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ORIGINAL PAPER

Direct imaging of salt effects on lipid bilayer ordering at sub-molecular resolution

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Abstract The interactions of salts with lipid bilayers are known to alter the properties of membranes and therefore influence their structure and dynamics. Sodium and calcium cations penetrate deeply into the headgroup region and bind to the lipids, whereas potassium ions only loosely associate with lipid molecules and mostly remain outside of the headgroup region. We investigated a dipalmitoylphosphatidylcholine (DPPC) bilayer in the gel phase in the presence of all three cations with a concentration of Ca^{2+} ions an order of magnitude smaller than the Na^{+} and K^{+} ions. Our findings indicate that the area per unit cell does not significantly change in these three salt solutions. However the lipid molecules do re-order non-isotropically under the influence of the three different cations. We attribute this reordering to a change in the highly directional intermolecular interactions caused by a variation in the dipole-dipole bonding arising from a tilt of the headgroup out of the membrane plane. Measurements in different NaCl concentrations also show a non-isotropic re-ordering of the lipid molecules.

Keywords Lipid model membranes · Membrane-ion interactions · Sodium cations · Calcium cations · Potassium cations

Introduction

Biological membranes in cells form a barrier between cell compartments and between the interior of the cell and its local environment. They serve as a support matrix for many proteins that play a crucial role in signal transduction, cell-cell recognition and cell fusion (Edidin 2003). In addition, various ions present in physiological solutions are known to influence the local and global properties of lipid bilayers. Salts induce the swelling of bilayers (Petrache et al. 2006a, b), changes in the ordering of the acyl chains, affecting the phase transition (Garcia-Manyes et al. 2005b), and an altering of the tilt angle of the headgroup (Sachs et al. 2004; Seelig et al. 1987). All effects are considered to be both ion specific and concentration dependent (Garcia-Manyes et al. 2005a). These highly complex lipid-ion interactions have been studied using an array of experimental techniques including neutron and X-ray scattering (Nagle and Tristram-Nagle 2000) as well as being the subject of molecular dynamics simulations (Berkowitz et al. 2006; Tu et al. 1996). Atomic force microscopy (AFM) studies have recently demonstrated submolecular imaging resolution in liquid (Fukuma et al. 2007a, b), which enables the investigation of the surface of a lipid membrane at the Ångstrom scale (Asakawa and Fukuma 2009). This enables researchers to locally study the ordering of lipid molecules, whereas scattering techniques inherently average over a long range, concealing molecular defects and directional changes in lipid ordering. In addition, in order to process scattering data, models of the sample are required. It is therefore possible to obtain information about the structure of the bilayer; however AFM directly images the membrane surface. Therefore, intermolecular distances can be acquired without relying on any assumptions about the sample. Importantly, due to the experimental setup of the AFM the bilayer is always fully hydrated.

Recent advances in computing power have allowed researchers for the first time to simulate lipid bilayers in the

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fluid phase. This has also included the study of lipid-ion interactions. This has changed the focus of lipid research towards membranes in the fluid phase. However, the gel phase remains a biological system worth studying since, for example, lung surfactant and skin lipids exist in the gel phase.

Many reports, particularly those based on theoretical studies that investigate the effect of salt on zwitterionic bilayers in the fluid phase, have shown that salt has a significant impact on membrane properties such as the area per lipid or chain ordering. Cations are thought to penetrate deeply into the headgroup region and bind between the carbonyl oxygen and the phosphate group (Berkowitz et al. 2006), whereas the anions only loosely associate with the choline group. The ion-lipid complexes cause a reduced diffusion of the lipid molecules in the membrane (Böckmann et al. 2003; Filippov et al. 2009) and increase the stiffness of the bilayer (Garcia-Manyes et al. 2005a). Monovalent sodium binds less strongly to the membrane than divalent calcium due to the lower charge. Ca^{2+} ions significantly shift the main phase transition of lipid membranes upwards, whereas monovalent cations have only a minor impact (Koynova and Caffrey 1998). It is also well established that the binding constant of calcium ions to zwitterionic bilayers is considerably higher than sodium ions (Tatulian 1987). In contrast, the binding affinity of potassium ions is substantially lower than that of sodium ions (Gurtovenko and Vattulainen 2008; Vácha et al. 2010). Two different explanations are given for this: firstly, the potassium ions cannot penetrate into the headgroup area because the ionic radius of potassium ions is large in comparison to sodium ions. Secondly, the charge density of potassium is lower than that of sodium as the diameter is larger but still carrying the same charge (Gurtovenko and Vattulainen 2008). This is thought to result in a weak cation-lipid interaction, which is also reflected as a minor effect on the area per lipid and the chain order parameter compared to the presence of other cations such as sodium or calcium (Cordomí et al. 2008).

The addition of salt to pure water adjacent to a membrane is reported to reduce the area per lipid (Böckmann et al. 2003; Cordomí et al. 2008; Gurtovenko 2005). However there remains some debate with respect to the impact of monovalent and divalent ions on bilayers at low salt concentrations (10 to $\sim$200 mM). A study by Uhríková et al. (2008) shows a decrease of the area per lipid upon the addition of calcium to pure water. Above 20 mM $CaCl_2$, the area per lipid increases monotonically until it reaches its pure water value at $\sim$80 mM. In addition, little is known about the effect of ions on zwitterionic bilayers in the gel phase and how the salt-induced change in the area per lipid affects the ordering of the lipid molecules.

