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What Can We Learn about Cholesterol's Transmembrane Distribution Based on Cholesterol-Induced Changes in Membrane Dipole Potential?

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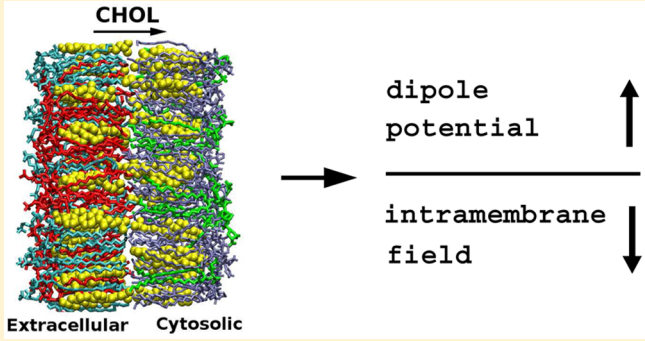
Supporting Information

ABSTRACT: Cholesterol is abundant in the plasma membranes of animal cells and is known to regulate a variety of membrane properties. Despite decades of research, the transmembrane distribution of cholesterol is still a matter of debate. Here we consider this outstanding issue through atomistic simulations of asymmetric lipid membranes, whose composition is largely consistent with eukaryotic plasma membranes. We show that the membrane dipole potential changes in a cholesterol-dependent manner. Remarkably, moving cholesterol from the extracellular to the cytosolic leaflet increases the dipole potential on the cytosolic side, and vice versa. Biologically this implies that by altering the dipole potential, cholesterol can provide a driving force for cholesterol molecules to favor the cytosolic leaflet, in order to compensate for the intramembrane field that arises from the resting potential.



Cholesterol molecules are vital for regulating a variety of cell membrane properties such as in-plane structural organization, fluidity, and lateral dynamics.[1,2] Given the outstanding importance of cholesterol, its effects on cell membrane structures have been explored for decades. The transmembrane distribution of cholesterol has, however, remained an open issue. One is tempted to assume that in eukaryotic plasma membranes cholesterol would reside mainly in the extracellular membrane leaflet,[3] because cholesterol is known to interact very favorably with sphingolipids,[4] which in turn are known to be most abundant on the extracellular side. However, there are also data that suggest cholesterol resides mainly in the cytosolic leaflet.[5−7] Because these studies have been carried out under quite complicated conditions due to the use of, for example, detergents and cold temperatures, the idea that cholesterol would reside mainly on the cytosolic side has not received general approval. Resolving this issue would be a quite simple feat if cholesterol's transmembrane distribution could be studied enzymatically, such as with phospholipids (via phospholipases) or sphingolipids (using sphingomyelinases). However, because of the lack of appropriate enzymes, this has not been possible. The distribution of cholesterol across plasma membranes has therefore remained an outstanding open question.[8]

Here we approach this problem from a different perspective. We ask ourselves how changes in cholesterol's transmembrane distribution influence the membrane's dipole potential. This potential develops within the membrane−water interface region and is considered to be essential for conformations and the function of membrane proteins, and more generally for interactions between a lipid membrane and other biological molecules embedded in a membrane.[9−11] It can also be relevant

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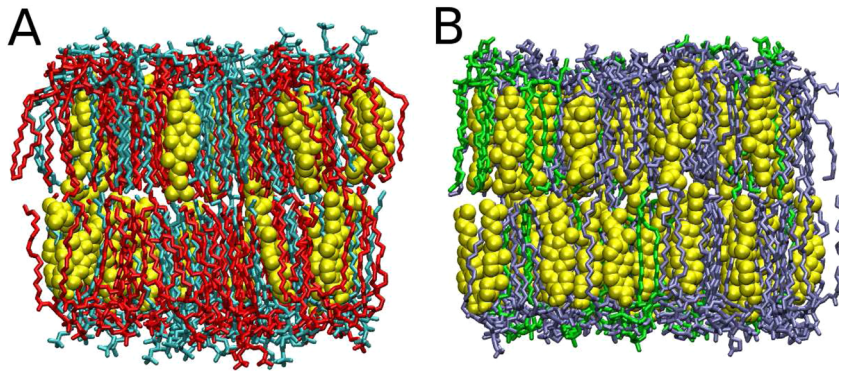

