

Accurate binding of calcium to phospholipid bilayers by effective inclusion of electronic polarization

Josef Melcr and Hector Martinez-Seara Monne

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague 6, Czech Republic

Pavel Jungwirth

*Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague 6, Czech Republic and
Department of Physics, Tampere University of Technology, P.O. Box 692, FI-33101 Tampere, Finland*

O. H. Samuli Ollila*

*Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague 6, Czech Republic and
Institute of Biotechnology, University of Helsinki*

(Dated: October 12, 2017)

Despite of the significant biological relevance, the binding details of cations in cellular lipid membranes are not fully understood. For example, consistent results for the binding affinities and stoichiometries of Na^+ and Ca^{2+} ions to phospholipid bilayers have not been achieved with different experimental and theoretical methods. As recently shown in the NMRlipids project, a Open Collaboration running at nmrlipids.blogspot.com, the ion binding details could be resolved with unprecedented detail by interpreting the experimental NMR data with classical molecular dynamics (MD) simulations. However, the accuracy of the existing lipid models for MD simulations was not sufficient for this. In this work we show that the binding details of Na^+ and Ca^{2+} ions to 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) bilayer can be accurately described with a MD simulation model having implicitly included electronic polarization. This is demonstrated by applying the electronic continuum correction (ECC) to a state of the art lipid model for MD simulations of POPC lipid bilayer. The introduced ECC-lipid model reproduces the experimentally measured structural parameters for the ion-free membrane, the response of lipid headgroup to the bound positive charge, and the binding affinities of Na^+ and Ca^{2+} ions. The imperceptible binding of Na^+ ions to POPC bilayer and interactions of Ca^{2+} mainly with phosphate oxygens in the ECC-lipid model give support to the early interpretations of the experimental spectroscopic data. On the other hand, Ca^{2+} ions form complexes with 1-3 lipid molecules with almost equal probabilities, suggesting more complicated binding stoichiometry than the simple binding model used to interpret the NMR data. The results in this work pave the way for MD simulations of complex biochemical systems with realistic electrostatic interactions in the vicinity of cellular membranes.

I. INTRODUCTION

Cation interactions with cellular membranes play a key role in several biological processes, such as in signal propagation in neurons and vesicle fusion. Since the direct measurements of ion-membrane interactions from biological systems are difficult, lipid bilayers are often used as models systems for cellular membranes. Especially the zwitterionic phosphocholine (PC) lipid bilayers are used to understand the role of ions in complex biological systems [1–3].

Interactions of biological cations, especially Na^+ and Ca^{2+} , with PC bilayers are widely studied in experiments [2–9] and classical MD simulations [10–14]. The details of ion binding are, however, not fully consistent in the literature. Interpretations of non-invasive spectroscopic methods, like nuclear magnetic resonance (NMR), scattering and infrared spectroscopy suggest that Na^+ ions exhibit negligible binding to PC lipid bilayers with submolar concentrations, while Ca^{2+} specifically binds to phosphate groups of two lipid molecules [4, 5, 7–9, 15–17]. Atomistic resolution molecular dynamics (MD) simulation models, however, predict significantly stronger binding for the cations than NMR experiments [18]. On the other hand, some experiments have also

been interpreted to support the predictions from MD simulations [10, 19]. Furthermore, interactions of Calcium ions with 3-4 lipids, including also interactions with carbonyl oxygens, have been reported from simulations [10, 11, 13, 14].

Recent work published by the NMRlipids project (nmrlipids.blogspot.fi) [18] made an attempt to resolve the apparent controversies. A direct comparison of ion binding affinities to PC bilayers was presented between simulations and experiments by using the electrometer concept, which is based on the experimental NMR data for the lipid headgroup order parameters [20]. Using massive amounts of data collected by Open Collaboration method, it was concluded that the accuracy of the current state of the art lipid models for MD simulations is not sufficient for the detailed interpretation of the cation interactions with PC lipid bilayers [18].

In this work we show that the cation binding behavior in MD simulations of 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) bilayer can be significantly improved by implicitly including the electronic polarizability in the polar region of lipid molecules. The electronic polarizability is included by using the electronic continuum correction (ECC) [21], which has been previously shown to improve the behaviour of MD simulations of ions in bulk water [22–24]. As a starting point we use the parameters from the Lipid14 model [25], which gave the best cation binding behaviour in the previous study [18]. The developed ECC-lipid parameters reproduce the ex-

*samuli.ollila@helsinki.fi

perimentally measurable structural parameters of an ion-free POPC lipid bilayer with the accuracy comparable to the other state of the art lipid models, while surpassing them significantly for reproducing the membrane binding affinities and induced structural effects of Sodium and Calcium ions.

II. METHODS

A. Electronic continuum correction for lipid bilayers

The lack of electronic polarizability in the standard MD simulation force fields has been considered a highly relevant issue since the early days of lipid bilayer simulations. In this work we circumvent the rather demanding explicit inclusion of electronic polarization effects [26] by implicitly including electronic polarizability in lipid bilayer simulations by using the electronic continuum correction (ECC) [21]. Technically, it is a similar approach to the phenomenological charge-scaling as applied in the early studies where a scaling factor one half was used [27, 28]. **1. We should also cite papers where empirical scaling was used ionic liquids - but there the factor is not 0.5.** However, the present concept of ECC is physically well justified and rigorously derived [21, 29, 30].

According to ECC, electronic polarizability can be implicitly included in classical MD simulations by placing all particles into a homogeneous dielectric continuum with a dielectric constant ϵ_{el} , which is the electronic part of the dielectric constant of the media [21]. Measurements of high frequency dielectric constant gives values of approximately $\epsilon_{el} \approx 2$ for almost any biomaterial [21?]. Such a dielectric continuum can be easily included in standard MD simulation by a formal transformation of partial charges

$$Q^{ECC} = f_q \cdot Q \quad (1)$$

with a constant scaling factor $f_q = \epsilon_{el}^{-1/2}$ effectively representing the newly introduced electronic continuum. Assuming globally a high frequency dielectric constant as measured in water (corresponding to the square of the refraction index), $\epsilon_{el} = 1.78$, results in a scaling factor of $f_q = 0.75$ [21?]. This scaling factor has been successfully used to improve the performance of force field for ions in solution [23? , 24] which then agree quantitatively with neutron scattering data [22–24].

While the scaling factor of $f_q = 0.75$ for ions in water improves their performance and is physically justifiable within the ECC theory [?], it is not clear whether the same factor should be used for partial charges in molecules, e.g., lipids in our case. Unlike the total charge of an atom or molecules, atomic partial charges within each molecule are not physical observables. There are several schemes for the assignment of partial charges for biomolecules. [31] Currently, the most commonly employed scheme is the restrained electrostatic potential (RESP) method [32, 33]. In practice, partial charges currently implemented in force fields may already include to some extend some of the solvent electronic polarizability effects, i.e., the RESP charges are often scaled to fit some ex-

perimental observables **2. This needs a citation.** Thus, we expect that the application of the ECC scaling factor, f_q , to the molecular partial charges included in the available force fields does not necessarily have to follow the relation $f_q = \epsilon_{el}^{-1/2}$, but instead it lies between 0.75 (no electronic polarizability in the partial charge calculation) and 1 (full electronic polarizability already included in the partial charge calculation).

In this work, we develop a phospholipid model for classical MD simulations that accurately describes the lipid head group response to varying concentrations of monovalent and divalent cations. This is a biologically highly relevant membrane feature, which is poorly reproduced by currently available models which can affect not only on membrane properties in the presence of ions but also modulates the interaction with charged moieties in the surface. Importantly, this response from simulations can be accurately compared against experimental NMR data [4, 5, 34], as discussed in Ref. 18 in section II B. To this end, we empirically explore the scaling factor parameter space, $f_q \in [0.75, 1.0]$ for the Lipid14 [25] force field. We selected this force field as a starting point because its response to bound ions was apparently the most realistic against NMR data in recent work by NMRlipids project (see Fig. 5 in Ref. 18). Also glycerol backbone and head group structures in Lipid14 model were relatively realistic when compared with other state of the art lipid models [35]. The ECC correction was applied to Lipid14 parameters by scaling partial charges of the head group, glycerol backbone and carbonyl regions, which are the most polar parts in lipids and are expected to have the largest contribution to the cation binding. However, we do not modify the hydrocarbon chain parameters, because they do not come in contact with salt ions and are already highly optimized and give generally a good description for hydrophobic part of lipid bilayers in most lipids models, including Lipid14 [36]. This is in contrasts with the behavior in glycerol backbone and head group order parameters which call for improvements in all available lipid models [35].

Exploring different scaling factor values, we found out that ion binding and related head group order parameter responses become weakened in general. The optimal behaviour of ion binding was observed with the scaling factor of $f_q = 0.8$. **3. JOE: following discussion shall be modified in the enlightenment of our recent ECC-discussions.** Interestingly, this scaling factor is in line with the estimate given by “implicitly polarized charges” (IPolQ) [37] combined with RESP calculations in vacuum and implicit solvent reported in [38]. IPolQ charges are obtained as the average of partial charges given by RESP calculation [32] in vacuum and in a solvent. Applying the scaling factor of 0.75 to IPolQ charges calculated from the data in Ref. [38], gives similar partial charges to ones obtained by scaling Lipid14 charges with a factor 0.8.

