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

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1.Abstract directly from Joe's conference abstracts. To be rewritten. Classical molecular dynamics simulations give detailed information about membrane structure and dynamics. However, there is still a room for improvements in current force fields it is known from the literature, that the binding of ions, especially cations, to phopholipid membranes is overestimated in all classical models [1]. We suggest that the membrane-ion interactions can be corrected by including implicit electronic polarizability into the lipid models through the electronic continuum correction (ECC) [2], which was already applied to monovalent and divalent ions yielding models that feature correct ion pairing [3]. Using the electrometer concept [3, 4] and x-ray scattering form factors, our simulations point out that our hypothesis is correct and ECC is indeed a missing important contribution in current classical lipid models. Moreover, the solid physical principles behind ECC are found not to hamper other relevant properties of a phospholipid bilayer. The new lipid model, "ECC-lipids", shows accurate binding affinity to sodium and calcium cations and head group order parameter response to bound charge. We also provide for the first time a realistic stochiometry of bound calcium cations to a POPC membrane, and their binding sites. This work will continue as an open collaboration project NMRlipids VI (http://nmrlipids.blogspot.fi).

I. INTRODUCTION

Cation interactions with cellular membranes play a key role in several biological processes, like in signal propagation in neurons and vesicle fusion. **2.This could be maybe extended.** Since the direct measurements of ion-membrane interactions from biological systems are difficult, lipid bilayers are often used as models systems for cellular membranes. The detailed results from simple model systems can be then used to understand the role of ions in complex biological systems.

Zwitterionic phosphocholine (PC) lipid bilayers are commonly used model systems for cellular membranes. Interactions of biological cations, especially Na^+ and Ca^{2+}, with PC bilayers are widely studied with experiments [1–8] and classical MD simulations [9–13]. The details of ion binding are, however, not agreed in the literature. Non-invansive spectroscopic methods, like nuclear magnetic resonance (NMR), scattering and infrared scpectroscopy, give accurate information about ion binding in PC lipid bilayers[1, 2, 6–8, 14–16]. Interpretation of these experiments suggests that Na^+ ions have negligible binding in PC lipid bilayers with submolar concentrations, while Ca^{2+} specifically binds to phosphate groups of two lipid molecules. Atomistic resolution molecular dynamics (MD) simulation models, however, predict significantly stronger binding for the cations and interactions with 3-4 lipids, including also interactions with carbonyl oxygens [9, 10, 12, 13]. Some experiments have also been interpreted to support the predictions from MD simulations [9, 17].

Recent work published by the NMRlipids project (`nmrlipids.blogspot.fi`) [18] made an attempt to resolve the apparent controversies. The study presented a direct comparison between ion binding affinity between simulations and experiments by using NMR data for lipid headgroup order parameters and the electrometer concept [19]. Using massive 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 electronic polarizability in the polar region of lipid molecules. The electronic polarizability is included by using the electronic continuum correction (ECC) [20], which has been previously shown to improve the behaviour of MD simulations of ions in bulk water [21**?** –23]. As a starting point we use the parameters from Lipid14 model [24], which gave the best cation binding behaviour in the previous study [18]. The developed ECC-lipid parameters reproduce the experimentally measurable structural parameters of POPC lipid bilayer with the accuracy comparable to the other state of the art lipid models.

Since the cation binding affinity and the headgroup order parameter changes are in good agreement with experiments in the proposed ECC-lipids, it can be used to interpret the related structural changes and ion binding stoichiometry. New lipid models with correct ion binding affinity in lipid bilayers are necessary in applications of MD simulations with physiological salt conditions. The overestimated cation binding in the current lipid models [18] may lead to significant artefacts in MD simulations. For example, artificially positively charged membranes overestimate interactions with biomolecules having opposite sign.

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II. METHODS

A. Electronic continuum correction for lipid bilayers

The lack of electronic polarizability in the standard MD simulation force fields has been considered higly relevant issue since the early days of lipid bilayer simulations. In this work we circumvent the rather demanding explicit inclusion of electronic polariization effects [25] by implicitly including electronic polarizability in lipid bilayer simulations by using the electronic continuum correction (ECC) [20]. 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 [26, 27]. However, the concept of ECC is physically well justified and rigorously derived [20, 28, 29], but significantly more simple than the explicit inclusion of electronic polarizability [25] both for derivation and application.

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 [20]. Measurements of high frequency dielectric constant gives values of approximately $\epsilon_{el} \approx 2$ for almost any biomaterial [20**?**]. Such a dielectric continuum can be easily included in standard MD simulation by a formal transformation of partial charges

$$Q^{ECC} = f_q \cdot Q \tag{1}$$

with a constant scaling factor $f_q = \epsilon_{el}^{-1/2}$ effectively representing the newly introduced electronic continuum. The value measured for water, $\epsilon_{el} = 1.78$, gives a scaling factor of $f_q = 0.75$ [20**?**], which has been successfully used to improve the performance of force field for ions [22**?** , 23].