Figure 1. Snapshots of (A) PC-SM-Chol and (B) PE-PS-Chol lipid membranes. POPC is shown in red, SM in cyan, POPE in ice-blue, POPS in green, and Chol in yellow; water molecules and ions are not shown.

for lipid-mediated hydrogel−colloid interactions.[12] Experimental studies have demonstrated that cholesterol leads to an increase of the dipole potential in model phosphatidylcholine membranes, the effect being more pronounced for saturated lipids as compared to unsaturated counterparts.[13−16] Cholesterol has been shown to induce a similar effect also in cell membranes and in reconstituted membranes composed of natural membrane lipids.[13,14]

However, the effect of cholesterol on the dipole potential of the separate extracellular and cytosolic leaflets of cell membranes is mostly unknown. It is established that the leaflets of plasma membranes differ in their lipid composition.[17] In general, in animal cells phosphatidylcholine (PC) and sphingomyelin (SM) lipids are localized mostly in the extracellular leaflet, whereas phosphatidylethanolamine (PE) and (anionic) phosphatidylserine (PS) are predominant lipids in the cytosolic leaflet.[18] Therefore, one can expect that the effect of cholesterol (Chol) could be different in these distinctly different leaflets. Such differences cannot be explored in single-component symmetric model membranes (vesicles) that are often employed in experimental measurements of the dipole potential.[14−16] Asymmetric lipid membranes would be more appropriate to consider this task, but they are still challenging from an experimental point of view.[8]

In this work, we use atomistic molecular dynamics (MD) simulations to clarify how changes in cholesterol's transmembrane distribution influence the dipole potential in asymmetric lipid membranes, whose composition is chosen to match eukaryotic plasma membranes to an adequate degree. Because the exact distribution of cholesterol molecules in cell membranes is not known, we vary the cholesterol distribution systematically. We also account for cholesterol flip-flops that are known to be frequent.[19,20] Therefore, instead of studying an asymmetric membrane directly, we consider dipole potential profiles in membranes comprising a pair of leaflets taken from the opposite leaflets of corresponding asymmetric membranes. This approach is justified given that the monolayer's dipole potential develops mostly within the lipid−water interface and is not affected to a large degree by the hydrophobic acyl chain region.[21]

Two types of lipid bilayer mixtures were studied, see Figure 1. The bilayers comprising palmitoyl-oleoyl-phosphatidylcholine (POPC) and SM were considered as a model of the extracellular leaflet. The cytosolic leaflet of plasma membranes is modeled by a bilayer composed of palmitoyl-oleoyl-phosphatidylethanolamine (POPE) and palmitoyl-oleoyl-phosphatidylserine (POPS). POPC, POPE, and SM are zwitterionic (neutral) lipids with polar lipid head groups, while POPS is anionic and characterized by a net charge ($-1e$). PC-SM bilayers consisted of 64 PC and 64 SM lipids, together with 0, 32, or 64 Chol molecules, leading to three PC-SM-Chol systems with molecular ratios 64:64:0, 64:64:32, and 64:64:64, respectively (corresponding Chol molar concentrations were 0, 20, and 33 mol %, respectively). The numbers of cholesterol molecules were the same in PE-PS bilayers, but the numbers of PE and PS lipids were slightly adjusted to ensure that the leaflet areas of PC-SM and PE-PS bilayers would match. This was necessary to eliminate possible finite size effects in asymmetric membranes assembled from monolayers with different lipid compositions (see refs 22 and 23). Consequently, we considered PE-PS-Chol bilayer systems with molecular ratios 118:30:0, 100:26:32, and 88:22:64, respectively (or with Chol molar concentrations 0, 20, and 37 mol %). The number of water molecules ranged between 5100 and 7700; thus, the hydration of the lipids was high. Simulations of every bilayer system were repeated twice: (i) under salt-free conditions and (ii) in a saline solution at a physiological concentration (∼150 mM) and an appropriate electrolyte composition[24−26] (NaCl for PC/SM/Chol bilayers and KCl for PE/PS/Chol bilayers).