While, the charge scaling improved the behaviour of lipid-ion interactions, it reduced the area per molecule of the lipid bilayer without ions below experimental values. Simulations with Lipid14 parameters having partial charges of head group, glycerol backbone and carbonyls scaled with 0.8 gave the area per molecule value of $\approx 60 \text{ \AA}^2$, which is smaller than the experimental value 64.3 \AA^2 ([4. missing REF for APL experiment) and the original Lipid14 value $(65.6 \pm 0.5) \text{ \AA}^2$ [25]. The decrease

of the area per lipid was found to arise from a lower hydration of the lipid head group region, which can be explained by the increased solvation free energy due to the lower polarity of molecules with scaled charges. The hydration can be increased back by reducing the effective radius of atoms by changing the σ parameters in the Lennard-Jones potential for the selected atoms similarly as done for free ions in solution [22–24]. **5. We should discuss how this can potentially affect the intermolecular interaction when mixing scaled and non scaled molecules.**

JOE: I think that we rather increasingly see that there's nothing like "fully non-scaled" with the exception of ions with integer charges. So the discussion shall be rather more about the interaction of our "scaled" (I'd still rather call it ECC-corrected or whatever) and "semi-scaled" models.

SAMULI: There is now a paragraph in the conclusions, which mentions this topic. This decreases the solvation free energy by allowing water molecules to approach closer to lipid atoms and have stronger electrostatic interactions with them. After reducing the σ parameters by a factor of $f_\sigma = 0.89$ for the same atoms for which charges were scaled, the area per molecule value was back in agreement with the experimental value (see Table I).

B. Electrometer concept

Ion binding in lipid bilayers was compared between experiments and simulations by using the lipid head group order parameters and the "electrometer concept" [18, 20], which is based on the experimental observation that the C-H bond order parameters of α and β carbons in PC lipid head group (see Fig. 1) are proportional to the amount of unit charge bound per lipid [20]. The change of order parameters measured with varying aqueous ion concentration can be then related to the amount of bound ions.

The concept can be used to compare the ion binding affinity in lipid bilayers between MD simulations and NMR experiments, because the order parameters can be accurately determined from both techniques [18, 36]. The order parameters for all C-H bonds in lipid molecules, including α and β segments in head group, can be accurately measured using ^2H NMR or ^{13}C NMR techniques [36]. From MD simulations the order parameters can be calculated using the definition

$$S_{\text{CH}} = \frac{3}{2} \langle \cos^2 \theta - 1 \rangle, \quad (2)$$

where θ is the angle between the bond and membrane normal and the average is taken over all sampled configurations [36].

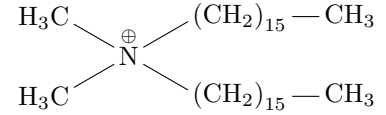
The relation between bound charge per lipid, X^\pm , and the head group order parameter change, ΔS_{CH}^i , is empirically quantified as [20, 40]

$$\Delta S_{\text{CH}}^i = S_{\text{CH}}^i(X^\pm) - S_{\text{CH}}^i(0) \approx \frac{4m_i}{3\chi} X^\pm, \quad (3)$$

where $S_{\text{CH}}^i(0)$ denote the order parameter in the absence of bound charge, i refers to either α or β carbon, m_i is an empirical constant depending on the valency and position of the bound charge, and the experimental value [41, 42], $\chi \approx 167 \text{ kHz}$, is used for the quadrupole coupling constant.

Atomic absorption spectra and ^2H NMR data gave $m_\alpha = -20.5 \text{ kHz}$ and $m_\beta = -10.0 \text{ kHz}$ for the binding of Ca^{2+} to POPC bilayer (in the presence of 100 mM NaCl) [5, 18, 36]. The slopes are negative, because the head group order parameters decrease with bound positive charge and increase with bound negative charge [18, 36]. This can be rationalized as a change of the lipid head group dipole tilt toward water phase with the bound positive charge and *vice versa* with the negative charge [20].

The measured order parameter change depends on both, the head group response to the bound charge and the amount of bound charge, i.e. m_i and X^\pm in Eq. 3, respectively. The former property has to be well quantified before the electrometer concept can be used to analyze the binding affinities, as done experimentally for a wide range of systems [20, 43]. To calibrate the head group order parameter response to the bound charge in simulations, we use the experimental data for dihexadecyldimethylammoniumbromide mixed within a POPC bilayer [34]. Dihexadecyldimethylammonium



is a cation surfactant having two acyl chains and bearing a unit charge in the hydrophilic end. Thus, it is expected to locate in the bilayer similarly to the phospholipids and the molar ratio then gives directly the amount of bound unit charge per lipid X^\pm in these systems [34].

C. Salt concentrations and binding affinity

The early experimental electrometer concept data for the lipid head group order parameter changes was reported as a function of the salt concentration in water before solvating the lipids [4]. The later study used atomic absorption spectroscopy and reported the order parameter changes as a function of the salt concentration in the supernatant after the solvation of lipids [5]. In this work we focus on POPC for which the latter definition was used [5]. The salt concentration in the aqueous bulk region was calculated from the farthest point from both lipid leaflets in the water phase. Note that in the previous study, Ref. 18, the ion concentrations were calculated in water before solvating the lipids as in the earlier experiments [4]. Despite of the measurable differences between these two definitions of concentrations for CaCl_2 systems, the qualitative conclusions in this or in the previous work [18] are not affected by this.

To quantify the ion binding affinity to a lipid bilayer, we calculated the relative surface excess of ions with respect to water, Γ_i^w [44]. This quantity was chosen because it does not depend on the position of the Gibbs dividing plane between two bulk regions. Here we assume that the interface locates between the hydrophobic interior of a lipid bilayer and the bulk water region far from the membrane. The bulk concentration of ions and water is zero inside the bilayer. The con-

centrations in bulk water region can be calculated from the farthest point from both lipid leaflets in the water phase. The region between these boundaries contains all the ions and water molecules in the simulation box. This setup provides a simplified relation for Γ_i^w in lipid bilayers simulations

$$\Gamma_i^w = \frac{1}{2A_b} \left(n_i - n_w \frac{C_i}{C_w} \right), \quad (4)$$

where n_w and n_i are the total number of waters and ions in the system; C_w and C_i are their respective bulk concentrations in the aqueous phase; and A_b is the size of the box in the membrane plane. The total area of the interface is twice the area of the membrane, $2A_b$, because bilayers have an interface at both leaflets.

D. Validation of lipid bilayer structure against experiments

The structure of lipid bilayers in simulations without ions were validated against NMR and x-ray scattering experiments by calculating the order parameters for C-H bonds and the scattering form factors. The former validates the structures sampled by individual lipid molecules in simulations with atomic resolution, while the latter validates the dimensions of the lipid bilayer (thickness and area per molecule) [36].

The order parameters were calculated from simulations for all C-H bonds in lipid molecules by using Eq. 2. Form factors were calculated from equation 6. **As Hector suggested, it might be better to write the simpler form for this equation.**

$$F(q) = \int_{-D/2}^{D/2} \left(\sum_{\alpha} f_{\alpha}(q_z) n_{\alpha}(z) - \rho_s \right) \exp(izq_z) dz, \quad (5)$$

where $f_{\alpha}(q_z)$ is the density of atomic scattering length, ρ_s is the density of solvent scattering length in the bulk region, $n_{\alpha}(z)$ is the number density of atom α and z is the distance from the membrane centre along its normal spanning until the water bulk region, D .

E. Simulation details

1. Simulations of POPC bilayers in aqueous ions

Simulations of POPC bilayer in pure water or in varying salt concentrations contained 128 POPC molecules and approximately 50 water molecules per each lipid in the periodic orthorhombic simulation box. As a default, water molecules were described by the OPC3 force field [45] which is currently the most accurate three site rigid water model **7. More justification for the choosing the OPC3 water model are needed. It might be good to show the comparison with the scattering data in bulk water in SI.** In order to test transferability of our newly developed ECC-lipids model, we also performed several additional simulations with OPC [46], SPC/E [47], TIP3p-FB and TIP4p-FB [48], and TIP4p/2005 [49] water models **8. The normal TIP3P was tested as well, right?** presented in Supporting Information (SI). The ECC-ion models

TABLE I: Area per lipid (APL) values of POPC with no ions from the Lipid14 simulation ran in this work and from the literature, from ECC-lipid model and from experiments.

model	APL (\AA^2)	Temperature [K]
Lipid14	65.1 ± 0.6	300
Lipid14 [25]	65.6 ± 0.5	303
ECC-lipids	62.2 ± 0.6	300
experiment [60] 18. REF	64.3	303

were used for Sodium, Calcium and Chloride ions [22, 24?]. Simulation with the Lipid14 model was also ran with the ion model by Dang et al. [50–52]. Simulation data for the Lipid14 model with Åqvist [53] ion model was taken directly from [54]. MD simulations were performed using the GRO-MACS [55] simulation package (version 5.1.4). The simulation settings used in this work are summarized in Table III. Simulation trajectories and parameters are available at [?]