While the scaling factor of $f_q = 0.75$ for ions in water is well justified and improves the model performance against experimental scattering data [21–23], it is not clear if the same factor should be used for partial charges in molecules, i.e, lipids in our case. Unlike the total charge of molecules, atomic partial charges within each molecule are not experimental observables. **3.Text should be clarified from here to the end of the paragraph** Although there have been proposed several schemes for the assignment of partial charges of molecules, methods targetting at fitting of electrostatic potentials are most popular among classical fixed charge force fields for biomolecules. [30] Currently the most commonly employed scheme is the RESP scheme [31, 32], which fits the electrostatic potential from quantum mechanical calculations. The resulting set of fixed partial atomic charges is hence the best fit to electrostatic potentials of an ensemble of conformations of a molecule. Such a physical meaning is especially well suited for the application of ECC, which targets at improving electrostatic interactions. Depending to the details, partial charge calculations may also partially include some of the solvent electronic polarizability effects. Thus, we expect that the correct ECC scaling factor, f_q, for molecular partial charges does not have to be stricly $f_q = \epsilon_{el}^{-1/2}$, but rather lies between this value (no electronic polarizability in the charge calculation) and 1 (full electronic polarizability already included in the charge calculation).

In this work, we empirically sample this parameter space $f_q \in [0.75, 1.0]$ to find an atomistic MD simulation force field parameters for lipids that accurately describe the lipid head group response to varying concentrations of cations when compared to NMR data [1, 2, 33]. Such data can be used to accurately asses ion binding in PC bilayers, as discussed in Ref, 18 and in section II B. We chose Lipid14 [24] force field parameters as a starting point, because their response to bound ions was apparently 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 [34]. 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. The hydrocarbon chain parameters are not modified, because they are already highly optimized and give generally a good description for hydrophobic part of lipid bilayers in various conditions in most lipids models, including Lipid14 [35]. In contrast, improvements in glycerol backbone and head group parameters are required in all available lipid models [34].

Exploring different scaling factor values, applied to head group, glycerol backbone and carbonyl charges of Lipid14 model, we found out that ion binding and related head group order parameter responses become weaker. The optimal behaviour of ion binding was observed with the scaling factor $f_q = 0.8$. Interestingly, this scaling factor is in line with the estimate given by "implicitly polarized charges" (IPolQ) [36] combined with RESP calculations in vacuum and implicit solvent reported in [37]. IPolQ charges are obtained as the average of partial charges given by RESP calculation [31] in vacuum and in a solvent. Applying the scaling factor of 0.75 to IPolQ charges calculated from the data in Ref. [37], 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 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 ≈60 Å^2, which is significantly smaller than the experimental value 64.3 Å^2 ([]**4.missing REF for APL experiment**) and the original Lipid14 value (65.6 ± 0.5) Å^2 [24]. The decrease of area 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 by reducing the effective radius of atoms by changing the the σ parameters in Lennard-Jones potential for the selected atoms similarly as done for free ions in solution [21–23]. This decreases the solvation free energy by allowing water molecules to approach closer to lipid atoms and have stonger electrostatic interactions with them. After reducing

the σ parameters with a factor of $f_\sigma = 0.89$ for the same atoms for which charges were scaled, the area per molecule value was again in agreement with experimental value (see Table II).

B. Electrometer concept

Ion binding was compared between experiments and simulations by using lipid head group order parameters and the "electrometer concept" [18, 19]. The concept 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, $X^\pm$ [19]. Change in the order parameters measured with varying aqueous ion concentration can be then related to the amount of bound ions.

The change of the head group order parameters is empirically quantified as [19, 39]

$$\Delta S^i_{\rm CH} = S^i_{\rm CH}(X^\pm) - S^i_{\rm CH}(0) \approx \frac{4m_i}{3\chi} X^\pm, \qquad (2)$$

where $S^i_{\rm CH}(0)$ 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 [40, 41], $\chi \approx 167$ kHz, is used for the quadrupole coupling constant. Atomic absorption spectra and ^{2}H NMR data gave $m_\alpha = -20.5$ kHz and $m_\beta = -10.0$ kHz for Ca^{2+} binding to POPC bilayer (in the presence of 100 mM NaCl) [2, 18, 35]. The slopes are negative, because recent analysis concluded that the order parameters decrease with bound positive charge and increase with bound negative charge when the signs are taken in account [18, 35]. This is rationalized as a change of lipid head group dipole tilt toward water phase with bound positive charge and *vice versa* with negative charge [19].

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 [35]. The order parameters for all C-H bonds in lipid molecules, including α and β segments in head group, can be accurately measured by using ^{2}H NMR or ^{13}C NMR techniques. From MD simulations the order parameters can be calculated by using the definition

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

where θ is the angle between the bond and membrane normal and the average is taken over all sampled configurations [35].

The measured order parameter change depends on the response of headgroup on bound charge, i.e. m_i in Eq. 2, and the ion binding affinity. Thus, the former property has to be well quantified before using using the electrometer concept to analyze binding affinities. This is done experimentally for wide range of systems [19, 42]. To calibrate the head group order parameter response also in simulations, we use experimental data for dihexadecyldimethylammonium ($C_{12}C_{16}{}^+N2C_1Br^-$) in POPC bilayer [33]. 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 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 [43].

C. Ion concentrations and binding affinity

The salt concentrations are reported in two different ways in the used experimental data for the lipid head group order parameters. The data for DPPC is reported by using solution concentrations before solvating the lipids [1], while the data for POPC [2] is reported by using supernatant concetration measured with atomic absorption spectroscopy after solvation of lipids. The former was used in comparison with experiments and calculated from simulations in previous work as [salt]=$N_c \times$[water]$/N_w$, where [water] = 55.5 M [18]. Here we use the latter, because the focus is on POPC bilayers. This is determined from the ion concentrations, which reach a constant value at the edges of large enough simulation box. The two concentrations differ somewhat for $CaCl_2$ systems, because some amount of ions bind in lipids. However, the small difference do not affect the conclusions in this or in previous work [18].

