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Molecular Dynamics Simulations of Mixed DOPC-β-Sitosterol Bilayers and their Interactions with DMSO

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Abstract

Cell membrane phospholipid bilayers can be damaged by the large amounts of dimethyl sulphoxide (DMSO) commonly used in cryopreservation. The interaction of DMSO with model bilayers consisting of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and β-sitosterol has been studied using molecular dynamics simulations. Initially the effect of sterol concentration and temperature upon bilayers solvated in pure water was determined, and membranes containing β-sitosterol were compared with membranes containing cholesterol. These simulations showed that the presence of sterols has a condensing effect on the phospholipids, causing a reduction in the area per lipid as the sterol concentration increases, up to a phospholipid-sterol ratio of 2:1. The incorporation of sterols into the bilayer also increased the thickness and order of the phospho-

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lipid acyl tails. DOPC-β-sitosterol bilayers at different relative concentrations were simulated in solutions of 2.5 and 25.0 mol % DMSO. The interaction of DMSO with the bilayers caused the bilayers to expand laterally, while thinning normal to the plane of the bilayer expansion. The same qualitative behaviour has been shown to occur in pure phosphocholine bilayers. However, the presence of sterols made the membranes more resistant to the effects of DMSO, to the extent that the membranes were able to maintain their integrity in 25.0 mol % DMSO, a concentration that would otherwise cause the destruction of a pure DOPC bilayer. Increasing the concentration of β-sitosterol within the bilayers reduced the rate of DMSO diffusion across the bilayer and, if the concentration was large enough, caused the diffusion mechanism to change. DMSO was observed to disorder the membranes enough to cause an increase in the number of sterol “flip-flops”. The findings of this work provide a more realistic description of how DMSO interacts with cell membranes and the role of the composition of the membrane.

**Keywords**

Molecular simulation, cryopreservation, cell membranes
Introduction

The storage of biological samples by means of cryopreservation\textsuperscript{1–7} is an increasingly important tool. By storing samples at liquid nitrogen temperatures (77K) any biological, chemical and physical processes occurring within the sample are slowed to the point that they are effectively suspended. This allows cryopreserved samples to be stored for decades without any deterioration. Unfortunately, the cryopreservation process itself can cause some damage to cells but the extent of this damage can be reduced by treating the samples, before and during cryopreservation, with an aqueous solution containing a number of cryoprotective agents (CPAs).\textsuperscript{8–12}

There are a number of different CPAs in use such as glycerol, ethylene glycerol, various sugars and DMSO.\textsuperscript{9,13,14} DMSO is one of the most widely used and important CPAs due to its ability to diffuse through cell membranes\textsuperscript{15} coupled with its effect on promoting the vitrification of aqueous solutions.\textsuperscript{16,17} The formation of a glassy state of water upon rapid cooling of the sample reduces the likelihood of the formation of intracellular ice crystals, which can cause significant damage to cells. DMSO has been shown to have other interesting properties, such as the ability to induce cell fusion\textsuperscript{18} and act as an anti-inflammatory\textsuperscript{19} and anesthetic.\textsuperscript{20} As such it is important to determine the nature of the interactions of DMSO with cell membranes at the molecular level.

The actions of DMSO on cell membranes have been investigated using both experimental and computational techniques.\textsuperscript{21–29} The authors recently performed a series of molecular dynamics simulations investigating the interaction of DMSO with homogeneous 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) membranes.\textsuperscript{27} The results of these and other simulations\textsuperscript{22–24,26,28} showed that at low concentrations DMSO causes these model bilayers to expand laterally while thinning
normal to the plane of the bilayer. Increasing the concentration of DMSO causes pores to form in the bilayer and, if the concentration is high enough, the bilayer structure to be destroyed. It was also shown that DOPC bilayers were more resistant to the effects of DMSO than the DPPC bilayers by being able to both withstand a higher concentration of DMSO before destruction and having a higher free energy barrier to the diffusion of DMSO molecules through the bilayer. Simulations have also shown that DMSO displaces water molecules at the bilayer interface making the membrane dehydrated.\textsuperscript{24, 27} Experimental studies have shown that as the concentration of DMSO is increased the spacing between bilayers is reduced.\textsuperscript{30–32} This reduction in the repeat spacing of membranes combined with the dehydration of the bilayers are the most likely reason why the gel-liquid crystal phase transition temperature of phospholipid bilayers increases with the mole fraction of DMSO present.\textsuperscript{30–32} Recently, de Ménorval \textit{et al.} performed simulations of a binary DOPC-cholesterol (at a single 20% cholesterol concentration) in a range of DMSO concentrations and compared the simulation results against experiment.\textsuperscript{33} The simulations showed that the bilayer behaved in similar manner to pure DOPC bilayers with three regimes (membrane thinning, pore formation and bilayer destruction) observed. For this 20% cholesterol bilayer it was found that 40 mol % DMSO would cause it to be destroyed. However, the simulations were relatively short, 45 ns, and it is possible that longer simulation times would show that the bilayers would also be unstable at lower DMSO concentrations. The experimental work confirmed many of the findings of the simulations, with the permeability of the bilayer increasing as the DMSO thinned the bilayers.

