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Cholesterol level affects surface charge of lipid membranes in saline solution

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Cholesterol is an important component of all biological membranes as well as drug delivery liposomes. We show here that increasing the level of cholesterol in a phospholipid membrane decreases surface charge in the physiological environment. Through molecular dynamics simulation we have shown that increasing the level of cholesterol decreases Na^+ ion binding. Complementary experimental ζ – potential measurements have shown a decreased ζ – potential with increasing cholesterol content, indicative of reduced surface charge. Both experiments and simulations have been carried out on both saturated 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and monounsaturated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membranes. This result is particularly important because membrane surface charge plays an important role in the interactions of biomembranes with peripheral membrane proteins and drug delivery liposomes with the immune system.

Cholesterol is a key component of the animal cell membrane. It is present in different proportions based on the type of membrane[1] and known to modify membrane properties in a number of important ways. Cholesterol affects the mechanical properties of membranes, increasing their mechanical strength, affecting membrane elasticity, and increasing the packing density of lipids via the so called "ordering and condensing" effects[2-5]. As a result of the above modifications, the membrane becomes less permeable to water, small molecules and ions[6-8]. Due to this, cholesterol is often included in the formulation of liposomes used in drug delivery. Of the ~15 liposome based drugs in phase III trials, it is present in all of them[9]. Cholesterol is also a key molecule involved in the formation of lipid nanodomains, known as rafts, which in turn are involved in numerous cellular processes like apoptosis, signaling, and cell differentiation[10-12]. Despite its paramount importance and the extensive studies that have been carried out, there are still some gaps in our knowledge concerning the effect of the presence of cholesterol in phospholipid membranes, both in the context of biomembranes and drug delivery liposomes. One of the less studied issues is the effect of cholesterol in the membrane on the properties of the water-membrane interface. It is known that cholesterol affects, for example, the binding of peripheral proteins like, e.g. cholera toxin[13], to both artificial and biological membranes, as well as the binding of small molecules, like neurotransmitters, at the water membrane interface[14]. Little is known, however, about the effect of cholesterol on the interaction between the lipid bilayer and ions found in the intercellular and intracellular media.

It is known that Na^+ cations bind to the carbonyl and phosphate groups of lipids[15-16]. Considering other biologically important cations, the binding of K^+ ions to lipids is much weaker, while the divalent cation Ca^{2+} binds very strongly[17]. Since the Cl^- anions of the salts commonly found in physiological conditions remain only loosely associated with the lipid bilayer[17], the result is an effective positive charge on the membrane surface. This will influence the interaction of the membrane with all molecules found in the fluid phase.

While the interaction of ions with lipid membranes has been studied both experimentally[18] and computationally[19], and rigorous computational study of the ordering effect of cholesterol on lipid membranes has been carried out[20], systematic study of the effects of cholesterol in the membrane on the interaction between the membrane and salt ions has, however, up until now not been carried out. In this study we have determined that increasing the level of cholesterol in the lipid membrane reduces Na^+ binding to lipid headgroups, reducing the surface charge of the membrane. To achieve this we have combined molecular dynamics simulations of membranes with experimental measurements of the surface (ζ) potentials of liposomes.

Results

In Figure 1A and 1B, the percentage of all Na$^+$ ions in the systems that are bonded to the lipid headgroups is shown. Ions were defined as bonded if they were within a distance of 0.325 nm from any oxygen atom of the lipid molecule[17]. Figure 1A and 1B shows a decrease of ions bonded with the lipid headgroups in both bilayer types. In the range of 0 to 50% of cholesterol the percentage of bound ions drops from 45–50% to 20%. In this manuscript all percentages referred in the analysis of the simulation results are percentages of the total number of ions of that particular element in the simulation system, not the molar percentage. This is an interesting observation as the DSPC bilayer at 310 K remains in the gel state while the POPC bilayer remains in the liquid crystalline state. While looking at the area per lipid, DSPC occupies about 0.43 nm^2 and is relatively constant, independent of cholesterol concentration while for the case of POPC the area drops from 0.673 to 0.623 nm^2 (See supplementary figure S2). This means that the area per lipid or membrane phase is not a primary factor affecting Na$^+$ binding.