In this report, we focus on the impact of monovalent and divalent cations and their concentration dependence on the structure of a DPPC bilayer in the gel phase. The addition of an order of magnitude smaller amount of calcium relative to sodium considerably alters the intermolecular distances in a highly directional way, while the area per unit cell stays the same. Potassium ions with the same concentration as sodium ions show a less pronounced effect on the lipid ordering although the direction of the structural change is identical. We link the non-isotropic re-ordering of lipid molecules to modifications in the dipole-dipole interactions of the lipid headgroups. The concentration-dependent measurements in sodium buffer solution show a similar impact on the membrane ordering in concentrations below a threshold value of 220 mM NaCl.

Materials and methods

Sample preparation

The buffer solutions were prepared by dissolving 20 mM, 130 mM, 220 mM, and 1,000 mM NaCl, 130 mM KCl and 100 mM NaCl + 10 mM $CaCl_2$ (all salts from Sigma-Aldrich, Dublin, Ireland) in pure water, respectively. All buffers were adjusted to pH 7.4 with 10 mM Hepes/NaOH. The DPPC bilayer on mica was formed using the vesicle fusion method. DPPC (Sigma-Aldrich, Dublin, Ireland) in powder form was dissolved in chloroform to a concentration of 20 mg/ml. A 50-µl aliquot was put in a glass vial and was dried out under an argon flow in a glass test tube until all liquid was evaporated. The sample was vacuum desiccated for at least 30 min to ensure the removal of all traces of organic solvent. Then 130 mM NaCl buffer was added to the test tube to a concentration of 1 mg/ml and subjected to cycles of vortexing and heating up in a 50°C water bath until a milky vesicle suspension was achieved. To reduce the diameter of the vesicles an extruder kit (Mini-Extruder, Avanti Alabaster, USA) was placed on a heating plate at 50°C. The pore size of the membrane (Nucleopore, Whatman, Kent, UK) was 50 nm. The vesicle solution was heated up to 50°C for 10 min, and afterwards, it was extruded 11 times through the membrane. DPPC solution (280 µl) was deposited onto a freshly cleaved mica disc (15-mm diameter, Mica Grade V-1, SPI Supplies, West Chester, PA). The sample was incubated at 55°C for 1 h in a humid environment to avoid dehydration. The sample was then rinsed five times with the appropriate buffer as used in the subsequent experiment. The DPPC bilayer coverage was typically more than 90% of the mica surface. This was confirmed by imaging the membrane over a 20 × 20 μm^2 area (data not shown) using a MFP-3D AFM (Asylum Research, Santa Barbara, CA).

FM-AFM imaging

In frequency modulation atomic force microscopy (FM-AFM), the cantilever is mechanically oscillated at its resonance frequency by maintaining a frequency shift of 90° between the detected tip oscillation and the driving force. The tip-sample interaction is detected from the resultant shift in the resonance frequency (Δf) caused by changes in the distance dependent intermolecular forces between the probe and the sample. Δf is used as a feedback parameter to control the tip-sample distance in order to maintain a constant force between the tip and the sample. The amplitude and phase are kept constant throughout the measurement, but in contrast to previous measurements (Fukuma et al. 2007a, b) this is not done by a self-oscillation circuit. Instead, both variables are controlled by digital feedback loops as described in Kilpatrick et al. (2009). All experiments were performed by driving the cantilever at the second eigenmode resulting in lower frequency noise and higher force sensitivity than that of the fundamental due to a higher Q-factor.

In this experiment, a bespoke low-noise FM-AFM was used for which the design and performance have been described previously (Fukuma and Jarvis 2006). All experiments described here were performed at room temperature (23°C) where the DPPC bilayer is in the gel phase. The oscillation amplitude of the cantilever was initially set to ~0.2 nm and was subsequently adjusted to achieve the best image quality. It has been shown that the use of extremely small oscillation amplitudes enhances the sensitivity of the measurement to short range interactions (Giessibl et al. 1999). Highly sensitive force spectroscopy measurements revealed that water molecules are bound to lipid membrane forming a hydration layer (Fukuma et al. 2007a). The force required to remove these layers can be measured by performing force curves. The setpoint for imaging in the data presented here was set to values higher than the binding force of water molecules adjacent to the lipid membrane. This ensures that the obtained images were DPPC bilayers and not ordered water molecules at the membrane-fluid interface. Gold coated silicon cantilever (SSS-NCH-AuD, Windsor, Slough Berkshire, UK) with a nominal spring constant of 42 N/m was used. The thermal frequency of the second eigenmode in buffer solution was ~650 kHz. All images were obtained at the same scan frequency (4.34 Hz), scan size (10×10 nm^2) and same number of pixels (512×512). Half of the images in both buffer solutions were recorded at a scan angle of 0°, the other half at 45°. This was done to minimise scanning artefacts due to piezo hysteresis resulting in an inaccurate expansion. All images shown are recorded at a scan angle of 0° except of image (a) in Fig. 2, which was recorded at 45°.