The results are based on two force fields whose outcomes were compared on equal footing for validation. All phospholipids and cholesterol molecules were first described by the united-atom force-field by Berger et al.[27] Water was then represented by the simple point charge (SPC) model,[28] and all systems were simulated in the NpT ensemble at the physiological temperature (T = 310 K) and pressure (1 bar). The velocity-rescaling thermostat[29] was used to control the temperature, while the Parrinello−Rahman scheme[30] was employed to keep the pressure constant through a semi-isotropic coupling. The time step used was 2 fs. All 12 bilayer systems were simulated for 1 μs each; the last 300 ns were used for analysis. Moving on, for validation and to explore the sensitivity of the simulation results to the force field employed, the simulations of all systems were repeated with the all-atom lipid force field CHARMM36.[31] The lipid composition and simulation conditions were kept as close as possible to those used in Berger simulations. The GROMACS suite was used for both simulation sets (version 4.5.6 for Berger lipids[32,33] and version 5.0.4 for CHARMM36 lipids).[34] Below we discuss the results based on the Berger force field unless mentioned otherwise.

The electrostatic potential of the lipid membranes was calculated from a MD simulation trajectory by integrating the one-dimensional (1D) Poisson equation twice, as outlined in

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ref 35. Each potential profile was then symmetrized with respect to the center of mass (COM) of the membrane, resulting in the electrostatic potential profile of one leaflet. Figure 2 depicts how these potential profiles for PC-SM-Chol and PE-PS-Chol monolayers are combined to mimic the electrostatic potential of an effectively asymmetric membrane.

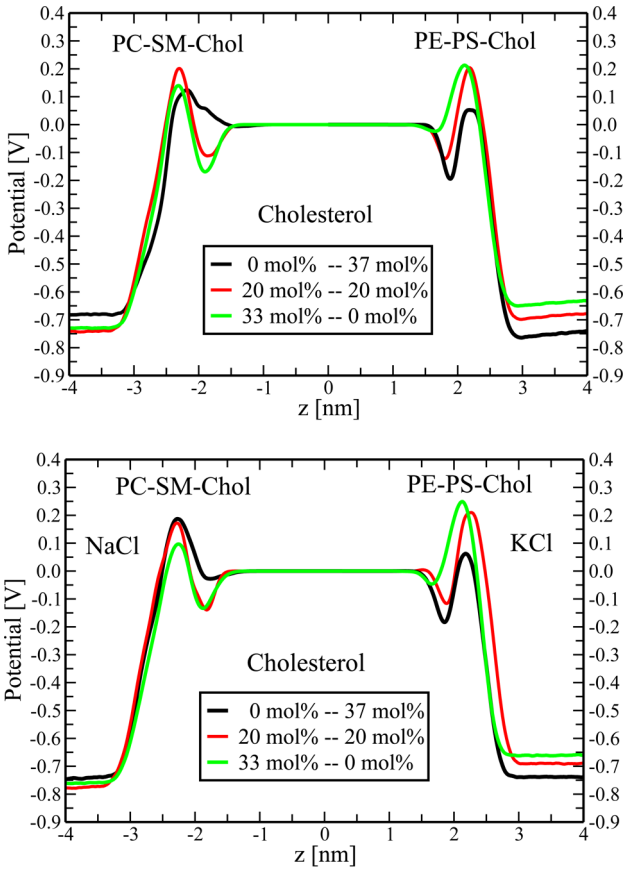

Figure 2. Combined electrostatic potential profiles of phospholipid membranes with cholesterol versus distance z from the membranes' center of mass (Berger lipids). Shown are profiles for PC-SM-Chol ($z < 0$) and PE-PS-Chol ($z > 0$) membranes in (top panel) salt-free and (bottom panel) saline conditions. For convenience, the potential was chosen to be zero at the membranes' center of mass.

The electrostatic potential difference between the membrane's COM and the bulk water phase (taken at a distance of 4 nm from the COM of a membrane) defines the potential known as the boundary potential (ψ_b). This boundary potential consists of the dipole potential (ψ_d) and the surface potential (ψ_s) that always appears at charged surfaces.[36,37] The surface potential was evaluated from the potential of mean force of ions at the closest plane corresponding to a zero surface excess of water following a procedure outlined in ref 38. Finally, the dipole potential was calculated as $\psi_d = \psi_b - \psi_s$.