If we look at the charge per unit area of the membrane, shown in figure 1C and 1D, we see a trend in agreement with the result of the number of bound ions. Additionally, if we plot the number of bound ions relative to the number of available PC groups in the membrane, we also see a decline with increasing cholesterol content, as shown in supplementary figure S9. The relationship is, however, not linear, for the POPC the number of bound ions per PC headgroup remains approximately constant between zero and 30% cholesterol.

We have also measured the number of hydrogen bonds between the cholesterol oxygens and the phospholipid headgroups[21], shown in supplementary figure S12 A–B. This result shows that the association between cholesterol oxygens and phospholipid headgroups increases as the cholesterol content in the membrane grows.

We then investigated the specific groups to which the bound Na$^+$ ions were associated. We found that binding to phosphate oxygens to be dominant, as shown in supplementary figure S6. Of particular note is the very small level of Na+ binding to the cholesterol oxygens. We thus see that as cholesterol level increases, strongly binding PC headgroups are replaced with more weakly binding cholesterol OH groups. This partly explains the decrease of the number of bound ions and is probably the only factor for the case of POPC in low cholesterol concentration, where the number of Na$^+$ ions bound per
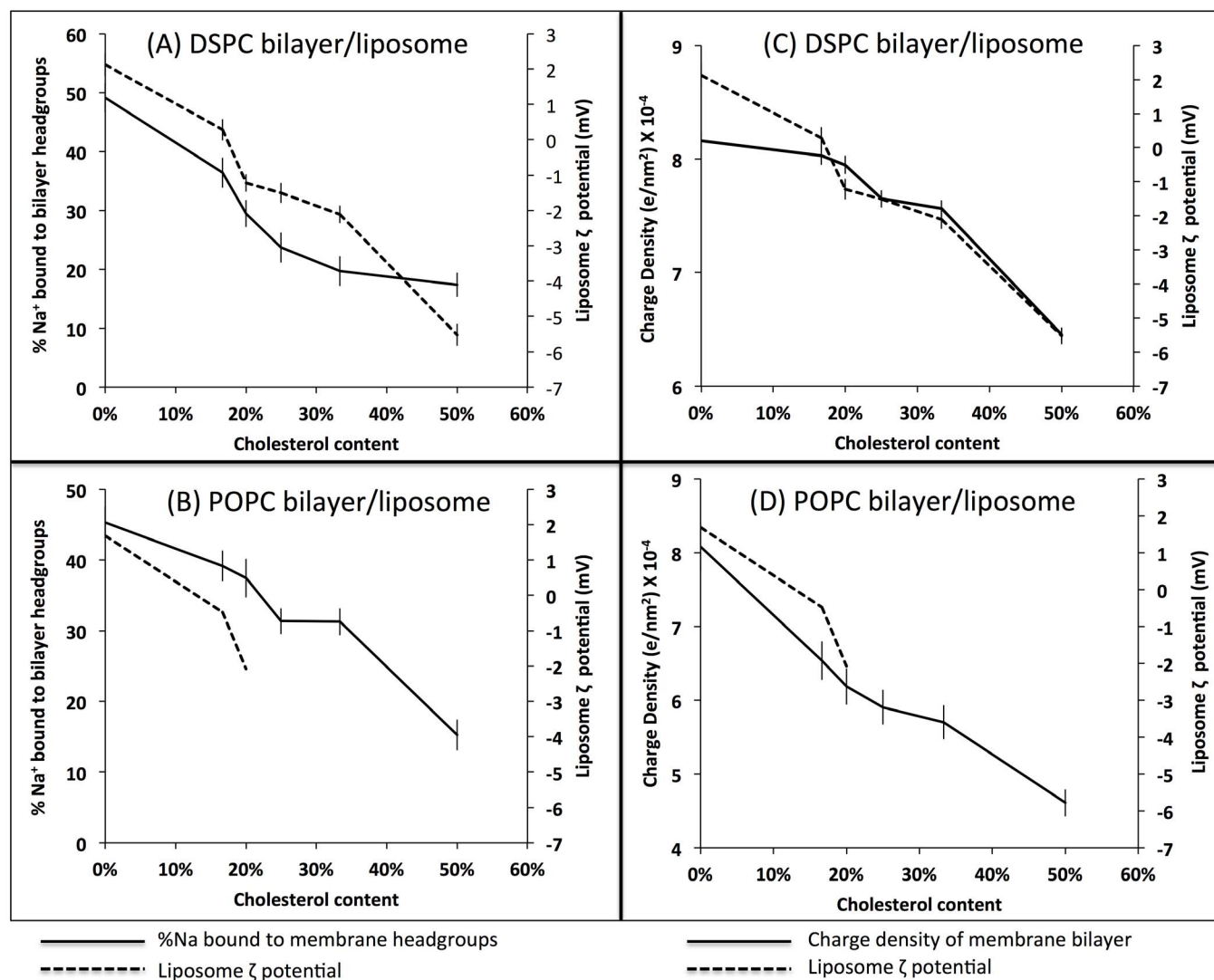

