

# 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 phospholipid 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 headgroup order parameter response to bound charge. We also provide for the first time a realistic stoichiometry 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. Zwitterionic phosphocholine (PC) lipid bilayers have been widely used as model systems to understand molecular level details of specific cation interactions with cellular membranes by using experimental [1–5] and theoretical methods [6?–8]. **2. We need to select references for these.** While relative binding affinity of different ions is agreed to follow Hoffmeister series, the molecular details of binding and binding energetics are not fully understood [1–3, 9? , 10]. Non-invasive spectroscopic methods, like nuclear magnetic resonance (NMR), scattering and infrared spectroscopy, give accurate information about ion binding in lipid bilayers [3, 11–15]. Molecular level interpretation of the results, however, requires assumptions about binding model and is often not fully conclusive [? ]. On the other hand, the accuracy of the state of the art atomistic resolution lipid and ion models have turned out insufficient for the detailed interpretation of cation binding details [10].

In this work we show that the accuracy of Calcium binding on zwitterionic PC lipid bilayer can be significantly improved by including electronic polarizability by using effective continuum correction (ECC) [16] for polar region of zwitterionic lipid molecules in classical MD simulation model. This is essentially physically well justified version of partial charge scaling implemented in early days of lipid and surfactant simulations [17, 18]. The approach has been previously shown in to improve bulk performance of ion models against neutron scattering data [19? , 20]. The better bulk behaviour was not,

however, sufficient to correct binding in lipid bilayers [10].

The proposed MD simulation model with ECC-corrected lipids and ions reproduce the experimental NMR data of Calcium binding in PC lipid bilayers and mainly supports the proposed ternary complex model (2 Ca<sup>2+</sup> bind in on POPC) [14, 15]. However, non-negligible contribution of one Ca<sup>2+</sup> cross bridging three POPC molecules is also observed. The overestimated specific cation binding observed in current lipid models [10] may lead to artificially positively charged membranes and significant artefacts in MD simulations. Thus, the proposed approach to improve the accuracy of cation binding in current MD models is highly useful for future MD simulations with physiological salt conditions.

## II. METHODS

### A. Electronic continuum correction for lipid bilayers

The lack of electronic polarizability from the classical MD simulation models has been considered highly relevant issue since the early days of lipid bilayer simulations and pioneering simulation studies scaled the partial charges by half to effectively include electronic polarizability [17, 18]. Also approaches that explicitly include electronic polarizability has been introduced, but this has turned out to be practically complicated [21]. In this work we effectively include electronic polarizability in lipid bilayer simulations by using the electronic continuum correction (ECC) [16]. The approach is more sophisticated version of early studies with scaling to half was used [17, 18], but significantly more simple than the explicit inclusion of electronic polarizability [21].

According to ECC, electronic polarizability can be included in classical MD simulations by placing all particles into a homogeneous dielectric continuum with a dielectric constant  $\epsilon_{el}$ ,

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which is the electronic part of the dielectric constant of the media [16]. Measurements of high frequency dielectric constant gives values of approximately  $\epsilon_{el} \approx 2$  for almost any biomaterial [16? ]. Such a 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. The value measured for water,  $\epsilon_{el} = 1.78$ , gives a scaling factor of  $f_q = 0.75$  [16? ], which has been successfully used to improve the performance of ion force fields [19? , 20].

The main goal of this work is to apply ECC correction to accurately describe the lipid headgroup response to  $\text{Na}^+$  and  $\text{Ca}^{2+}$  concentrations in MD simulation models when compared with NMR data [10]. The Lipid14 [22] force field parameters were used as a starting point, because their response to bound ions was most realistic against NMR data (see Fig. 5 in Ref. 10). Also glycerol backbone and headgroup structures in Lipid14 model were relatively realistic when compared with other state of the art lipid models [23].