The ion concentrations with constant value at the simulation box edges are also used to quantify the ion binding affinities is different simulation models by using the relative surface excess of ions with respect to water Γ^w_i. This is defined as [44]

$$\Gamma^w_i = \Gamma_i - \Gamma_w \frac{C_i - C'_i}{C_w - C'_w} \qquad (4)$$

$$\Gamma_a = \frac{n_a - C_a V - C'_a V'}{A} \; ; \; a = i, w\,, \qquad (5)$$

where index i and w denote ions and water respectively, prime accent denotes the phase inside bilayer, whereas non-prime symbols account for bulk water phase, n is the total amount of water or ions, C denotes bulk concentrations, V is the volume of the respective phase separated by the Gibbs divinding plane, A is the area of the interface, and Γ_a is the surface excess of water or ions. As Γ^w_i does not depend on the definition of the interfacial region and the position of the Gibbs dividing plane [44], we consider the whole simulation box as an interface. Because bilayers have an interface in both leaflefts, the total area of the interface in the simulation is given by the simulation box area multiplied by two, $2A_b$. The concentrations of water and ions inside the bilayer can be expected to vanish, thus $c'_{i,w} = 0$. This provides us a simplified relation for the relative surface excess of lipid bilayers in simulations

$$\Gamma^w_i = \frac{1}{2A_b}\left(n_i - n_w \frac{C_i}{C_w}\right), \qquad (6)$$

where the bulk concentrations, C_w and C_i, are concentrations with constant value at the simulation box edges.

D. Validation of lipid bilayer structure against experiments

Lipid bilayer structure without ions was validated against NMR experiments by order parameters for C-H bonds and x-ray scattering experiments by using form factors. The former validates the structures sampled by individual lipid molecules in simulations with segmental resolution, while the latter validates the dimensions of the lipid bilayer (thickness and area per molecule) [35].

The order parameters were calculated from simulations for all C-H bonds in lipid molecules by using Eq. 3. Form factors were calculated from equation

$$F(q) = \int_{-D/2}^{D/2} \left(\sum_{\alpha} f_\alpha(q_z) n_\alpha(z) - \rho_s \right) \exp(izq_z) \mathrm{d}z, \quad (7)$$

where $f_\alpha(q_z)$ is the density of atomic scattering length, ρ_s is the density of solvent scattering length, $n_\alpha(z)$ is the number density of atom α and z is the distance from the membrane centre along its normal spanning the membrane with thickness D.

E. Simulation details

1. *Simulations with aqueous ions*

The simulated systems consisted of 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) bilayer and an aqueous salt solutions of varying concentrations. In particular, the periodic orthorhombic simulation box conatined 128 POPC molecules and approximately 50 water molecules per each lipid. Water molecules were described by OPC3 model [45] as it is currently the most accurate three site rigid water model. In order to test transferability of the newly developed ECC-lipids model, we also performed several additional simulations with water models OPC [46], SPC/E [47], TIP3p-FB and TIP4p-FB [48], and TIP4p/2005 [49] presented in Supporting Information (SI). We used ECC-ions model for ions [21, 23**?**]. Simulations with Lipid14 use ion models by Dang [50–52], and by Åqvist [53]. Classical molecular dynamics simulations were performed using the GROMACS [54] simulation package (version 5.1.4). The simulation settings used in this work are summarized in Table I, and they are based on previously used settings in [18] available at [55]. **5.As far as remember, I used there Langevin dynamics instead of thermostated MD, because this is done in Amber by default. If this is correct, the information in the table do not match with this sentence. Based on semi-extensive testing I made few years ago this do not change anything. Anyway, this should be reported consistently.** Simulation trajectories and parameters are available at [**?**] **6.To be uploaded to Zenodo.**

2. *Simulations with cationic surfactants*

Automated topology builder [62] was first used to create the structure of dihexadecyldimethylammonium bromide, $C_{12}Cl_{16}{}^{+}N2C_1Br^{-}$, molecule. AmberTools program [63] was then used to generate the Amber-type force field parameters. The parameters were converted to the Gromacs format by using acpype tool [64]. The partial charges were then manually modified to approximately correspond to their equivalent segments in Lipid14 [24]. The surfactants were randomly placed among the lipids to form bilayer structures with mole fractions 10%, 20%, 30%, 42% and 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. The systems were simulated for 200 ns using Lipid14 model for POPC. First 20 ns were omitted from the analysis.

The same systems were also simulated with ECC-lipid model for POPC using the same setup. 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, $f_q = 0.8$, and by using the atom types with reduced σ parameters from ECC-lipids.

TABLE I: Simulation parameters

simulation property	parameter
time-step	2 fs
equilibration time	100 ns
simulation time	200 ns
temperature	313 K
thermostat	v-rescale [56]
barostat	Parrinello-Rahman, semi-isotropic [57]
long-range electrostatics	PME [58]
cut-off scheme	Verlet [59]
Coulomb and VdW cut-off	1.0 nm
constraints	LINCS, only hydrogen atoms [60]
constraints for water	SETTLE [61]

7.This could be moved to SI. Only simulation lengths needs to be mentioned in the main paper.

