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Free Energy Landscapes of Sodium Ions Bound to DMPC-Cholesterol Membrane Surfaces at Infinite Dilution†

Jing Yang,[a] Massimiliano Bonomi,[b] Carles Calero,*[c] and Jordi Martí,*[a]

Exploring the free energy landscapes of metal cations on phospholipid membrane surfaces is important for the understanding of chemical and biological processes in cellular environments. Using metadynamics simulations we have performed systematic free energy calculations of sodium cations bound to DMPC phospholipid membranes with cholesterol concentration varying between 0% (cholesterol-free) and 50% (cholesterol-rich) at infinite dilution. The resulting free energy landscapes reveal the competition between binding of sodium to water and to lipid head groups. Moreover, the binding competitiveness of lipid head groups is diminished by cholesterol contents. As cholesterol concentration increases, the ionic affinity to membranes decreases. When cholesterol concentration is greater than 30%, the ionic binding is significantly reduced, which coincides with the phase transition point of DMPC-cholesterol membranes from a liquid-disordered phase to a liquid-ordered phase. We have also evaluated the contributions of different lipid head groups to the binding free energy separately. The DMPC's carbonyl group is the most favorable binding site for sodium, followed by DMPC's phosphate group and then the hydroxyl group of cholesterol.

1 Introduction

The interactions between metal cations and phospholipid membranes have drawn great attention during last decades [1,2]. Several experiments revealed that metal cations are bound to the negative charged head groups of phospholipid membranes [3–6]. Meanwhile, numerous molecular dynamics (MD) simulations have been performed to study the binding of metal cations at phospholipid membranes from an atomic point of view [6–13]. However, most of the experiments and simulation works on ionic binding to phospholipid membranes have been devoted to cholesterol-free environments. Cholesterol is a crucial component in mammalian cell membranes, constituting up to 50% of their weight [14]. Cholesterol can modulate the structural and mechanical properties of membranes and can induce a phase transition from a liquid disordered phase to a liquid ordered phase [15–17]. Therefore, the study of the binding of metal cations to cholesterol-containing membranes is of interest to understand ionic binding in realistic cellular environments. Only recently, experiments by Iraolagoitia et al. showed that cholesterol significantly reduced the Ca^{2+} binding to membranes [18]. More recently, Magarkar et al. performed both experiments and simulations, revealing that increasing cholesterol concentration decreased Na^+ binding [19].

The binding of metal cations at membranes in aqueous solution can result in several possible bound configurations, as a consequence of the loss of water molecules and the gain of lipid atoms in the ion's first hydration shell. Exploring the binding processes and bound states of metal cations at phospholipid membrane surfaces is important for the understanding of chemical and biological processes such as binding, hydration, leakage, and dissociation in cellular environments. Nevertheless, a comprehensive study of the relative stabilities of different bound states is a difficult experimental task, and also difficult for classical MD simulations because of the high free energy barriers among various bound states. To circumvent such difficulty, free energy calculations applying enhanced sampling techniques can be employed.

Recently, we have proposed a general methodology to explore the free energy landscapes for ions at biological interfaces and obtained the relative stabilities of different bound states for biologically relevant cations Na^+, K^+, Ca^{2+}, and Mg^{2+} at cholesterol-free membranes [20]. In this work, we further apply this methodology to cholesterol-containing membranes and have performed systematic free energy calculations of Na^+ bound to phospholipid membranes of various cholesterol concentrations at infinite ion dilution. The resulting free energy landscapes further validate our methodology at membrane interfaces with higher complexity and unveil the cholesterol effects on Na^+ binding at phospholipid membranes.

