J Membrane Biol (2011) 244:35–42
DOI 10.1007/s00232-011-9395-1

NaCl Interactions with Phosphatidylcholine Bilayers Do Not Alter Membrane Structure but Induce Long-Range Ordering of Ions and Water

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Received: 12 August 2011 / Accepted: 19 September 2011 / Published online: 21 October 2011

Abstract It is generally accepted that ions interact directly with lipids in biological membranes. Decades of biophysical studies on pure lipid bilayer systems have shown that only certain types of ions, most significantly large anions and multivalent cations, can fundamentally alter the structure and dynamics of lipid bilayers. It has long been accepted that at physiological concentrations NaCl ions do not alter the physical behavior or structure of bilayers composed solely of zwitterionic phosphatidylcholine (PC) lipids. Recent X-ray scattering experiments have reaffirmed this dogma, showing that below 1 M concentration, NaCl does not significantly alter bilayer structure. However, despite this history, there is an ongoing controversy within the molecular dynamics (MD) simulation community regarding NaCl/PC interactions. In particular, the CHARMM and GROMOS force fields show dramatically different behavior, including the effect on bilayer structure, surface potential, and the ability to form stable, coordinated ion–lipid complexes. Here, using long-timescale, constant-pressure simulations under the newest version of the CHARMM force field, we find that Na^+ and Cl^- associate with PC head groups in a POPC bilayer with approximately equal, though weak, affinity, and that the salt has a negligible effect on bilayer structure, consistent with earlier CHARMM results and more recent X-ray data. The results suggest that interpretation of simulations where lipids interact with charged groups of any sort, including charged proteins, must be carefully scrutinized.

Keywords Ions · Lipid bilayer · MD simulation

Introduction

Biological membranes serve multiple functional roles in signal transduction, for example the organization and segregation of proteins (both soluble and membrane bound) and the sequestration of ions to establish ionic gradients. It has long been accepted that some ions interact directly with certain lipids in membranes, and that these interactions have the capacity to influence a wide array of physiological processes. Thus, much attention has been paid to understanding the full biophysical detail of how ions and lipids interact, how that interaction influences the behavior of water in a membrane's hydration shell, and how collectively these interactions perturb the underlying structure of the membrane. These efforts have been critical in determining the physical roles ions play in regulating membrane–protein structure and function.

Somewhat surprisingly, as the resolution of experimental and computational methods for studying atomic-scale interactions has increased, one of the most firmly entrenched assumptions regarding how NaCl interacts with membranes has been challenged. Historically, the defining experiments were carefully done electrophoretic mobility measurements that showed the surface (ζ) potential of phosphatidylcholine (PC) vesicles was completely unaffected by NaCl, with the interpretation that NaCl was not binding to the head groups (Bangham 1968; Eisenberg et al. 1979; Hanai et al. 1965; McDaniel et al. 1984; Winiski et al. 1986). Further, after decades of experimental

Electronic supplementary material The online version of this article (doi:10.1007/s00232-011-9395-1) contains supplementary material, which is available to authorized users.

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structural measurements, any suggestion of NaCl-induced structural changes in PC bilayers has been, at best, extremely sparse. Recently, numerous molecular dynamics (MD) simulations have been utilized to study local and global effects of ion–lipid interactions (Bockmann et al. 2003; Khavrutskii et al. 2009; Lee et al. 2008; Pandit et al. 2003; Sachs and Woolf 2003; Sachs et al. 2004; Vacha et al. 2009) and to corroborate a wide range of experimental results (Clarke and Lupfert 1999; Cunningham et al. 1988; Loosley-Millman et al. 1982; Macdonald and Seelig 1988; McDaniel et al. 1984; Pabst et al. 2007; Parsegian et al. 1979; Petrache et al. 2004, 2005, 2006; Roux and Bloom 1990; Rydall and Macdonald 1992; Szekely et al. 2011; Tatulian 1987; Winiski et al. 1986). However, disagreements have emerged in the data from various simulations, most notably about whether NaCl binds tightly to PC head groups and whether this binding induces significant structural changes in PC bilayers.