While the scaling factor of  $f_q = 0.75$  for ions in water is well justified and shown to improve model performance against scattering data [19? , 20], it is not clear if the same factor should be used for lipid headgroups 3., because ?? . Here we estimated the correct scaling factor for Lipid14 model by using so called implicitly polarized charges (IPolQ) [24], which are the average of partial charges given by RESP [? ] calculations in vacuum and in implicit solvent. The partial charges in original Lipid14 parametrization were derived by fitting the electrostatic potential to its model quantum chemistry representation (RESP[? ]) in vacuum, while charges from calculations in an implicit solvent vary most for the polar moieties [25]. Here we estimate the appropriate scaling factor for lipid hedgroups and glycerol backbone by comparing IPolQ charges (average of RESP calculation in vacuum and in implicit solvent) to the RESP results in vacuum. By using the results reported in [25] we conclude that the IPolQ charges can be achieved from vacuum calculations by scaling roughly with the factor of  $f_q = 0.8$ , which is the used to derive ECC-corrected Lipid14 model. 4.SAMULI: please check that this is correctly described. [43]

Here we apply ECC correction with the proposed scaling factor only to the headgroup, 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 highly optimized in Lipid14 and other lipids models and give generally a good description for hydrophobic part of lipid bilayers in various conditions [26], in contrast to glycerol backbone and headgroup regions which require some improvement in all available lipid models [23]. The scaling of partial charges in the polar region was found to decrease area per molecule to ??5.find the value, which is significantly smaller than the experimental value ([1]) and the original Lipid14 values ([1])6.Add the values. The decrease of area was found to arise

from lower hydration of the lipid headgroup region, which can be explained by the increased solvation free energy due to the lower polarity of molecules with scaled charges. The solvation free energy can be decreased and hydration increased by reducing the radius of beads by changing the the  $\sigma$  parameters in Lennard-Jones potential for the selected atoms. Here we reduce  $\sigma$  with a scaling factor of  $f_\sigma = 0.89$  for the same atoms for which charges was scaled. This increased the area per molecule back to a level close to experimental and original Lipid14 values.

## B. Comparison of ion binding affinity to experiments by using the electrometer concept

Ion binding was compared between experiments and simulations by using lipid headgroup order parameters and "electrometer concept" introduced by Seelig et al. [10, 28]. The concept is based on the experimental observation that the order parameters of  $\alpha$  and  $\beta$  carbons in lipid headgroup (see Fig. ?? 7.Figure with chemical structure and labeling to be added) are proportional to the amount of bound charge in lipid bilayer [28]. More recent analysis included also the order parameter signs and concluded that the order parameters decrease with bound positive charge and increase with bound negative charge [10, 26]. The observations are rationalized as a change of lipid headgroup dipole tilt to more vertical orientation with bound positive charge and *vice versa* for negative charge [28]. Order parameters for C-H bonds can be accurately measured for each C-H bond of lipids by using  $^2\text{H}$  NMR or  $^{13}\text{C}$  NMR techniques and calculated from MD simulations directly from definition

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

where  $\theta$  is the angle between the bond and membrane normal and average is taken over all sampled configurations [26].

The change of the headgroup order parameters as a function of the amount of bound charge per lipid  $X^\pm$  can be written as [29]

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

where  $S_{\text{CH}}^i(0)$  denote the order parameter in the absence of bound charge,  $i$  refers to either  $\alpha$  or  $\beta$  carbon,  $m_i$  is an empirical constant depending on the valency and position of bound charge, and the value of the quadrupole coupling constant is  $\chi \approx 167 \text{ kHz}$ . Combination of atomic absorption spectra,  $^2\text{H}$  NMR experiments and information about order parameter signs gave  $m_\alpha = -20.5$  and  $m_\beta = -10.0$  for  $\text{Ca}^{2+}$  binding to POPC bilayer (in the presence of 100 mM NaCl) [10, 15, 26].

Recent work published by the NMRlipids project showed that the concept works qualitatively also in simulations and can be used to compare ion binding affinity between simulations and experiments [10]. However, it also turned out that also the sensitivity of the order parameters response to bound

charge has to be quantified more accurately for quantitative comparison of binding affinity. In this work we first we first quantify the response of headgroup order parameters against experimental data from cationic surfactants embedded in PC bilayer [42]. Essentially all surfactants locate in lipid bilayers in these experiments, thus the amount of bound charge is known and can be plotted against order parameter change.