[a] Department of Physics and Nuclear Engineering, Technical University of Catalonia-Barcelona Tech, B4-B5 Northern Campus, Jordi Girona 1-3, 08034 Barcelona, Catalonia, Spain; E-mail: jordi.marti@upc.edu

[b] Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom

[c] Center for Polymer Studies and Department of Physics, Boston University, 590 Commonwealth Avenue, Boston, Massachusetts 02215, United States; E-mail: ccalero@bu.edu

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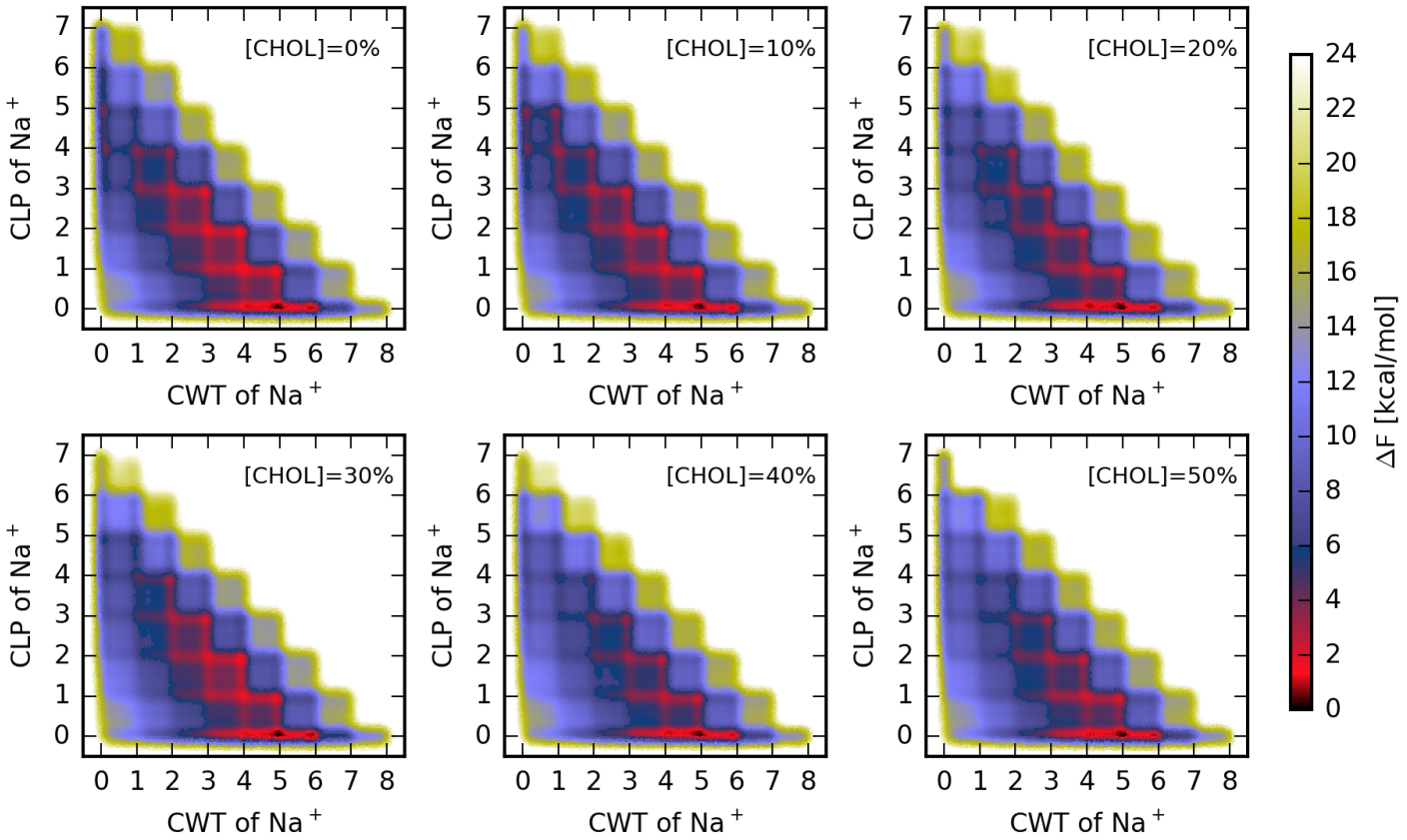

Fig. 1 Binding free energy difference ΔF as a function of the coordination number of lipid oxygens (CLP) and the coordination number of water oxygens (CWT) for Na^+ bound to DMPC membranes with cholesterol concentrations [CHOL] varying between 0% and 50%. The origin of free energy is given by the binding state with minimum free energy, with CLP=0 and CWT=5.