Results

Figure 1a–d shows representative images of a mica-supported DPPC bilayer in 20 mM, 130 mM, 220 mM and 1,000 mM NaCl buffer. The lipid headgroups can be clearly distinguished, and their phosphate and choline subunits are also often resolved (see yellow arrows in Fig. 1e,f). The DPPC molecules show a high degree of ordering over the whole 10×10 nm^2 image. The arrangement of the individual lipids is highlighted by the yellow circles in Fig. 1b. The lipid molecules highlighted by the yellow arrows in Fig. 1e, f show two subunits, of which one has a brighter contrast indicating headgroups generally point in the same direction. This difference in contrast is most likely explained by a different headgroup-tip interaction due to opposite charges of the phosphate and choline group or alternatively by a tilt of the headgroup. Hence, the brighter submolecular moiety cannot be unambiguously identified as one of the two subgroups. However it can be concluded that the lipid headgroups generally point in the same direction. This is depicted in the sketch of the molecular ordering in Fig. 2e. The orientation of the phosphate and choline groups is not determined and is only shown to highlight the general ordering of the lipid headgroups. The angle Φ as defined in Fig. 2e lies between distance A and B is always measured to be less than 90°. The unit cell was defined in accordance to Tu et al. (1996) and Sun et al. (1994). The headgroups pack in a distorted orthorhombic two-dimensional lattice with the lattice parameters A and B (see Fig. 2e). The distances A and B between the lipid headgroups were measured by drawing a line over as many lipids as possible and dividing the length by the number of covered molecules, thereby minimising the relative error. One set of data was read out per image. The area per unit cell was calculated by multiplying the two intermolecular distances and the sine of Φ. The values of A, B, Φ and the area per unit cell for all buffer solutions are listed in Table 1. Fifty-five images of bilayers in 20 mM NaCl were analysed in order to gain the parameters of intermolecular distances, 32 for 130 mM NaCl, 47 for 220 mM NaCl, 75 for 1,000 mM NaCl, 98 for 130 mM KCl and 40 for 10 mM $CaCl_2$ + 100 mM NaCl, respectively.

Figure 2 shows a DPPC membrane in 130 mM NaCl (a), in 10 mM $CaCl_2$ + 100 mM NaCl (b) and in 130 mM KCl (c). Image (a) exhibits two different directionalities of the lipid ordering highlighted by the yellow circles.

The distance A is within the error margin the same in 130 mM, 220 mM and 1,000 mM NaCl. The other buffer solutions show a significant difference. The distance B is the same within the error margin in all buffer solutions except in 1,000 mM NaCl. The area per unit cell increases