Figure 1 | (A) and (B) Percentage of cations bound to the phospholipid bilayer in the molecular dynamics simulations and ζ – potential result from the experiment on liposomes vs. cholesterol content in the bilayer for a) DSPC and b) POPC. The plot shows that as the cholesterol content in the phospholipid bilayer increases, the percentage of Na$^+$ ions bound to the membrane and the ζ – potential of the liposomes both decrease. (C) and (D) Charge density (the integral of the charge through the membrane cross section (the region of the membrane where the membrane headgroup is located, according to the mass density profile) divided by the area of the membrane, in units of electron charge per nm^2) in the lipid layer as a function cholesterol concentration of cholesterol showing decline parallel to the decline in number of bound ions.

PC headgroup is constant. In addition, PC headgroups become increasingly associated with the cholesterol headgroups, further decreasing the number of binding sites for cations. The increase in the level of cholesterol in the membrane increases the hydrophobicity of the membrane, further decreasing the membrane affinity for cations. This mechanism of expulsion of Na^+ ions from the water membrane interface is similar to that proposed in the "Umbrella model"[22]. In this model the changes induced by the cholesterol in the bilayer properties are proposed to result from the tendency of PC headgroups to prevent unfavorable cholesterol interaction with water at the membrane water interface. We have also shown the binding stoichiometry in figure S7, where we see significant bridging between DSPC phosphate groups, some bridging between the POPC phosphate groups and the cholesterol oxygens, and some ions binding to more than two groups.

To better show the effect of cholesterol on the interaction between ions and lipids, mass density plots from all simulations for Na^+ with the phosphate group peaks aligned are shown in Figure 2. As can be seen, cholesterol affects the amplitude of the Na^+ peak in keeping with the observed decrease in the density of ions at the membrane interface. In addition, the position of the peak is gradually shifted towards the water phase. A Mass density plot including the Cl^- ion distribution is included in the supplementary materials as supplementary figure S4. The distribution of Cl^- ions is in qualitative agreement with previous results[16,23], and, as shown in supplementary figure S10 and S11, there is no evidence of chlorine ions binding to the membrane headgroups. We also measured the water ordering along the membrane normal, and showed this in figure S13.

