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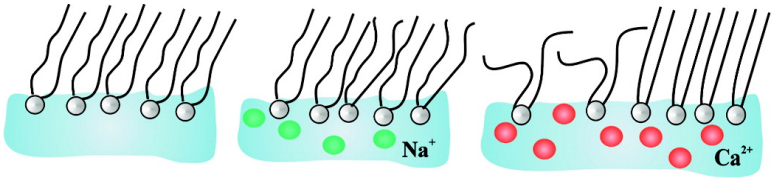

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Calcium-Induced Phospholipid Ordering Depends on Surface Pressure

Maria Sovago, George W. H. Wurpel,† Marc Smits, Michiel Müller,‡ and Mischa Bonn*

Contribution from the FOM Institute for Atomic and Molecular Physics (AMOLF), Kruislaan 407, 1098 SJ, Amsterdam, The Netherlands, and Swammerdam Institute for Life Sciences, University of Amsterdam, P.O. Box 94062, 1090 GB Amsterdam, The Netherlands

Received February 23, 2007; Revised Manuscript Received July 10, 2007; E-mail: bonn@amolf.nl

Abstract: The effect of sodium and calcium ions on zwitterionic and anionic phospholipids monolayers is investigated using vibrational sum-frequency generation in conjunction with surface pressure measurements and fluorescence microscopy. Sodium ions only subtly affect the monolayer structure, while the effect of calcium is large and depends strongly on the surface pressure. At low surface pressures (~5 mN/m), the presence on Ca^{2+} results in the unexpected appearance of ordered domains. For pressures between ~5 and ~25 mN/m, Ca^{2+} ions induce disorder in the monolayer. For pressures exceeding 25 mN/m, calcium cations expand the monolayer, while simultaneously ordering the lipid chains. Interestingly, effects are similar for both zwitterionic lipids and negatively charged lipids. In both vibrational sum-frequency generation and surface tension measurements, the molecular signature of the association of Ca^{2+} with the lipids is evident from Ca^{2+}-induced changes in the signals corresponding to area changes of 4 Å²/lipid–precisely the surface area of a Ca^{2+} ion, with evidence for a change in lipid Ca^{2+} complexation at high pressures.

Introduction

The cell's plasma membrane consists of many different phospholipids that are asymmetrically distributed over the two bilayer leaflets. The cytosolic leaflet is enriched in anionic phosphatidylserine (PS) phospholipids, whereas the outer cellular leaflet contains mostly neutral, zwitterionic lipids. The properties of membrane lipids depend strongly on the environmental conditions such as pH and ionic strength. The interactions between membrane lipids and ions—the subject of this study—play an important role in many biological processes, such as membrane fusion, enzyme regulation, and signal transduction.[1−4] Because of its negative charge, PS phospholipids attract cations, such as sodium and calcium. This can be explained by the Gouy−Chapman theory of the electric double layer, which predicts a local increase in ion concentration near the negatively charged surface.[5] The interaction between metal ions and the headgroups of zwitterionic phosphatidylcholine (PC) lipids on the other hand is expected to be relatively weak compared with PS. However, the binding constants of ions to PC and PS were found to be similar, after correcting for the differences in electric surface potential.[6] Moreover, small changes in the PC headgroup orientation can significantly alter the electrical properties of the membrane surface.[7,8]

Apart from a fundamental interest, the binding of cations to PC membranes has received special attention for more practical applications.[9,10] Recently, it was shown that the interaction between DNA and zwitterionic lipids is strongly enhanced by electrostatic interactions in the presence of Ca^{2+}. Therefore, they might serve as suitable vectors for DNA transfection, as zwitterionic lipids are nontoxic and biocompatible, in contrast to most cationic lipids.