The used experimental data report order parameters as a function of equilibrium cation concentration in the bulk solvent [14, 15]. Such a condition is reached in simulations by adjusting the simulation box size to dimensions large enough that ion concentration reaches a clear plateau in the bulk solvent. The concentrations in the units of mol/l were then determined as

$$C_{eq} = \frac{C_{plateau}}{0.602}, \quad (4)$$

where plateau concentration is the number density in the units of  $\text{nm}^{-3}$ . **8.SAMULI: Once we have to final results, we can probably say that the repeat distance is not far from the experimentally measured distance [4, 5]**

### C. Comparison of lipid bilayer structure to experimental data

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 latter validates the dimensions of the lipid bilayer (thickness and area per molecule) [26]. Form factor was calculated from simulations as

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

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

### D. 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. Water molecules were described by OPC3 model [30]. In order to test transferability of the newly developed ECC-lipids model, we also performed several additional simulations with water models OPC [31], SPC/E [32] and TIP4p/2005 [33] presented in Supporting Information (SI). We used ECC-ions model for ions [20, 34? ]. Simulations with Lipid14 use ion model by Dang [35–37]. **9.Table about simulations details and some**

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

model	APL ( $\text{\AA}^2$ )	Temperature [K]
Lipid14 [22]	$65.6 \pm 0.5$	303
ECC-lipids		
$(4.6 \cdot 5.1 \text{ nm}^2)$ , 72 lipids patch, OPC3	63.2	313
$(6.4 \text{ nm})^2$ , 128 lipids patch, OPC3	64.2	313
$(6.4 \text{ nm})^2$ , 128 lipids patch, SPC/E	65.1	313
$(6.4 \text{ nm})^2$ , 128 lipids patch, OPC	64.4	313
$(6.4 \text{ nm})^2$ , 128 lipids patch, TIP4p/2005	66.8	313
experiment [41] <b>13.REF</b>	64.3	303
experiment	67.3	323

**related text is deleted in commit [https://github.com/ohsOllila/NMRLipids\\_V1-NewIonModel/commit/6526079062ca322e90f8ea0654597394b023041e](https://github.com/ohsOllila/NMRLipids_V1-NewIonModel/commit/6526079062ca322e90f8ea0654597394b023041e) Maybe this is done accidentally, or is there some other plan with this?**

#### 2. Simulations with cationic surfactants

Automated topology builder [?] was first used to create the structure of dihexadecyldimethylammonium bromide,  $\text{C}_{12}\text{Cl}_{16}^{+}\text{N}_2\text{C}_1\text{Br}^{-}$ , molecule. The code AmberTools [38] was then used to generate the Amber-type force field parameters. The parameters were converted to the Gromacs format by using acpype tool [39]. The partial charges were then manually modified to approximately correspond to their equivalent segments in Lipid14 [22]. 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. These systems were ran with original Lipid14 POPC model and with ECC-corrected model. **10.Describe the changes in surfactant model used with ECC-corrected lipids.**

## III. RESULTS AND DISCUSSION

### A. Lipid bilayers without ions

The scattering form factors, NMR order parameters and area per lipid calculated using the ECC-lipids model for POPC are compared to experiments and original Lipid14 results in Fig. 1 and in Table I. The structural quality is comparable to the state of art lipid models available in literature [26], thus we conclude that the ECC-lipids model reproduces the lipid bilayer structure in liquid disordered phase with similar accuracy to other available models.

Headgroup order parameter response to the bound charge was evaluated against experimental data measured with the cationic surfactant (dihexadecyldimethylammonium bromide,  $\text{C}_{12}\text{Cl}_{16}^{+}\text{N}_2\text{C}_1\text{Br}^{-}$ ) [42]. The exact amount of bound charge in the membrane is known in these systems, because practically all cationic surfactant molecules are embedded in the lipid bilayer due to their amphiphilic nature. Thus, the changes of headgroup order parameter as a function of mole