Originally, we ran short timescale CHARMM simulations of POPC lipids with NaCl, and our results suggested that, at physiological concentrations, while Na^+ does have affinity for PC head groups, it is quite weak (Sachs et al. 2004). We also found that NaCl causes no detectable change in bilayer structure. Concurrently, several groups were using the GROMOS force field to investigate similar questions. The GROMOS results, somewhat loosely supported at the time by fluorescence correlation spectroscopy (FCS) measurements that showed reduced lipid diffusion in the presence of NaCl, were quite alarming: Na^+ ions at physiological levels, it was claimed, have a high affinity for PC head groups. In fact, Na^+ binding and complexation (with 3–4 lipids/ion) was so strong that it creates a positively charged layer in each monolayer's head group region and induces a strong, nonzero ζ potential. Additionally, the results showed that NaCl thickens the PC bilayer by $\sim$2 Å, thereby reducing the area per lipid (A_L) by $\sim$4.9 Å^2 and significantly increasing the chain order (Bockmann et al. 2003; Lee et al. 2008; Pandit et al. 2003). These GROMOS results have been quite widely propagated in the literature over the past several years. Not surprisingly, it has been shown that the interaction between Na^+ and/or Cl^- and PC head groups, as well as the observed bilayer structural effects, are highly dependent on the ionic parameterization within the MD force field (Cordomi et al. 2008).

In the meantime, a recent X-ray diffraction study has provided further, high-resolution experimental evidence that NaCl does not have a large effect on bilayer structure. The measurements were able to detect changes in bilayer structure, but found that significant changes occur only at NaCl concentrations greater than 1 M (Pabst et al. 2007). At lower, physiological concentrations ($\sim$200 mM NaCl), small but statistically irresolvable structural changes were observed, including a decrease in A_L (<1 Å^2) as well as an increase in bilayer thickness (<1 Å). The reported order parameter remained unchanged at 200 mM NaCl. It should be noted that these results do not preclude the possibility that NaCl at physiological concentrations can interact directly, or bind to lipid head groups. The X-ray diffraction methods typically rely upon Fourier reconstruction of incomplete and truncated data, a model-dependent approach that may not reflect slight structural changes without an accurate model for reconstruction (Sachs et al. 2003).

Our aim here has been to update our older simulations using the newest CHARMM force field (Klauda et al. 2010) at longer timescales in order to determine whether our earlier observations were an artifact of undersampling and ensemble choice (constant as opposed to flexible surface area). We find that both Na^+ and Cl^- ions do interact transiently with the head groups. But, consistent with our earlier simulations and the scattering data, there is negligible effect on bilayer structure. For comparison, we have also run GROMOS simulations which recapitulate the earlier claims of tight, essentially irreversible, ion binding and associated dramatic changes in bilayer structure.

Materials and Methods

CHARMM Simulations

Systems were setup using the CHARMM molecular mechanics package (Brooks et al. 2009) with each system containing 128 POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, 16:0–18:1 PC) lipids fully hydrated ($\sim$42 TIP3P waters/lipid) in a solution without and with NaCl at a NaCl:H_2O ratio of 1:180. This setup is comparable to previous simulations (Bockmann and Grubmuller 2004; Bockmann et al. 2003), and were simulated for 300 ns using the NAMD molecular dynamics package (Phillips et al. 2005) (v. 2.7b2) with the CHARMM36 force field (Klauda et al. 2010) at $T = 300$ K with flexible unit cells (isothermal–isobaric, NPT, ensemble). Large systems contained 128 POPC lipids, $\sim$125 TIP3P waters/lipid without and with NaCl again at a NaCl:H_2O ratio of 1:180 (90 each Na^+ and Cl^- ions), and were simulated for 300 ns as before. The large system containing NaCl was simulated using the NBFIX parameters (Roux and Luo 2010), an ion-pairing parameter correction for accurate simulation at high electrolyte concentration. A separate system without NBFIX was also simulated for 300 ns. All CHARMM results shown include the NBFIX parameters, though there was no noticeable difference in electron density profile or salt distribution (data not shown). That the NBFIX parameters did not influence either electron density profile or salt distributions is likely a result of low average

electrolyte concentrations (less than 0.5 M NaCl) in our systems. The NBFIX parameter is critical for solutions with high electrolyte concentration (greater than 1 M NaCl) where deviations in osmotic pressure between experiment and simulation are apparent (Roux and Luo 2010). However, NBFIX parameter was used to correct for potentially high local concentrations of NaCl within the system.