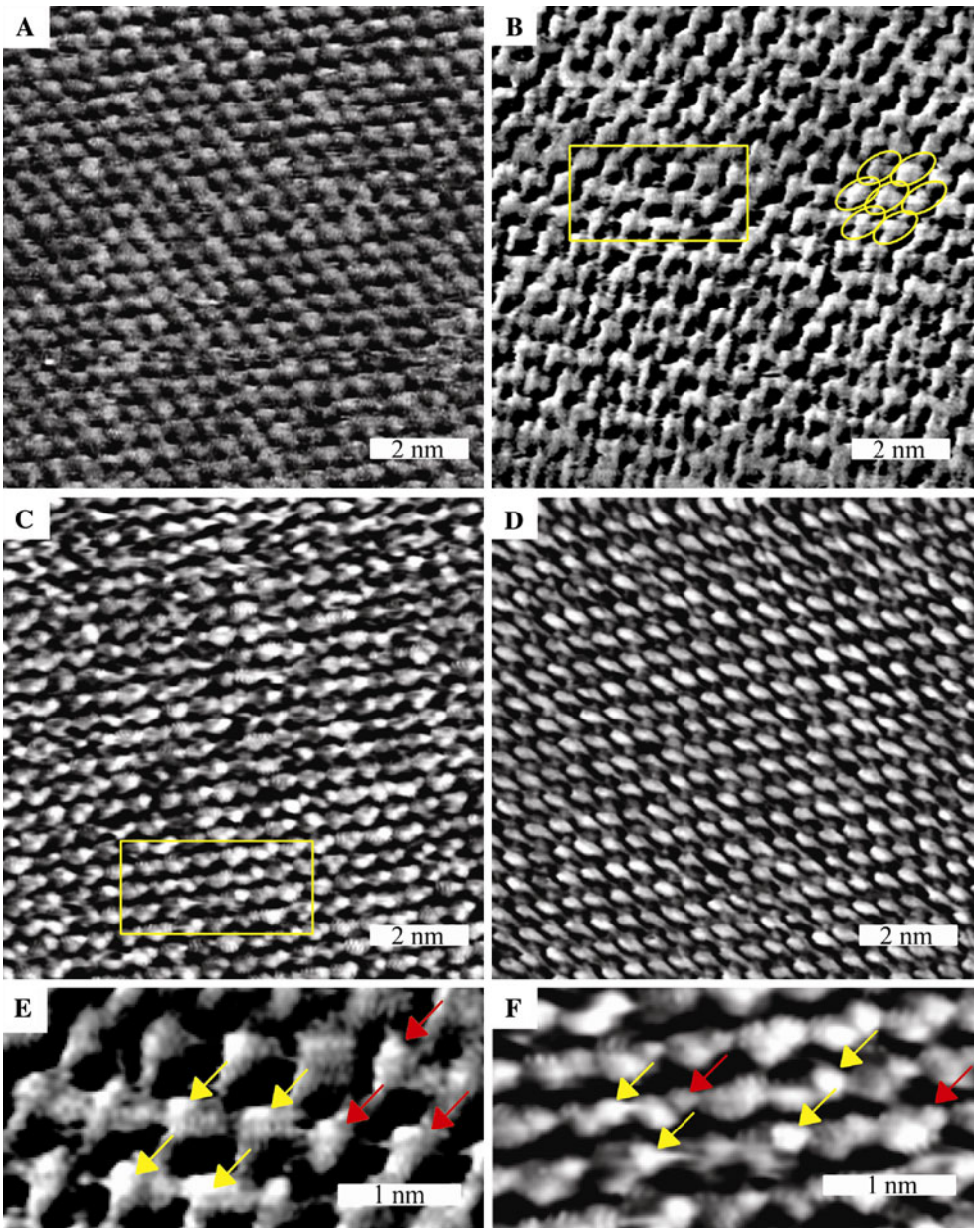

Fig. 1 Images of a mica-supported DPPC bilayer in various NaCl buffer solutions using FM-AFM; **a** 20 mM, **b** 130 mM, **c** 220 mM and **d** 1,000 mM. **e** and **f** show a magnified view of the area marked by the yellow rectangle in (**b**) and (**c**), respectively. Individual headgroups of lipid molecules and their phosphate and choline groups are depicted throughout the magnified images. Some lipid headgroups highlighted by the *yellow arrows* in images **b** and **c** show two subunit, of which one has a higher contrast. The *red arrows* point to lipid molecules that show a different directionality compared to their neighbours (**b**) and whose headgroups have directionality opposite to the ones marked by the *yellow arrows* (**f**). Fast and slow scan direction is from left to right and bottom to top, respectively

with increasing NaCl concentration whereby a plateau may appear in the region of 130–220 mM NaCl concentration. The measurements in 130 mM NaCl, 130 mM KCl and 10 mM $CaCl_2$ + 100 mM NaCl show no significant difference in the area per unit cell. The values of the angle Φ are often quite close to each other and, together with rather large uncertainties, there is no significant difference between most buffer solutions. However, the angle Φ measured in lowest and highest sodium concentration show a significant difference as well as 130 mM NaCl and 10 mM $CaCl_2$ + 100 mM NaCl.

Discussion

The images in Figs. 1 and 2 show well-ordered structures over whole displayed area and non-periodic features on the Ångstrom scale. The orientation of the lipid headgroups is