2 Methods

2.1 System Setup and Equilibration.

Six sets of simulations were performed to study the binding of Na^+ to neutral zwitterionic phospholipid membranes with different cholesterol content. CHARMM-GUI[21,22] was employed to generate six sets of lipid bilayers with cholesterol concentrations [CHOL] varying between 0% and 50%. Each system consisted of 72 cholesterol / dimyristoylphosphatidylcholine (DMPC)[23,24] molecules, 3600 water molecules described by the modified TIP3P model[25,26], and a pair of Na^+ and Cl^- described by the CHARMM36 force field[27,28]. In contrast to other force fields (such as GROMOS), the CHARMM36 force field generates a less pronounced adsorption of Na^+ ions on phosphatidylcholine (PC) membranes, which was shown to be consistent with structural X-ray measurements and experiments on chain ordering of PC lipid bilayers in the presence of NaCl[29]. To achieve such agreement with experiment, in CHARMM36 the Lennard-Jones parameters characterizing the interaction of Na^+ with selected groups in the phospholipid are not calculated following the standard arithmetic combining rules, but they are determined specifically for each atom pair (NBFIX)[30]. The need for a specific description of certain pair interactions shows the limitation of point charge force fields, which cannot account for charge transfer or polarization effects[31].

The molar ratios of cholesterol/DMPC were set to 0/72 (0%), 8/64 (11.1%), 14/58 (19.4%), 22/50 (30.6%), 30/42 (41.7%), and 36/36 (50%). Due to the limited size of the system, our simulations can only capture the effect of short wave undulations on the adsorption of ions to the membrane. However, we believe that, being ion-binding presumably a local effect with characteristic length scales in the order of the nanometer, the relevant undulations are the short wavelength modes, which are well accounted for even by small systems[32]. We have investigated the effect of the limited size of the simulated system by comparing the occupancy distribution of the lower ion binding states obtained from unbiased simulations of systems containing 72 and 128 DMPC/cholesterol molecules with 0%, 20%, and 30% of cholesterol (see Fig. S3 in the Supplementary Information). We obtain very similar occupancy distributions for the lower free energy states sampled by unbiased simulations, discarding a large size effect of our simulated system.

Each membrane system was equilibrated for 100 ns in the NPT ensemble at 1 atm and 303 K. MD simulations for equilibration

were performed using NAMD 2.9[33]. A time step of 2 fs was used. Covalent bonds with hydrogen atoms of lipids were kept rigid using SHAKE[34], and water molecules were kept rigid using SETTLE[35]. The particle mesh Ewald method was employed to compute long-range electrostatic interactions[36]. The cutoff for Lennard-Jones interactions was set to 12 Å, with a switching distance of 10 Å. Pressure was controlled by the Langevin piston Nosé-Hoover method and the ratio of the unit cell in the x-y plane was kept constant[37]. Temperature was controlled by the Langevin dynamics with a damping coefficient of 1 ps^{-1} [38].

2.2 Collective Variables.

For membranes in ionic solutions, there exists a competition between ion binding to water molecules and to certain binding sites of phospholipids[9,20]. Therefore, a bound state can be characterized by the ion's coordination number with lipid binding sites and its simultaneous coordination number with water molecules. A number of experiments[3–6] and simulation works[6–13] have indicated that metal cations bind directly to the oxygen atoms of the negative charged phosphate (PO_4^-) and carbonyl (C=O) groups of lipid molecules. Accordingly, we defined two collective variables (CVs) to describe the ion's bound states: the coordination number between a Na^+ ion and lipid (including cholesterol) oxygens (CLP), and the coordination number between a Na^+ ion and water oxygens (CWT).