GROMOS Simulations

All simulations run under the GROMOS force field are defined as follows. Systems were run using GROMACS v4.5.3, with each system containing 128 Berger POPC lipids (Berger et al. 1997) fully hydrated using SPC water molecules (van Buuren et al. 1993) ($\sim$42 SPC water molecules per lipid). The starting bilayer configuration was taken from a preequilibrated 128 POPC lipid system provided by the Tieleman group (http://moose.bio.ucalgary.ca/). Ions were added to bulk water solution to match the CHARMM systems (NaCl:H_2O ratio of $\sim$1:180). Systems were run at $T = 300$ K with flexible unit cells (isothermal–isobaric, NPT, ensemble). Simulations were carried out for 200 ns and analysis was performed on equilibrated systems (100–200 ns) using GROMACS and Perl.

Simulation Analysis

Analysis was performed on equilibrated trajectories (50–300 ns) using CHARMM and Perl, utilizing an autocorrelation function as previously described (Lee et al. 2008) to determine the number of independent samples. Equilibration was determined by convergence of both A_L and the Na^+ distribution with respect to the bilayer normal. Electron density profiles were calculated by first recentering the bilayer and applying periodic boundary conditions to water and NaCl. Electron density was calculated by normalizing to the bin volume determined by a z-bin size of 0.2 Å (z, the dimension of the bilayer normal) and the variable x- and y-box dimensions of the system at each step. Results represent the time averaged electron density as a function of the distance from the center of the bilayer ($z = 0$). The 250 ns CHARMM simulation trajectories and 100 ns GROMOS simulation trajectories were output and analyzed at every 10,000 steps, or 20 ps increments. Na^+ and Cl^- molar concentrations were calculated based on the number density of each ion and the calculated bin size.

Lipid order parameters, S_{CD}, were calculated for all atom CHARMM trajectories (using Perl) and for unified atom GROMOS system (using g_order). Results shown represent the average order parameter as a function of carbon number over the entire equilibrated trajectory. Na^+–lipid coordination numbers were calculated based on Na^+ distance to lipid carbonyl and phosphate oxygen atoms. Radial distribution functions between these groups were generated, and Na^+ ions closer than the minimum after the first peak in the RDF (cutoff at 3.22 Å) were considered as interacting with the head group.

The estimation of error for area per lipid (see Supporting Information, Fig. SI6) and lipid order parameter (see Supporting Information, Fig. SI7) were estimated using block sampling analysis as previously described (Grossfield and Zuckerman 2009). Briefly, error estimation of area per lipid and order parameter was calculated from converged trajectories considering a full range of block sizes. The block standard error (BSE) increases monotonically with larger block sizes and asymptotes at large block sizes; error was calculated based on the asymptotic value. The results for block sampling to estimate error for area per lipid and order parameter are shown in the Supporting Information (Figs. SI6 and SI7, respectively).

The water dipole was calculated as previously described (Gurtovenko 2005; Sachs et al. 2004) where theta (θ) represents the angle between the water–dipole moment and the z-axis (normal to the bilayer). Results presented are the time-averaged $\langle \cos(\theta) \rangle$, as a function of z-distance from the bilayer center. Full isotropic rotation of water molecules corresponds to $\langle \cos(\theta) \rangle = 0$, and is observed in the absence of head group dipoles and salts.

Results

Figure 1 illustrates the electron density profiles for the POPC systems containing $\sim$42 H_2O/lipid and either 0 or 30 NaCl salts (30 of each ion). Using the CHARMM force field, the addition of NaCl causes only a subtle change in the electron density profile (Fig. 1a), with a negligible effect on thickness (D_{HH}). Increased electron density in the water region is due to the added ions. Similar to our previous CHARMM simulations (Sachs et al. 2004), Na^+ ions are somewhat enriched near the head group region ($\sim$60% relative to their density at the limits of the water box). Cl^- ions show slightly less, though still nonnegligible enrichment outside the head group region (Fig. 1b). Quite distinctly, the GROMOS simulations show that Na^+ ions have a significantly increased affinity for POPC lipids, having a 500% enrichment within the lipid head group region relative to the level at the simulated box's boundary. As expected, this causes a significant thickening of the bilayer (Fig. 1c), consistent with previous GROMOS results (Bockmann et al. 2003; Lee et al. 2008; Pandit et al. 2003) (component electron density profiles for these systems are available in Supporting Information). In all cases, it is clear that these simulated boxes, the same approximate size as all previous salt/bilayer simulations, are not large