To validate the findings from the computational studies, we performed experimental measurement of the ζ – potential of liposomes composed of DSPC or POPC and cholesterol. We have considered this to be crucial due to reported discrepancies in quantitative picture of ions binding to the membrane surface obtained from various force fields. We could formulate the DSPC liposomes with 0%, 16.66%, 20%, 25%, 33.33% and 50% cholesterol. However for POPC only liposomes containing 0%, 16.66% and 20% cholesterol where formulated; attempts at cholesterol density above this resulted in liposome aggregation. The particle size of the liposomes was ~100 nm for the liposomes containing cholesterol and ~200 nm for pure DSPC and

POPC liposomes. The surface potential of the Pure DSPC liposome in saline buffer is positive (~2 mV) but as the cholesterol content is increased in the liposomes, the surface potential drops to negative values. The negative ζ – potential result in a system composed of neutral lipids is not a surprising result, in light of previous published results[24]. This showed that there is a definite trend, which can be attributed to the change in the cholesterol content.

Discussion

Results shown above indicate that the presence of cholesterol decreases Na^+ ion binding with the lipid headgroup, releasing ions from the water membrane interface to the water phase. This leads to a more neutral surface charge of the membrane as seen in the observed changes in the ζ – potential. Since we see this effect in both DSPC and POPC lipid systems we see that this is a robust effect that will be present in both biological systems and drug delivery liposomes. The selected lipid compositions cover the whole range of lipid phases present in biological membranes and liposomes, including: 1) the liquid disordered phase for the case of pure POPC 2) the liquid ordered phase for the case of the bilayer with the higher cholesterol concentration and 3) the gel phase for the case of the pure DSPC bilayer. The presence of Na^+ ions at the membrane interface plays an important role in both the interaction between 1) biomembranes and peripheral membrane proteins and 2) drug delivery liposomes and serum opsonins[20] in the bloodstream. Our results indicate that the addition of cholesterol to the liposome membrane reduces the binding of Na^+ ions to the membrane. Thus, this could 1) for the case of biomembranes play a role in governing the interaction between the membrane and peripheral membrane proteins and 2) for the case of drug delivery liposomes, decrease interactions with serum opsonins, hence increasing the circulation time of the liposome[25].

We should add that regarding ion binding properties of membranes, it is known that there is some discrepancy between the results obtained using the different potential sets[26,27]. While these discrepancies will clearly have a quantitative effect, as shown in the referenced papers, we do not expect a qualitative effect, i. e. while the extent of the phenomenon may differ in the experimental system, the phenomenon itself has been correctly identified.
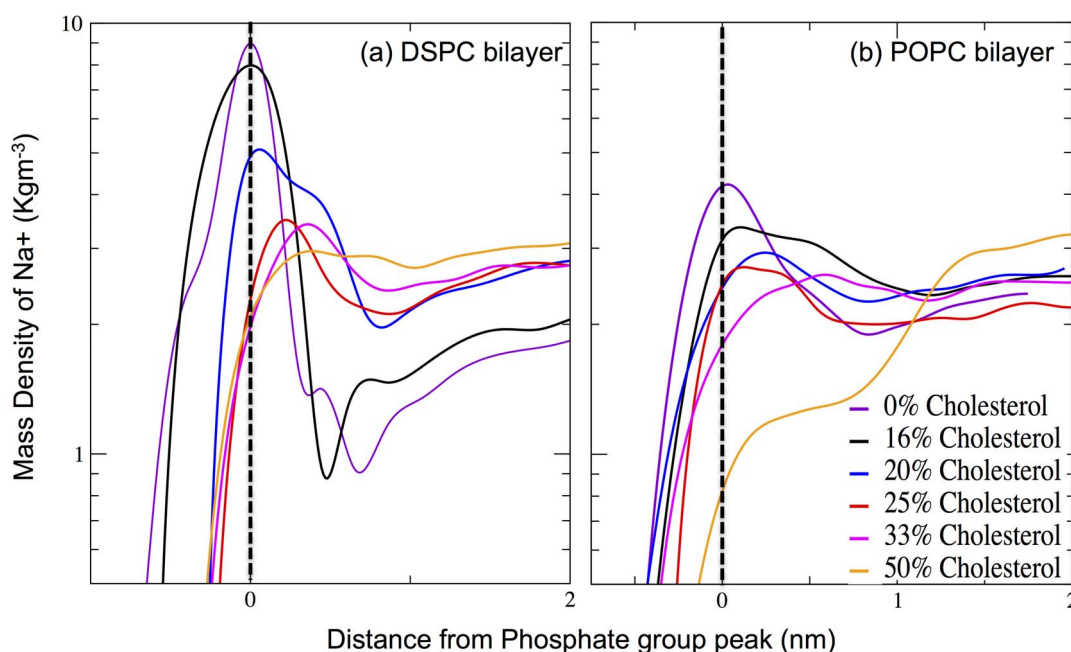