The coordination number[39] is defined as

$$s = \sum_{i \in G_1} \sum_{j \in G_2} s_{ij}, \tag{1}$$

where

$$s_{ij} = \frac{1 - \left(\frac{|\mathbf{r}_i - \mathbf{r}_j| - d_0}{r_0}\right)^6}{1 - \left(\frac{|\mathbf{r}_i - \mathbf{r}_j| - d_0}{r_0}\right)^{12}}. \tag{2}$$

For CLP, G_1 is the Na^+ ion, G_2 is all the lipid oxygens (including oxygen atom in cholesterol for cholesterol-containing membranes), d_0 = 2.3 Å, and r_0 = 0.25 Å. For CWT, G_1 is the Na^+ ion, G_2 is all the water oxygens, d_0 = 2.35 Å, and r_0 = 0.25 Å. The values of d_0 and r_0 were determined from the radial distribution function g(r), which was calculated from unbiased MD simulations on membrane systems contained 128 DMPC lipids, 6400 water molecules, and 46 pairs of Na^+ and Cl^- ions (corresponding to the ionic concentration of 0.4 M). d_0 is the position of the first peak of g(r), and r_0 is the width at half maximum of the peak. The determination of the parameters for coordination number, i.e., d_0 and r_0, is essential for the accuracy of the resulting free energy surfaces. More details on the determination of d_0 and r_0 can be referred to our previous work[20].

2.3 Well-tempered Metadynamics Simulations.

To obtain binding free energy landscapes of Na^+ at DMPC-cholesterol membranes, we applied well-tempered metadynamics simulations[40], a variant of metadynamics[41,42] capable of enhancing the sampling of coordination numbers in multiple CV dimensions. Two-dimensional (2D) well-tempered metadynamics simulations on six membrane systems with different cholesterol contents were performed based on the previously defined CVs using NAMD 2.9[33] together with PLUMED2 plugin[43] and the CHARMM36 force field[27,28]. After equilibration for 100 ns in the NPT ensemble as described above, 1000 ns well-tempered metadynamics simulations were performed on each system in the NVT ensemble at the temperature of 303K. The Gaussian widths for both CLP and CWT were set to 0.2. The initial Gaussian deposition rate was 0.3 kcal/mol per ps, with a bias factor of 5. Despite the NPT ensemble is preferred in simulations of phospholipid membranes, we employed the NVT ensemble in applying well-tempered metadynamics due to technical reasons[43]. However, the 100ns NPT equilibration run brings the membrane to its equilibrium area per lipid (see Figure S1 in the Supplementary Information), which is then used in the production run to minimize the possible artifacts caused by the application of the NVT ensemble.

3 Results and discussion

The resulting 2D free energy surfaces (FES) of Na^+ bound to DMPC-cholesterol membranes with cholesterol concentrations [CHOL] = 0-50% are shown in Figure 1. Na^+ is considered bound to the membrane for CLP > 0 and unbound for CLP $= 0$. There are a number of bound states (CLP > 0) and several unbound states (CLP $= 0$) in each FES, and each state can be indexed by (CWT, CLP). A common feature for all the [CHOL] cases is the global minimum of the FES located at the (CWT=5, CLP=0) state, revealing that the hydration with 5 water molecules in the aqueous solution is most favorable for Na^+. In addition, we observe a staircase pattern in all of the FES, which is the consequence of the ionic binding competition between water and lipids. As cholesterol concentration increases, the stable bound states (represented by red color in the FES), which are $\sim 1-4$ kcal/mol higher than the global minimum, shift from the region with CLP$\in [1,5]$ and CWT$\in [0,5]$ for [CHOL]=0-10% to the region with CLP$\in [1,4]$ and CWT$\in [1,5]$ for [CHOL]=20-30%, and further to the region with CLP$\in [1,3]$ and CWT$\in [2,5]$ for [CHOL]=40-50%. This visible shifts of stable bound states clearly reveal that the binding competitiveness of lipid head groups has been diminished by cholesterol contents, and that the affinity of Na^+ to DMPC membranes becomes less favorable as cholesterol concentration increases, which is in agreement with experiments[19]. Although our results are strictly valid only at infinite ion dilution, a similar behavior should be expected at moderate ion concentrations, where ion-ion correlations are not important[44].