Most experimental studies to date have aimed mainly at quantifying intrinsic binding constants between ions and lipids and at the characterization of membrane surface potentials. Apart from molecular dynamics (MD) studies,[11−16] insights into the molecular details of the interaction between lipids and ions have remained limited. MD simulations have shown that sodium ions bind to the PC bilayers specifically by interacting with phosphodiester oxygens;[11] upon deeper penetration the ions may co-

† Current address: Molecular Biophysics, Utrecht University, P.O. Box 80000, 3508 TA, Utrecht, The Netherlands.
‡ Swammerdam Institute for Life Sciences.

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ordinate with the carbonyl group.[13] Divalent cations, such as calcium, on the other hand, do not penetrate so deeply into the anionic PS bilayers, being localized in a narrow region around the phosphate group.[14] When cations bind to the membrane, the lipid headgroups have been suggested to undergo a conformational change followed by an increase in the lipid-tail order.[11,13,14]

Despite intensive efforts using MD simulations, there are still some controversial issues,[12,15,16] originating partially from different simulations times, and presumably the choice of potentials in the different simulations. Moreover, the reports to date are limited to one specific lipid density, that is, a single area per molecule. Experimental investigations of ion−lipid interactions, in particular at various lipid densities, is therefore desirable, to complement existing theoretical efforts. We address here the interaction between PC and PS lipids and mono- and divalent cations for different lipid densities and phases. In lipid monolayers at the air−water interface, we study the formation of ion−phospholipid complexes and possible ion-induced lipid reorganization in single composition membranes over a range of lipid densities.

The cation−lipid monolayer systems were investigated using a combination of three complementary techniques that provide information on different length scales: surface pressure measurements, fluorescence microscopy, and vibrational sum-frequency generation (VSFG) spectroscopy. The surface pressure-versus-area isotherms form a macroscopic measure of the phase behavior of the entire monolayer. With fluorescence microscopy[17−19] lipid domains can be visualized with ∼200 nm resolution, while VSFG provides information on the molecular orientation and conformation of the lipids,[20−24] albeit averaged over a probing area of $\sim 10^{-2}$ mm^2. Note that SFG is most sensitive to ordered domains, as these provide a significantly larger signal than disordered regions, owing to the decreased symmetry upon ordering.

Lipid monolayers are widely used as model systems for biological membranes, since the cell membrane can be considered as two weakly coupled monolayers.[25] The study of lipid monolayers at the air−water interface furthermore allows for experimental control over the surface pressure. To observe the subtle effects of cations on the monolayer organization, relatively high (1 M) concentrations of cations are used. Previous studies have indicated that such high concentrations are required to obtain a significant effect of the interaction of ions with the majority of lipid molecules.[26,27] Although we report here results for 1 M concentrations, control experiments indicate that the effects reported here affect the majority of lipids at ion concentrations above 50 mM.

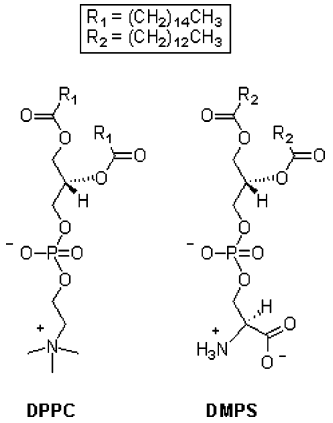

Figure 1. The chemical structures of DPPC and DMPS.

Cation−lipid interactions are investigated for both the zwitterionic lipid *L*-1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine (DPPC) and the negatively charged lipid *L*-1,2-dimyristoyl-*sn*-glycerol-3-phosphoserine (DMPS). The structures of the lipids are shown in Figure 1. DMPS and DPPC monolayers exhibit very similar phase behavior, both displaying distinct coexistence regions.[28] Both DPPC and DMPS have saturated alkyl chains, with slightly different lengths of the hydrocarbon chains. As such, these two different types of lipids present good model systems where cation−lipid interactions can be followed through different phases, and the effect of lipid headgroup charge can be revealed.