Figure 2 (top) and Table 1 highlight that Chol makes a difference for the membrane dipole potential: cholesterol molecules increase the boundary potential of a membrane to a significant extent. As the effect of cholesterol on the surface potential is minor (≤10 mV, see Table 1), this result also holds for the dipole potential. We find that adding 20 and 33 mol % of cholesterol to PC-SM membranes increases the dipole potential by 61 and 48 mV, respectively. The same trend is found for PE-PS membranes, where adding 20 and 37 mol % of cholesterol to a membrane increases the dipole potential by 55 and 118 mV, respectively. Cholesterol clearly increases the membrane's dipole potential in a manner that is dependent on membrane lipid composition.

Table 1. Electrostatic Properties of Membrane Systems Considered

system	C_{chol} [mol %][a]	salt	ψ_b [mV][b]	ψ_s [mV][c]	ψ_d [mV][d]
PC-SM-Chol	0	–	682	0	682
	20	–	743	0	743
	33	–	730	0	730
PC-SM-Chol	0	NaCl	747	19	728
	20	NaCl	778	11	767
	33	NaCl	761	10	751
PE-PS-Chol	0	–	632	−35	667
	20	–	677	−45	722
	37	–	740	−45	785
PE-PS-Chol	0	KCl	661	−21	682
	20	KCl	688	−20	708
	37	KCl	739	−19	758

[a]Cholesterol concentration (mol %). [b]Boundary potential (with zero defined in bulk water). [c]Surface potential (the sign of the potential reflects the sign of the surface charge). [d]Dipole potential.

Under physiological conditions membranes are exposed to salt, whose composition is different on the extracellular and cytosolic sides. Figure 2 (bottom) and Table 1 demonstrate the influence of adding 150 mM of NaCl (KCl) to PC-SM-Chol (PE-PS-Chol) membrane systems with varying content of cholesterol. The overall effect of cholesterol remains unchanged: cholesterol gives rise to a substantial increase in the membrane dipole potential, although the increase is here smaller as compared to salt-free systems. This is an important conclusion given that salt-free conditions do not occur under relevant experimental circumstances (e.g., in experiments done in vivo). In PC-SM-Chol membranes the increase due to 20 and 33 mol % of cholesterol is 39 and 23 mV, respectively. The corresponding changes for PE-PS-Chol membranes with 20 and 37 mol % of cholesterol are 26 and 76 mV, respectively.

While the effect of cholesterol is strong, the influence of salt is less pronounced. Typically, the absolute values of the dipole potential are not affected much by salt ions.[39] We can draw largely the same conclusion given that Table 1 indicates the dipole potential to change less than ∼20−25 mV because of added salt. The only exception is the cholesterol-free PC-SM membrane in which adsorption of sodium ions gives rise to membrane compaction.[26] The surface potential, on the other hand, is quite sensitive to both the type of salt cations and the ionic strength. Cations of different types have different affinities to anionic phospholipids,[37] while the ionic strength affects the surface potential through a change of the Debye screening length. Both effects are well-known and often analyzed in terms of the Gouy−Chapman−Stern theory, see e.g. ref 36 for a review. While a detailed description of the effects of salt ions on the membrane's electrostatic potential is beyond the scope of this paper, let us mention that the observed drop in the surface potential of anionic PE-PS-Chol bilayers (Tables 1 and S1) is a signature of a salt-induced decrease in the electrostatic screening length in the bilayer system.

For validation, we repeated the above-discussed simulations and the analysis using the CHARMM36 force field.[31] Figures S1 and S2 (see the Supporting Information) show that the

