Water Order Profiles on Phospholipid/Cholesterol Membrane Bilayer Surfaces

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Abstract: Water is pivotal in the stabilization of macromolecular biological structures, although the dynamic ensemble structure of water near to molecular surfaces has yet to be fully understood. We show, through molecular simulation and fluorescence measurements, that water at the membrane surface is substantially more ordered than bulk water, due to a loss of hydrogen bonding between water molecules, coupled with an alignment of lipid and water dipole moments. Ordering of the water leads to a gradient in the effective dielectric permittivity, which is evident in both the molecular simulations and the fluorescence measurements. A lower effective dielectric permittivity was correlated with a decreasing degree of hydrogen bonding over the same spatial range. The water molecules closest to the lipid headgroup oxygen atoms form hydrogen bonds which exhibit a mean lifetime of 6.3 ps, compared with a mean lifetime of water-water hydrogen bonds of less than 2 ps. Membranes made up purely of phosphatidylcholine (PC) were compared with those made with a PC/cholesterol ratio relevant to cell membranes. Clear differences were found between these membrane configurations. These observations point to molecular structural differences in the surface environments of membranes and may underlie regional differences in the surface biophysical properties of membrane microdomains.


Key words: membranes; cholesterol; classical molecular dynamics; dielectric permittivity; order of water

Introduction

Membranes play an important part in the selectivity of molecular trafficking into and out of cells. The interaction of water with phospholipids is critical in maintaining the macromolecular organization of the membrane. The reciprocal influence of the membrane on the water structure is, however, less well understood. Processes that are affected by the water structure include inter-cellular signalling, endo/exocytosis, and the cellular entry and exit of important solutes.1–3

The ordering of molecular components, both proteins and lipids, within membranes allows localized behavior.3 In vivo, biological membranes possess phospholipids, sphingolipids, and cholesterol, which are important in the formation of raft regions, where high concentrations of cholesterol are found and where the molecular components adopt a liquid-ordered (l_o) structure, as opposed to the rest of the membrane, which is found in the liquid-disordered (l_d) phase.1 In vitro, high concentrations of cholesterol form these raft regions, where intra-membrane protein structure is strongly affected and the measured dipole potential is higher than the corresponding cholesterol-free membrane bilayer.4,5 Given this ordering in the membrane interior, it is highly likely that the water at the interface of the lipid headgroups will also show significant order and contribute to the dipole potential.6 Estimations of the effective dielectric permittivity of water at the interfacial region have been reported, using both fluorescent molecular probes and atomic force microscopy.7,8 Computational studies have complemented these findings, using both continuum models9 and fully atomistic models.10

Atomistic simulations of increasingly complex systems are furnishing more detailed understanding of molecular properties and influences not easily accessible from experiment.11–14 Geometrical and electrostatic effects can be readily determined and can provide new insight into experimental observables for a variety of biomolecular systems, including phospholipid bilayers. Woolf and Roux12 demonstrated that the boundary between the lipid headgroups and bulk solvent was much broader than was previously thought, highlighting the utility of combined experi-

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mental and theoretical approaches in such investigations. An explicit characterization of the nature of intermolecular interactions between the gramicidin A channel (protein) and the membrane was possible: tryptophan residues located on the interface between the protein and membrane hydrogen bond to the ester carbonyl group of the membrane lipids, suggesting the important contribution of such interactions in stabilising membrane proteins. Furthermore, it would seem likely that the nature of water close to membrane ion channels would also be extremely influential in the loading and unloading of the ions within the channel. Thus, studies with the gramicidin channel in membrane bilayers highlighted the importance of the dipole potential\textsuperscript{15} and other studies have illustrated how water may be involved in the transport process as single-file water through the narrow channel.\textsuperscript{16} Recently, simulations have shown that S1–S4 voltage transport process as single-file water through the narrow channels (200 on each layer), with bulk solvent added comprising force-field.\textsuperscript{22} The first system consisted of 400 DPPC lipid molecules. The unit cell was allowed to fluctuate anisotropically. Producing piston oscillation period of 200 fs, with a decay period of 100 ns was allowed to equilibrate for 5 ns, using a Nose–Hoover Langevin piston oscillation period of 200 fs, with a decay period of 100fs. The unit cell was allowed to fluctuate anisotropically. Production dynamics were carried out for a further 70 ns.