9. To be uploaded to Zenodo.

2. Simulations of POPC bilayers with cationic surfactants

An automated topology builder [56] was first used to create the structure of dihexadecyldimethylammonium. The Amber-Tools program [57] was then used to generate the Amber-type force field parameters. The parameters were converted to the Gromacs format by using the acpype tool [58]. The partial charges were then manually modified to approximately correspond to their equivalent segments in Lipid14 [25]. The parameters are available at [?] **10. To be added.** The surfactants were randomly placed among the lipids to form bilayer structures with mole fractions of 10%, 20%, 30%, 42%, or 50% of surfactant in the POPC bilayer. All systems contained 50 POPC molecules per leaflet, 6340 TIP3P water molecules and 6, 14, 21, 35, or 50 surfactants per leaflet. Chloride counter ions were used in simulations, because parameters for bromide, the counterion in the experimental data [34], were not available in the standard Gromacs files for Amber force field. The Lipid14 model was used for POPC. The first 20 ns of the total simulation time of 200 ns was considered as an equilibration time and was omitted from the analysis. A reasonable lipid neighbor exchange occurred during the simulation.

The same systems were also simulated with the ECC-lipid model for POPC using the same setup. **11. Which water model was used in these simulations?** In these simulations the ECC correction was also applied to the cationic surfactant by scaling all charges with the same factor as for ECC-lipids, i.e., $f_q = 0.8$, and by using the atom types with reduced σ parameters from ECC-lipids. **12. Parameters are available at ??**

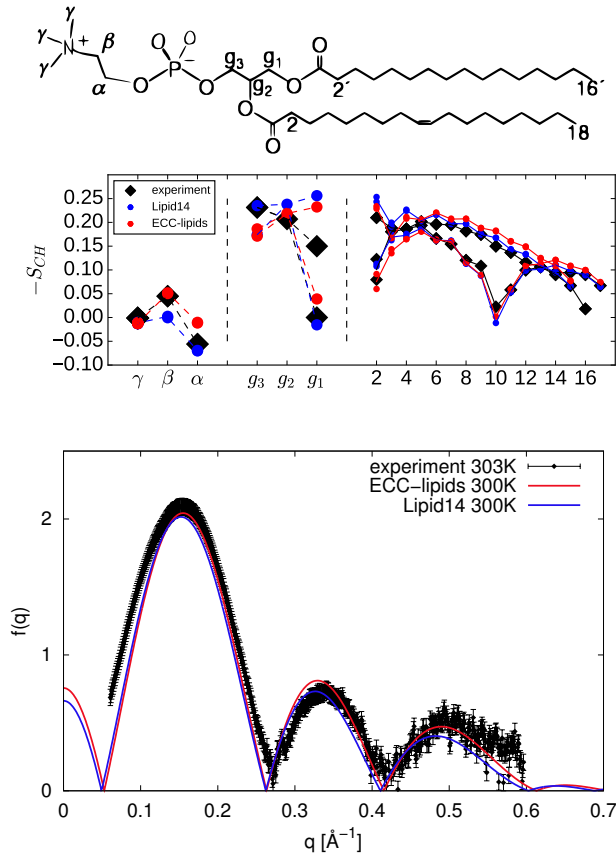


FIG. 1: Top: The chemical structure of POPC and the labeling of the carbon segments. Middle: Order parameters of POPC head group, glycerol backbone and acyl chains from simulations with the Lipid14 [25] and the ECC-lipid models compared with the experimental values from [59]. The size of the points for the head group order parameters correspond to the error bars (± 0.02 for experiments [35, 36] and ?? for simulations). The size of the points for acyl chains are decreased by a factor of 3 to improve the clarity of the plot. Bottom: X-ray scattering form factors from simulations with the Lipid14 [25] and the ECC-lipid models compared with experiments [?].

13. Increase the height of the order parameter figure

14. $sn-1$ and $sn-2$ order parameters should be somehow labelled in the order parameter figure. Maybe empty and filled points?

15. Add size of the error bars in simulations to the caption.

16. Would it be possible to increase the size of points for acyl chain order parameters only in z-direction such that it would correspond the error bars?

17. x-axis in form factor plot from 0 to 0.6, where experimental data ends. y-axis from 0 to ~ 2.5 to remove the empty space.

III. RESULTS AND DISCUSSION

A. POPC membrane structure and dynamics

The ECC-lipid and Lipid14 models reproduce the experimental x-ray scattering form factors of POPC bilayer with the similar accuracy in Fig. 1. The area per lipid values from the Lipid14 model is slightly larger than the experimental value in Table I, while the value from ECC-lipid model is slightly smaller. Area per lipid values of the ECC-lipid model show

some variation when simulated with different water models in Table II (Supplementary Information), however, all the values are close to the experimentally reported values. In conclusion, the ECC-lipid model reproduces the experimental dimensions of POPC lipid bilayer with the accuracy comparable to the other state of the art lipid models [36].

The acyl chain order parameters of the Lipid14 model [25] and the ECC-lipid model agree with the experimental values within the error bars in Fig. 1, although the ECC-lipid model gives slightly larger values for $sn-1$ chain. Notably, the experimentally measured forking and small order parameter values of C_2 segment in $sn-2$ chain are relatively well reproduced by the both models. This has been suggested to indicate that the carbonyl of $sn-2$ chain is directed towards the water phase, in contrast to the carbonyl in $sn-1$ chain, which would orient more along the bilayer plane [61–63]. While this may be an important feature for the ion binding details, it is not necessarily reproduced by the available lipid models [36].

The order parameters of α and β carbons in the headgroup are slightly larger in the ECC-lipid model than in the Lipid14 model, which is apparently related to the P-N vector orienting 7° more toward the water phase in the ECC-lipid model, see Fig. 2. With the current data we cannot, however, conclude which one of the models give the more realistic headgroup conformations. The ECC-lipid model gives the β carbon order parameter value closer to experiments, while value for α carbon is better in the Lipid14 model. Despite of some deviations from the experimental order parameter values in Fig. 1, the accuracy of the both models in the glycerol backbone region is comparable to the other state of art lipid models available in literature [35].

19. Dynamics check is missing: MSD (Hector/Joe)

B. Response of POPC head groups to bound charge

Before proceeding to the ion binding affinity, we quantify the response of the headgroup order parameters to the amount of bound charge by using mixtures of monovalent cationic surfactants (dihexadecyldimethylammonium) and POPC [34]. The amount of bound charge per PC in these systems is given by the molar fraction of cationic surfactants, because essentially all surfactants with two hydrophobic acyl chains can be assumed to locate in the lipid bilayers. The experimental data for these systems can be used to validate the sensitivity of lipid headgroup order parameters to the amount of bound charge in simulations.

The changes of the headgroup order parameters with an increasing amount of the cationic surfactant is compared between experiments [34] and simulations in Fig. 2. Approximately linear decrease of the order parameters, as expected from Eq. 3, is observed in simulations and experiments at least for the mole fractions below $\sim 30\%$. The slope is, however, too steep in the Lipid14 model indicating that the headgroup order parameters respond too sensitively to the bound positive charge. The slope in the ECC-lipid model is in very good agreement with experiments for the α segment, while the slope is slightly underestimated for the β segment.

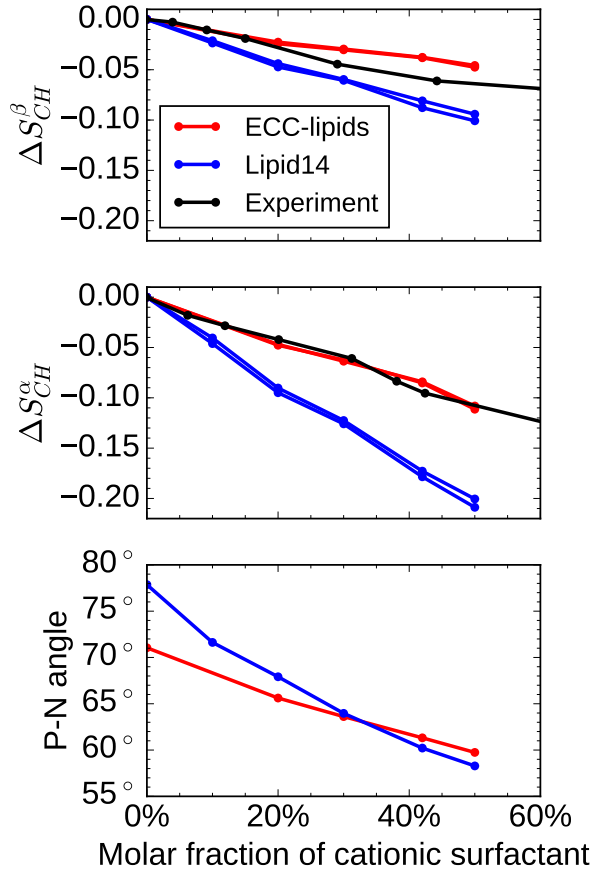


FIG. 2: The changes of headgroup order parameters and P-N vector orientation as a function of cationic surfactant (dihexadecyldimethylammonium) in POPC bilayer from simulations and experiments [34].

- 20. Labeling should be consistent with the previous figure, i.e. experimental data with diamonds.
- 21. x-axis scale from -1 to 51 to make the point in zero fully visible and to remove empty space.
- 22. Empty space between figures and from the right column could be reduced.