Most previous simulations of DMSO with phospholipid bilayers have investigated bilayers consisting solely of a single phosphocholine (PC) lipid species. The cell membranes of real biological samples contain a multitude of different
lipid species as well as proteins and sterols. The effect of sterols on the properties of cell membranes has been investigated experimentally in some detail.\textsuperscript{34–42} The presence of sterols makes a membrane more ordered and plays an important role in determining the fluidity and mechanical properties of a membrane. Differential scanning calorimetry (DSC) of mixed DPPC-sterol bilayers showed that the presence of sterols caused the transition between the gel and liquid crystal phases to both broaden and weaken.\textsuperscript{34, 43} The phase transition temperature of the bilayer changes from $\sim$313-315 K for pure DPPC to 307-320 K for a bilayer with a sterol concentration of 27 mol %. This effect increased with the sterol concentration of the bilayers, leading to the complete elimination of the phase transition at $\sim$33% sterol. These results lead to the conclusion that bilayers containing certain sterol species (including cholesterol and $\beta$-sitosterol) are able to form a liquid-ordered phase, $l_o$, in which the lipids have nearly the same translational and rotational freedom that they do in the liquid-disordered, $l_d$, (or liquid-crystalline) phase, but with a degree of conformational order comparable to the solid-ordered, $s_o$, (or gel) phase.\textsuperscript{38–40} More recently Kamal and Raghumathan determined the phase behaviour of mixed DPPC-sterol bilayers for a number of different sterols from X-ray diffraction data.\textsuperscript{42} Their findings are largely consistent with the DSC studies. In bilayers containing 5-10 mol % sterol the transition temperature remains roughly constant at $\sim$310 K but the gel phase coexists with a sterol-induced modulated phase, $P_{\beta}$. For bilayers with sterol concentrations between 12.5 and 20.0 mol % the main transition temperature decreases with the increasing sterol concentration and the gel phase no longer exists, with the bilayers below the transition temperature being in the $P_{\beta}$ phase. Increasing the sterol concentration further to more than 25 mol % leads to the eradication of any phase boundaries, with the system being in the fluid phase for all temperatures between 283-318 K.
Sterols also have an effect on the permeability of bilayers, generally reducing the ability of water, ions and small organic molecules to cross the membrane. The effectiveness of different sterol species at reducing permeability remains an open question and depends strongly on the species of lipids present in the membrane. In animal cells cholesterol is the main sterol species, while in plants β-sitosterol and stigmasterol are much more common in combination with a higher degree of unsaturation in the acyl chains of the phospholipids. These differences can mean that a sterol species may have little effect on the permeability of a membrane consisting of one lipid species and yet have a significant effect on a membrane consisting of a different species. Sterols also play important roles in determining the elasticity of a bilayer and the formation of lipid rafts within membranes. The amount of sterols that are present in membranes is difficult to determine accurately and a wide range of values have been given, but a number of studies quote that in animal cells cholesterol typically makes up 30 to 50% of the bilayer. In plant cells the ratio of sterols to lipids is less well characterised and seems to vary considerably.

Several previous simulations have investigated the effect of a variety of different sterols on phospholipid bilayers, and two recent reviews provide a good overview of the subject. In nearly all simulations the condensing effect of the sterols has been observed, with the addition of sterol molecules leading to a decrease in the area per lipid (APL) of the lipids present in the bilayer. In addition, the incorporation of sterols into the membrane leads to a thickening of the bilayer. Due to the large number of variables that such simulations have to take into account (e.g. amount of sterol, lipid species, sterol species, etc.), comparisons between different simulations can be difficult. The most popular sterol to simulate is cholesterol but other species have been

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A comparison of cholesterol, ergosterol and lanosterol simulated at 11% showed little structural difference in the bilayers of 1,2-dimyristoyl-snglycero-3-phosphocholine (DMPC). In contrast, a simulation of the same three sterols at a concentration of 40% revealed significant differences between the three sterols, with ergosterol packing closer to the lipids than cholesterol, which in turn packed more closely than lanosterol. Systems containing plant sterols have not been as widely investigated but a recent study of DPPC-stigmasterol and DPPC-β-sitosterol membranes showed that plant sterols have the same condensing effect on lipids as cholesterol. The study also found, in agreement with experimental results, that β-sitosterol was more effective at ordering the bilayer than stigmasterol. The chemical structures of cholesterol and β-sitosterol are shown in Figure 1.