Figure 2 | Partial mass density profiles comparison: (A) DSPC (B) POPC: The black dashed line represents the position of phosphate head groups. As the cholesterol content in the bilayer increases, the association of Na^+ ions with the membrane head group decreases.

Clearly the blood plasma is more complex than our simple picture of it comprising of water, ions and opsonins, and both our experimental and computational models are very simplified models that only capture one aspect of this complex system. In spite of this we propose that our results, however, provide new insight into the effect of cholesterol on a lipid membrane with dissolved salts in its solvent, and this in turn provides insight into the role cholesterol plays in lipid membranes both biologically and pharmaceutically.

Methods

In our molecular dynamics simulations, we prepared ten phospholipid bilayers for a total of 288 lipid molecules with varying proportions of saturated 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) or monounsaturated 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and cholesterol in proportions: 6:0, 5:1, 4:1, 3:1, 2:1 and 1:1. The exact composition of the simulated systems is given in Table S1. All simulations were performed using GROMACS 4.5.5 software[28]. The simulation length was 200 ns, with the initial 100 ns considered as the equilibration period based on an analysis of the surface area per lipid and the number of ions binding to the membrane interface, as shown in supplementary figure S3 and S5 respectively. In addition we measured the dynamics of ion exchange between the bonded and unbonded states, shown in supplementary figure S8. These show an increase in the exchange dynamics with increasing level of cholesterol, and faster dynamics for POPC than DSPC. The longest binding time for the pure DSPC system is in agreement with results in our previous work[16]. In all cases the data was fit to a multi-exponential[29] and our interpretation of this multiexponential fit is found in the supplementary material.

The final 100 ns of the trajectory were used for all analysis. The molecular dynamics simulations were conducted under constant pressure (1 Bar) controlled through a Parrinello−Rahman barostat[30] using a semi-isotropic pressure scheme. The temperature (310 K) was controlled by Nosé-Hoover thermostat[31,32]. The temperatures of the solute and solvent were controlled independently. For parameterization of all molecules and ions we used the OPLS[33] all atom force field (OPLS-AA) as described in our previous publications[13–15]. For water we used the TIP3 water model[34]. Periodic boundary conditions with the usual minimum image convention were used in all three directions. In order to preserve the covalent bond lengths, the linear constraint solver (LINCS) algorithm[35] was employed and a 2 fs time step was used in all simulations. The Lennard-Jones Interactions were cut off at 1.0 nm, and for the electrostatic interactions we employed the particle mesh Ewald method[36]. All error bars were calculated using the block method[37].

For ζ – potential measurements of liposomes DSPC and POPC liposomes were prepared. Both DSPC and POPC were purchased from Lipoid® GmbH Germany, cholesterol (99% pure) and Sodium Chloride (99% pure) were obtained from Sigma-Aldrich. Other reagents and solvents were of analytical grade. Water obtained from a MilliPore system with a conductivity of 18 MΩ cm⁻¹ was used for the preparation of the liposomes, using the thin film hydration followed by extrusion[38] method to achieve a uniform size of liposomes. The hydration media contained 140 mM NaCl. The liposomal suspensions were stored at 4°C and analyzed for particle size and ζ – potential.

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Acknowledgments

Author contributions

A.M. and T.R. built the computational models of the systems and performed the simulation V.D. and M.E. performed the experiments and analyzed the data P.K. and T.V. designed the experiments, A.M., T.R. and A.B. wrote the manuscript.

Additional information

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