The headgroup P-N vector angle with respect to the membrane normal is also shown in Fig. 2 as a function of the mole fraction of the cationic surfactant. As suggested previously [20], the headgroup orients more towards the water phase with the increasing amount of positive charge in a PC lipid bilayer. The effect is more pronounced in the Lipid14 model, for which the addition of 50% mole fraction of the cationic surfactant leads to the decrease of 20° of the P-N vector angle, while the corresponding change in the ECC-lipid model is 11°. The difference is in line with the smaller order parameter changes and the reduced charge–dipole interactions in the latter model. The lesser sensitivity of the P-N vector angle response in the ECC-lipid model can be considered to be more realistic, because the changes of the headgroup order parameters as a function of the bound positive charge are in better agreement with experiments in this model. The results also imply that the validation and improvements of MD sim-

ulation models are generally required for the reliable simulation studies of the lipid headgroup responses to ions or other biomolecules, as also concluded previously [35].

C. Cation binding affinities to POPC

The changes of the lipid bilayer headgroup order parameter from different simulations and experiments [4, 5] are shown in Fig. 3 as a function of NaCl and CaCl₂ concentrations. These results can be used to compare the ion binding affinities to lipid bilayers between simulations and experiments by using the electrometer concept, because the order parameters decrease proportionally to the amount of bound positive charge [18, 20]. The recent comparison of different simulation models to the experimental data revealed that most models significantly overestimate the Na⁺ ion binding to PC lipid bilayers and that none of the available models correctly reproduce the details of the binding of Ca²⁺ ions [18]. A positive exception was the Lipid14 model [25] simulated with Åqvist ions, as seen from the data replotted from Ref. 18 in Fig. 3. The model reproduced the experimentally measured small order parameter changes with NaCl and imperceptible Na⁺ binding to PC bilayers [4, 5]. However, the changes of the headgroup order parameters as a function of CaCl₂ concentration were overestimated by the same combination of force field parameters.

Since significant artefacts are reported in simulations with Åqvist ions is water [?], we also simulated the Lipid14 model with the ion models by Dang et al. [50–52] and ECC-ions [22, 24?] having more realistic bulk behaviour. Instead of the improvement in the binding behaviour, we observed overbinding also of Na⁺ with these ions models, while the results with Ca²⁺ were barely affected, as seen in Figs. 3 and S???. 31. Add OP-response of Lipid14+ECC-ions plot in SI. The results are in line with the previous work [18], suggesting that the improvements in the lipid parameters are required to correctly describe the divalent cation binding to PC lipid bilayers.

To improve the cation binding behaviour to POPC lipid bilayer we implicitly included the electronic polarization to the Lipid14 model by using the ECC approach as described in section II A. The ECC-lipid model simulations with the ECC-ion models [?] exhibit a good agreement with the experimental changes of the lipid headgroup order parameters as a function of NaCl and CaCl₂ concentrations in Fig. 3. Since also the headgroup order parameter response to the bound positive charge the ECC-lipid model was in good agreement with experiments in section III B, we conclude that the model correctly reproduces the binding affinity of Na⁺ and Ca²⁺ ions to POPC lipid bilayer.

The density distributions of water and ions along the membrane normal are shown in Fig. 4. 32. Densities and relative surface excess from NaCl systems to be added. The discussion below to be finished after this. To quantify the binding affinities in different models we calculated the relative surface excess from Eq. 4. The value for Calcium in the ECC-lipid model, $\Gamma_i^w = 0.07 \pm 0.01 \text{ nm}^{-2}$, is significantly smaller than the values for the Lipid14 model with Åqvist ions, $\Gamma_i^w = 0.13 \pm 0.01 \text{ nm}^{-2}$, or with Dang ions,

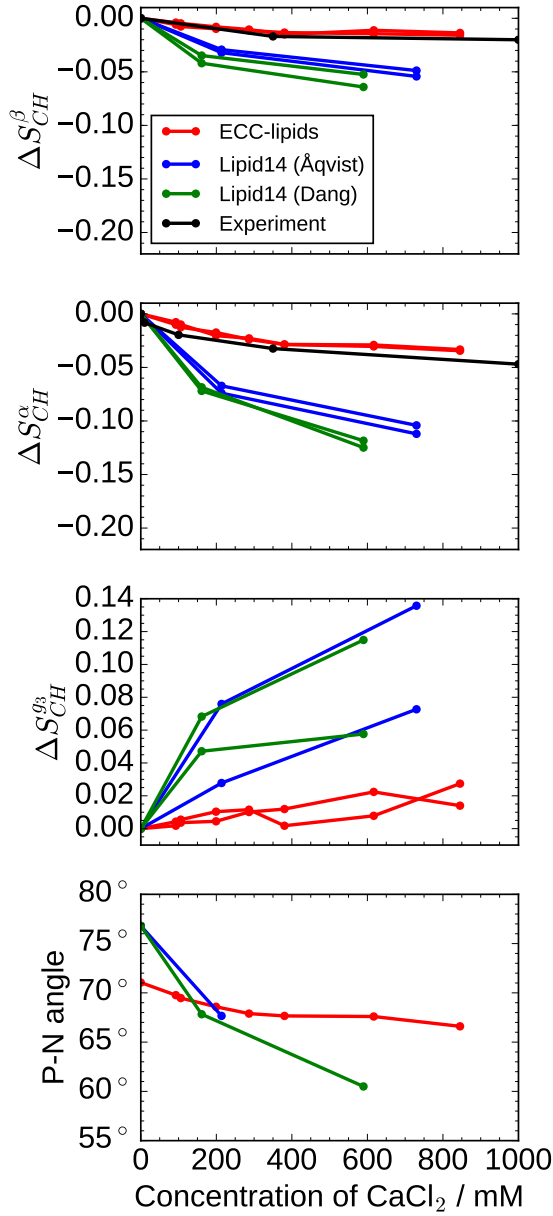


FIG. 3: Changes of head group order parameters of POPC bilayer as a function of CaCl_2 concentrations are shown from simulations with different force fields together with experimental data (DPPC [4] and POPC [5]). Ion concentrations in bulk water are shown in x-axis. Values from simulations are calculated from the of cation number density C_{np} from the region at the simulatin box edge with the constant ion concentration as $[\text{ion}] = C_{np}/0.602$. Simulation data with Lipid14 and Åqvist ion parameters is taken directly from Ref. [18].

23.I think that we must show the NaCl results in the main paper. I think that the best would be to use similar two column format, which we had before. This is used also in NMRlipids II and makes the comparison easier

24.The DPPC and POPC should be shown and properly labelled as we had previously.

25.The labeling should be consistent with previous figures, i.e., experiments with diamonds.

26.Start x-axis scale from -1 to make the point in zero fully visible.

27.Start y-axis scale of the two top figures from -0.13 to remove the empty space.

28.y-axis scale of the bottom figure from 59 to 78 to remove the empty space.

29.Empty space between figures and from the right column could be reduced.

30.Experimental values from [4] to be put in the g_3 figure: the value of g_3 order parameter of DPPC was -0.214 in the absence of ions [average of two closely spaced splittings] and -0.211 in the presence of 0.35 M CaCl_2 (at 59 Celcius). No effect of ions could be detected on DPPC bilayers labeled at the C-2 segments of both fatty acyl chains.”

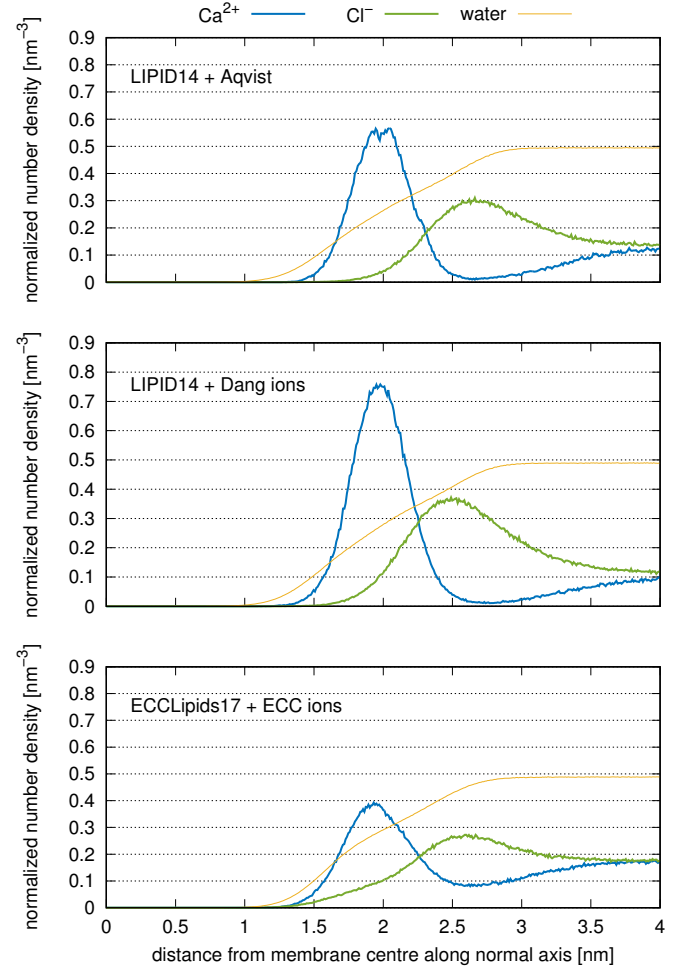


FIG. 4: Number density of Ca^{2+} and Cl^- as a function of membrane normal axis for different force fields. Data for Lipid14 with Åqvist ions are taken directly from Ref. 18. Densities of Cl^- and water are divided with 2 and 200, respectively, to visualize them with the same scale as Ca^{2+} . The molar concentration of the ions in water is 350 mM in all systems presented here.