The range of lipids that have been investigated is also quite wide, with simulations having been performed on bilayers containing DMPC, DPPC, 2-oleoyl-1-palmitoyl-snglycero-3-phosphocholine (POPC), DOPC and sphingomyelin (SM). The presence, and location, of an unsaturated double bond within the acyl chain has been observed to cause significant structural differences in lipid-sterol bilayers, with POPC being less able to pack around sterol molecules than DPPC. This is consistent with conventional wisdom that the addition of unsaturated bonds to acyl chains increases fluidity and lowers the transition temperature to a much larger extent than the decrease in length of the acyl chains. A number of studies have observed the formation of small subunits consisting of one sterol and two lipids or one sterol to one lipid. The ability of sterol-containing membranes to resist the effect to electroporation showed that cholesterol increases the cohesion of the membrane and that, as the percentage of cholesterol within the membrane was increased, the electric field needed for the formation of a pore also increased. Although the majority of work has investigated binary
mixtures there is an increasing amount of simulations of ternary systems,\textsuperscript{53,63,65} where the preference of sterols for different lipid species can be evaluated. In one study of mixed DOPC-SM-cholesterol bilayers it was found that cholesterol tended to arrange itself so that the side of the sterol with the protruding groups would lie next to DOPC molecules.\textsuperscript{63}

The aims of this work are twofold. Firstly to determine if there are any significant differences between those membranes containing sterols typically found in animals (cholesterol) with membranes containing sterols that are typically found in plants (\(\beta\)-sitosterol). Secondly, to determine what effect the presence of sterols within a bilayer has when the membrane is exposed to DMSO. A recent study evaluating the type and amount of sterols and phospholipids present in three plant species found that the majority of the phosphocholine lipids had acyl tails at least eighteen carbon atoms long and that the degree of unsaturation of the lipids was significant.\textsuperscript{66} The same study also found that in all three species the most abundant sterol was \(\beta\)-sitosterol. Considering these findings it was decided that DOPC would be used for the lipid species while cholesterol and \(\beta\)-sitosterol would be used as the representative animal and plant sterol species, respectively. As the amount of \(\beta\)-sitosterol within plant bilayers can vary considerably a range of sterol concentrations were simulated.

\textbf{Methods}

The time- and length-scales required for these systems to be simulated fully make them unsuitable for simulation with and all atom force-field. Instead the lipids and sterols molecules were modelled using the united-atom GROMOS 53A6f forcefield,\textsuperscript{67–69} part of the GROMAS 54A7 force-field.\textsuperscript{70} While the use of a united-atom force-field will mean that some detail is lost from the simulation,
lipid united-atom force-fields contain enough information that they are able to provide an accurate description of lipid bilayers. The parameters for the 54A7 force-field have been fitted to allow the phospholipids to reproduce a broad range of membrane properties.\textsuperscript{71,72} The SPC model\textsuperscript{73} was used for the water molecules, DMSO was represented using a united-atom with the parameters taken from the GROMOS 53A6 and 54A7 force-fields.\textsuperscript{70,74}

The simulations were performed using GROMACS v.3.3.3.\textsuperscript{75} The equations of motion were integrated using the leap-frog algorithm and a timestep of 2 fs was used.\textsuperscript{76} The non-bonded interactions were evaluated using twin-range cutoffs, with interactions within 0.8 nm calculated every step while interactions within 1.4 nm and the pair list were updated every five timesteps. The electrostatic interactions were computed using the reaction-field method with the relative dielectric permittivity constant, $\varepsilon_{RF}=62$, suitable for SPC water.\textsuperscript{73} All simulations were performed in the NpT ensemble, and the lipids/sterols and solvent molecules were independently coupled to external temperature baths with a coupling constant of 0.1 ps using the Berendsen thermostat.\textsuperscript{77} The pressure of the system was kept constant at 1 bar using the Berendsen barostat\textsuperscript{77} with a coupling constant, $\tau_P$, of 1 ps and the isothermal compressibility of the system was set to $4.6 \times 10^{-5}$ bar$^{-1}$. For the simulations of the preformed bilayers, the box dimensions parallel and perpendicular to the plane of the bilayer were independently coupled to a pressure bath, while in the case of the simulations starting from randomly distributed lipids/sterols (see below) each box dimension was independently coupled to a pressure bath.