A more quantitative representation of the above results is given in Figure 2, where the relative free energy ΔF as a function of CWT for several integer lipid binding levels (CLP) is extracted from the FES of Figure 1. Here we only represent the two extreme cases of [CHOL] = 0% (continuous lines) and [CHOL] = 50% (dashed lines). For CLP=0, the hydration free energies of Na^+ unbound to the membrane are exactly the same for both [CHOL] cases, indicating that, as expected, the hydration of Na^+ in the aqueous solution is not affected by the content of the membrane. There are several hydration free energy basins, and the most stable one is coordinated with five water molecules (which we establish as the reference state), in agreement with the Na^+

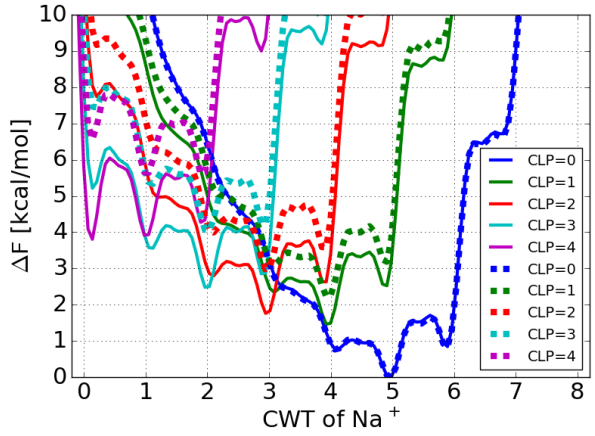

Fig. 2 Relative free energy ΔF as a function of CWT at CLP $= 0-4$ for Na^+ bound to DMPC membranes with cholesterol concentration [CHOL] of 0% (continuous lines) and 50% (dashed lines). The data are extracted from Figure 1.

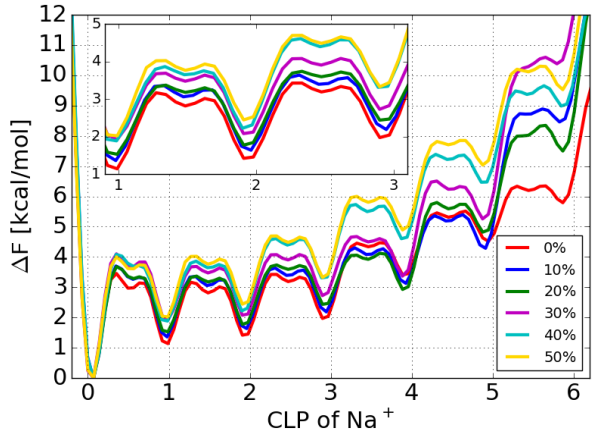

Fig. 3 Relative free energy ΔF as a function of CLP for Na^+ bound to membranes with cholesterol concentration [CHOL] varying between 0% and 50%. (Inset) Zoom in the low CLP region.

hydration number measured by experiments[45,46]. When bound to the membrane (CLP>0), the curves for the two cases become different. For each given CLP, the free energy profiles for [CHOL] = 50% case increase $1\sim2$ kcal/mol compared to the cholesterol-free case ([CHOL] = 0%). Such increase evidences the decrease of Na^+ affinity to the membrane with high cholesterol content. However, for both [CHOL] cases, the corresponding free energy minima for CLP = 1, 2, 3, 4, are located at CWT = 4, 3, 2, 1, respectively, keeping 5 the total coordination number, which is the same as experimental hydration number of Na^+ in aqueous solution[45,46].

In Figure 3, we represent the dependence of the relative binding free energy ΔF on CLP for membranes with various cholesterol concentrations after integrating out CWT according to

$$\Delta F(s_1) = -k_B T \log \int \exp^{-\frac{\Delta F(s_1,s_2)}{k_B T}} ds_2, \qquad (3)$$

where s_1 and s_2 are CVs, k_B is the Boltzmann constant, and T is

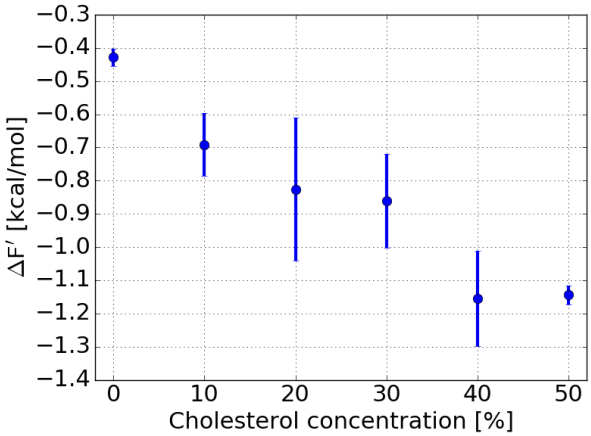

Fig. 4 Average values and standard deviations of ΔF', the free energy difference between unbound states (CLP <0.5) and bound states (CLP >0.5) for different cholesterol concentrations.