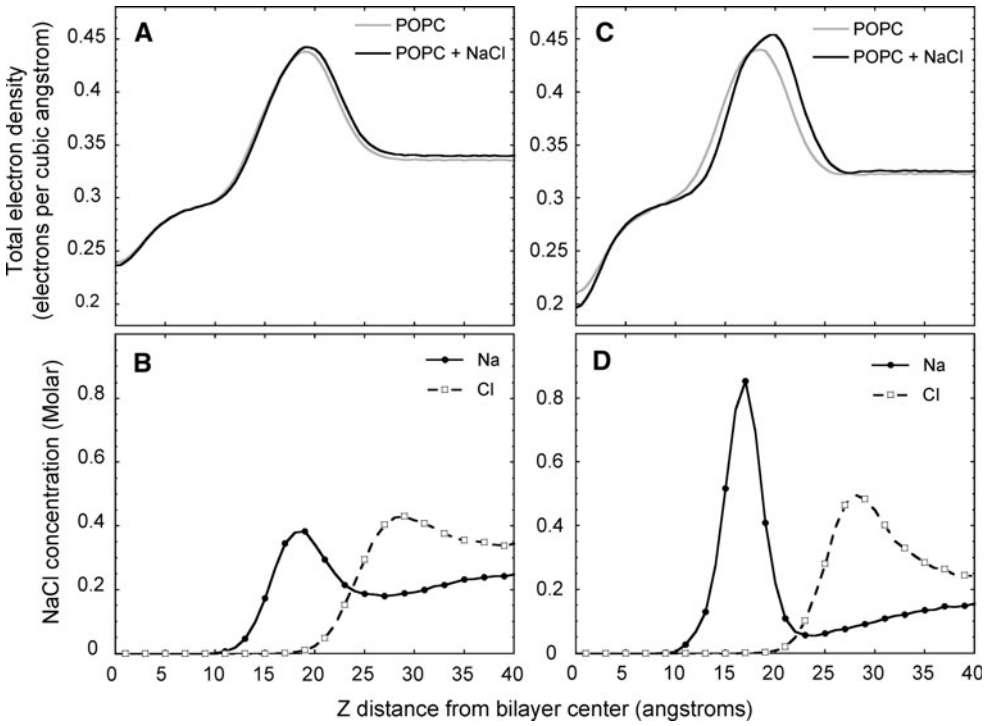

Fig. 1 Simulations demonstrate that Na^+ and Cl^- have an affinity for POPC bilayer, but the level of salt binding as well the effect on bilayer structure is highly dependent on force field. **a** Long-timescale simulations of POPC using the CHARMM force field in the absence (*gray line*) and presence of NaCl (*black line*) show little structural effect of physiological levels of NaCl. **b** The distribution of Na^+ (*solid line*) and Cl^- (*dashed line*) show the affinity of ions for POPC, with an enrichment of Na^+ ions in the head group region and an enrichment of Cl^-outside the head group region. **c** Identical POPC bilayer systems run using the GROMOS force field show a marked difference in the bilayer thickness in the presence of NaCl (*black line*) compared to the pure POPC bilayer (*gray line*). **d** The ion distribution demonstrates under the GROMOS force field a high enrichment of Na^+ ions (*solid line*) deep in the head group region and a similar enrichment of Cl^- ions (*dashed line*) outside the head group

Table 1 Structural effects of NaCl

System	POPC	NaCl	Simulation time (ns)	Area per lipid Å^2 (% change)		D_{HH} Å (% change)		$\|S_{CD}\|$ (Palm. C6)		Ion–lipid coordination nos.
CHARMM										
Pure	128	−	300	67.16	−	37.6	−	0.206	−	−
NaCl	128	+	300	65.67	(−2.22%)	38.1	(+1.33%)	0.209	(+1.46%)	1.75
GROMOS										
Pure	128	−	200	64.41	−	36.6	−	0.198	−	−
NaCl	128	+	200	59.39	(−14.63%)	39.4	(+7.65%)	0.229	(+15.64%)	3.1

enough to reach bulk NaCl levels ($[Cl^-] > [Na^+]$ at $z = 40$ Å), an issue that will be addressed further below.