As for the comparison of ion−lipid interactions between DPPC and DMPS, the binding constant for calcium with neutral lipids has been estimated[6] to be $12\ M^{-1} < K < 20\ M^{-1}$. Thus, on a 1 M $CaCl_2$ solution, more than 90% of the DPPC lipids will be bound to calcium. On DMPS, one needs to account for the negative surface charge, which will attract the cations. However, since for the neutral monolayer already nearly all surface sites are bound, this will not significantly change the fraction of bound lipids. Thus, at high electrolyte concentration (1 M) DPPC and DMPS can be meaningfully compared.

We find that Na^+ does not greatly affect the DPPC monolayers, while calcium perturbs the lipid organization significantly. Surprisingly, the effect of calcium on lipid organization (i.e., inducing order or disorder) depends strongly on surface pressure, π. At low surface pressures (∼5 mN/m) the presence of Ca^{2+} triggers the occurrence of ordered domains, at intermediate pressures Ca^{2+} tends to weakly disorder the monolayer, and at high pressures (π > 20 mN/m) substantial order is induced in the lipid tails through interactions with the headgroup. These effects are similar for both DPPC and DMPS, which suggests that the Ca^{2+} ion interacts with the same headgroup moiety, most likely the phosphate group (see Figure 1). Calcium and phosphate are known to form a strong ion pair in water,[29] and the strength of this interaction is likely to be increased in the lipid headgroup region where the dielectric permittivity of the surrounding (and thus the electrostatic screening of charges)

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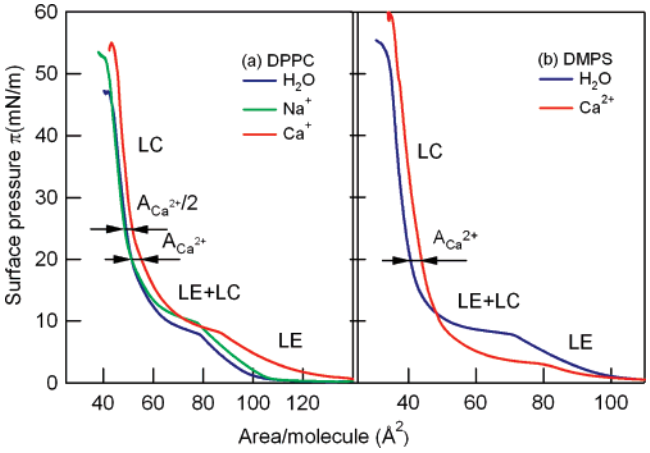

Figure 2. Pressure−area (π-A) isotherms of DPPC (a) and DMPS (b) on different subphases: neat water (blue), 1 M NaCl solution (green), 1 M $CaCl_2$ solution (red). The liquid expanded phase (LE), liquid condensed phase (LC) and the coexistence region of the two phases (LE + LC) are indicated along the isotherms. $A_{Ca^{2+}}$ represents the area of a calcium ion.

is reduced. This does not rule out any interactions of Ca^{2+} with the negative charge on the serine group of DMPS, but if this occurs it has no influence on the ordering of the lipid tails. The presence of Na^+ has a more significant effect on DMPS, where in contrast to Ca^{2+}, the lipid order is reduced at high pressures.

Results and Discussions

First, compression isotherms obtained from the surface pressure measurements are presented, revealing macroscopic differences between lipid monolayers on water, sodium, and calcium solutions. Then we visualize the domain formation using fluorescence microscopy along the compression isotherm, and we follow how the domains change when Ca^{2+} ions bind to the monolayer. Finally, VSFG is employed to elucidate the cation−lipid interaction at the molecular level.

Compression Isotherms. Langmuir isotherms for DPPC and DMPS monolayers on different subphases (neat water, 1 M NaCl and 1 M $CaCl_2$ solution) are shown in Figure 2. For the 1 M NaCl and 1 M $CaCl_2$ solutions used here, the concentration of chloride ions in the subphase differs. As the coordination of the chloride anions with the phospholipids is expected to be weak,[11] possible effects due to the difference in chloride concentration can be neglected, as confirmed by control experiments using 0.5 M $CaCl_2$ solution.