the absolute temperature. As cholesterol concentration increases, the free energy profiles corresponding to bound states (CLP>0) raise monotonically. To understand these changes, we should remember the well-known condensing effect of cholesterol on lipid bilayers, which produces higher membrane rigidity and ordering[47]. For [CHOL] $\leq 20\%$, there are overlaps of the free energy profiles at low binding (CLP ≤ 3), indicating that Na^+ is easily bound to the membranes with low cholesterol concentration. When [CHOL] $\geq 30\%$, the ionic binding is significantly reduced (free energy increases). Such transition in the ionic binding behavior at [CHOL] $\approx 30\%$ coincides with the phase transition point of DMPC-cholesterol membranes, in which membranes change from a liquid-disordered phase to a liquid-ordered phase[17].

We monitor the convergence of the FES by calculating ΔF', the free energy difference between unbound states (CLP <0.5) and bound states (CLP >0.5) from the free energy profiles of CLP as the simulations proceed (see Figure S8 in the Supplementary Information). The average values and standard deviations of ΔF' calculated from the last 100 ns of well-tempered metadynamics simulations are shown in Figure 4. The monotonic trend indicates that the energy gap between unbound and bound states increases with the increasing cholesterol concentration, which reduces the affinity of Na^+ to cholesterol-containing membranes. This is in good agreement with the results obtained from the previous Figures 1, 2, and 3.

The above 2D FES (Figure 1) and 1D free energy ΔF as a function of CLP (Figure 3) are based on the consideration that all the oxygen atoms from DMPC lipids (i.e. phosphate and carbonyl groups) and cholesterol (hydroxyl group in the polar head) are equivalent binding sites for Na^+. In order to understand the contributions of different head groups separately, we calculate the relative free energy ΔF as a function of CLP between Na^+ and oxygen atoms from different head groups by applying a reweighting technique[48]. As shown in Figure 5, for a given CLP, the binding free energy follows the order of C=O $<$ PO_4^- $<$ -OH. Therefore, C=O is the most favorable binding site for Na^+, followed by PO_4^- and then the -OH group of cholesterol. For