33.PAVEL: draw phosphate position with its variance, add water density (scaled) and include the number of Γ -surface access.

34.JOE: Change the figure so that it contains a membrane background

$$\Gamma_i^w = 0.3 \pm 0.03 \text{ nm}^{-2}.$$

In conclusion, the results suggest that the ECC-lipid model together with the ECC-ion parameters gives a realistic description for the binding affinities of Na^+ and Ca^{2+} ions to POPC lipid bilayer. Furthermore, the overestimated head-group order parameter changes of POPC in the Lipid14 model as a function of CaCl_2 concentration arise from both, the overestimated binding affinity and sensitivity of the headgroup tilt to the bound positive charge. This probably applies also to the other lipid models considered in the previous study [18], emphasizing the importance of the comparison of the lipid head-group order parameter response to the bound charge between simulations and experiments as done in section III B.

35.SAMULI: Maybe we should discuss the repeat distances and area per molecules measured at [8, 9, 64]

D. Binding stoichiometry

This section is rough and will likely require editing. Data – up to noted exceptions – shall be all there, however. **36.SAMULI:** I think the we should make clear that the current binding constants in, e.g., Marsh's handbook are based on the headgroup order parameter changes interpreted with ternary complex binding model. I.e. the experimental raw data is exactly the same as we have in this work. Our simulations enable much more versatile interpretation of the binding phenomena. I think that this is one the main points of this work, so this comment applies to the previous section, introduction, abstract and conclusions as well.

Binding stoichiometry of Ca^{2+} and POPC was thoroughly studied in the experimental work [5], in which the head group order parameter changes to cation binding are determined. Several binding models were proposed and tested of which only one, ternary complex binding model, provided a good fit of the experimental observations.

Simulations allow us to directly evaluate the stoichiometry by calculating relative propensities of various $\text{Ca}^{2+}:n \times \text{POPC}$ clusters by evaluating contacts between cations and lipids with a cut off radius 0.3 nm. In Figure 5 we see that ternary complex is indeed the most probable binding mode of calcium at 285 mM concentration. Apart from this complex, we also find complexes with 1 and 3 lipids occurring with only a slightly lower but similar probability. The fractions of $\text{Ca}^{2+}:n \times \text{POPC}$ complexes at 285 mM concentration are then in order: 42% for two lipids, 30% for one lipid, and 28% for three lipids.

Several binding models were proposed and tested [5] of which only one, ternary complex binding model, provided a good fit of the experimental observations. In such a model, it is assumed that Ca^{2+} cations bind to a POPC membrane with a stoichiometry 2 POPC:1 Ca^{2+} . In a later work [65], a Langmuir adsorption model (i.e. stoichiometry 1 POPC:1 Ca^{2+}) was found to provide as good fit as ternary complex model, when only low concentrations of CaCl_2 are considered. Ternary complex model also provides a good fit to our simulations with ECC-lipids (see Fig. 7 in SI and its caption for details). The symmetry of the distribution of complexes from simulation – i.e. almost equal probabilities of complexes with 1 or 3 lipids that behave in the total average picture as complexes with 2 lipids – provides clues why ternary complex binding model fits both simulation and experimental results relatively well, although it is apparently incorrect.

In addition, we estimated relative binding affinities of several moieties in POPC towards Ca^{2+} . The dominant contribution to the binding of Ca^{2+} to POPC membranes comes from the phosphate group. Such a finding is supported by the calculated number of contacts between Ca^{2+} and POPC oxygen atoms. The average number of contacts between Ca^{2+} and any oxygen atom in POPC is 59.8, whereas if only phosphate oxygen atoms are considered the average number of contacts decreases only by a tiny amount to 57.2. We corroborate this finding with the probability isodensity contours in Fig. 6, which are easier to interpret.

The residence time of Ca^{2+} bound to a POPC membrane is experimentally estimated to be lower than 10 μs [5]. From recent theoretical work with long enough simulations this time

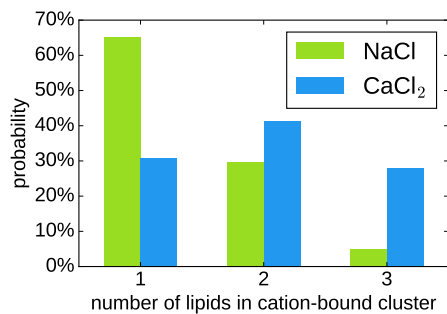


FIG. 5: Relative probabilities of existence of Na^+ or Ca^{2+} complexes with a certain number of POPC lipids. Na^+ complexes were evaluated from the simulation with 1 M concentration; and Ca^{2+} complexes were evaluated from the simulation with 287 mM concentration.

can be roughly estimated in the order of 1–10 μs [14]. This is in contrast to our model, ECC-lipids, which gives a mean residence time in the order of 10 ns, **37.evaluate this number, mean residence time, accurately based on the contacts data.** i.e. at least two orders of magnitude lower than previous estimates. Such a finding changes the point of view of calcium binding from very tight long-term stable binding with rare exchanges to a relatively frequent exchange of cations in equilibrium between membrane and solvent.

38.Finalize stoichiometry analysis for Na^+ , Ca^{2+} , their interaction energies with the lipid membrane, etc, and finalize the discussion after these results.

IV. CONCLUSIONS

By using the electrometer concept we show that the binding of Na^+ and Ca^{2+} ions to a POPC lipid bilayer can be accurately described with the classical MD simulation force field, where the electronic polarization is implicitly included using the electronic continuum correction (ECC) [21]. The proposed ECC-lipids model is a significant improvement over other available lipid models, which all overestimate specific cation binding affinities [18]. While the structural details of a POPC lipid bilayer simulated with the ECC-lipids model agree with experiments with the comparable accuracy to the other state of the art lipid models, it also reproduces the experimental lipid head group order parameter responses to the cationic surfactant, NaCl and CaCl_2 concentrations.

The good agreement with experiments enables the atomic resolution interpretation of NMR experiments by using MD simulations. In agreement with previous interpretations of experimental data [7, 15–17], the Ca^{2+} ions mainly interact with phosphate oxygens. However, the stoichiometry of the binding is significantly more complicated than in the previous interpretation of the NMR data based on the ternary complex model, where one calcium binds to two POPC molecules [5]. The complexes of one calcium bound to two lipids are the most probable also in the ECC-lipids model, but the complexes of one or three lipids per one calcium were observed to be almost equally likely. While the success of the ternary

Finland via the FiDiPro award.

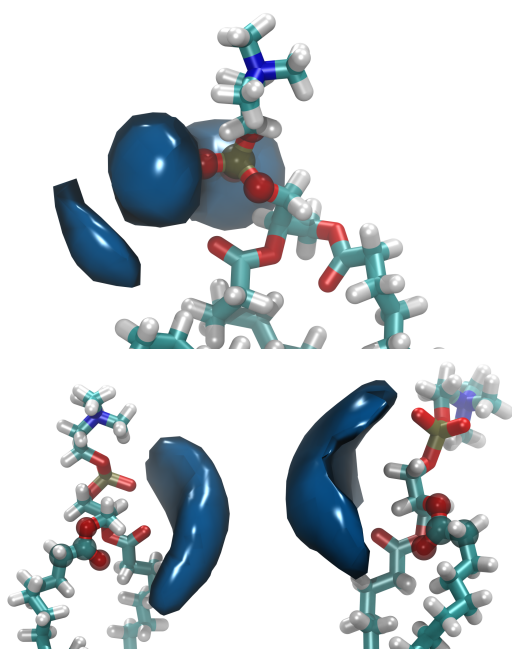


FIG. 6: Contours of probability isodensities of Ca^{2+} with respect to various moieties fixed in space (highlighted with transparent spheres): phosphate moiety, side chain 1 carbonyl group and side chain 2 carbonyl group. Shown contours suggest that the dominant contribution to Ca^{2+} binding comes from the phosphate oxygens, whereas the interactions with any of the two carbonyl groups are considerably milder.

39.JOE: I'll update this figure with some ensemble of configuration to support binding preference of Ca^{2+}

complex model is understandable based on the simulation results, a simple binding model cannot detect the complex binding observed in the simulation.

The improved cation binding behaviour to POPC bilayer paved the way for simulations of complex biochemical systems with correctly described electrostatic interactions in the vicinity of cellular membranes. The ECC-lipids model is built by scaling the partial charges and the LJ-radius of the head-group, glycerol backbone and carbonyl atoms of the Lipid14 POPC model [25]. While the Lipid14 model is compatible with the AMBER force field family, the compatibility of the ECC-lipids model may be compromised due to the changes in intermolecular interactions of the scaled atoms. On the other hand, a fully consistent ECC-force field should include the correction also in other than lipid molecules, including water. The work toward this direction and the extension to other lipid molecules and force fields is left for the future studies.

This work can be reached as a repository containing all data at [zenodo.org:\dots\dots\dots](https://zenodo.org/records/10000000).