On water, the phase behavior of DPPC has been well documented.[23,25] With decreasing surface area, the following regions have been identified: a gas phase, a liquid expanded (LE) phase, a plateau characteristic of the coexistence of the LE and the liquid condensed (LC) phase, and the pure LC phase.

With sodium ions present in the subphase, the compression isotherm for DPPC shifts to slightly higher surface pressures in the LE and LE + LC regions. The same effect has been observed previously[30] and was explained by disorder of the lipid chains induced by ions binding to the LE phase. At higher surface pressure, sodium has no effect on the compression isotherm, indicating no significant interaction of Na^+ with the LC phase: the ions are probably being "squeezed out" from the headgroup region.

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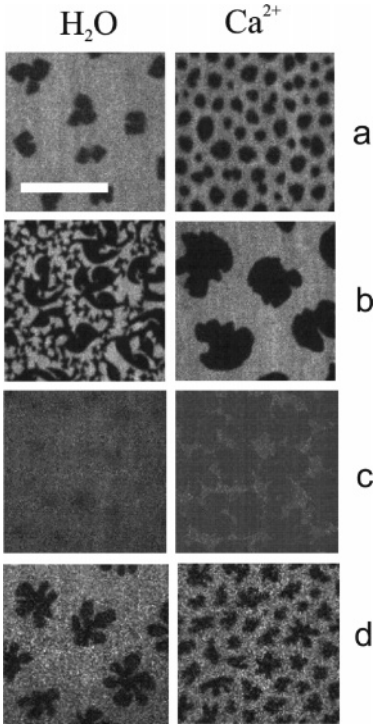

Figure 3. Fluorescence images for a DPPC (a,b,c) and DMPS (d) monolayer on water (left images) and 1 M $CaCl_2$ solution (right images) for π = 4 mN/m (a), π = 6.5 mN/m (b), π = 35 mN/m (c), and π = 2 mN/m (d). Monolayers were labeled with 5 mol % Rhodamine−PE: the light regions correspond to the LE phase, and the dark regions are LC domains. The scale bar represents 15 μm. The contrast is the same for all images.

A more dramatic effect is observed for a $CaCl_2$ solution subphase. Here, the surface pressure becomes finite at much lower densities (~140 Å^2/molecule), while the transition from the LE phase to the LE + LC coexistence region is less pronounced. In the LC region (π > 20 mN/m), the presence of calcium causes the lipid to take up 4 Å^2/lipid additional room—precisely the footprint of a Ca^{2+} ion.[31] This indicates that at surface pressures exceeding 20 mN/m, the water hydrating the headgroups is replaced by calcium ions. At still higher surface pressures (>25 mN/m), the additional area drops by approximately 2 Å^2/lipid. The changes in molecular area difference can be interpreted in two ways: (1) a transition from a 1:1 to a 2:1 lipid/Ca^{2+} complex, as proposed previously from MD simulations;[11] (2) a change in the conformation of the lipid headgroup.[6,23]

For DMPS the compression isotherm on water (Figure 2b) shows the same phase behavior as DPPC, with a LE, LE + LC, and LC region. However, upon compression, a smaller area per molecule can be reached. This indicates that PS is more compressible than PC, in contrast to the intuitive notation that Coulombic repulsive interactions between neighboring charged headgroups should expand the area per PS molecule. It was shown that strong intermolecular coordination of charged moieties of the neighboring PS headgroups plays a role in the area reduction.[16]

For DMPS monolayers on a 1 M Ca^{2+} solution, the LE + LC plateau is lowered to a pressure of π = 5 mN/m compared to the π = 10 mN/m pressure for the coexistence region