The initial starting configurations of the pure DOPC bilayers in water were taken from previous work.\textsuperscript{27,69} For the bilayers consisting of a mixture of lipids and sterols the membranes were generated by randomly replacing a number of DOPC lipids by sterol molecules (an equal number of sterol molecules were
placed in each leaflet). The sterol-lipid interactions were initially “soft” and were
gradually “hardened” to their proper values over a 50 ps run, thus removing any
unfavourable contacts. The systems were then simulated for 300 ns at both
303 and 350 K to ensure they were fully equilibrated. The final configurations
of the lipid-sterol systems in water were used as the starting configurations
for the simulations of the same bilayers in the aqueous DMSO solution. The
total number of solvent molecules was fixed at 5841, representing ~46 solvent
molecules per lipid and/or sterol. The final 60 ns of each simulation were used
for analysis.

In addition to systems set up in a bilayer formation, a simulation of randomly
distributed lipids and sterols in water was performed in order to determine if
such systems would spontaneously form a bilayer and, if so, that the properties
of such a bilayer would be equivalent to the pre-formed systems. In this case 64
DOPC and 64 β-sitosterol molecules were solvated in 9045 water molecules. The
system was simulated at 303K for 400 ns with the final 60 ns of the simulation
used for analysis.

As mentioned above the primary motivation for this work is to provide a
better understanding of the effect that DMSO has on cell membranes, partic-
ularly plant cell membranes, during the cryopreservation process. In order to
make the model bilayers more representative of plant cell membranes, a sterol
commonly found in plants, β-sitosterol, and an unsaturated lipid, DOPC, have
been used. For these β-sitosterol-containing bilayers, membranes consisting of
10, 33, 40 and 50 % β-sitosterol were simulated in both water and 2.5 mol %
DMSO solutions. The bilayers consisting of 10, 33 and 50% β-sitosterol were
also simulated in 25.0 mol % DMSO solution. As the primary aim of the work
is to investigate the effect of DMSO on lipid bilayers for the purpose of better
understanding the actions of this cryopreservant, and since 25.0 mol % (59 wt
DMSO is already a significantly more concentrated solution than is typically used in cryopreservation protocols (5-15 wt %, <5 mol %), concentrations in excess of 25.0 mol % were not simulated. DMSO is known to cause an increase of the liquid crystalline-gel phase transition temperature of DPPC and POPE membranes.\textsuperscript{30-32} Due to this behaviour many simulations of phospholipid bilayers in the presence of DMSO have been performed at 350 K in order to ensure the bilayer is in the liquid crystalline phase. While to the authors knowledge the effect of DMSO upon DOPC membranes has not been investigated experimentally, one would predict to see a similar effect, although the presence of unsaturated acyl tails will lead to a lower phase transition temperature than for DPPC. The addition of sterols to the bilayer increases the complexity of the system further. As a result, in order to allow comparison with previous simulations the simulations of the mixed DOPC-\(\beta\)-sitosterol with DMSO in this study were also performed at 350K. The bilayer containing cholesterol was only simulated in water. The details of the simulation runs are given in Table 1.

\section*{Results and Discussion}

\subsection*{Mixed DOPC/sterol bilayers in water}

For a bilayer containing only DOPC the APL is predicted to be 0.653 nm\(^2\) and 0.683 nm\(^2\) at 303 and 350 K, respectively. At 303 K the experimental values of the APL are in a range of 0.674-0.725 nm\(^2\), showing that the predicted APL is somewhat below this figure but is still reasonable, and falls within the range (0.651-0.660 nm\(^2\)) reported for other simulations of DOPC at 303 K.\textsuperscript{67,68} The presence of either cholesterol or \(\beta\)-sitosterol causes the cross-sectional area of the bilayer to be reduced at both 303 and 350 K. Figure 2 shows the area of
the different bilayers over the last 100 ns of the simulations. The area of the systems remains approximately constant and shows no drift, indicating that the bilayers are equilibrated. It is apparent that the greater the amount of sterol in the bilayer the greater the reduction in area. There are a number of different methods that have been used to determine the area per lipid in a mixed lipid-sterol system.\textsuperscript{49,50,53,54,64} In this work the method of Höfsäk has been used.\textsuperscript{50} Here the APL of a lipid is determined from

\[ A_L = \frac{2A}{N_L} \left( 1 - \frac{N_S V_S}{V - N_W V_W} \right) \]  