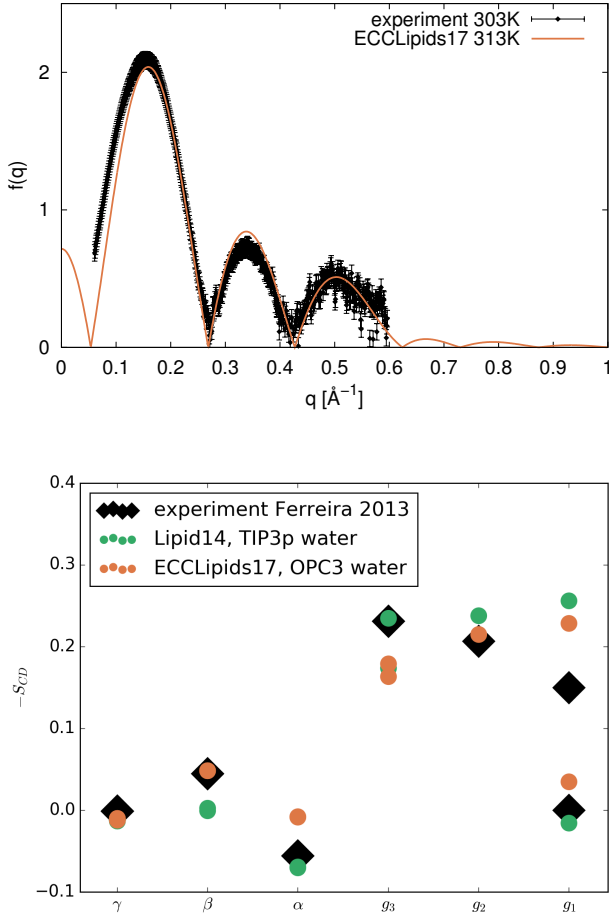


FIG. 1: X-ray scattering form factors from experiments [?] and simulations using Lipid14 [22] and ECC-lipids models. Headgroup and glycerol backbone order parameters from simulations with Lipid14 [22] and ECC-lipids models compared with experimental order parameters from [40].

11.Add acyl chain order parameters, POPC chemical structure

12.We should run a simulation for the system without ions with the same (or close) temperature as the experimental data. The NMR data is at 300K so that would be good. I think that this would be also close enough for the scattering data at 303K.

fraction of cationic surfactants (Fig. 2) gives also the order parameter changes as a function of the amount of bound cations.

14.Split the OP-changes figure in two: cationic surfactant -and- binding of cations. The separate figure with the cationic surfactant shall be additionally accompanied with a plot of Head group tilt (PN-vector?) change/response.

The headgroup order parameter response to the bound cation concentration is in experiments approximately linear up to  $\sim 0.3$  mole fraction of the cation [42]. The linearity is also observed in simulations with original Lipid14 and with newly derived ECC-lipids models. Quantitative comparison, however, reveals that the response is overestimated in Lipid14 for both segments, while the ECC-lipids model gives a good agreement for the change of  $\alpha$  segment order parameter, and slightly underestimates the  $\beta$  segment order parameter change. Such a finding is not surprising, as the  $\Delta S^\alpha / \Delta S^\beta$

ratio of the response of Lipid14 model is found to be slightly larger than in experiments (VALexp vs VALsim 15.Add values of  $S^\alpha / S^\beta$  response for sim and experiment SAMULI: Maybe these could be put in the same figure with the order parameter response and P-N vector angle change with surfactants. ), and this property is also kept by the newly derived Lipid14-based model, ECC-lipids. The present comparison of headgroup order parameter changes suggests that the observed overestimated response of all models to increasing  $\text{CaCl}_2$  concentration in [10] can be explained at least in part by a high sensitivity of the headgroup order parameter response to the bound charge.

## B. Cation binding to POPC membrane

Headgroup order parameter response to increasing  $\text{CaCl}_2$  concentration from experiments, original Lipid14 model and ECC-lipids model are shown in Fig. 2. The order parameter response is significantly overestimated in original Lipid14 model, while results from ECC-lipids model are in a good agreement with the experiments. This is a significant improvement over current models, which in general overestimate the head group order parameter response to  $\text{CaCl}_2$  concentration [10]. The good agreement with experiments indicate that the binding details of  $\text{Ca}^{2+}$  are realistic in the ECC-lipids model and, hence, it can be used to study lipid-ion interaction in detail.

Ion density profiles from simulations with different models are compared in Fig. 3. The density profiles from simulations with original Lipid14 [22] and Dang ions [35–37] show a pronounced peak in the position of the phosphate moieties of POPC. The use of a ECC-ion model [19, 20] or the standard Amber ion model by Åquist along with Lipid14 does not significantly change it (Headgroup order parameter responses for these models in SI). 16.Add OP-response of Lipid14+ECC-ions into the OP-changes plot in SI. The new ECC-lipids model with ECC-ions exhibits on the other hand smaller density in this region suggesting overall weaker binding of cations (Fig. 2). Together with the accurate head group order parameter response (Fig. 2), the decrease in cation density of ECC-lipids demonstrates that ion binding to zwitterionic phospholipid membranes can be accurately described with classical MD simulation models that account for electronic polarizability.