(31) Ionic radius of Ca^{2+} ion is r = 1.14 Å, therefore its area is ~4 Å^2

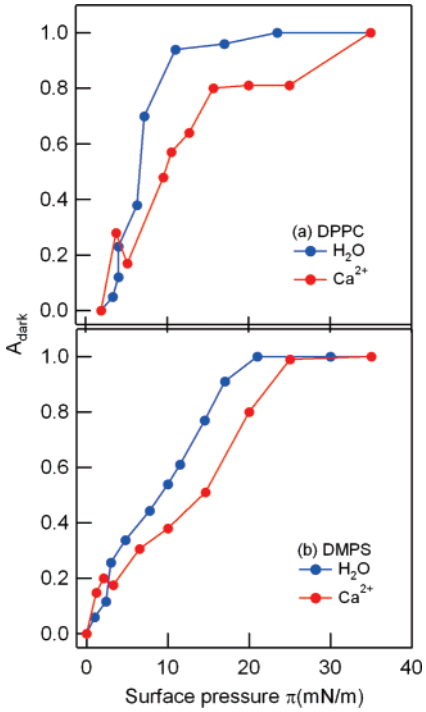

Figure 4. The fractional area of dark, ordered LC domains, A_{dark}, from the fluorescence images as a function of surface pressure for DPPC (a) and DMPS (b) monolayers on neat water (blue) and on 1 M $CaCl_2$ solution (red). Note the local maximum in A_{dark} around ~3 mN/m for both lipids in the presence of Ca^{2+} ions.

observed for pure water; at higher surface pressures (π > 20 mN/m) the displacement of the two isotherms remains equal to the Ca^{2+} footprint, 4 Å^2/lipid, and no apparent transition to a 2:1 lipid/Ca^{2+} complex is observed.

Fluorescence Microscopy. Coexisting phases within lipid monolayers can be visualized using fluorescence microscopy. Fluorescence images obtained for DPPC and DMPS monolayers, labeled with a fluorescent probe, along the isotherm, are displayed for three typical surface pressures in Figure 3. The dark areas correspond to ordered (LC) domains and the bright areas to disordered (LE) domains. For the neat water subphase (left column in Figure 3), the LC domains at low surface pressure are "bean"-shaped for DPPC (Figure 3a)[19] and "flower"-shaped for DMPS (Figure 3d). As the surface area decreases, the LC domains become interconnected by narrow regions of ordered lipids (Figure 3b)[33] before coalescing into a continuous LC phase (Figure 3c). The normalized area of the ordered dark regions, A_{dark}, obtained from an analysis of the fluorescence images (Figure 4), shows that the LC phase increases upon monolayer compression and covers the entire monolayer for π > 10 mN/m.

When calcium is present in the subphase, small ordered domains are observed at low surface pressure for both PC and PS monolayers. A much larger area of the monolayer is covered by these ~3.5 μm ordered domains for Ca^{2+} when compared to water (see Figure 4). These small domains at low surface pressure are thus calcium-induced and they occupy ~30% (for PC) and 20% (for PS) of the surface. The presence of these domains is also evident from VSFG measurements presented

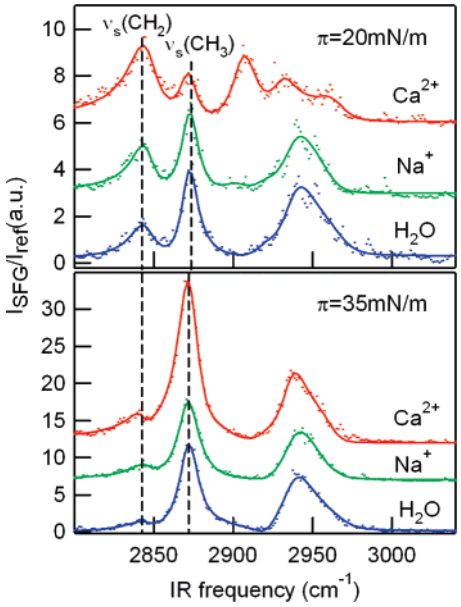

Figure 5. VSFG spectra of DPPC at π = 20 mN/m (top panel) and π = 35 mN/m (bottom panel). The vertical lines indicate the positions of methylene symmetric stretch ($\nu_s CH_2$) and methyl symmetric stretch (ν_s-CH_3). All spectra are normalized to a reference signal from *z*-cut quartz. The solid curves represent fits to the data using a Lorentzian model.

below. Such Ca^{2+}-induced domains have thus far only been observed in mixed monolayer systems.[34,35] At intermediate surface pressures (5 mN/m < π < 25 mN/m), it is evident from Figure 4 that the effect of Ca^{2+} is to reduce the density of ordered domain.