(1)

where \( V \) is the volume of the system, \( A \) is the area of the bilayer in the plane of the bilayer, \( N_L \), \( N_S \) and \( N_W \) are the total number of lipids, sterols and water molecules in the system, respectively, and \( V_S \) and \( V_W \) are the volume of a sterol molecule and a water molecule, respectively. The volume of a water molecule was determined from simulations of a box of water and is 0.0309 nm\(^3\) at 303 K and 0.0325 nm\(^3\) at 350 K. Previous simulations have estimated the volume of a sterol molecule from the crystal structure; however, in this work a different approach was taken, with the volume of the sterol molecules being determined from the simulation of small systems consisting of 80 sterol molecules in 3680 water molecules. The volumes calculated using this approach are 0.715 nm\(^3\) and 0.674 nm\(^3\) for \( \beta \)-sitosterol and cholesterol, respectively, at 350 K. In a previous work\textsuperscript{52} the volume of cholesterol, averaged over three published crystal structures, was given as 0.619 nm\(^3\), which is somewhat lower than the value calculated above but probably not significantly so when the effects of temperature and the different structures (crystal vs. hydrated sterols) are considered. The APLs of the different systems are given in Table 2. As in previous simulations, the presence of the sterols causes the APL to decrease. At 10\% no difference between cholesterol and \( \beta \)-sitosterol is observed, while at 50\% cholesterol has
a slightly greater condensing effect. For \( \beta \)-sitosterol the APL decreases with increasing sterol concentration up to the point where a third of the bilayer is \( \beta \)-sitosterol, and above this point the APL is approximately constant. This behaviour occurs at both 303 and 350 K. The reason for this change in behaviour is likely due to the fact that at concentrations above one sterol per two lipids, sterol-sterol interactions start to occur and the phospholipids cannot solvate the sterols completely, a behaviour also observed experimentally.\(^{34,36}\)

The thickness of the membrane normal to the plane of the bilayer, \( D_{HH} \) (calculated as the distance between the two peaks in the lipid density profile) is given in Table 3. It is clear that the more sterol is added to the membrane the greater the increase in the membrane thickness. The difference between the two sterols was minor.

The density profiles of the bilayers are shown in Figure 3. It is clear that the presence of sterol molecules in the bilayer makes the membrane more ordered (this is especially true when the sterol concentration is 50%), the interdigitation of the acyl chains of the two leaflets of the bilayer is also reduced, causing the trough in the density profile at the intersection of the two leaflets to become deeper. The density profiles also show that both cholesterol and \( \beta \)-sitosterol take up a position in the membrane below that of the DOPC headgroups. The headgroups of the phospholipids form an “umbrella” over the hydroxyl group of the sterol, shown in Figure 4. The lipid density profile of the random 50% \( \beta \)-sitosterol system does not show any significant differences to that of the 50% \( \beta \)-sitosterol system starting from a bilayer, but the sterol density profile is more smoothed out, indicating that the sterol molecules are not quite as well ordered.

The ordering of the acyl tails of the phospholipids can be determined from the deuterium order parameter, \( S_{CD} \), which provides a measure of the relative orientation of C-D bonds with respect to the bilayer normal. \( S_{CD} \) can be
calculated from

\[ S_{CD} = \frac{1}{2} < 3 \cos^2 \theta - 1 > \]  

(2)