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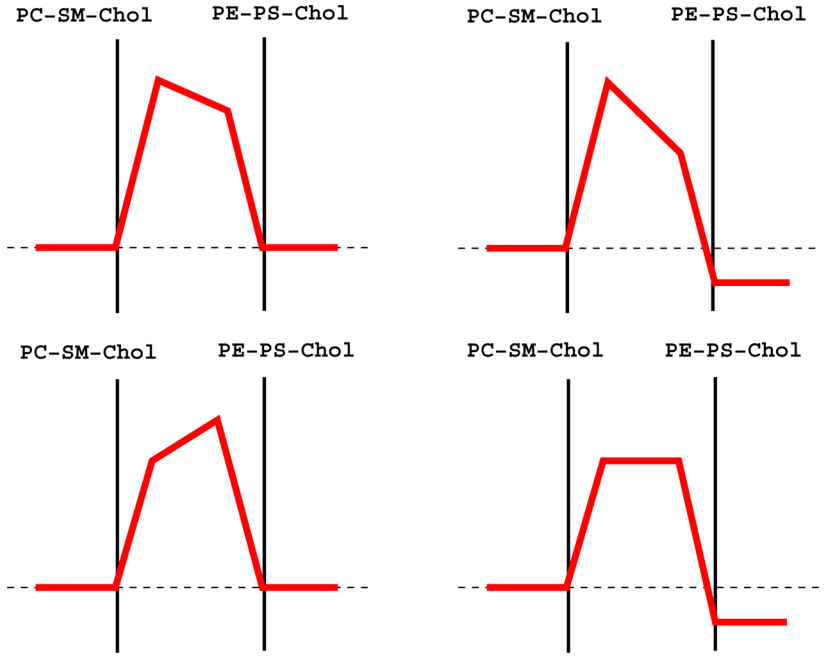

Figure 3. Schematic representation of the electrostatic potential of an asymmetric lipid membrane (right column) with and (left column) without an external resting potential. Shown here are the situations where the boundary potential of the extracellular PC-SM-Chol leaflet (top panels) exceeds or (bottom panels) is smaller than the potential of the cytosolic PE-PS-Chol leaflet. The surface potential is not shown.

conclusions made above are not sensitive to the choice of the force field. In all considered systems, the additional simulations also revealed that cholesterol elevates the membrane dipole potential (Table S1). The cholesterol-dependent effect with CHARMM36 was stronger compared to simulations with the Berger force field, which stems from a different treatment of lipid hydrocarbon chains in the two force fields.[27,31]

How does cholesterol give rise to the observed changes in membrane dipole potential? To answer this question, we calculated component-wise contributions to the boundary potential profiles depicted in Figure 2. In salt-free systems, the contribution of zwitterionic lipids to the potential decreases progressively with increasing cholesterol content, while the contribution of cholesterol (arising from its hydroxyl group) increases, see Tables S2 and S4. Perhaps surprisingly, the drop in the contributions of zwitterionic lipids is not related to the reorientation of their head groups; the tilt angle between the lipids' PN vector and the outward bilayer normal is not sensitive to cholesterol concentration (Tables S3 and S5). Instead, it is likely caused by the "dilution" of polar head groups on the bilayer surface: cholesterol molecules increase the distance between zwitterionic lipids and reduce their surface number density, see Tables S3 and S5. Correspondingly, the contribution of water molecules (and anionic lipids together with counterions in the case of PE-PS-Chol systems) that compensates the contribution of zwitterionic lipids also decreases. Meanwhile, the surface number density of cholesterol increases with its concentration, thereby strengthening cholesterol's contribution to the boundary potential, see Tables S3 and S5. This molecular-scale picture captures the essence of the cholesterol-dependent potential; however, additional subtle interactions such as hydrogen bonding between zwitterionic lipids and cholesterol are also expected to play a role and complement the above effect. For instance, in PC-SM-Chol systems the increase in the boundary and dipole potentials is not a linear function of cholesterol content (see Table 1). Overall, the total cholesterol-induced increase in the boundary potential is of the same order of magnitude as the contribution of cholesterol molecules into the potential. This key conclusion remains unchanged upon adding monovalent salt into the bilayer system, see Tables S6–S9.