III. RESULTS AND DISCUSSION

A. POPC membrane and its structure

In order to validate the newly developed model, ECC-lipids, we compared our simulation results without any ions to NMR order parameters measurements and x-ray scattering form factors (Fig. 1 and Table II). The tail order parameters being already highly optimized in the original Lipid14 model [24] are found to match the experimental values nicely. **14.The discussion about acyl chain and headgroup order parameters needs to be finished when the value for C2, the scale are fixed in the figure and size of the points are fixed in the figure.** The headgroup order parameters are slightly larger in the ECC-lipid model, which is in line with larger P-N vector angle in Fig. 2. With the current data we cannot, however, conclude which is the more realistic conformation since in both models other headgroup order parameter agrees with experiments within error bars and other does not. Slight

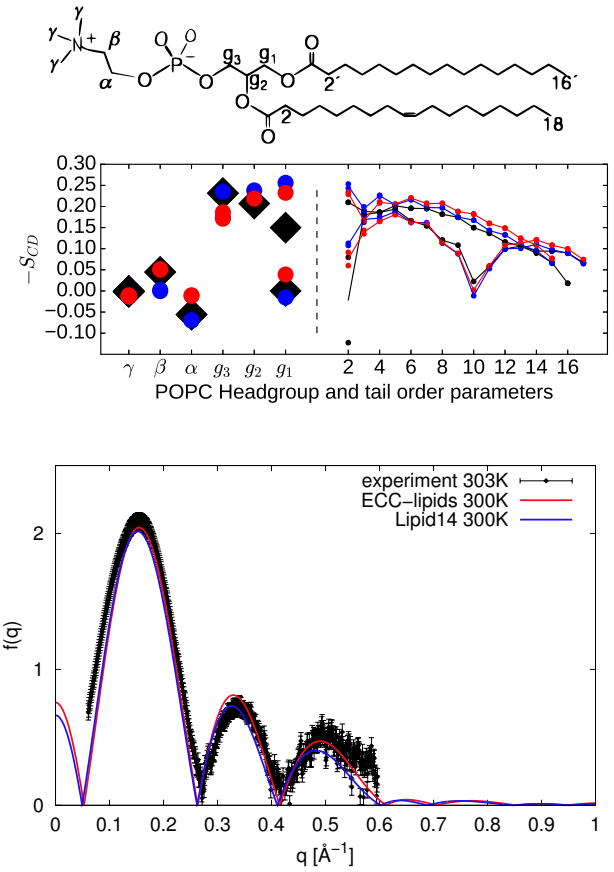

FIG. 1: X-ray scattering form factors from experiments [**?**] and simulations using Lipid14 [24] and ECC-lipids models. Order parameters of head group, glycerol backbone and sn-1 and sn-2 tails from simulations with Lipid14 [24] and ECC-lipids models compared with experimental order parameters from [65].

8.There seems to a typo in experimental value for other order parameter for C2 carbon in sn-2 chain; it should be negative. The scale of the y-axis should be then zoomed when this is fixed.

9.I would put the acyl chain order parameters also for original Lipid14; JOE: Already done, check whether your figures are up to date. I usually push only *.eps.

10.x-axis scale in form factor figure would be better from 0 to 0.6. The experimental data is available for this range.

11.$S_{CD} -> S_{CH}$

changes are also observed in glycerol backbone order parameters, bu the accuracy of both models is comparable to the state of art lipid models available in literature [35]. The agreement between the x-ray scattering form factors and the areas per molecule from simulations and experiments confirm that the membrane structural properties are well captured. A structural comparison of ECC-lipids with Lipid14 can be found in SI along with results with other water models.

B. Response of POPC head group to bound charge

Before proceeding to the ion binding affinity studies, we quantify the response of headgroup order parameters to the

TABLE II: Area per lipid (APL) from different models of POPC without ions

model	APL (Å^2)	Temperature [K]
Lipid14 [24]	65.6± 0.5	303
ECC-lipids		
$(4.6 \cdot 5.1\,\mathrm{nm}^2)$, 72 lipids patch, OPC3	63.2	313
(6.4 nm)^2, 128 lipids patch, OPC3	64.2	313
(6.4 nm)^2, 128 lipids patch, SPC/E	65.1	313
(6.4 nm)^2, 128 lipids patch, OPC	64.4	313
(6.4 nm)^2, 128 lipids patch, TIP4p/2005	66.8	313
experiment [66]**12.REF**	64.3	303
experiment	67.3	323

13.SAMULI: I would put here Lipid14 in 303K, ECC-lipid in 303K and experiment in 303K. Rest in SI. The best experimental value would be the one analyzed from the form factor shown in previous figure, if available.