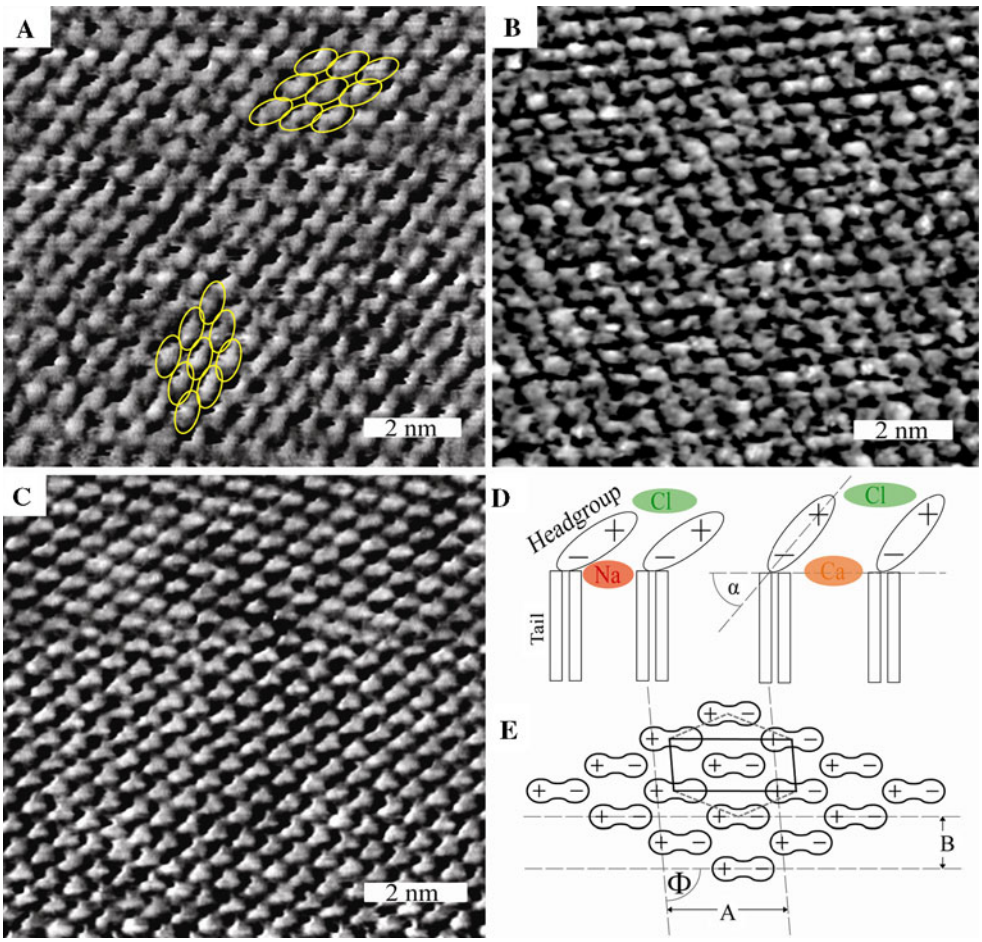

Fig. 2 Images of a mica-supported DPPC bilayer in 130 mM NaCl buffer (**a**), 10 mM $CaCl_2$ + 100 mM NaCl buffer (**b**) and 130 mM KCl (**c**). Individual headgroups are visible throughout the three images. In addition, the phosphate and choline groups can be clearly distinguished in many headgroups. Fast and slow scan direction is from *left* to *right* and *bottom* to *top*, respectively. **d** shows a sketch of a side view of the upper leaflet of a DPPC membrane and **e** the ordering of the lipid headgroups indicated by the AFM images. The + signs symbolise the choline group and the − signs the phosphate group, respectively. The directionality of the lipid molecules is arbitrary and is just shown to emphasise the uniform ordering. The distances *A* and *B* as well as the angle Φ are defined in (**e**). The unit cell [distorted rectangle in (**e**)] is defined in accordance to Tu et al. (1996) and Sun et al. (1994)

Table 1 Overview of all obtained intermolecular distances

Sample	Distance *A* (nm)	Distance *B* (nm)	Angle Φ (°)	Area per unit cell (nm^2)
20 mM NaCl	0.85 ± 0.07	0.42 ± 0.04	84 ± 1	0.36 ± 0.02
130 mM NaCl	0.95 ± 0.01	0.42 ± 0.03	85 ± 3	0.40 ± 0.03
220 mM NaCl	0.91 ± 0.05	0.43 ± 0.05	79 ± 5	0.39 ± 0.04
1,000 mM NaCl	0.94 ± 0.04	0.64 ± 0.08	71 ± 7	0.55 ± 0.08
130 mM KCl	0.87 ± 0.04	0.44 ± 0.02	82 ± 3	0.38 ± 0.02
10 mM $CaCl_2$ 100 mM NaCl	1.01 ± 0.02	0.43 ± 0.04	78.3 ± 0.5	0.42 ± 0.04

The distances *A* and *B* and the angle Φ are defined in Fig. 2 (e)

not perfectly uniform but has some variation, which indicates true sub-molecular resolution. This can be seen in the three molecules marked by the red arrows in Fig. 1e, which are orientated in a different direction than most of the lipid headgroups in Fig. 1b.

Whilst snapshots of molecular simulations suggest a much higher degree of disorder (Tu et al. 1996) than shown in Figs. 1 and 2, an AFM image is a time-averaged topology. The time the tip stays in a location corresponding to one pixel is approximately 1,000 times longer than the

total simulation time. Therefore, we suggest the disorder of the lipid orientation is averaged with respect to time. Yet, defects are still visible, such as the directionality of the lipid headgroups in Fig. 1f marked by the red arrows, which point in the opposite direction than its neighbours indicated by the yellow arrows.

The local ordering of the lipids could be influenced by the cantilever tip as there is a repulsive interaction between the tip and the lipids during imaging. This force, which is in the order of magnitude required for the removal of the first layer of water molecules adjacent to the surface (Fukuma et al. 2007a), may influence the motion of lipid molecules and subsequently their ordering and orientation. However this effect appears to be negligible because the directionality of the lipid headgroups varies within images (see for example the two directionalities of the bilayer in Fig. 2a), while intermolecular distances remained unaffected. The geometry of the tip might also influence the measurement. This effect, too, can be neglected since many cantilevers of the same type were used to obtain the images. Every tip is slightly different due to nano-asperities, but results achieved using different tips are the same.

In a few cases, the sharpness of the tip required for high-resolution AFM could be preserved, and it was possible to measure the same sample in two different ion solutions using the same cantilever. This strongly indicates that changes in the molecular ordering must be induced by different types of ions interacting with the membrane and not by the probe. Since the concentration of Cl^- anions is approximately the same in the sodium, potassium and calcium buffer solutions, the changing intermolecular distances are caused by the three different cations. In addition, Cl^- anions are thought to only weakly interact with the zwitterionic PC headgroup (Gurtovenko and Vattulainen 2008; Miettinen et al. 2009; Pandit et al. 2003).