Acknowledgments

P.J. acknowledges support from the Czech Science Foundation (grant no. 16-01074S) and 600 from the Academy of

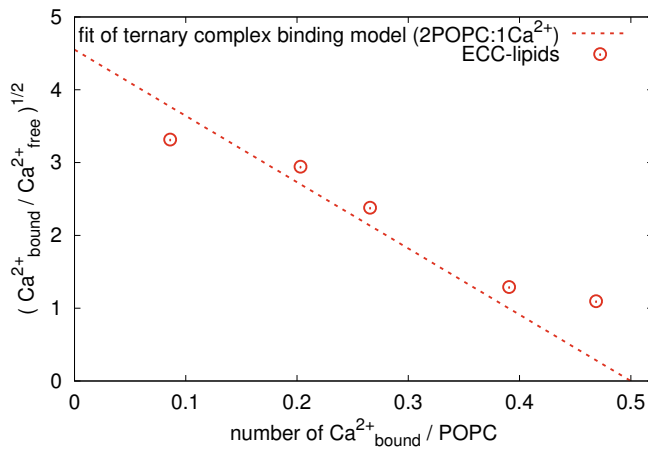


FIG. 7: Ternary complex binding model of Ca^{2+} to a POPC membrane that assumes the stoichiometry of 2 POPC:1 Ca^{2+} (details in reference 5) provides a good fit to experimental measurements [5] and it also provides a good fit to our simulation data. Note that the units in the reference 5 are different from the units presented here, and, hence, the observed slope of the linear relationship is different.

SUPPLEMENTARY INFORMATION

It was found in the original work [5] that a ternary complex binding model (i.e. 2 POPC:1 Ca^{2+}) provides the best fit to experimental measurements of all considered models in that study. In such a model, there is a linear relationship between quantities C_b , mole fraction of bound Ca^{2+} per POPC, and $\sqrt{C_b/C_I}$, where C_I is the concentration of free cations at the plane of ion binding [5]. The concentration C_b was obtained from an extrapolation of linear relation between deuterium NMR measurements and atomic absorption spectroscopy for low concentrations of CaCl_2 . Such an extrapolation is valid as long as the mode of Ca^{2+} binding remains constant throughout the extrapolation range. The concentration C_I is determined by using the surface potential by using the Boltzmann equation. However, Boltzmann theory yields inaccurate results for divalent cations like Ca^{2+} [66]. An atomistic simulation, on the other hand, provides these quantities directly without severe assumptions. **40. Did you really calculate the C_I from simulations without severe assumptions? Note that this concentration at the plane of binding, which do not equal the concentration of free cations.** Hence we hypothesise that the discrepancy between the results in the experiment [5] and our simulations likely lays in the fact that the assumptions and relations used for determining concentrations C_b and C_I in the experiment [5] gradually do not hold for higher concentrations of Ca^{2+} .

- [1] P. Scherer and J. Seelig, The EMBO journal **6** (1987).
- [2] J. Seelig, Cell Biol. Int. Rep. **14**, 353 (1990), URL [http://dx.doi.org/10.1016/0309-1651\(90\)91204-H](http://dx.doi.org/10.1016/0309-1651(90)91204-H).
- [3] G. Cevc, Biochim. Biophys. Acta - Rev. Biomemb. **1031**, 311 (1990).
- [4] H. Akutsu and J. Seelig, Biochemistry **20**, 7366 (1981).
- [5] C. Altenbach and J. Seelig, Biochemistry **23**, 3913 (1984).
- [6] J.-F. Tocanne and J. Teissie, Biochim. Biophys. Acta - Reviews on Biomembranes **1031**, 111 (1990).
- [7] H. Binder and O. Zschörnig, Chem. Phys. Lipids **115**, 39 (2002).
- [8] G. Pabst, A. Hodzic, J. Strancar, S. Danner, M. Rappolt, and P. Laggner, Biophys. J. **93**, 2688 (2007).
- [9] D. Uhrkov, N. Kuerka, J. Teixeira, V. Gordeliy, and P. Balgav, Chemistry and Physics of Lipids **155**, 80 (2008).
- [10] R. A. Böckmann, A. Hac, T. Heimburg, and H. Grubmüller, Biophys. J. **85**, 1647 (2003).
- [11] R. A. Böckmann and H. Grubmüller, Ang. Chem. Int. Ed. **43**, 1021 (2004).
- [12] M. L. Berkowitz and R. Vacha, Acc. Chem. Res. **45**, 74 (2012).
- [13] A. Melcrov, S. Pokorna, S. Pullanchery, M. Kohagen, P. Jurkiewicz, M. Hof, P. Jungwirth, P. S. Cremer, and L. Cwiklik, Sci. Reports **6**, 38035 (2016).
- [14] M. Javanainen, A. Melcrova, A. Magarkar, P. Jurkiewicz, M. Hof, P. Jungwirth, and H. Martinez-Seara, Chem. Commun. **53**, 5380 (2017), URL <http://dx.doi.org/10.1039/C7CC02208E>.
- [15] H. Hauser, M. C. Phillips, B. Levine, and R. Williams, Nature **261**, 390 (1976).
- [16] H. Hauser, W. Guyer, B. Levine, P. Skrabal, and R. Williams, Biochim. Biophys. Acta - Biomembranes **508**, 450 (1978), ISSN 0005-2736, URL <http://www.sciencedirect>.

TABLE II: Area per lipid (APL) from different models of POPC with no ions

model	APL (\AA^2)	Temperature [K]
Lipid14	65.1 ± 0.6	300
Lipid14 [25]	65.6 ± 0.5	303
ECC-lipids		
OPC3	62.2 ± 0.6	300
OPC3	64.2 ± 0.6	313
SPC/E	65.1 ± 0.6	313
OPC	64.4 ± 0.6	313
TIP4p/2005	66.8 ± 0.6	313
experiment	62.7	293
experiment [60] 41.REF	64.3	303
experiment	67.3	323
experiment	68.1	333

42. Result with normal TIP3P missing?

- com/science/article/pii/0005273678900913.
- [17] L. Herbertte, C. Napolitano, and R. McDaniel, Biophys. J. **46**, 677 (1984).
- [18] A. Catte, M. Giryach, M. Javanainen, C. Loison, J. Melcr, M. S. Miettinen, L. Monticelli, J. Maatta, V. S. Oganessian, O. H. S. Ollila, et al., Phys. Chem. Chem. Phys. **18** (2016).
- [19] R. Vacha, S. W. I. Siu, M. Petrov, R. A. Böckmann, J. Barucha-Kraszewska, P. Jurkiewicz, M. Hof, M. L. Berkowitz, and P. Jungwirth, J. Phys. Chem. A **113**, 7235 (2009).
- [20] J. Seelig, P. M. MacDonald, and P. G. Scherer, Biochemistry

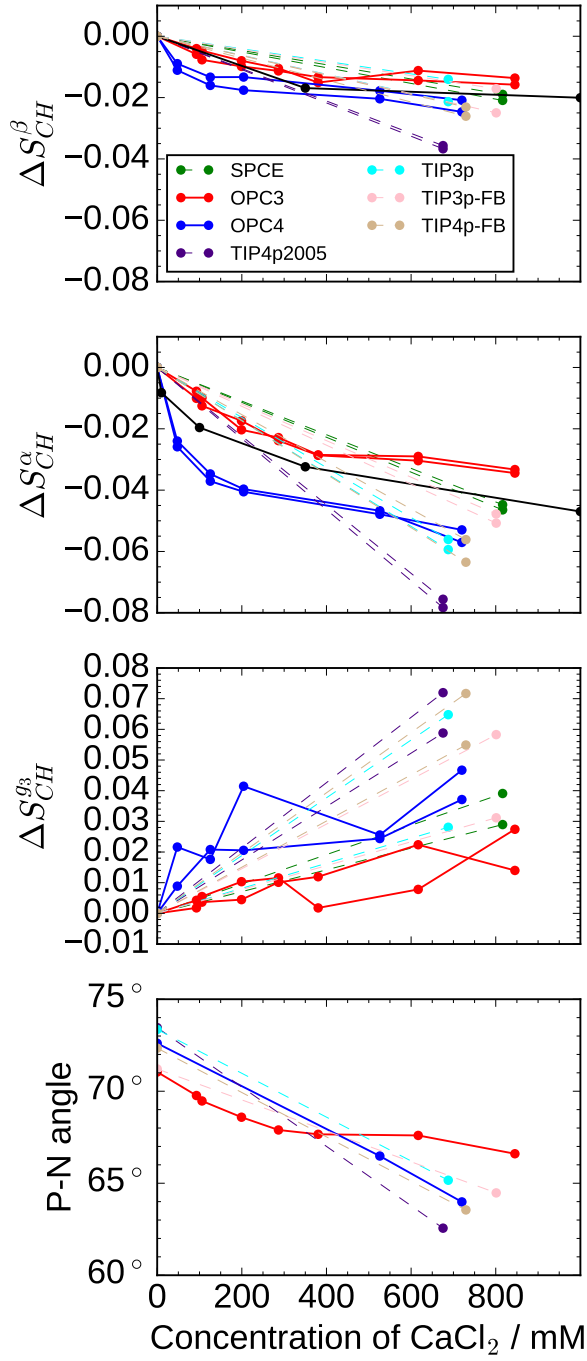


FIG. 8: Changes of head group order parameters of POPC bilayer as a function of CaCl_2 concentrations are shown from simulations with different force fields and water models together with experimental data (DPPC [4] and POPC [5]). Ion concentrations in bulk water are shown in x-axis. Values from simulations are calculated from the of cation number density C_{np} from the region at the simulation box edge with the constant ion concentration as $[\text{ion}] = C_{np}/0.602$.