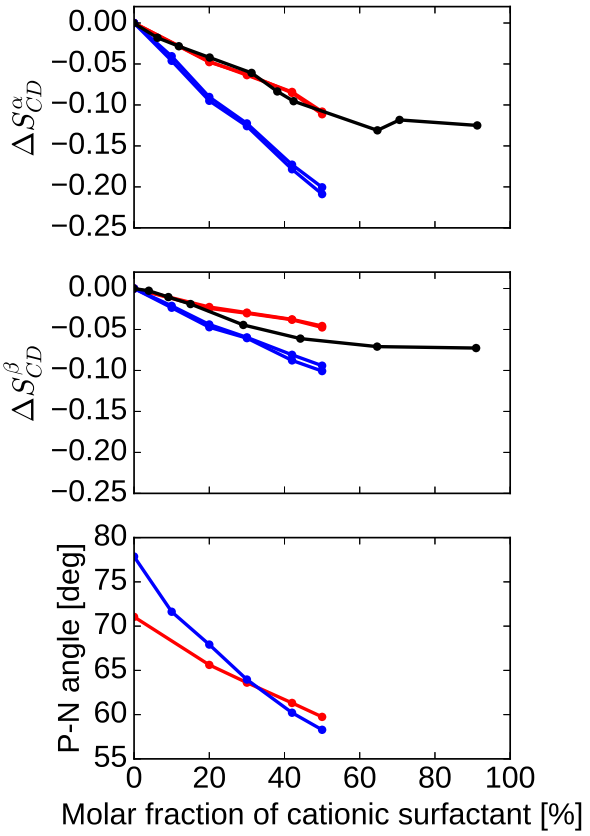

FIG. 2: Headgroup order parameter changes and P-N vector orientation as a function of cationic surfactant (dihexadecyldimethylammonium bromide, $C_{12}Cl_{16}{}^{+}N2C_1Br^{-}$) in PC bilayer from simulations and experiments [33].

15.Labels are missing.

16.Order should be the same as in other figures, i.e, β segment on top.

17.I would put x-axis from 0 to 51 and maybe zoom y-axis little bit as well.

amount of bound charge by using mixtures of monovalent cationic surfactants and POPC [33]. The amount of bound charge per PC in these systems is given by the molar fraction of cationic surfactants, because essentially all surfactants locate in the lipid bilayers. 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 headgroup order parameter changes with increasing amount of cationic surfactant dihexadecyldimethylammonium bromide ($C_{12}Cl_{16}{}^{+}N2C_1Br^{-}$) is compared between experiments [33] and simulations in Fig. 2. The observed order parameter decrease is simulations and experiments can be approximated to be linear at least with the mole fractions below $\sim$30%, as expected from Eq. 2. The slope is, however, too steep in Lipid14 model indicating that the head group order parameters are too sensitive to a bound charge. The ECC-lipids model gives a slope in very good agreement with experiments for the α segment, while the slope is slightly underestimated for the β segment. **18.SAMULI: We could calculate the slopes from simulations, but I am not sure if we would actually learn anything useful from this.**

The headgroup P-N vector angle with respect to the membrane normal is also shown as a function of cationic surfactant mole fraction in Fig. 2. The headgroup orients more parallel to the membrane normal with increasing amount of bound cations, as expected [19]. The effect is more pronounced in Lipid14 than in ECC-lipids model, which is in line with the order parameter results and the reduced charge–dipole interactions in the ECC-lipid model. The response of α-order parameter to bound positive charge in ECC-lipid model is in good agreement with experiments. The model can be thus used to study changes of lipid P-N vector in varying conditions.

C. Cation binding affinity in POPC

The binding affinity of aqueous cations in lipid bilayers can be measured by using the headgroup order parameters, because they decrease linearly with the bound positive charge [18, 19]. The headgroup order parameter responses to aqueous NaCl and $CaCl_2$ concentrations are shown in Fig. 3 from experiments (DPPC [1] and POPC [2]) and different simulation models for POPC.

Negligible changes of the headgroup order parameters are measured with submolar concentrations of NaCl due to the very low affinity of Na^+ in PC bilayers [1]. While Na^+ binding and the related headgroup order parameter changes were overestimated in almost all the available simulation models, the low affinity and negligible order parameter changes were reproduced by Lipid14 model when simulated with Åqvist ions [18]. However, the same combination of force field parameters overestimated the headgroup order parameter response to $CaCl_2$ concentration, which was the case also in all other models tested in Ref. 18. Using ion model by Dang et al. [50–52] or ECC-ions [21, 23**?**] with more realistic bulk behaviour did not improve the results for the $CaCl_2$ interactions with Lipid14 model, as seen in Figs. 3 and **??** (in SI), respectively. **19.Add OP-response of Lipid14+ECC-ions plot in SI**. The results support the conclusion of the previous work [18] that improvements also in lipid models are needed to correctly describe cation binding in PC bilayers.

Significant improvement can be achieved by using the ECC approach also for lipids. The headgroup order parameter changes as a function of $CaCl_2$ concentration from ECC-lipid model with ECC-ions show a good agreement with experiments in Fig. 3. As discussed in previous section, the model gives also a good agreement with experiments for the headgroup response to bound charge. Thus, the model can used for more detailed analysis of the binding affinity.

The binding affinities are quantified by using the ion density profiles along membrane normal shown in Fig. 4. The density profiles show larger Ca^{2+} density peak in lipid headgroup region for Lipid14 model with Dang and ECC-ions than for the ECC-lipid model. The relative surface excess calculated from Eq. 6 gives $\Gamma_i^w = 0.07 \pm 0.01 nm^{-2}$ for the ECC-lipid model, which is significantly smaller than $\Gamma_i^w = 0.13 \pm 0.01 nm^{-2}$ for Lipid14 with Åqvist and $\Gamma_i^w = 0.3 \pm 0.03 nm^{-2}$ with Dang ions.