The basic lipid structure is a distorted orthorhombic lattice, as previously observed by Tu et al. (1996). Parallel to this notion, it is often assumed that lipid ordering in the gel phase is based on a (distorted) hexagonal lattice (Tardieu et al. 1973). However this is still consistent with the lipid structure defined in Fig. 2e (dashed lines).

Measurements in sodium, calcium and potassium buffer solutions

The reported effects of salts on the properties of a membrane are diverse. For example, the binding of ions to the membrane is thought to reduce lateral diffusion within the membrane due to the increased size of the lipid-ion complexes compared to the individual constituents (Böckmann et al. 2003; Filippov et al. 2009). In addition the reported increases in the ordering of the lipid acyl chain, in the rigidity (Pabst et al. 2007) and in the stiffness of the bilayer (Garcia-Manyes et al. 2005a) are likely to stem from the interaction of salt with the membrane. Several simulations attribute the reduction of the area per lipid to the binding of the cations between the carbonyl oxygen of the lipid backbone and the oxygen of the phosphate group (Cordomí et al. 2008; Gurtovenko and Vattulainen 2008; Pandit et al. 2003). A Na^+ ion is thought to bind three lipids (Lee et al. 2008) and a Ca^{2+} ion two to three lipids (Altenbach and Seelig 1984; Pabst et al. 2007).

While simulations indicate a monotonic decrease in the area per lipid with increasing Na^+ concentration (above $\sim$30 mM) for a bilayer consisting of PC lipids in the fluid phase (Cordomí et al. 2008; Pabst et al. 2007), similar studies using Ca^{2+} solutions demonstrate inconsistency in the literature. Pabst et al. (2007) saw no change in the area per lipid for low calcium concentrations (10 mM), and Filippov et al. (2009) reported no change in the lateral diffusion for concentrations of $CaCl_2$ in the range of 0–100 mM, which suggests that ions are not interacting with the membrane. In contrast, Uhríková et al. (2008) observed, after an initial decrease in the area per lipid between 0 and 2 mM $CaCl_2$, a monotonic increase in both the gel and fluid phase of a DPPC bilayer in a neutron scattering study. The lateral diffusion showed the opposite behaviour to the area per lipid.

While there is some controversy over the effect of calcium on a lipid bilayer, the reports studying the influence of potassium on a membrane generally come to the same conclusion. The interaction of K^+ ions with the membrane is believed to be weaker than Na^+ ions due to the bigger ionic radius of K^+ ions compared to the Na^+ ions, while the charge is the same (Gurtovenko and Vattulainen 2008). The size of the Ka^+ cations stericly hinders them from penetrating into the headgroup region. Consequently, the K^+ ions only loosely bind to the lipid bilayer, resulting in no distinctive maximum of the probability of location in the headgroup region (Gurtovenko and Vattulainen 2008; Vácha et al. 2010). In addition, evidence of a weak interaction can be seen from the coordination number of potassium with lipid molecules, which is 1, as compared to 3–4 of Na^+ ions (Lee et al. 2008). Accordingly, potassium has a lower impact on the change of the area per lipid than sodium, whilst calcium shows the strongest (Cordomí et al. 2008, 2009).

In this study, the addition of Ca^{2+} ions did not change the area per unit cell within experimental error. This finding appears counterintuitive since divalent calcium ions are believed to have a stronger effect on the lipid bilayer than the monovalent Na^+ ions, which are known to reduce the area per unit cell. However, a detailed analysis reveals that there is a significant difference in the molecular ordering under the influence of the two salt solutions. The distance along the long axis of the lipid headgroup

A increases in the presence of Ca^{2+} ions. The decrease of the angle Φ while *B* remains constant gives rise to the unchanged area per unit cell. In summary, there is a clear response of the lipid structure to the presence of a relatively small amount of Ca^{2+} ions. This is not surprising since the binding affinity of Ca^{2+} ions is more than 200 times higher than that of Na^+ ions, which means that Ca^{2+} ions can displace the bound Na^+ ions. The strong interaction between membrane and Ca^{2+} ions has been demonstrated by Pedersen et al. (2006) who found in their simulation of a charged phospholipid bilayer that all Ca^{2+} ions present in the simulation cell were bound to charged moieties of the lipid headgroup.

A similar method of lipid re-ordering can be seen in the presence of K^+ ions. The area per unit cell and the distance *B* did not change within experimental error compared to the case of Na^+ ions, while the distance *A* is smaller than in presence of Na^+ ions. The angle Φ also remains unchanged. If the results of the measurement in potassium solution are compared to the calcium solution, a significant difference is observed in all parameters except in the distance *B*, which remains unchanged in all three buffer solutions. The magnitude of the distance *A* in the presence of the three different ions followed the order $Ca^{2+} > Na^+ > K^+$, which is similar to observations of various physical properties such as area per lipid (Cordomí et al. 2008). This behaviour follows the strength of interaction predicted by the Hofmeister series.