Vibrational Sum-Frequency Generation Spectroscopy (VSFG). The molecular origin of this altered phase behavior in the presence of Ca^{2+} was further investigated with VSFG, a nonlinear vibrational spectroscopic technique that provides surface specific information on conformational order and orientation of interfacial molecules.[21,22] To quantify the effect of ions on the conformation of the lipid tails, VSFG spectra were collected in the C−H stretch region. Spectra were measured for several surface pressures along the isotherms; spectra for the three different subphases at 20 mN/m and 35 mN/m are displayed in Figure 5 for the DPPC monolayer. Five vibrational modes can be observed: the peaks centered at 2876, 2970, and 2938 cm^{-1} have been assigned to the CH_3 symmetric stretch, CH_3 antisymmetric stretch, and CH_3 Fermi resonance, respectively.[22] The CH_2 symmetric and antisymmetric stretch frequencies are found around 2846 and 2920 cm^{-1}. VSFG experiments on deuterated DPPC lipid monolayers (d62 and d75 DPPC) revealed very low-signal for the choline C−H stretches.

Upon compression of the monolayer, a marked decrease of the CH_2 intensities is observed. This can be understood from symmetry arguments: when the methylene groups in an alkyl chain change from cis to trans conformation, a local center of inversion appears which renders the CH_2 modes SFG inactive.[23] The CH_3 intensities on the other hand increase sharply as the monolayer is compressed, which is due to a narrowing of the angular distributions of chain tilt angles. The CH_3 and CH_2 symmetric stretch oscillator strengths can be obtained from fits to the data. Given the preceding arguments, the ratio *R* of the

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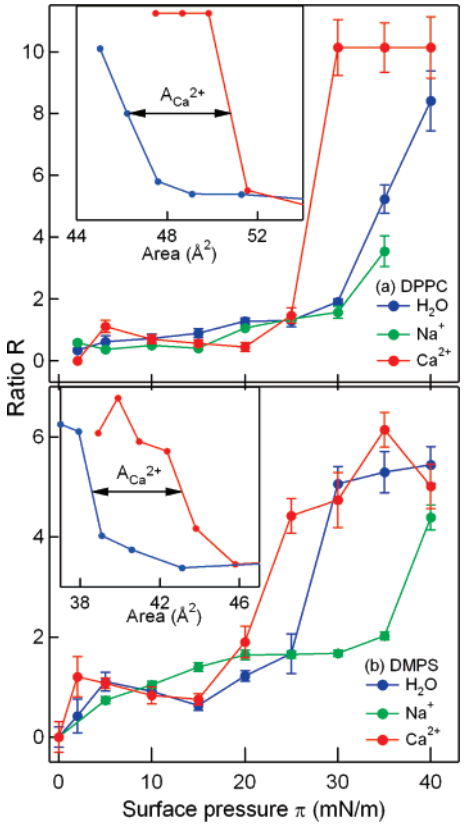

Figure 6. The ratio R of the CH_3 and CH_2 symmetric stretch oscillator strengths (a measure for the lipid molecular order) as a function of surface pressure for DPPC (a) and DMPS (b) monolayers: pure water (blue), 1 M NaCl solution (green), and 1 M $CaCl_2$ solution (red). The ratio R could not be determined for Na^+−DPPC at $\pi = 40$ mN/m, because of the collapse of the monolayer. The error bars are larger at higher pressures because of uncertainties in the amplitude of $\nu_s CH_2$ for a compressed monolayer. Insets show the ratio R displayed as a function of area per lipid. Note the ∼4 Å²/lipid displacement (interpreted as the Ca^{2+} footprint), in agreement with the isotherms in Figure 2.