Computational Details

We have performed MD simulations using the CHARMM\textsuperscript{20} and NAMD\textsuperscript{21} programs, employing the CHARMM36 all-atom lipid force-field.\textsuperscript{22} The first system consisted of 400 DPPC lipid molecules (200 on each layer), with bulk solvent added comprising 20,000 TIP3P water molecules\textsuperscript{23} and was assembled using the CHARMM-GUI web-based service.\textsuperscript{24} The RATTLE algorithm\textsuperscript{25} was used for all bonds involving hydrogen. The second system comprised 280 DPPC lipid molecules and 120 cholesterol molecules, with 20,000 TIP3P water molecules. Minimization was performed using the steepest descent algorithm to remove bad contacts between atoms that were unphysically close, followed by conjugate gradient minimization. Nonbonded interactions were truncated at 12 Å, and the long-range electrostatics were treated with the particle mesh Ewald summations.\textsuperscript{26} Dynamics, using a 2 fs timestep, were propagated using the NPT ensemble. Initially, the lipid headgroups and water molecules were harmonically constrained; 0.5 ns of dynamics was performed at 310 K to allow the lipid tails to melt. The constraints were removed, and the system was allowed to equilibrate for 5 ns, using a Nosé-Hoover Langevin piston oscillation period of 200 fs, with a decay period of 100 fs. The unit cell was allowed to fluctuate anisotropically. Production dynamics were carried out for a further 70 ns.

The full electrostatic potential profile was calculated as the double integral of the charge density, given in eq. (1).

$$\phi(z) - \phi(0) = -\frac{4\pi}{\varepsilon_0} \int_0^z \int_0^z \rho_n(z') dz' dz$$

(1)

following Feller et al.\textsuperscript{27}

The effective dielectric permittivity was computed from the MD simulations using eq. (2).\textsuperscript{28} Because the membrane system is anisotropic, we refer to an effective dielectric permittivity rather than simply a dielectric permittivity; the latter would only be strictly justified for an isotropic medium. For the purposes of analysis, the water was partitioned (postsimulation) into seven overlapping slices with a height of 6 Å, effectively giving a running average. The slices started at the outer aqueous edge and continued to the boundary described in Figure 1.

$$\varepsilon = 1 + \frac{4\pi \left( \langle M^2 \rangle - \langle |M| \rangle^2 \right)}{332.072 \langle \varepsilon_0 V D^2 \rangle}$$

(2)

Here, $M$ and $V$ refer to the dipole moment and volume of the slice, respectively. $T$ is the temperature and $D$ is the Debye constant.

The hydrogen bond lifetimes were calculated as the average time a hydrogen bond existed over the course of the trajectory. A maximum hydrogen bond distance of 3.15 Å and a bond angle of 45° between the vectors of the donor group and acceptor group were used in this study. The rotational correlation time of water, $\tau$, was calculated by fitting the exponential decay of the corresponding time correlation function, $C(t)$, to an exponential function of the form

$$C(t) = Ae^{-t/\tau}$$

Figure 1. Probability distribution for lipid and water atoms. A bin size of 0.1 Å was used. The solid black line represents the “boundary” between water and lipids used in the analysis of these simulations, chosen to represent the position of the DPFPE probe. The solid line is for DPPC oxygen atoms; dashed line for DPPC nitrogen atoms; dotted line for DPPC phosphorous atoms, and the dot-dashed line is for the TIP3P oxygen atoms.
Results and Discussion