Good agreement of ECC-lipids model with experiments encourages us to analyse the binding details from MD simulations. Direct analysis of contacts between ions and lipids from simulations suggest that the most abundant POPC: $\text{Ca}^{2+}$  complex have stoichiometry of 2 POPC:1  $\text{Ca}^{2+}$ . As shown in Fig. 4 this is in agreement with the ternary complex model suggested based on headgroup order parameter experiments [15]. In addition to the ternary complexes, there also is a non-negligible probability of one  $\text{Ca}^{2+}$  cross-bridging three POPC molecules. Technical details of the analysis are in the SI.

20.Put details of the cation-binding stoichiometry analysis to SI. 21.Update the binding isotherm figure with new simulations 22.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. 23.SAMULI: I would also analyze how much there is contact between ions and different parts of the lipid (phosphate, carbonyl,



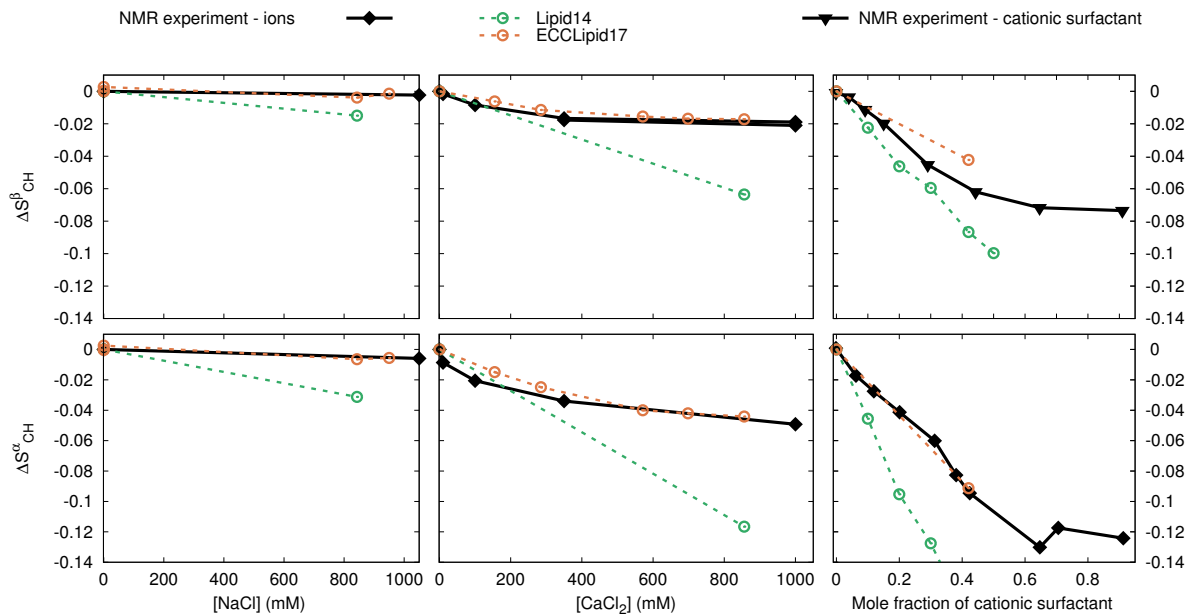


FIG. 2: Headgroup order parameter changes as a function of NaCl, CaCl<sub>2</sub> concentration and cationic surfactant (dihexadecyldimethylammonium bromide, C<sub>12</sub>Cl<sub>16</sub><sup>+</sup>N<sub>2</sub>C<sub>1</sub>Br<sup>-</sup>) mole fraction from simulations and experiments (DPPC [14], POPC [15], surfactant [42]).

etc.). 24.SAMULI: I think we should quantify this, i.e. how much there are these. Maybe also the other possible complexes? Maybe also the correlation between complexes and binding sites, if it is not too much work. JOE: This looks like a careful work for the next paper to me. I'd only add a relatively simple analysis of binding sites and probably the propensity of 1-2-3 membered clusters.. 25.Finalize stoichiometry analysis for Na<sup>+</sup>, Ca<sup>2+</sup>, their interaction energies with the lipid membrane, etc, and finalize the discussion after these results.