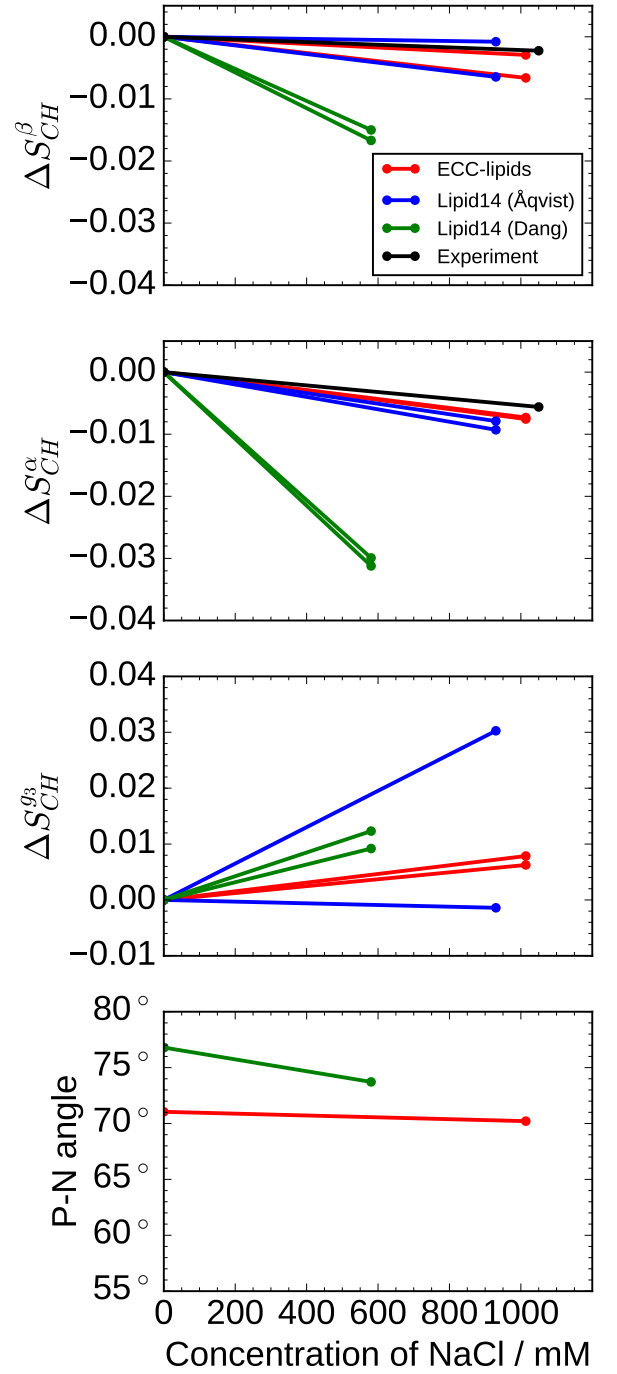


FIG. 9: Changes of head group order parameters of POPC bilayer as a function of NaCl concentrations are shown from simulations with different force fields together with experimental data [4]. Ion concentrations in bulk water are shown in x-axis. Values from simulations are calculated from the of cation number density C_{np} from the region at the simulation box edge with the constant ion concentration as $[\text{ion}] = C_{np}/0.602$. Simulation data with Lipid14 and Åqvist ion parameters is taken directly from Ref. [18].

TABLE III: Simulation parameters

simulation property	parameter
time-step	2 fs
equilibration time	100 ns
simulation time	200 ns
temperature	313 K
thermostat	v-rescale [67]
barostat	Parrinello-Rahman, semi-isotropic [68]
long-range electrostatics	PME [69]
cut-off scheme	Verlet [70]
Coulomb and VdW cut-off	1.0 nm
constraints	LINCS, only hydrogen atoms [71]
constraints for water	SETTLE [72]

- 26, 7535 (1987).
- [21] I. Leontyev and A. Stuchebrukhov, Phys. Chem. Chem. Phys. **13**, 2613 (2011).
- [22] E. Pluhaová, H. E. Fischer, P. E. Mason, and P. Jungwirth, Molecular Physics **112**, 1230 (2014), ISSN 0026-8976, URL <http://www.tandfonline.com/doi/abs/10.1080/00268976.2013.875231>.
- [23] M. Kohagen, P. E. Mason, and P. Jungwirth, J. Phys. Chem. B **118**, 7902 (2014).
- [24] M. Kohagen, P. E. Mason, and P. Jungwirth, J. Phys. Chem. B **120**, 1454 (2016).
- [25] C. J. Dickson, B. D. Madej, A. Skjervik, R. M. Betz, K. Teigen, I. R. Gould, and R. C. Walker, J. Chem. Theory Comput. **10**, 865 (2014).
- [26] J. Chowdhary, E. Harder, P. E. M. Lopes, L. Huang, A. D. MacKerell, and B. Roux, J. Phys. Chem. B **117**, 9142 (2013).
- [27] B. Jonsson, O. Edholm, and O. Teleman, J. Chem. Phys. **85**, 2259 (1986).
- [28] E. Egberts, S.-J. Marrink, and H. J. C. Berendsen, European Biophysics Journal **22**, 423 (1994).
- [29] I. V. Leontyev and A. A. Stuchebrukhov, The Journal of chemical physics **130**, 085102 (2009), ISSN 1089-7690, URL <http://scitation.aip.org/content/aip/journal/jcp/130/8/10.1063/1.3060164>.
- [30] I. V. Leontyev and A. A. Stuchebrukhov, Journal of Chemical Theory and Computation **6**, 1498 (2010), ISSN 1549-9618, URL <http://dx.doi.org/10.1021/ct9005807>.
- [31] H. Hu, Z. Lu, and and Weitao Yang*, Journal of Chemical Theory and Computation **3**, 1004 (2007), ISSN 1549-9618, URL <http://dx.doi.org/10.1021/ct600295n>.
- [32] C. C. I. Bayly, P. Cieplak, W. D. Cornell, and P. a. Kollman, The Journal of Physical ... **97**, 10269 (1993), ISSN 0022-3654, 93/2091- 10269\$04.00/0, URL <http://pubs.acs.org/doi/abs/10.1021/j100142a004>.
- [33] U. C. Singh and P. A. Kollman, Journal of Computational Chemistry **5**, 129 (1984), ISSN 1096987X.
- [34] P. G. Scherer and J. Seelig, Biochemistry **28**, 7720 (1989).
- [35] A. Botan, F. Favela-Rosales, P. F. J. Fuchs, M. Javanainen, M. Kanduč, W. Kulig, A. Lamberg, C. Loison, A. Lyubartsev, M. S. Miettinen, et al., J. Phys. Chem. B **119**, 15075 (2015).
- [36] O. S. Ollila and G. Pabst, *Atomistic resolution structure and dynamics of lipid bilayers in simulations and experiments* (2016), in Press, URL <http://dx.doi.org/10.1016/j.bbamem.2016.01.019>.
- [37] D. S. Cerutti, J. E. Rice, W. C. Swope, and D. A. Case, The Journal of Physical Chemistry B **117**, 2328 (2013), pMID: 23379664, <http://dx.doi.org/10.1021/jp311851r>, URL <http://dx.doi.org/10.1021/jp311851r>.
- [38] A. Maciejewski, M. Pasenkiewicz-Gierula, O. Cramariuc, I. Vattulainen, and T. Rog, J. Phys. Chem. B **118**, 4571 (2014).
- [39] (???)
- [40] T. M. Ferreira, R. Sood, R. Bärenwald, G. Carlström, D. Topgaard, K. Saalwächter, P. K. J. Kinnunen, and O. H. S. Ollila, Langmuir **32**, 6524 (2016).
- [41] A. Seelig and J. Seelig, Biochemistry **16**, 45 (1977).
- [42] J. H. Davis, Biochim. Biophys. Acta - Reviews on Biomembranes **737**, 117 (1983).
- [43] G. Beschiaschvili and J. Seelig, Biochim. Biophys. Acta - Biomembranes **1061**, 78 (1991).
- [44] D. K. Chattoraj and K. S. Birdi, *Adsorption at the Liquid Interface from the Multicomponent Solution* (Springer US, Boston, MA, 1984), pp. 83–131, ISBN 978-1-4615-8333-2, URL https://doi.org/10.1007/978-1-4615-8333-2_4.
- [45] S. Izadi and A. V. Onufriev, Journal of Chemical Physics **145**, 074501 (2016), ISSN 00219606, URL <http://aip.scitation.org/doi/10.1063/1.4960175>.
- [46] S. Izadi, R. Anandakrishnan, and A. V. Onufriev, The Journal of Physical Chemistry Letters **5**, 3863 (2014), ISSN 1948-7185, 1408.1679, URL <http://pubs.acs.org/doi/10.1021/jz501780a>.
- [47] H. J. C. Berendsen, J. R. Grigera, and T. P. Straatsma, Journal of Physical Chemistry **91**, 6269 (1987), ISSN 0022-3654, URL <http://links.isiglobalnet2.com/gateway/Gateway.cgi?GWVersion=2\&\&SrcAuth=mekentosj\&\&SrcApp=Papers\&\&DestLinkType=FullRecord\&\&DestApp=WOS\&\&KeyUT=A1987K994100038\&\&5Cnpapers2://publication/uuid/17978EF7-93C9-4CB5-89B3-086E5D2B9169\&\&5Cnhttp://pubs.acs.org/doi/pdf/10.1021/>.
- [48] L. P. Wang, T. J. Martinez, and V. S. Pande, Journal of Physical Chemistry Letters **5**, 1885 (2014), ISSN 19487185, URL <http://pubs.acs.org/doi/abs/10.1021/jz500737m>.
- [49] J. L. Abascal and C. Vega, The Journal of chemical physics **123**, 234505 (2005), ISSN 00219606, URL <http://aip.scitation.org/doi/10.1063/1.2121687>.
- [50] D. E. Smith and L. X. Dang, J. Chem. Phys. **100** (1994).
- [51] T.-M. Chang and L. X. Dang, J. Phys. Chem. B **103**, 4714 (1999), ISSN 1520-6106, URL <http://dx.doi.org/10.1021/jp982079o>.
- [52] L. X. Dang, G. K. Schenter, V.-A. Glezakou, and J. L. Fulton, J. Phys. Chem. B **110**, 23644 (2006), ISSN 1520-6106, URL <http://dx.doi.org/10.1021/jp064661f>.
- [53] J. Aqvist, The Journal of Physical Chemistry **94**, 8021 (1990), URL <http://dx.doi.org/10.1021/j100384a009>.
- [54] M. Gyrch and O. H. S. Ollila, *Popc-amber-lipid14-verlet* (2015), URL <http://dx.doi.org/10.5281/zenodo.30898>.
- [55] M. J. Abraham, T. Murtola, R. Schulz, S. Páll, J. C. Smith, B. Hess, and E. Lindah, SoftwareX **1-2**, 19 (2015), ISSN 23527110, URL <http://www.sciencedirect.com/science/article/pii/S2352711015000059>.
- [56] A. K. Malde, L. Zuo, M. Breeze, M. Stroet, D. Poger, P. C. Nair, C. Oostenbrink, and A. E. Mark, Journal of Chemical Theory and Computation **7**, 4026 (2011).
- [57] D. Case, D. Cerutti, T. Cheatham, III, T. Darden, R. Duke,

