10), even when the nominal surface charge increases. Saturation in the Stern layer results from a molecular rearrangement of both water and lipid molecules within the Stern layer at elevated charge densities, as a result of the strong interaction between surface charges and counterions. Sigmoidal fits to the alignment of water in the Stern and diffuse layer (Table 1) show that the alignment of water in the Stern layer displays a more protracted saturation (smaller inverse rate and thus a more moderate slope of the sigmoid) than that of water in the diffuse layer. The expulsion of water from the Stern layer and penetration of the counterions into the lipid headgroup layer is less abrupt, smearing out the transition.

Figure 5 summarizes the results of this study: For the studied lipid surfaces and ions, over the charge density range reported here, the Stern layer changes its molecular nature: it is gradually converted from an interface consisting of three layers: charged surface/water/counterion to an interface increasingly consisting of two layers: charged surface/counterion (21). Hence, our results reveal that both the lipids and counterions can restructure, such that condensed counterions can fully neutralize an increase in the headgroup charge density at high surface charge densities. In turn, our results suggest that a saturation of the water response to the ionic electric field (the first scenario described above) at the very interface does not significantly contribute to the observed saturation of the overall water alignment. Hence, for the presently studied lipid membranes, there is no indication for a uniform rigid water structure in the plane perpendicular to the surface normal, particularly, for water molecules in the direct vicinity of the headgroups. This rigid ordering of water has been observed for solid interfaces (21) and has been assumed to impose an additional potential barrier for transport of charged residues across membranes (40). Thus, although a rigid water structure may locally hinder transport, our results suggest that a barrier due to structured water is not uniformly present for the lipid interface (50). Also for processes taking place at protein interfaces, like the acceleration of the conformational changes of proteins with increasing surface charge (51), our results imply that these cannot be solely attributed to the attractive forces between charged lipid and the dipole moment of the protein in the diffuse layer. In this regard, any quantitative approach taking only the lipid charge density into account is inadequate for describing protein-lipid interaction. Already at moderate surface charge densities, charged residues on lipids (and presumably proteins as well) will be screened, suggesting that an effective charge density should be less than 0.1 C/m\(^2\).

In summary, we have reported SFG measurements of water alignment at lipid layers with varying charge densities. Our results show that the water orientation is saturated for surface charges exceeding \( \pm 0.1 \text{ C/m}^2 \), whereas the water alignment simply scales with surface charge for low surface charge. MD simulations reveal the different sensitivity of water in the diffuse and Stern layers to the surface charges; the water orientation is rapidly saturated in the diffuse layer because of counterion condensation, whereas the water orientation is affected more gradually by the increasing surface charge in the Stern layer. The saturation of water alignment observed in the Stern layer can be attributed to a molecular-level structural transition of both the lipid and the counterion within the Stern layer: Counterions approach the charged headgroups at elevated surface charge densities. Above \( \pm 0.15 \text{ C/m}^2 \), the lipids rearrange and counterions penetrate into the lipid headgroup region, which allows for an effective compensation of the surface charge. Our results thus show that the soft nature of lipid interfaces—not captured in any classical double-layer models—makes lipid interfaces distinctively different from solid-liquid interfaces, and the reported molecular rearrangement of the interface is relevant to both reactions occurring at biological membranes and transport of molecules (such as cell metabolites) across membranes.

**MATERIALS AND METHODS**

**Sample preparation**

DPTAP chloride salt, DPPG sodium salt, 1,2-dipalmitoyl-\(sn\)-glycero-3-ethylphosphocholine (chloride salt) (DPEPC), 1,2-dipalmitoyl-\(sn\)-glycero-3-phosphocholine (sodium salt) (DPPA), DOTAP chloride salt, and DOPG sodium salt were purchased from Avanti Polar Lipids Inc. and used without further purification. The lipids were dissolved...
in a 9:1 mixture of chloroform (stabilized with amylene, >99%; Thermo Fisher Scientific) and methanol (99.8%; VWR Chemicals) at a concentration of 4.3*10^{-4} M. The lipid solutions were then mixed at different ratios, always yielding a total volume of 200 μl. D$_2$O (99.9%) was obtained from Euriso-Top and used as received. NaCl (≥99.5%; Sigma-Aldrich) was baked at 650°C for 8 hours and dissolved in D$_2$O immediately after cooling down. KCl (≥99.0%; Sigma-Aldrich), CsCl (≥99.9%; Carl Roth GmbH), and sodium thiocyanate anhydrous (>95%; Thermo Fisher Scientific) were dissolved in D$_2$O as received. Lithium chloride anhydrous (99%; Sigma-Aldrich) was weighed in a glovebox before dissolving in D$_2$O. H$_2$O was deionized with a Millipore unit (resistivity ≥18.2 megohm cm).