The structural effects of NaCl using these two force fields are summarized in Table 1. Notably, the effects on A_L, D_{HH} and lipid order are much greater under the GROMOS force field than under CHARMM. Consistent with previous results, the GROMOS force field shows a remarkable 5 Å^2 NaCl-induced decrease in A_L. This is compared to a 1.5 Å^2 decrease using CHARMM. Consistently, D_{HH} increases by 2.8 Å under GROMOS compared to a 0.5 Å under CHARMM. The observed structural changes due to NaCl using the GROMOS force field cause a noticeable shift in the X-ray scattering form factor (see Supporting Information), a result that is not supported by experimental evidence at comparable NaCl concentrations. Conversely, using the CHARMM force field, the small structural changes observed upon salt addition are within the error of X-ray scattering measurements.

Further, coordination numbers were calculated based on the localization of Na^+ ions near lipid carbonyl and phosphate oxygen atoms (with a cutoff based on the radial distribution function, data not shown). The ion–lipid

coordination number, the number of lipid carbonyl or phosphate groups surrounding a Na^+ ion in the head group region, as previously described (Lee et al. 2008), is markedly different using CHARMM, ~1.75, compared to GROMOS, ~3.1, consistent with the higher Na^+ enrichment in the head group region using GROMOS (detailed analysis of coordination number available in Supporting Information). Interestingly, using the CHARMM force field, there is a strong correlation between lipid–ion coordination and residence time; Na^+ ions with longer residency time have a higher coordination number—Na^+ ions with a residency time greater than 5 ns are most often coordinated to 2 or 3 carbonyl or phosphate oxygen atoms (see Supporting Information). This suggests that the high degree of lipid–ion coordination observed using GROMOS is not precluded by using CHARMM, but occurs only rarely and correlates with ions becoming trapped by complexation with several head groups.

As stated above, recent EPR measurements support long-standing knowledge that NaCl concentrations up to 300 mM cause no detectable change in lipid ordering. We examined the effect of NaCl on order parameter, S_{CD}, and the force field-dependent effect of NaCl on lipid chain order is dramatic. Under the CHARMM force field, the addition of NaCl has little or no effect on chain ordering (Fig. 2a), and the presence of NaCl with GROMOS causes a significant increase in lipid chain order (Fig. 2b). The observed ordering effect induced by NaCl using GROMOS coincides with bilayer thickening and a reduction in A_L. Using CHARMM, despite the small changes in A_L and bilayer thickness, there is no observable change in lipid tail ordering upon the addition of NaCl (see Supporting Information for error estimation).

Because both Na^+ and Cl^- ions have a noticeable affinity for PC head groups, we observed in Fig. 1 that the salt concentration in the fully hydrated bilayer (~42 H_2O/lipid) fails to reach a bulk concentration. This led us to question whether the addition of more water and ions would change the level of enrichment of either Na^+ or Cl^- in or near the PC head group region, and to establish how large a water box is necessary for these types of simulations. Large systems were constructed to contain the same number of lipids (128 equilibrated POPC lipids) but with three fold more water and ions (~125 H_2O/lipid and 90 NaCl, thus keeping the NaCl:H_2O ratio at 1:180). These bilayers, with and without NaCl, were run for 300 ns using the CHARMM force field, and analysis was performed on equilibrated systems as before. Figure 3 illustrates that the addition of more water and NaCl has a negligible effect on the bilayer thickness (Fig. 3a) compared to the small system. The time-averaged distribution of ions (Fig. 3b) shows that the addition of more NaCl does not increase the enrichment of Na^+ in the head group region. Further, we