We have sought to provide a more definitive description of the nature of water in near proximity to a membrane surface as well as to clarify the differences in magnitude of the membrane dipole potential in membranes made up of different lipid compositions. We summarise our investigation below by outlining the profile of the membrane dipole potential across a membrane bilayer and beyond. The system was equilibrated, leading to average area per lipid of 59.4 Å² for the pure DPPC system and 47.4 Å² for the DPPC/cholesterol bilayer. The evolution of the area per lipid during the equilibration phase is given in Figure 2. The computed electron density profiles of both the pure DPPC bilayer and the DPPC/cholesterol bilayer are shown in Figure 3. In both cases, the curves are nearly symmetrical, as would be expected from a stable MD simulation. The peak for each simulation corresponds to the lipid headgroup region, where the electron density is high, due to the presence of phosphorus atoms. The distance between the peaks in the electron density profile is equal to the membrane thickness, which for the pure DPPC system is 38.6 Å. In contrast, the mixed DPPC/cholesterol system has a bilayer thickness of 43.6 Å.

The membrane dipole potential profile (Fig. 4) was calculated for each of the two systems over the course of the MD simulations. The experimentally determined values for the pure DPPC and the mixed DPPC/cholesterol (30%) bilayer were ~300 mV and ~400 mV, respectively. The calculated potentials agree with other simulations of the membrane dipole potential, although these are higher than the experimentally determined values. The relative difference between the two systems (~100 mV) is in good agreement with experiment. In both types of bilayer that we studied (i.e., with and without cholesterol), the contribution to the total dipole potential from the interfacial region dominates. The calculated increase in the membrane dipole potential upon the addition of cholesterol is consistent with experimental observations. The difference in the dipole potentials between the two systems appears to arise from the presence of cholesterol molecules. It seems that cholesterol induces a tighter packing in the membrane interior, leading to a stronger contribution to the overall dipole potential from the lipid region. The effect of cholesterol on membranes has been studied for many years, particularly on the phase behavior, and

Figure 2. Evolution of the area per lipid for the pure DPPC bilayer (top) and the mixed DPPC/cholesterol bilayer (bottom).

Figure 3. Electron density profile along the membrane normal calculated for the pure DPPC bilayer (solid line) and the DPPC/cholesterol bilayer (dashed line).

Figure 4. Calculated membrane dipole potential for the pure DPPC bilayer (solid line) and the mixed DPPC/cholesterol bilayer (dashed line).

Figure 5. The calculated deuterium order parameter, $S_{2D}$, of the pure DPPC (solid line) and mixed DPPC/cholesterol (dashed line) bilayers for the C-H bonds along the DPPC hydrocarbon chain.

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lipid packing and these earlier experiments are consistent with our current observations. Likewise, NMR has been used extensively to study lipid order of membranes. One can define the deuterium order parameter, $S_{CD}$, as

$$S_{CD} = \frac{1}{2} \left( 3 \cos^2 \theta - 1 \right)$$

where $\theta$ is the angle between the C-D bond vector and the bilayer normal for each of the carbon atoms along the hydrocarbon chain of DPPC. Figure 5 shows more order along the hydrocarbon chain for the DPPC/cholesterol bilayer compared with the pure DPPC system.

Figure 6. Calculated (circles) and experimentally determined (triangles) effective dielectric permittivity profiles for the DPPC bilayer. The FPE probe, using different tether lengths, is shown schematically alongside DPPC. The spacer groups took one of two chemical forms: (i) NC[CH$_2$]$_n$C$^-$ and (ii) NC[CH$_2$]$_m$[CH$_2$]$_n$[CHOH]$_m$C$^-$, where $n$ ranged from 3 to 18 and $m = n/3$. The length of the probe was estimated from a simple count of C-C bonds. The fluctuation bars represent the maximum and minimum values obtained computationally and experimentally for different regions of the membrane.