20.Below analysis is done in a stupid way to get some idea. I would fine it useful to do this analysis by using the density profiles, but it is not necessary. Rough estimates for the free energy difference between bound and unbound cation are given by

$$\Delta G = \mathrm{k_b T} \log(\frac{p_o}{p_i}), \tag{8}$$

where p_o and p_i are estimated from the Ca^{2+}densities in bulk water and in the maximum density in bilayer, respectively. The density profiles in Fig. 4 give $\sim$ 0.8 $\mathrm{k_b T}$ for the free energy difference between bound and unbound Ca^{2+} ions in ECC-lipid model and $\sim$ 1.4 $\mathrm{k_b T}$ in Lipid14 with Åqvist.

21.SAMULI: Maybe we should discuss the repeat distances and area per molecules measured at [7, 8, 67]

Since the lipid headgroup order parameter responses to the amount of bound charge and to the aqueous ion concentrations are both in good agreement with experiments in the ECC-lipid model with ECC-ion parameters, we consider the Na^+ and Ca^{2+} binding affinities to be realistic in this model. On the other hand, the Ca^{2+} binding affinity is overestimated by Lipid14 model when simulated with all the tested ion models. Similar conclusions were previously made based only on the headrogup order parameter data with aqueous cations [18]. However, the discrepancies with experiments in previous work could partly arise also from the inaccurate sensitivity of the headgroup to bound charge. Here we quantify this effect and conclude that the improvement due to ECC is partly, but not completely, caused by more realistic headgroup sensitivity to bound charge. This indicates that the issue should be carefully considered also when the electrometer concept is used to compare ion binding between experiments and simulations wiht other models.

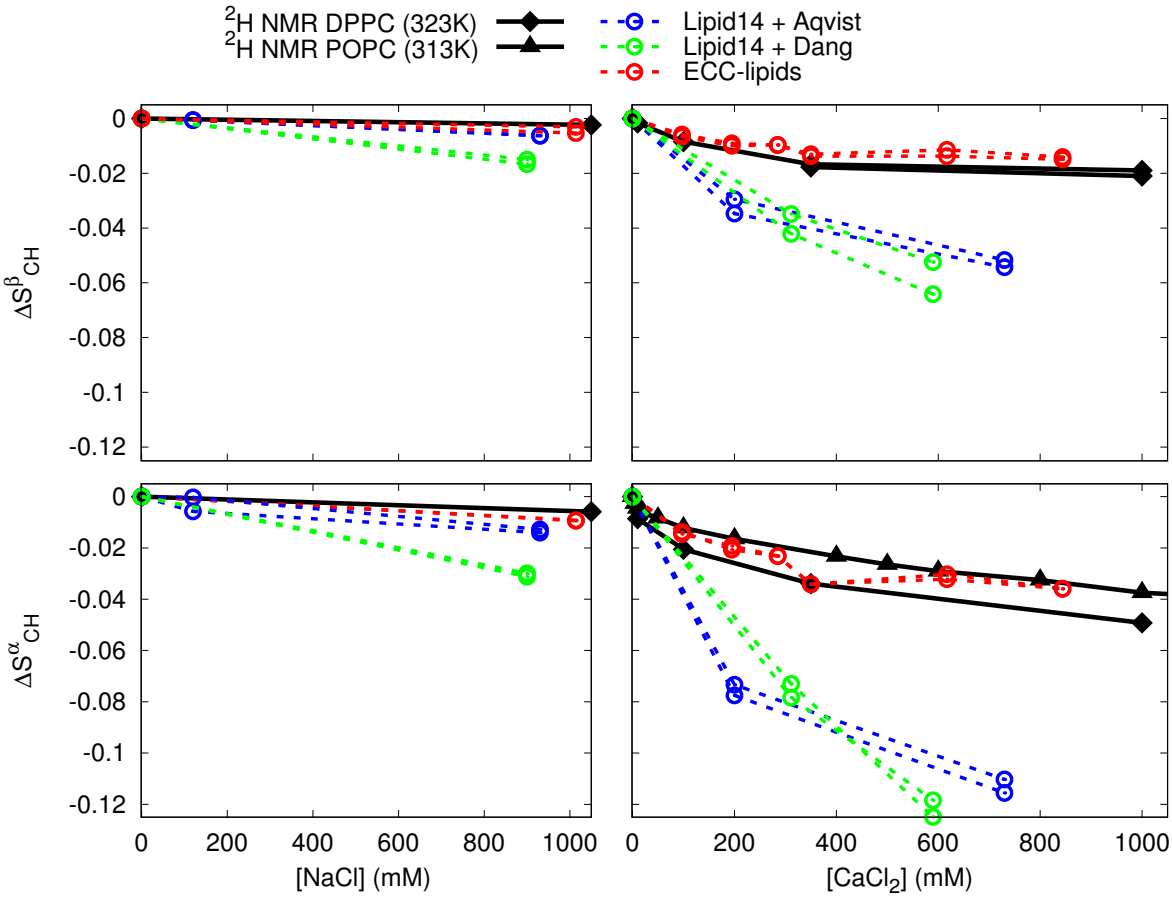

FIG. 3: Changes of head group order parameters of POPC bilayer as a function of NaCl and $CaCl_2$ concentrations are shown from simulations with different force fields together with experimental data (DPPC [1] and POPC [2]). Ion concentrations in bulk water are shown in x-axis. Values from simulations are calculated from the of cation number density C_{np} from the region at the simulatin box edge with the constant ion concentration as [ion]=$C_{np}/0.602$. Simulation data with Lipid14 and Åqvist ion parameters is taken directly from Ref. [18].