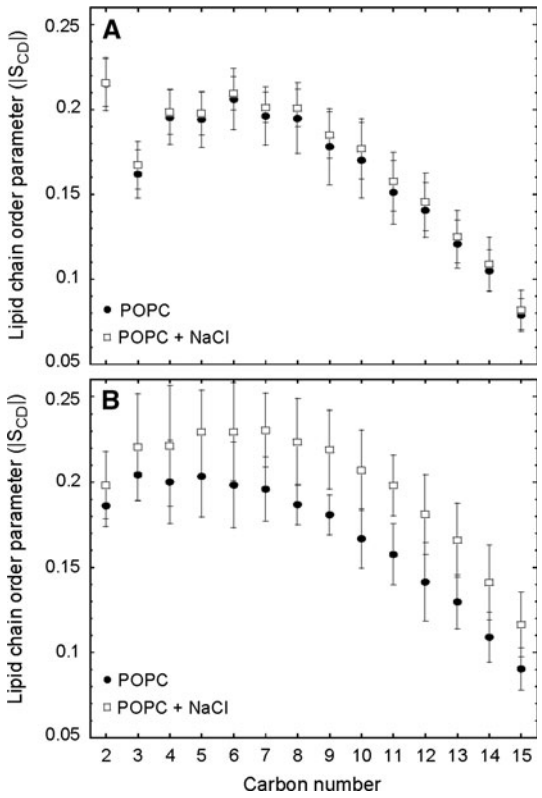

Fig. 2 The effect of NaCl on lipid ordering using CHARMM and GROMOS. **a** CHARMM force field shows little induced ordering in the palmitoyl chain upon the addition of NaCl (*open squares*) compared to the pure system (*solid circles*). **b** Using the GROMOS force field, the addition of NaCl (*open squares*) induces ordering of the palmitoyl chain, consistent with the other structural changes shown in Table 1, but inconsistent with experimental data. Error was estimated using block sampling analysis (see Supporting Information, Fig. SI7)

note that the addition of NaCl causes a marked increase in the distance at which the electrostatic effects of the bilayer can be observed by measuring the water dipole orientation (Fig. 3c) as previously described (Gurtovenko 2005; Sachs et al. 2004). Notably, in the pure POPC system the water orientation reaches an isotropic distribution ($\langle \cos\theta \rangle = 0$, Fig. 3c, dashed line) approximately 30 Å from the bilayer center, whereas the addition of ~300 mM bulk NaCl to the system causes further ordering of water molecules out to ~50 Å, approximately consistent with the distance at which both Na^+ and Cl^- concentrations reach their bulk values. Collectively, these results suggest that bilayer simulations in the presence of salt should contain significantly more water than has been previously considered full hydration (~42 H_2O/lipid), a factor that is particularly important when using MD simulations with periodic boundary conditions to study ion adsorption to a bilayer, and the resulting charge distribution and surface potential of a bilayer.