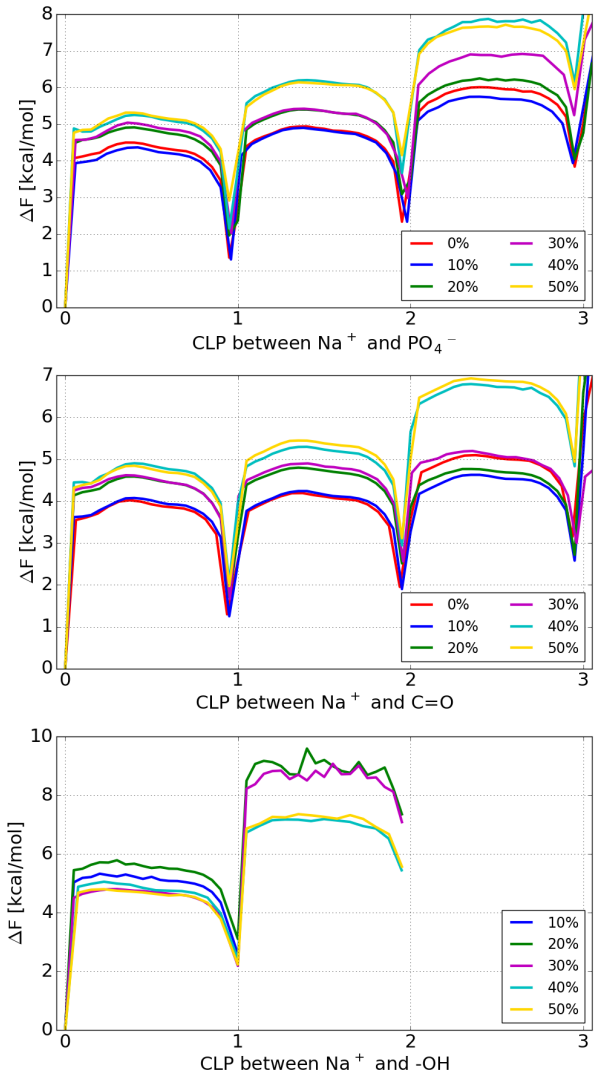

Fig. 5 Relative free energy ΔF as a function of CLP between Na^+ and oxygen atoms from different lipid head groups with cholesterol concentration [CHOL] varying between 0% and 50%. The three different lipid head groups are: DMPC's phosphate group PO_4^- (top figure); DMPC's carbonyl group C=O (middle figure); and cholesterol's hydroxyl group -OH (bottom figure).

DMPC binding sites (C=O and PO_4^-), we observe higher binding free energy basins and higher binding free energy barriers for higher cholesterol concentration, which is in accordance with above results obtained when different CLP are considered equivalent. The situation for the CLP between Na^+ and cholesterol is radically different. At low cholesterol concentration (10%), only one cholesterol oxygen can be attached to Na^+. For [CHOL] $\geq 20\%$, it would be possible for Na^+ to bind up to two cholesterol oxygens. However, the free energy barriers for high cholesterol concentrations $(40-50\%)$ are lower than those for the medium ones $(20-30\%)$, which is in contrast to the trend observed for DMPC binding sites where higher free energy barrier corresponds to higher cholesterol concentration. It should be mentioned that the results reported in Figure 5 are influenced by the use of the new CHARMM36 force field, which uses specific parameters to describe the interaction of Na^+ with selected groups in the phospholipid (NBFIX) [30].

Due to the high computational demands of the 2D well-tempered metadynamics simulations, we used a small membrane, containing 72 DMPC/cholesterol molecules. Although our tests using unbiased simulations of systems with 72 and 128 DMPC/cholesterol molecules suggest that large size effects can be discarded in describing ion binding to the membrane, a possible contribution from the finite size of the system cannot be completely ignored. The effect of the limited size of the system can also be relevant in regards to the heterogeneity of the membrane, which is important to ensure a proper sampling of the DMPC/cholesterol configurations of the membrane. We have run long enough simulations to allow complete mixing of lipids, but there might be size effects on the mixing of DMPC and cholesterol molecules which our approach was unable to evaluate.

4 Conclusions

We have performed systematic free energy calculations of Na^+ bound to DMPC-cholesterol membranes by means of well-tempered metadynamics simulations with two collective variables being the ion's coordination number to lipids (CLP) and to water (CWT). The free energy surfaces reveal the competition between binding of ion to water and to lipids. However, the binding competitiveness of lipid head groups is diminished by cholesterol contents. As cholesterol concentration increases, the ionic affinity to the membrane decreases, which is in agreement with experiments [19]. When cholesterol concentration [CHOL] $\geq 30\%$, the ionic binding is significantly reduced. Such transition in the ionic binding behavior at [CHOL] $\approx 30\%$ coincides with the phase transition point of DMPC-cholesterol membranes, in which membranes change from a liquid-disordered phase to a liquid-ordered phase. In contrast, the hydration free energies of Na^+ in aqueous solution are not affected by the cholesterol content of membranes. The most stable hydration for Na^+ with five water molecules is in good agreement with experiments. We have also evaluated the contributions of different lipid head groups to the binding free energy separately. The DMPC's carbonyl group (C=O) is the most favorable binding site for Na^+, followed by DMPC's phosphate group (PO_4^-) and then the hydroxyl group (-OH) of cholesterol.

The method employed can be widely applied to explore the free energy landscapes of ions at complex biological interfaces. Furthermore, provided the importance in a variety of biological processes of the interaction of ions and charged interfaces in aqueous solution, our approach could be extended to explore other problems in colloidal chemistry and biology, and could be helpful to deepen our understanding of specific ion effects on soft matter and biological systems.

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