- T. Giese, H. Gohlke, A. Goetz, D. Greene, et al., *AMBER 2017* (2017), university of California, San Francisco.
- [58] A. W. SOUSA DA SILVA and W. F. VRANKEN, *ACPYPE - AnteChamber PYthon Parser interfAcE*. (2017), manuscript submitted.
- [59] T. M. Ferreira, F. Coreta-Gomes, O. H. S. Ollila, M. J. Moreno, W. L. C. Vaz, and D. Topgaard, *Phys. Chem. Chem. Phys.* **15**, 1976 (2013).
- [60] J. P. M. Jämbbeck and A. P. Lyubartsev, *J. Phys. Chem. B* **116**, 3164 (2012).
- [61] A. Seelig and J. Seelig, *Biochim. Biophys. Acta* **406**, 1 (1975).
- [62] H. Schindler and J. Seelig, *Biochemistry* **14**, 2283 (1975).
- [63] K. Gawrisch, D. Ruston, J. Zimmerberg, V. Parsegian, R. Rand, and N. Fuller, *Biophys. J.* **61**, 1213 (1992).
- [64] H. I. Petrache, S. Tristram-Nagle, D. Harries, N. Kucerka, J. F. Nagle, and V. A. Parsegian, *J. Lipid Res.* **47**, 302 (2006).
- [65] P. M. Macdonald and J. Seelig, *Biochemistry* **26**, 1231 (1987).
- [66] D. Andelman, in *Handbook of biological physics* (Elsevier Science, 1995), vol. 1, chap. 12, pp. 603–642, URL <http://hwiki.liebel-lab.org/wiki/images/9/90/AndelmannReview.pdf>.
- [67] G. Bussi, D. Donadio, and M. Parrinello, *J. Chem. Phys.* **126** (2007).
- [68] M. Parrinello and A. Rahman, *J. Appl. Phys.* **52**, 7182 (1981).
- [69] T. Darden, D. York, and L. Pedersen, *J. Chem. Phys.* **98** (1993).
- [70] S. Páll and B. Hess, *Computer Physics Communications* **184**, 2641 (2013), ISSN 0010-4655, URL <http://www.sciencedirect.com/science/article/pii/S0010465513001975>.
- [71] B. Hess, H. Bekker, H. J. C. Berendsen, and J. G. E. M. Fraaije, *J. Comput. Chem.* **18**, 1463 (1997).
- [72] S. Miyamoto and P. A. Kollman, *J. Comput. Chem.* **13**, 952 (1992).

ToDo

1. We should also cite papers where empirical scaling was used ionic liquids - but there the factor is not 0.5. 2
2. This needs a citation 2
3. JOE: following discussion shall be modified in the enlightenment of our recent ECC-discussions. 2
4. missing REF for APL experiment 2
5. We should discuss how this can potentially affect the intermolecular interaction when mixing scaled and non scaled molecules. JOE: I think that we rather increasingly see that there's nothing like "fully non-scaled" with the exception of ions with integer charges. So the discussion shall be rather more about the interaction of our "scaled" (I'd still rather call it ECC-corrected or whatever) and "semi-scaled" models. SAMULI: There is now a paragraph in the conclusions, which mentions this topic. 3
6. As Hector suggested, it might be better to write the simpler form for this equation. 4
7. More justification for the choosing the OPC3 water model are needed. It might be good to show the comparison with the scattering data in bulk water in SI. 4
8. The normal TIP3P was tested as well, right? 4

18. put original references, not Slipids param. paper. 4
9. To be uploaded to Zenodo 4
10. To be added 4
11. Which water model was used in these simulations? 4
12. Parameters are available at ?? 4
13. Increase the height of the order parameter figure 5
14. sn-1 and sn-2 order parameters should be somehow labelled in the order parameter figure. Maybe empty and filled points? 5
15. Add size of the error bars in simulations to the caption. 5
16. Would it be possible to increase the size of points for acyl chain order parameters only in z-direction such that it would correspond the error bars? 5
17. x-axis in form factor plot from 0 to 0.6, where experimental data ends. y-axis from 0 to ~2.5 to remove the empty space. 5
19. Dynamics check is missing: MSD (Hector/Joe) 5
20. Labeling should be consistent with the previous figure, i.e. experimental data with diamonds. 6
21. x-axis scale from -1 to 51 to make the point in zero fully visible and to remove empty space. 6
22. Empty space between figures and from the right column could be reduced. 6
31. Add OP-response of Lipid14+ECC-ions plot in SI 6
32. Densities and relative surface excess from NaCl systems to be added. The discussion below to be finished after this. 6
23. I think that we must show the NaCl results in the main paper. I think that the best would be to use similar two column format, which we had before. This is used also in NMRlipids II and makes the comparison easier 7
24. The DPPC and POPC should be shown and properly labelled as we had previously. 7
25. The labeling should be consistent with previous figures, i.e., experiments with diamonds. 7
26. Start x-axis scale from -1 to make the point in zero fully visible. 7
27. Start y-axis scale of the two top figures from -0.13 to remove the empty space. 7
28. y-axis scale of the bottom figure from 59 to 78 to remove the empty space. 7
29. Empty space between figures and from the right column could be reduced. 7
30. Experimental values from [4] to be put in the g_3 figure: the value of g_3 order parameter of DPPC was -0.214 in the absence of ions [average of two closely spaced splittings] and -0.211 in the presence of 0.35 M CaCl_2 (at 59 Celcius). No effect of ions could be detected on DPPC bilayers labeled at the C-2 segments of both fatty acyl chains." 7
33. PAVEL: draw phosphate position with its variance, add water density (scaled) and include the number of Γ -surface access. 7
34. JOE: Change the figure so that it contains a membrane background 7
35. SAMULI: Maybe we should discuss the repeat distances and area per molecules measured at [8, 9, 64] 7

36. SAMULI: I think the we should make clear that the current binding constants in, e.g., Marsh's handbook are based on the headgroup order parameter changes interpreted with ternary complex binding model. I.e. the experimental raw data is exactly the same as we have in this work. Our simulations enable much more versatile interpretation of the binding phenomena. I think that this is one the main points of this work, so this comment applies to the previous section, introduction, abstract and conclusions as well.	8
37. evaluate this number, mean residence time, accurately based on the contacts data.	8
38. Finalize stoichiometry analysis for Na^+ , Ca^{2+} , their interaction energies with the lipid membrane, etc, and finalize the discussion after these results.	8
39. JOE: I'll update this figure with some ensemble of configuration to support binding preference of Ca^{2+} . . .	9
40. Did you really calculate the C_I from simulations without severe assumptions? Note that this concentration at the plane of binding, which do not equal the concentration of free cations.	10
41. put original references, not Slipids param. paper. . .	10
42. Result with normal TIP3P missing?	10