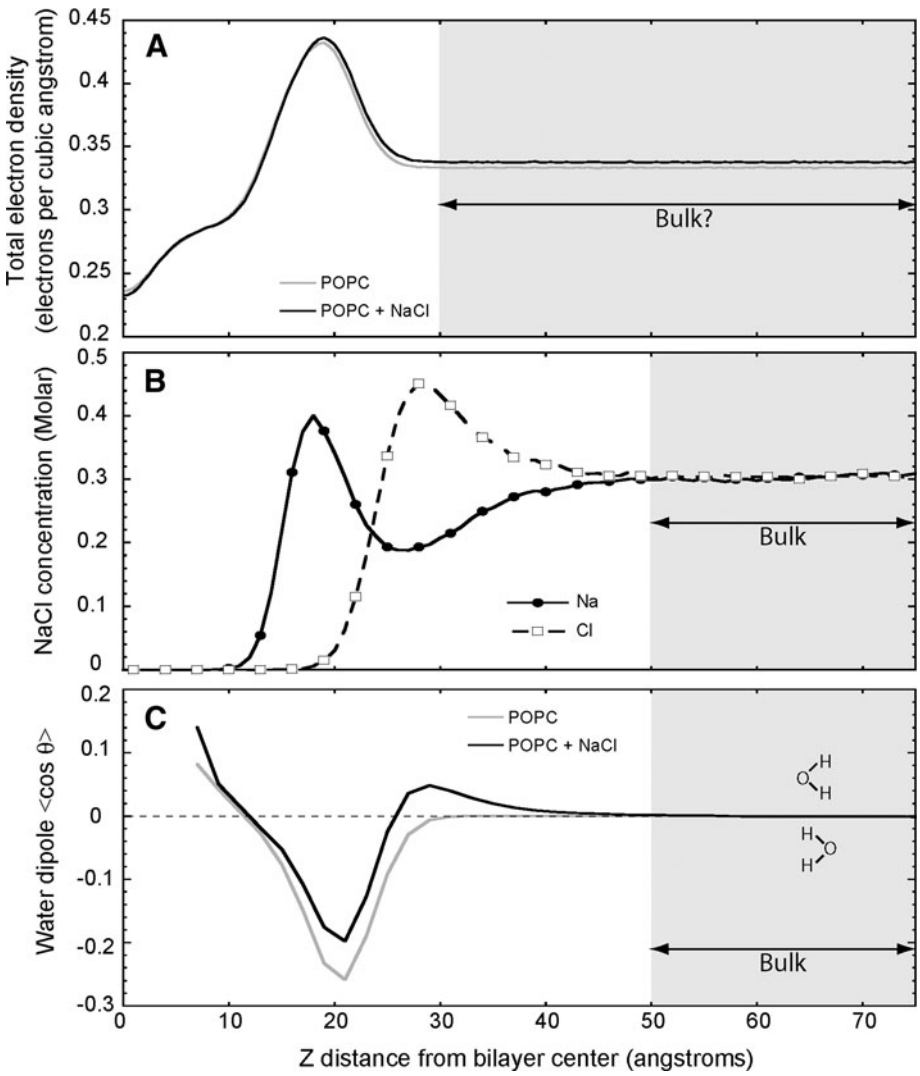

Fig. 3 Large, long-timescale simulations of POPC with additional water, both in the absence and presence of NaCl. **a** Electron density profiles of pure POPC (*gray*) or POPC + NaCl (*black*) demonstrate that the addition of more water and ions has little effect on bilayer structure. **b** The distribution of Na^+ (*solid line, closed circles*) and Cl^- (*dashed line, open squares*) ions shows the layering effect of NaCl, and the overall ion concentration reaches bulk ~50–60 Å from the bilayer center. **c** The water dipole compared to the bilayer normal suggests the pure POPC bilayer affects water ordering out to ~30 Å from the bilayer center whereas POPC in the presence of NaCl causes further ordering of water molecules out to ~50 Å. In each figure, the perceived bulk of POPC in the presence of NaCl is shown in *gray*

Discussion

Our MD simulations using the CHARMM force field demonstrate that the addition of NaCl to a POPC bilayer yield structural results that are within the range of those observed in X-ray experiments. The Na^+ and Cl^- affinity for PC head groups is clearly and highly dependent on the selection of parameter set, a somewhat unsettling, but not surprising result. Using CHARMM36, the affinity of Na^+ for PC head groups is markedly lower than is observed using GROMOS, shown here and previously described (Pabst et al. 2007), and is only slightly higher than was observed using CHARMM27 with fixed A_L (Sachs et al. 2004). The effects on bilayer structure upon the addition of NaCl using CHARMM36 appear to more accurately reflect experimental observables; the slight decrease in lateral A_L and increase in bilayer thickness are within the range of the X-ray scattering uncertainty. In the CHARMM simulations where sufficient water is added (Fig. 3) we observe equivalent enrichment of Cl^- ions outside the head group region as Na^+ within the head group region, resulting in an electrical double layer and suggesting that the experimentally observed neutrality of PC vesicles in electrolyte solution may be a result of equal, but weak, cation and anion adsorption. That the total charge density integrates to zero by ~50 Å from the bilayer center is thus, in a somewhat complicated way, consistent with electrophoretic mobility measurements. Reconciliation of the FCS data that suggested NaCl slows lipid diffusion remains a conundrum, though perhaps weak binding is sufficient to have this effect without the associated structural changes.

It is important for the simulation community that we demonstrated that lipid bilayers in the presence of NaCl exhibit long range ordering of both ions and water molecules—up to 60 Å from the bilayer center—as a result of the ion adsorption. Further simulations used to calculate charge-based distributions, such as potential gradients across such bilayers, need to take into account the

magnitude with which the lipid bilayer influences its surroundings, including the distribution of charged molecules and water. The significant differences in interaction between charged groups and lipids using CHARMM and GROMOS suggest that careful scrutiny of both parameters and system size are necessary depending on the system of interest, including not only various lipid–ion systems but potentially the simulation of membrane proteins with charged residues. If charged ions bind too tightly to lipid head groups in MD simulations, in all likelihood, so do charged amino acids.

Acknowledgments The authors would like to thank the Minnesota Supercomputing Institute for computational resources, B. Roux, and R. Pastor for valuable discussion, and A. Grossfield for discussion regarding error estimation.

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