**SFG spectroscopy**

The SFG spectra were recorded on a setup using a Ti:sapphire regenerative amplifier (Spitfire Ace, Spectra-Physics) that generated pulses with a wavelength of 800 nm with a pulse duration of around 40 fs, a repetition rate of 1 kHz, and a pulse energy of 5 mJ. The broadband IR pulse was generated by pumping an optical parametric amplifier with a collinear difference frequency generation (DFG) stage (TOPAS-C, Spectra-Physics) with around 1.7 mJ of the laser output. In parallel, the visible pulse was generated by passing another part of the laser output through a Fabry-Perot etalon (SLS Optics Ltd.) to achieve pulses of 15 cm$^{-1}$ full width at half maximum. Both the IR and the visible beam were focused onto the sample. The generated SFG pulse was subsequently collimated, focused onto a spectrograph (Acton, Princeton Instruments), and detected with an electron-multiplied CCD (Newton, Andor Instruments). All SFG spectra shown here were collected in ssp polarization (s-polarized SFG, s-polarized visible, and p-polarized IR beam); spectra recorded for ppp and pss polarization combinations show the same behavior and are shown in the Supplementary Materials. The beams were set to an angle of 34° for the visible and 36° for the IR. All data were normalized to the nonresonant signal taken from z-cut quartz after subtraction of a background spectrum, which had been acquired by blocking the IR.

For the SFG experiments, approximately 20 ml of a D$_2$O solution containing 10 mM NaCl was contained in a Teflon-coated trough with a surface area of 50 cm$^2$. The concentration of 10 mM NaCl was chosen to control the ionic strength and keep it constant upon changing the ratio of positively/negatively charged lipids. Subsequently, the lipids were deposited on the D$_2$O surface by a click syringe. Most sets of experiments were performed with a fixed number of 0.5 μl drops for each mixture, that is, at a constant area per molecule expressed in square angstrom (Å$^2$). If not otherwise indicated, the experiments were performed at 54 Å$^2$. The experiment with NaSCN in the subphase was performed in two different ways; on the one hand by gradually increasing the amount of DPTAP on the surface between the measurements, on the other hand by varying the DPTAP/DPPG ratio at a constant surface area per molecule. To avoid steady-state heating of the water surface due to the laser beam, we rotated the trough with an electromotor resulting in a linear speed of 0.05 m/s when moving between two consecutive measurement spots. In this manner, two subsequent laser shots did not hit the sample surface at the same spot (52). Before each measurement, the film was left to equilibrate for 10 min; during that time and during the SFG measurement, the setup was flushed with N$_2$ to avoid IR absorption by CO$_2$.

**MD simulations**

MD simulations have been conducted with the GROMACS 5.0.8 code (53). We used the three-atom simple point charge (SPC) force field model for water (54) and the GROMOS 54a7 force field for the ions and POPG sodium salt. For DOTAP, we obtained force field parameters compatible with the GROMOS 54a7 force field developed by Zhao and co-workers (55). We used periodic boundary conditions in all three dimensions and the smooth particle mesh Ewald method for computing the long-range part of the electrostatic interactions. The size of the simulation cell was 7 nm × 7 nm × 17 nm. The systems to simulate different lipid ratios were set up with packmol (56). One of the two vacuum-water interfaces of the water slab was covered with 80 lipids (DOTAP and/or POPG), resulting in a surface area per molecule of 61 Å$^2$. Note that the other interface is a water-vacuum interface. 10,820 water molecules were contained in the simulation cell. The counterions Na$^+$ or Cl$^-$ were included to neutralize the charge of the whole system. This means that, for example, a system with 80 DOTAP molecules contained 80 Cl$^-$ ions and that a system with 80 POPG contained 80 Na$^+$ ions. The 40 DOTAP-40 POPG mixture
contained no coulterers. Simulations were run for nine systems with DOTAP/POPG ratios of 0:100, 20:80, 40:60, 45:55, 50:50, 55:45, 60:40, 80:20, and 100:0.

We used 2-fs time steps for integrating the equation of motion in the canonical ensemble. The bond lengths were fixed using the SETTLE algorithm (57), and a temperature of 300 K was maintained with a stochastic velocity rescaling thermostat (58). We ran MD simulations over 10 ns for equilibrating the system and then conducted 100-ns production runs. These 100-ns trajectories were used in the subsequent analysis.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/3/eaap7415/DC1

section S1. Brewster angle microscopy

section S2. Sigmoidal fits

section S3. SFG data analysis

section S4. SFG experiments with different electrolytes in the subphase

section S5. Calculation of interference between $\chi^{(3)}$ and $\chi^{(3)}$ terms

section S6. SFG experiments measured with SSP and PSS polarization combination

section S7. SFG experiments of different lipid mixtures, different layer preparation, and isotopic dilution of the subphase

section S8. SFG experiments with different lipid surface area per molecule and Brewster angle microscopy measurement

section S9. SFG experiments of lipid mixtures on pure D$_2$O

section S10. SFG experiments with NaSCN in the subphase

section S11. Supplementary information to MD simulations and additional MD simulations

section S12. Calculation of another orientational metric

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