#### IV. CONCLUSIONS

We show that the Na<sup>+</sup> and Ca<sup>2+</sup> binding in phospholipid bilayers can be accurately described with classical MD simulation models, where electronic polarization is effectively included by using electronic continuum correction. This is a significant improvement over other available lipid models, which all overestimate specific cation binding affinities [10]. The newly proposed model, which we denote as "ECC-lipids 17", reproduces the lipid bilayer structural details with similar accuracy as other state of the art lipid models. ECC-lipids 17 exhibit accurate headgroup order parameter response to bound cations, monovalent Na<sup>+</sup> and cationic surfactant dihexadecyldimethylammonium bromide, and divalent Ca<sup>2+</sup>. Several water models (OPC3[30], OPC [31], SPC/E [32] and TIP4p/2005 [33]) 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 [15]. In this model 1 calcium binds to 2 POPC molecules, which together form a ternary complex.

The electronic continuum correction is applied here on

Lipid14 POPC model [22], but we expect that the correction can be generalized also for other lipids and force fields.

This work will serve as a foundation stone of a new open-collaboration project NMRlipids VI in [nmrlipids.blogspot.fi](http://nmrlipids.blogspot.fi).

#### Acknowledgments

## SUPPLEMENTARY INFORMATION

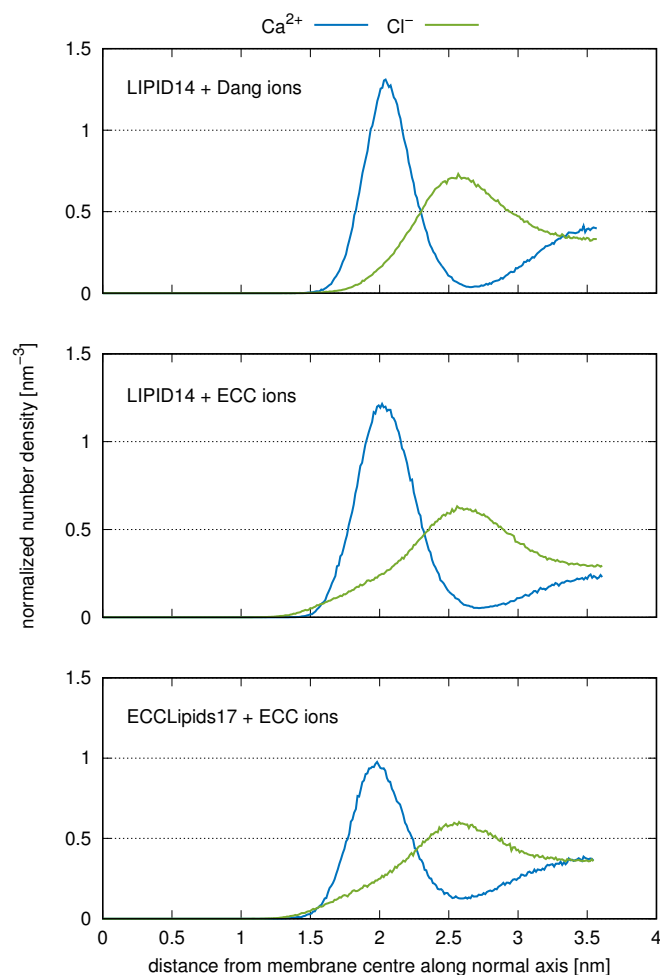


FIG. 3: Density profiles of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  for Lipid14 model with Dang ions and with ECC-ions and ECC-lipids model with ECC-ions.

17. Change the figure so that it contains a membrane background

18. What are the concentrations (calculated as in NMRlipids II) in this figure?

19. These should be also compared to the results with qvist ions, which are shown in NMRlipids II. NMRlipids II have results with 350mM and 1000mM. If we have the distributions with the same concentrations qvist results could be added also in this figure. If not, the comparison has to be made some other way.