CH_3 and CH_2 symmetric stretch oscillator strengths provides a sensitive, empirical measure for the order of the lipid chains.[24] To reliably extract R, the data are analyzed globally using a Lorentzian multipeak model (see eq 2, Experimental Section in Supporting Information), allowing only the peak amplitudes to vary as the surface pressure is modified. R for DPPC and DMPS is plotted as a function of surface pressure in Figure 6.

For DPPC monolayers on pure water and NaCl subphase, R varies in a similar fashion with pressure, with slightly, but reproducibly, smaller values of R in the presence of sodium. This indicates, as the pressure−area isotherms also show, that DPPC monolayers are slightly less ordered in the presence of Na^+, in agreement with previous reports pertaining to high concentration of NaCl in the subphase,[30] but at odds with molecular dynamic simulations,[11,13] which indicate an ordering effect at ∼10 mN/m surface pressure.

For DPPC on a subphase containing Ca^{2+} ions, R exhibits a small but reproducible peak at 5 mN/m, followed by a slight decrease between 10 and 20 mN/m and then increases sharply around 25 mN/m. The brief increase in order at low surface tension (∼5 mN/m) is in good agreement with the results from fluorescence microscopy, which indicate an increase in order at these pressures as well (Figure 6). In the following, we will attempt to provide a unified picture of the monolayer phase behavior, using the results from surface tension measurement, fluorescence microscopy, and VSFG measurements. We will first discuss the specific results for DPPC, followed by DMPS.

The ordered domains induced by Ca^{2+}, observed around 3 mN/m in the fluorescence images, are averaged out in the VSFG measurement as the probing area is $\sim 10^{-2}$ mm², but give rise to a peak in the R value, as the average order in the monolayer is increased. Compressing further, the induced domains coalesce into larger ones. Sinultaneously A_{dark} increases, but remains 20% lower for Ca^{2+} than for water (Figure 4a, 10 mN/m $< \pi <$ 25 mN/m). This is again in good agreement with the VSFG spectra that indicate more disorder in the lipid tails for these surface pressures, as the CH_2 symmetric and antisymmetric stretches at 20 mN/m are more prominent with Ca^{2+} in the subphase (Figure 5).

As the surface pressure reaches 25 mN/m, R increases sharply to reach a constant, large value. Interestingly, this sharp increase of R concurs with the position in the isotherm at which the lipid area decreases by 2 Å²/lipid (see Figure 2), which we attribute (see above) to the transition from 1:1 lipid/Ca^{2+} complex into a 2:1 complex. This implies that the increase of order, signified by the increase in R, involves a rearrangement of the headgroups, caused by a distinct change in the Ca^{2+}-lipid interaction. The 4 Å²/lipid surface areas associated with the Ca^{2+} ion footprint is also evident from the different points at which the increase in R values occurs, when comparing Ca^{2+}-solution with pure water. The increase in R is shifted by precisely one unit of $A_{Ca}{}^{2+}$ when R is plotted versus area per lipid (inset Figure 6).

At the point where ratio R increases sharply for the Ca^{2+}−DPPC system ($\pi = 25$ mN/m), 20% of the monolayer area is still covered by LE phase. The increase in the ratio R obtained from VSFG is not due to the disappearance of gauche-defects in the lipid tails, but can be attributed to the reorientation of alkyl chains in the LC phase toward smaller angles θ between the chains and the surface normal. This can be concluded (details in the Supporting Information) from the ratio between the amplitudes of the $\nu_s CH_3$ and methyl antisymmetric stretch[12] which reveal that θ is 10° smaller for DPPC on Ca^{2+}- solution than for DPPC on pure water. At these pressures the presence of calcium ions causes a cooperative tilting of lipids toward the surface normal.