D. Binding stoichiometry

This section is rough and will likely require editing. Data – up to noted exceptions – shall be all there, however.

Binding stoichiometry of Ca^{2+} and POPC was thoroughly studied in the experimental work [2], 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 Ca^{2+}:$n\times$POPC clusters by evaluating contacts between cations and lipids with a cut off radius $0.3\,\mathrm{nm}$. 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 occuring with only a slightly lower but similar probability. The fractions of Ca^{2+}:$n\times$POPC complexes at 285 mM concetration are then in order: 42% for two lipids, 30% for one lipid, and 28% for three lipids.

Several binding models were proposed and tested [2] 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 [68], 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+}. **26.Add a simple analysis using number of contacts.** Based on the probability isodensity contours

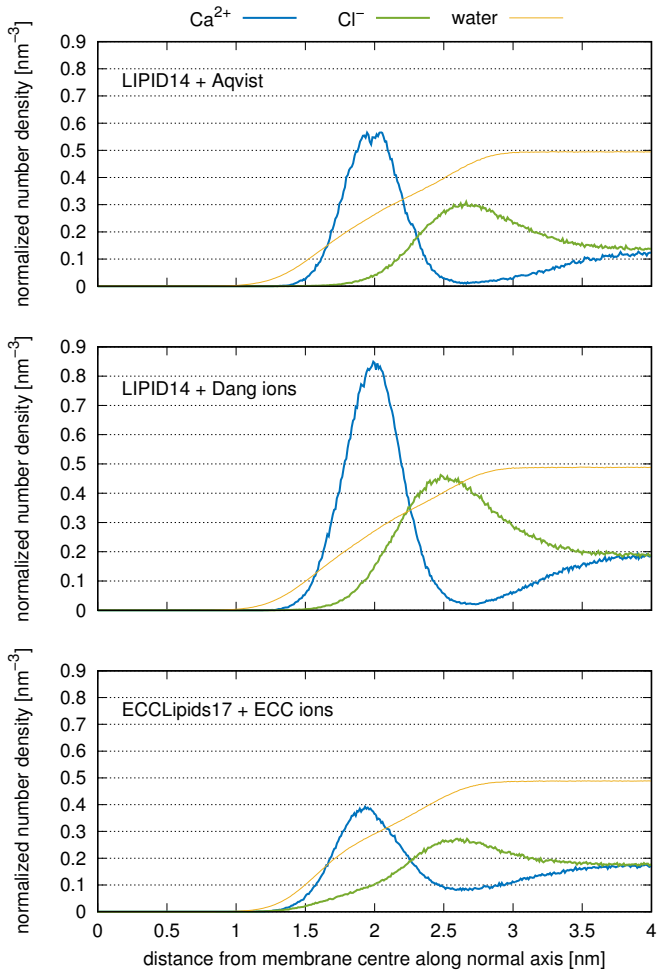

FIG. 4: Number density of Ca^{2+} and Cl^- as a function of membrane normal axis for different force fields. Data for Lipid14 with Åqvist ions are taken directly from Ref. 18. Densities of Cl^- and water are divided with 2 and 200, respectively, to visalize them with the same scale as Ca^{2+}. The molar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

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

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

24.The current data for Dang simulation seems to contain more ions than others.

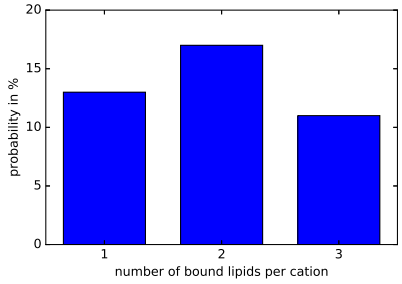

FIG. 5: Histogram of relative probabilities of existence of complexes of Ca^{2+} and n POPC lipids.

25.Change this figure so that it contains the relative probabilities of finding the particular clusters.

(see Fig. 6), we estimate that the largest contribution to the binding of Ca^{2+} to POPC membranes comes from the phosphate group. Although the isodensity plots are relatively easy to interpret, the contours shown in Fig. 6 cannot conclusively tell on the details of Ca^{2+} binding to any of the two carbonyl moieties but their apparently lower affinity compared to phosphate oxygens.

The residence time of Ca^{2+} bound to a POPC membrane is experimentally estimated to be lower than $10\,\mu$s [2]. From recent theoretical work with long enough simulations this time can be roughly estimated in the order of 1–$10\,\mu$s [13]. This is in contrast to our model, ECC-lipids, which gives a mean residence time in the order of 10 ns, **27.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.

28.SAMULI: The same authors have also literature, where they say that ternary complex may not be the only option. I will recheck and come back to this. SAMULI: This is written in [68]: "Ca^{2+} binding to POPC bilayers over the whole concentration range can be best described in terms of formation of a ternary complex involving complexation of two lipids to one calcium ion (Altenbach and Seelig, 1984). The addition of a sodium competition term has not changed this conclusion. However, if Ca^{2+} concentrations up to 100 mM are considered, the data can be equally well explained by a 1:1 binding mechanism (cf. Figure 7). In contrast, the Ca2+ binding to POPC-POPG mixtures can be best described by assuming a 1:l stoichiometry regardless of the range of Ca2+concentrations." We might or might not want to discuss about this. JOE: I think this is adressed/acknowledged enough now.