The penetration of Na^+, Ca^{2+} and K^+ ions into the headgroups induces a change in the lipid-lipid interaction, which is highly directional since the re-ordering is not isotropic. Binding via water bridges (Pasenkiewicz-Gierula et al. 1997) and charge pairing (Pasenkiewicz-Gierula et al. 1999) in a DMPC bilayer was found to be ubiquitous and plays an important role in stabilising the membrane; however these interactions have no specific directionality. In contrast, dipole-dipole interactions are highly directional and very strong. The dipole moment caused by the phosphate and choline groups is 24 Debye or approximately 10 times the dipole moment of water (Wohlert and Edholm 2004). Therefore, the dipole-dipole interactions between lipid headgroups are likely to cause changes in the lipid ordering. The dipole-dipole potential V_{dip} in SI units is:

$$V_{\mathrm{dip}}(\boldsymbol{r_{12}}) = \frac{1}{4\pi\varepsilon\varepsilon_0}\left[\frac{\boldsymbol{p_1 p_2}}{r_{12}^3} - \frac{3(\boldsymbol{p_1 r_{12}})(\boldsymbol{p_{12} r_{12}})}{r_{12}^3}\right]$$

ε_0 is the electric permittivity of vacuum, ε the relative permittivity, $\boldsymbol{p_i}$ the dipole moment vector and $\boldsymbol{r_{12}}$ the vector connecting dipole 1 and 2. The presence of ions does not directly alter the dipole-dipole interaction apart from screening effects that are thought to be isotropic. That is why salt screening is unlikely to induce directional changes in the lipid structure. If the ions alter the tilt angle α of the lipid headgroup, the dipole-dipole forces are affected depending on the angle between the lipids (see Fig. 2d). Equation 1 shows that the first term is unaffected by a change of the tilt angle for interactions parallel and perpendicular to the long axis of the lipid headgroup, assuming all headgroups behave in the same way. The second term is approximately zero for the dipole-dipole force perpendicular to headgroup since $\boldsymbol{p_i}$ is almost perpendicular to $\boldsymbol{r_{12}}$. A change of the tilt angle α out of the membrane plane reduces the attractive interaction of the second term as the angle between $\boldsymbol{p_i}$ and $\boldsymbol{r_{12}}$ increases. Less attractive forces along the direction of the long axis of the lipid headgroup result in an increased distance. Perpendicular to the direction of the P–N vector, the gap between headgroups remains unchanged since the force does not change. This is in very good agreement with our findings. The distance *B* remains unchanged, and *A* increases due to a reduced attractive force with increasing strength of the lipid-cation interaction. The change of the angle Φ is a consequence of the increase of the distance *A*, while distance *B* remains constant.

Our findings suggest a change of the tilt angle of the lipid headgroup, which has also been indicated theoretically. For example, Wohlert and Edholm (2004) show in their simulation of a DPPC bilayer in the fluid phase that dipole components perpendicular to the bilayer plane repel and parallel ones attract each other. The more ions that are bound to the lipids, the more the phosphate-choline vector is shifted out of the membrane plane (Cordomí et al. 2008, 2009). Another approach to this dipole-ion interaction is that the bound cations and the layer of diffusive Cl^- ions together form a capacitor producing a considerable electric field normal to the bilayer, which forces the dipole vector out of the membrane plane (Kotulska and Kubica 2005). A tilt of the headgroup out of the bilayer plane was also observed if lipids with a cationic or anionic headgroup were added to a DPPC membrane in the gel and fluid phase (López Cascales et al. 2006; Zhang et al. 2006).

Since calcium carries double the charge of sodium, the evolving electric field between cations and Cl^- anions is stronger, tilting the phosphate-choline dipole even more out of the membrane plane than in the solution containing only Na^+ ions (see Fig. 2d). The tilt angle α is smaller in the presence of K^+ ions than of Na^+ ions since only a small number of K^+ ions penetrate into the headgroup region (Gurtovenko and Vattulainen 2008). Tu et al. (1996) found a bimodal distribution in their simulation of a PC bilayer in pure water. Both configurations at 41° and 84° of the headgroup angle with the bilayer normal are evenly populated. At higher temperatures, the distribution becomes uniform with an orientation spanning 0–135° (Saiz and Klein 2002). As mentioned previously, ions shift the tilt angle out of the membrane plane. This would mean that the

peak corresponding to a headgroup tilt of 41° becomes more populated at the expense of the other one. Calcium ions induce a stronger redistribution than sodium or potassium ions, which results in the observed re-ordering. This behaviour was also observed in simulations of phosphatidylcholine lipid membranes in fluid phase (Vácha et al. 2009; Cordomí et al. 2008, 2009) where the change in the area per lipid and in the headgroup tilt according to the presence of the different cation.