Table 1. Effect of Dielectric Permittivity Upon the Emission Spectroscopy of FPE.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dielectric permittivity</th>
<th>$\lambda_{max}$/nm</th>
<th>Distance from membrane surface</th>
<th>Equivalent dielectric permittivity based on spectra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octan-1-ol</td>
<td>10.3</td>
<td>522.6</td>
<td>$-10,\AA$ (i.e., membrane interior)</td>
<td>2</td>
</tr>
<tr>
<td>Ethanol</td>
<td>24.3</td>
<td>517.8</td>
<td>Membrane surface</td>
<td>25</td>
</tr>
<tr>
<td>Methanol</td>
<td>32.6</td>
<td>515.5</td>
<td>5</td>
<td>25-30</td>
</tr>
<tr>
<td>Water</td>
<td>78.5</td>
<td>510.0</td>
<td>10</td>
<td>50-60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15</td>
<td>$\sim$78.4</td>
</tr>
</tbody>
</table>

As the length of the FPE tether is reduced, a gradient of effective dielectric permittivity from $\varepsilon_24$ (bulk water) to $\varepsilon_24$ was observed. Care was taken during the course of these studies to ensure (or at least consider) that the tether itself did not lead to changes of the local effective dielectric permittivity. One concern was, that for the longer lengths of spacer required to probe distances farther away from the membrane surface, the fluorescent reporter could not be guaranteed to locate itself at its maximum distance away from the molecular surface of the membrane. Clearly, a molecular structure that possessed a significant nonpolar moiety, especially if tethered to the membrane, would most likely become organized so that the aliphatic moiety was actually as close as possible to the nonpolar membrane interior. In fact, in unpublished work some years ago, we found that the experimental molecular dynamics determined using time-resolved fluorescence anisotropy methods$^{33}$ allowed the fluorescence emission spectroscopy of fluoresceinphosphatidylethanolamine (FPE). Comparison with emission data measured in several solvents of differing effective dielectric permittivity (Table 1) allows estimates of the effective dielectric permittivity at different distances from the membrane interior.

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cent moiety to be significantly close to the membrane for some fluorescent phospholipids. To ameliorate this problem, methanol groups were used to replace methyl groups on alternating unit spacers. We believe this led to a more accurate determination of the surface effective dielectric permittivity based on the spectral properties of the fluorescent moiety. For short spacers, the dielectric environment of the fluorophore was very similar for the methanolic and methyl spacers, but for longer distances, the two systems became quite different.

The effective dielectric permittivity was calculated from the MD trajectory, as illustrated in Figure 6. Both systems exhibit an increase in their effective dielectric permittivity as the distance from the lipid surface increases. Within experimental error, the curves are consistent with each other close to the molecular surface of membrane. At distances more remote from the membrane surface, the simulations yield a higher effective dielectric permittivity than the equivalent experimental point obtained with methyl-spacers (data not shown) but with the methanolic-spacers they are in reasonable agreement. Clearly, however, there is some further level of inaccuracy with the wet experimental system, as the “starting position” of the “zero-spacer” fluorophore is not defined to the same level of accuracy that we can implement with the computational study. Although there is a degree of fluctuation in the calculation of the effective dielectric permittivity, the gradient of effective dielectric permittivity persists. The computed effective dielectric permittivity at the outer aqueous edge is lower than that expected for bulk water using the TIP3P model, suggesting that there is still some order at this edge.