Although experimental data for the dipole potential of PC-SM-Chol and PE-PS-Chol membranes have not been reported yet, we can compare the computational results with experimental data available for phospholipid membranes. The restriction that we have to take into account, though, is the condition that in experiments one cannot measure absolute values of the dipole potential because it is a variant of the Galvani potential. Therefore, the comparison has to be based on cholesterol-induced relative changes in the dipole potential. Then, it was experimentally shown[14] that removing 80% of cholesterol from membrane vesicles composed of natural membrane lipids (phospholipids, cholesterol, and other lipids extracted from the kidney and brain of eight vertebrate species) resulted in a reduction in the dipole potential of ∼50 mV. A similar difference was observed for unsaturated dioleoyl-phosphatidylcholine vesicles with 30 mol % cholesterol.[14] Interestingly, the influence of cholesterol on the dipole potential was found to be much more pronounced for vesicles composed of saturated dimyristoyl-phosphatidylcholine with about 40 mol % cholesterol (more than 400 mV).[14] Despite the differences in lipid compositions (the lipid membrane mixtures considered in this work do not contain saturated lipids), one can conclude that the simulation models correctly reproduce the right ballpark and the trends of the experimentally observed cholesterol-induced changes in the dipole potential, and even quantitative agreement is reasonable.

Careful consideration of Figure 2 brings out highly relevant insight regarding the implications of an asymmetric transmembrane distribution and the role of cholesterol in this context. First, it is obvious that the electrostatic potential profiles of asymmetric membranes are also asymmetric,

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implying that a *nonzero* difference in the electrostatic potential develops across the membrane in line with previous experimental[40] and computational studies.[22] Second, moving cholesterol from the extracellular leaflet to the cytosolic side increases the boundary potential and, consequently, also the dipole potential on the cytosolic side of a membrane (and vice versa).

To emphasize the importance of the nonzero transmembrane potential difference in cell membranes, Figure 3 sketches two relevant scenarios. The boundary potential difference develops within the lipid−water interface region, while the potential difference between the water phases on the opposite sides of a membrane is governed by the resting potential. We recall that the resting potential is due to a charge difference in the cytosolic and extracellular compartments and has passive and active components. The former is a Gibbs−Donnan-type potential; the latter is generated by the action of Na^+−K^+ pumps.[41] When the resting potential is zero (see the left-hand side column in Figure 3), the boundary potential of the extracellular (PC-SM-Chol) leaflet can be either larger or smaller than the boundary potential of the cytosolic (PE-PS-Chol) leaflet. However, in animal cells the resting potential of membranes is not zero (instead it is ∼50−100 mV), and it is known to be negative on the cytosolic side of a membrane.[42,43] Taking this into account, let us now apply a nonzero resting potential to a membrane, see the right-hand side column in Figure 3. One can conclude that the field inside a membrane is minimized when the boundary potential of the cytosolic (PE-PS-Chol) leaflet exceeds the corresponding boundary potential on the extracellular (PC-SM-Chol) side. As seen from Figure 2, this is indeed the case for most asymmetric membranes where cholesterol molecules are in the cytosolic (PE-PS-Chol) leaflet only. This conclusion also applies to the dipole potential because cholesterol has almost no effect on the surface potential, as discussed above.

Because of rapid (passive) translocation of cholesterol between the opposite leaflets, it is often considered that cholesterol might be distributed evenly between the two monolayers, with some preference to the cytosolic leaflet.[7] Theoretical considerations have suggested that cholesterol would be exposed to a driving force toward the cytosolic leaflet to reduce the membrane's bending energy because of the presence of PE in the cytosolic leaflet.[8,44] In this work, we have shown that there is yet another potential physical mechanism: given that cholesterol increases the dipole potential, it can provide a driving force for cholesterol molecules to favor the cytosolic leaflet, in order to compensate for the intramembrane field that arises from the resting potential.

In native cell membranes it is not only passive cholesterol translocation but also active transport that contributes to the transmembrane distribution of cholesterol. The challenge to unravel the transmembrane distribution of cholesterol in native (or native-like) cell membranes therefore remains still to be clarified. Nonetheless, the present results suggest a new paradigm for assessing how cholesterol is distributed in cells. If membrane potential measurements could be performed rapidly enough, one could measure how the membrane potential responds to changes in cholesterol transmembrane distribution. Such experiments would likely be based on detergents to deplete cholesterol in an asymmetric fashion, but despite this subtle issue, experiments could then generate a more solid understanding as to the distribution of cholesterol.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jpclett.6b02123.

Results of additional MD simulations with an alternative lipid force field (CHARMM36); component-wise contributions to the boundary potential, surface number densities of lipids and cholesterol, and orientation of lipid head groups (Berger lipids) (PDF)

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Notes

The authors declare no competing financial interest.

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