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

IV. CONCLUSIONS

We show that the Na^+ and Ca^{2+} binding in phospholipid bilayers can be accurately described with classical MD simulation models, where electronic polarization is effectively included by using electronic continuum correction (ECC) [20]. This is a significant improvement over other available lipid models, which all overestimate specific cation binding affinities [18]. The newly proposed model, which we denote as "ECC-lipids 17", exhibits accurate head group order parameter response to bound cations, monovalent Na^+ and cationic surfactant dihexadecyldimethylammonium bromide, and divalent Ca^{2+} also quantifying their binding affinities. Moreover, ECC-lipids 17 reproduce the lipid bilayer structural details with similar accuracy as other state of the art lipid models [18]. Several water models (OPC3[45], OPC [46], SPC/E [47] and TIP4p/2005 [49]) were used to exemplify the transferability of the parameters of the new ECC-lipids 17 force field.

Direct analysis of calcium binding details from MD simulations is in agreement with ternary complex model, which is suggested based on NMR data [2]. In this model 1 calcium binds to 2 POPC molecules, which together form a ternary complex. **31.Continue summary using previous section once it is finished.**

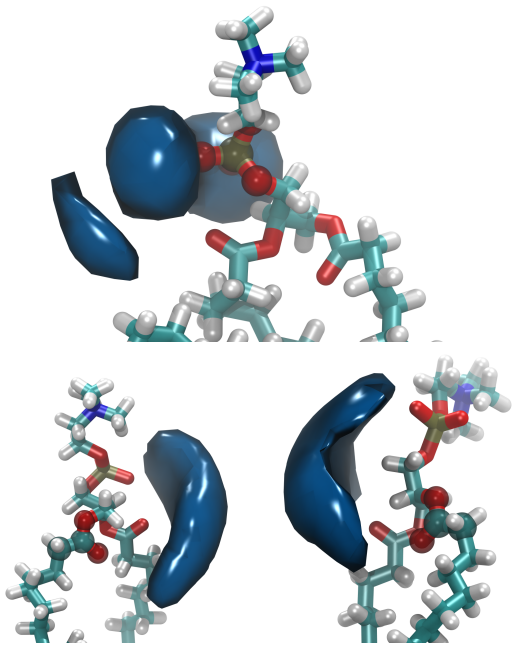

FIG. 6: Contours of probability isodensities of Ca^{2+} with respect to various moieties fixed in space (highlighted with transparent spheres): phosphate moiety, side chain 1 carbonyl group and side chain 2 carbonyl group. Shown contours suggest that the dominant contribution to Ca^{2+} binding comes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

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

The electronic continuum correction is applied here on Lipid14 POPC model [24], but we expect that the correction can be generalized also for other lipids and force fields. The parameters can be used with existing standard nucleic acid and protein force fields, e.g. AMBER-FB15 [69]. We suggest using state of the art water models like OPC3[45] or OPC [46], which yield higher accuracy than the traditional TIP3p water model [70].

This work can be reached as a repository containing all data at `zenodo.org:\dots\dots\dots` and as project NMRlipids VI in `nmrlipids.blogspot.fi`.

Acknowledgments

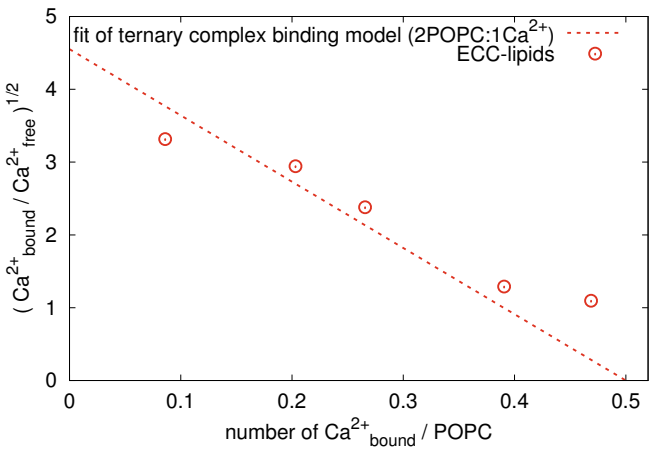

FIG. 7: Ternary complex binding model of Ca^{2+} to a POPC membrane that assumes the stoichiometry of 2 POPC:1 Ca^{2+} (details in reference 2) provides a good fit to experimental measurements [2] and it also provides a good fit to our simulation data. Note that the units in the reference 2 are different 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 [2] 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 [2]. The concentration C_b was obtained from an extrapolation of linear relation between deuterium NMR measurements and atomic absorption spectroscopy for low concetrations 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+} [71]. An atomistic simulation, on the other hand, provides these quantities directly without severe assumptions. Hence we hypothesise that the discrepancy between the results in the experiment [2] and our simulations likely lays in the fact that the assumptions and relations used for determinig concentrations C_b and C_I in the experiment [2] gradually do not hold for higher concentrations of Ca^{2+}.

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