For DMPS, the results are remarkably similar to those observed for DPPC, for both Na^+ and Ca^{2+} ions, despite its different headgroup structure and charge. As the length of the apolar hydrocarbon chain is slightly shorter for DMPS (14 carbons for DMPS and 16 carbons for DPPC), lower R values are found for a compressed monolayer of DMPS when compared to DPPC. A direct correlation between the length of the alkyl chain and the relative order of the monolayer have been reported previously.[36]

With Ca^{2+}, the ratio R shows a similar trend for the DMPS monolayer as for the DPPC monolayer: a peak is present at low surface pressure (∼3 mN/m), and a sharp increase occurs at higher surface pressure ($\pi = 30$ mN/m), with an onset of $A_{Ca}{}^{2+}$ toward higher areas per molecule (inset Figure 6). Again, this is in good agreement with the isotherm data and fluorescence images. For DMPS at high surface pressures ($\pi >$ 30

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mN/m), R is found to be independent of the presence of calcium in the subphase, suggesting a similar acyl chain ordering at high pressures.

Three differences between the two lipids may be noted: first of all, in the region 5 mN/m $< \pi <$ 25 mN/m, the Ca^{2+}-induced decrease in order observed for DPPC is apparent for DMPS in the fluorescence results, but less so in the VSFG results. Second, the transition from a 1:1 to a 2:1 lipid/Ca^{2+} complex observed for DPPC is not apparent for DMPS, although $A_{Ca^{2+}}$ appears in both the fluorescence and the VSFG data. Third, whereas Na^+ induces disorder in both lipids at high pressure ($\pi >$25 mN/m), this effect is significantly larger for DMPS. At present, we do not have a satisfactory explanation for these observations, other than that the difference must originate from differences in the headgroup structure.

Despite these differences in some of the details of the ion−lipid interactions between DPPC and DMPS, it is evident that Ca^{2+} induces ordered domain formation in both lipids at low surface pressure, disorder at intermediate surface pressures (for DPPC), and high degree of order at the highest surface pressures. For both lipids, clear evidence is observed for complex formation between the lipids and Ca^{2+} but not present for Na^+. Sodium affects the lipid monolayers to a much lesser extent, mostly inducing disorder at high surface pressures (most notably for DMPS). Additional similarities between the two lipids are observed when investigating the orientation of water molecules near the headgroups. VSFG spectra for DPPC and DMPS on D_2O subphase in the O−D stretch vibrations (data not shown) indicate that hydrating water molecules are oriented in the same manner near anionic and zwitterionic headgroups. This indicates that PC behaves as an anionic lipid as the headgroup of DPPC is oriented with its choline group toward the interface.[32] Therefore, it is not surprising that calcium interacts similarly with PC and PS phospholipids, most probably coordinated with the phosphate moiety.

Conclusions

Summarizing, we use a combination of three complementary techniques to investigate the effect of cations on PC and PS monolayers. Our results indicate that sodium ions have very little effect on DPPC monolayers, mainly expanding the lipid monolayer. On the other hand, calcium has a pronounced effect, depending strongly on the surface pressure. At low surface pressures calcium ions induce the formation of small, ordered lipid domains, which coalesce into larger ones as the surface pressure increases. At high surface pressures, Ca^{2+} induces a "condensation effect" on both zwitterionic lipid and on anionic lipid monolayers, indicating that ions preferentially interact with the phosphate moiety in the lipid headgroup. In both VSFG and surface tension measurements, the molecular signature of the association of Ca^{2+} with the lipids is evident from changes in the signals between monolayers on water and Ca solution corresponding to area changes of 4 Å^2/lipid—precisely the surface area of a Ca^{2+} ion.

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Supporting Information Available: Experimental Section, VSFG spectra for DMPS monolayers, and molecular orientation analyses using sum−frequency generation. This material is available free of charge via the Internet at http://pubs.acs.org.

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