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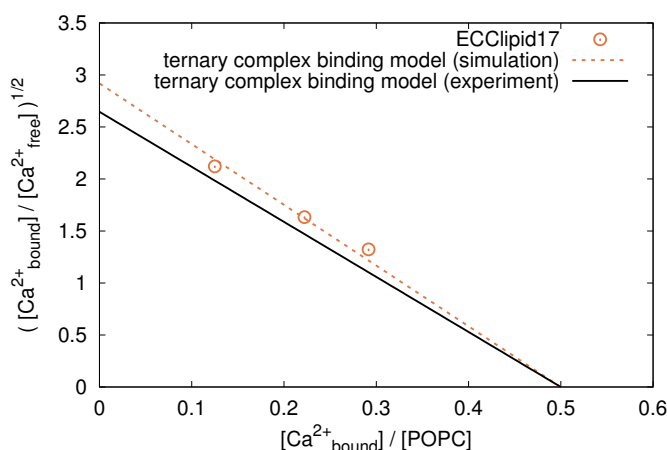


FIG. 4: Binding isotherm assuming stoichiometry of 2 POPC:1  $\text{Ca}^{2+}$  as used in [15] fits our simulation data well.

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- [43] Depending on which QM method you arrive at values from 0.76 to 0.83, averages across atom types being around 0.78–0.80. Even though the methods are almost identical, authors of Lipid 14 find lower partial charges in vacuum than here – so I prefer the higher value. As the choice of charges is arbitrary anyway, I use 0.80 as an approximate round value. The use of 0.78–.79 might be more appropriate, though.

## ToDo

- |  | P. |
|--|----|
| 1. Abstract directly from Joe's conference abstracts. To be rewritten. . . . .   | 1  |
| 2. We need to select references for these . . . . .  | 1  |
| 3. , because ?? . . . . .  | 2  |
| 4. SAMULI: please check that this is correctly described. . . . .  | 2  |
| 5. find the value . . . . .  | 2  |
| 6. Add the values . . . . .  | 2  |
| 7. Figure with chemical structure and labeling to be added . . . . .   | 2  |
| 8. SAMULI: Once we have to final results, we can probably say that the repeat distance is not far from the experimentally measured distance [4, 5] . . . . . | 3  |
| 13. put original references, not Slipids param. paper. . . . .   | 3  |

9. Table about simulations details and some related text is deleted in commit <a href="https://github.com/ohsOllila/NMRlipids_VI-NewIonModel/commit/6526079062ca322e90f8ea0654597394b023041e">https://github.com/ohsOllila/NMRlipids_VI-NewIonModel/commit/6526079062ca322e90f8ea0654597394b023041e</a> Maybe this is done accidentally, or is there some other plan with this? . . . . .	3	22. 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. . . .	4
10. Describe the changes in surfactant model used with ECC-corrected lipids. . . . .	3	23. SAMULI: I would also analyze how much there is contact between ions and different parts of the lipid (phosphase, carbonyl, etc.). . . . .	5
11. Add acyl chain order parameters, POPC chemical structure . . . . .	4	24. SAMULI: I think we should quantify this, i.e. how much there are these. Maybe also the other possible complexes? Maybe also the correlation between complexes and binding sites, if it is not too much work. JOE: This looks like a careful work for the next paper to me. I'd only add a relatively simple analysis of binding sites and probably the propensity of 1-2-3 membered clusters. . . . .	5
12. We should run a simulation for the system without ions with the same (or close) temperature as the experimental data. The NMR data is at 300K so that would be good. I think that this would be also close enough for the scattering data at 303K. . . . .	4	25. Finalize stoichiometry analysis for $\text{Na}^+$ , $\text{Ca}^{2+}$ , their interaction energies with the lipid membrane, etc, and finalize the discussion after these results. . . . .	5
14. Split the OP-changes figure in two: cationic surfactant -and- binding of cations. The separate figure with the cationic surfactant shall be additionally accompanied with a plot of Head group tilt (PN-vector?) change/response. . . . .	4	17. Change the figure so that it contains a membrane background . . . . .	6
15. Add values of $S^\alpha/S^\beta$ response for sim and experiment SAMULI: Maybe these could be put in the same figure with the order parameter response and P-N vector angle change with surfactants. . . . .	4	18. What are the concentrations (calculated as in NMRlipids II) in this figure? . . . . .	6
16. Add OP-response of Lipid14+ECC-ions into the OP-changes plot in SI . . . . .	4	19. These should be also compared to the results with qvist ions, which are shown in NMRlipids II. NMRlipids II have results with 350mM and 1000mM. If we have the distributions with the same concentrations qvist results could be added also in this figure. If not, the comparison has to be made some other way. . . . .	6
20. Put details of the cation-binding stoichiometry analysis to SI. . . . .	4		
21. Update the binding isotherm figure with new simulations . . . . .	4		