Measurements in buffer solutions with different NaCl concentration

The mechanism for the interaction of cations and lipid molecules and its impact on lipid ordering established above can be used to explain the observed concentration dependent effect of NaCl buffer solution on the bilayer structure. The difference between the 20 mM and 130 mM NaCl solution on the lipid spacing is only observed as a change in distance *A*. Since distance *B* remains unchanged, the area per unit cell is larger for the buffer solution with higher Na^+ concentration, as more cations can bind to the membrane to subsequently increase the tilt angle α and thereby the distance *A*. At 220 mM NaCl concentration, there is no significant change in any of the four observed parameters. This may be due to all binding sites being occupied by Na^+ ions. Garcia-Celma et al. (2007) reported that saturation of sodium binding to a PC membrane occurs in the range of 30 mM, and Tatulian (1987) gives a dissociation constant of Na^+ ions to phosphatidylcholine of (0.15 ± 0.10) M^{-1}. In contrast, the measurements in 1,000 mM NaCl buffer solution showed a different behaviour: it was the only system where the distance *B* was significantly altered. The distance *A* showed remained unchanged when the saturation of binding of Na^+ cations was reached. This may indicate a mechanism that was not observed in all the other solutions studied. One possible explanation is that anion interactions with the membrane in high salt concentration solutions become significant. The anion-bilayer interaction is thought to be very weak (Vácha et al. 2010); however at high Cl^- concentration a significant effect on the membrane structure may still be visible. This however needs further investigation.

The area per unit cell was observed to increase with increasing NaCl concentration. This is in contrast to simulations of DPPC bilayers in the fluid phase where the area per lipid is smaller for higher ionic strength NaCl solutions (Böckmann et al. 2003; Cordomí et al. 2008). However Uhríková et al. (2008) observed an increase in the area per lipid above $\sim$20 mM $CaCl_2$ in the gel and fluid phase in their study of a DPPC bilayer using small-angle neutron scattering.

The absolute value of the area per unit cell in 130 mM NaCl solution (0.40 nm^2) is significantly smaller than the 0.48 nm^2 reported for X-ray measurements of DPPC bilayer in pure water (Sun et al. 1994). Uhríková et al. (2008) observed that the area per lipid did not reach its original pure water value until a concentration of around 80 mM $CaCl_2$. Assuming similar behaviour occurs in the presence of Na^+ ions and keeping in mind that the interaction of Na^+ ions with the membrane is weaker than with Ca^{2+} ions, one would expect that the value of the area per unit cell was reached at considerably higher sodium concentrations. Our measurements suggest that this value was achieved at concentrations between 220 mM and 1,000 mM NaCl.

A measurement of the intermolecular spacing in pure water would have been very revealing in order to verify the behaviour observed by Uhríková et al. (2008) and to compare with the value reported by X-ray measurements (Sun et al. 1994). However the absence of ions at the lipid-fluid interface reduces the electrostatic screening and results in an increase of the Debye length. The corresponding reduction of the force gradient experienced by the AFM tip increased the frequency noise, rendering it unfeasible to obtain images of the bilayer at the required submolecular resolution.

An alternative explanation for the difference between the values of the area unit cell observed in this work and those found in the literature is the influence of the mica substrate. Johnson et al. (1991) showed that there is a thin water layer (approximately 30 Å) that uncouples the bilayer from the substrate. This effect is considered to be small since a reduction in interaction between the substrate and lower leaflet of the bilayer is not expected to affect the upper leaflet since the leaflets are not coupled.

The measurements in calcium and potassium buffer solutions fit the general model described herein and are consistent with the observations obtained in different ion concentrations. Calcium ions that interact more strongly with the membrane increase the area per unit cell more than potassium ions, which only weakly bind to the headgroup region of the lipid molecule. The strength of ion-lipid interactions reflected in the change of the distance *A* follows the predictions of the Hofmeister series.

Conclusions

We have investigated the ordering of lipid molecules of a DPPC bilayer in the gel phase in different salt solutions using high-resolution AFM. The Hofmeister series provides an indication of the anticipated strength of the lipid-ion interaction, which is well represented by our measurements of the membrane structure in potassium,

sodium and calcium buffer solutions. The distance *A* is smallest in the presence of K^+ ions having the lowest ionic strength and increases when measured in sodium buffer solution. The addition of an order of magnitude smaller amount of Ca^{2+} cations to a NaCl solution increases the distance *A* even further. The distance *B* and the area per unit cell do not significantly change in the three buffer solutions within experimental error. This gives rise to a non-isotropical re-ordering of the lipids that, we suggest, is caused by a change in the dipole-dipole interaction. On average, the calcium ions shift the headgroups more out of the membrane plane whereby the attractive interaction along the direction of the P–N vector is reduced, resulting in the reported change. Since the interaction along the distance *B* is unaffected by a headgroup tilt, this distance remains constant and is in very good agreement with our measurements. The mechanism established above can be applied to explain the re-ordering of lipid bilayers in sodium buffer solution of different ionic strength. Hereby, the P–N vector is not tilted as much out of the membrane plane in a low concentration regime since fewer cations bind to the lipid bilayer. With increasing ion concentration, a saturation effect was observed. At 1,000 mM NaCl, a different mechanism was observed as it was the only experiment where the distance *B* was significantly different than all other measurements. We attribute this finding to an anion-lipid interaction, which merely induces significant changes at high concentration since the anions are thought to only weakly interact with the lipid bilayer.

Our findings reveal new insights into the interaction of lipid bilayers and ions, where the ions induce a re-ordering of lipid molecules in a non-isotropical way. This highlights the complex interactions of bilayers and salts and their effect on the membrane structure, which potentially influences any biological processes such as membrane fusion or protein binding.

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