The Kirkwood equation for the effective dielectric permittivity is:

$$\frac{(\varepsilon - \varepsilon_{\infty})}{\varepsilon(\varepsilon_{\infty} + 2)} = \frac{4\pi N \beta^2}{9k_B T \varepsilon_0} \rho_0$$

where $\mu$ is the gas-phase dipole moment of an isolated molecule of interest and $\varepsilon_{\infty}$ is the high-frequency dielectric limit. The Kirkwood $g$-factor, $g_0$, accounts for the disorder of a system relative to some external direction. Suresh and Naik showed that, for a system where hydrogen bonding is important, $g_0$ is related to the degree of hydrogen bonding, $\rho$, by

$$g_0 = 1 + \frac{4\rho}{3 - \rho}$$

where

$$\rho = \frac{n_b}{2N_{\text{water}}}$$

and $n_b$ is the average number of hydrogen bonds over the trajectory and $N_{\text{water}}$ is the total number of water molecules in the system. $\varepsilon_{\infty}$ was taken to be unity, and the data were plotted to calculate $g_0$ as the gradient. Table 2 shows the degree of hydrogen bonding for each slice for which the effective dielectric permittivity was calculated; a lower effective dielectric permittivity is correlated with a decreasing degree of hydrogen bonding. The water molecules closest to the lipid headgroup oxygen atoms form hydrogen bonds that have a calculated mean lifetime of 6.3 ps, compared with a mean lifetime of water-water hydrogen bonds of less than 2 ps. Bulk water has been measured to possess a hydrogen bonded network with lifetimes of $\sim$1 ps. The longer-lived intermolecular bonds cause the rotational correlation time of the water near the membrane to increase to 0.52 ps, compared with 0.45 ps further away from the lipid headgroups. This hydrogen bonding pattern is responsible for the ordering of the water, which, in turn, contributes to the dipole potential in Figure 4.

In a bulk liquid, one would expect $\langle |M| \rangle^2$ to tend to zero as this property converges, as this term is a measure of order. The simulation of bulk water confirms this, with convergence to zero. As the distance from the headgroup decreases, the contribution of the $\langle |M| \rangle^2$ term in eq. (2) increases, thus lowering the calculated effective dielectric permittivity. The angular distribution of the water dipoles and the overall dipole moments for the boxes of water over time (Fig. 7) reveals an increasing alignment of the overall dipole for the water molecules close to the surface with the dipole moment of the lipids. Particularly, there is an increase in the alignment of the water dipole with respect to the membrane normal, which causes a nonvanishing mean dipole moment of the box of water.

**Table 2.** The Degree of Hydrogen Bonding as a Function of the Distance From the Membrane Surface.

<table>
<thead>
<tr>
<th>Distance from membrane/Å</th>
<th>0.0</th>
<th>5.0</th>
<th>10.0</th>
<th>Bulk water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of hydrogen bonding, $\rho$</td>
<td>0.66</td>
<td>0.71</td>
<td>0.74</td>
<td>0.95</td>
</tr>
</tbody>
</table>

**Figure 7.** Average cosine of the angle between the dipole moments of the water molecules with the membrane normal, for the pure DPPC bilayer (solid line) and the DPPC/cholesterol bilayer (dashed line).
as a result of increased hydrogen bonding. The accuracy of our calculations is sufficient to substantiate our present conclusions, but future consideration of many-body polarization effects will be likely to reduce to some extent the calculated membrane dipole potential,\textsuperscript{10,19} and it would be of interest to investigate the behavior of water models other than TIP3P.\textsuperscript{36,37} Quantum chemical calculation of the fluorescence of probe molecules in or close to the bilayer is also of interest.\textsuperscript{18} The development and application of more accurate computational methods to investigate changes in these gradients could provide a fully quantitative understanding of the effect of the dielectric effect upon ionic and other solute transport as well as protein trafficking through the membrane. Thus, defining the water structure around localized membranes such as rafts,\textsuperscript{1} the many signalling and receptor systems associated with membranes may well impact on activities as diverse as drug development to cell differentiation. Similarly, this study will have some bearing on developing a better understanding of the factors that dictate macromolecular structure on and within membranes. Particularly, a better insight into the structure and environment provided by the extra-cellular matrix/glycocalyx may well result from consideration of the membrane-associated water and the dipole potential.

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**References**


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