where \( \theta \) is the angle between a C-D of a methylene in the chain and the bilayer normal. As the force-field used in this work is a united-atom one, the positions of the deuteriums are derived from the positions of the neighbouring carbons and assuming a tetrahedral geometry of the methylenes. The calculated order parameters for both the \( sn-1 \) and \( sn-2 \) chains of the DOPC lipids for the different bilayer compositions are shown in Figure 5. The presence of an unsaturated bond in the acyl chains of DOPC is responsible for the drop in order at carbons 9 and 10. The addition of sterols into the bilayer forces the acyl chains to become more ordered. For the bilayers consisting of 10 mol % sterol the increased ordering is relatively minor but at 50 mol % sterol the acyl tail ordering is substantial so that the order profile looks rather like that which would be found for a membrane in the gel-phase. At higher temperatures the ordering of the lipids is reduced due to the higher thermal motion present in the system. It might be expected that the bulkier tail group of \( \beta \)-sitosterol would be responsible for a difference in the ordering at the end of the lipid tails. However, cholesterol and \( \beta \)-sitosterol show very similar order parameters for carbon atoms at C10 and above. Instead, for the bilayers which are half sterol in composition the ordering of the first eight carbon atoms in the DOPC tails is greater for cholesterol. The order parameters of the S50R system are slightly lower than for the S50 system, indicating that while the initial random distribution of lipids and sterols leads to the formation of a bilayer, it is not quite as well ordered as a system that is initially set up in a bilayer. However, the difference between the two systems is minor, showing that setting up the systems in a bilayer configuration does not lead to an unrealistic configuration.

The tilt angle of a sterol is defined as the angle between the bilayer normal,
directed away from the bilayer, and the vector formed between two atoms within the sterol. For the sterol head tilt angle the vector is defined between the carbon atoms at the top (C₃) and bottom (C₁₇) of the ring structure, and for the tail tilt angle the vector is defined between the bottom carbon of the ring and the second to last carbon in the tail of the sterol (C₂₅). The distributions of the tilt angles between the sterol and the bilayer normal are given in Figure 6. For the systems containing 10% sterol the sterol molecules tend to lie at a slight angle to the bilayer normal, and no significant difference between cholesterol and β-sitosterol is observed. As the amount of sterol in the system increases the distribution is shifted to lower angles, suggesting that the sterol molecules are aligning themselves nearly parallel to the membrane normal. At 50% sterol the differences between cholesterol and β-sitosterol become more apparent. While the peak in the distribution of the head tilt angle is the same for both species, the peak tail distribution of cholesterol is lower than that of β-sitosterol. This difference arises due to the bulkier tail of β-sitosterol. A higher temperature causes the distribution to widen but the position of the peaks does not change. In the case of S50R the distribution of the tilt angles is slightly shifted to higher angles when compared to S50, due to the fact that the bilayer is not quite as well ordered.

It is also possible to calculate the tilt angle between the bilayer normal and the vector between the P⁻→N⁺ dipole moment, ζ_{PN}, which indicates the orientation of the lipid headgroups. The distribution of ζ_{PN} is shown in Figure 7. For the systems at 303 K the presence of sterols causes some significant changes in the distribution. For bilayers that are 33% sterol or less the peak of the distribution is shifted to slightly lower angles. For bilayers with a 1:1 ratio of lipids and sterols the peak of the angle distribution is shifted to higher angles and the distribution narrows slightly. The reason for this variable behaviour
is explained in part by Figure 4. As mentioned previously, the headgroups of DOPC act as “umbrella” for a sterol molecule, resulting in the $P^{-}\rightarrow N^{+}$ dipole moment of the DOPC molecule acting as an umbrella being almost parallel to the plane of the bilayer. At high sterol concentrations the majority of DOPC molecules act as umbrellas, resulting in the angle distribution being shifted towards 90°. At lower sterol concentrations only a few lipid molecules will be acting as umbrellas, but the reduced APL of the lipids result in the headgroups of the remaining lipids tending to lie more out of the plane of the bilayer, thus reducing the peak of the angle distribution.

**DOPC/β-sitosterol bilayers in DMSO**

In the presence of DMSO a pure DOPC bilayer expands laterally, while thinning normal to the plane of the bilayer. If the concentration of DMSO is high enough (~15-20 mol %) then pores spontaneously form in the bilayer structure. These pores are stable on the timescales reached by molecular simulations. However, at a 25 mol % DMSO the pores become so large that the structure of the membrane is weakened to such extent that the bilayer falls apart.

In the case of mixed DOPC-β-sitosterol bilayers the presence of DMSO causes the bilayers to expanded laterally to the plane of the bilayer. The lateral area of the membrane, the relative increase in the lateral area and the thickness of the membrane are given in Table 4. This matches the behaviour of 20% cholesterol bilayers. At low concentrations (2.5 mol %) the relative increase in the lateral area of the bilayer appears to be largely independent of the amount of β-sitosterol in the membrane. The S10 and S33 systems show a similar ~9% increase in the area of the membrane compared to that of the pure DOPC bilayer. For the S40 and S50 bilayers the increase is slightly lower and the degree
of membrane thinning is likewise reduced slightly. At DMSO concentrations of 25 mol % the effect of the sterol presence in the bilayer is seen more clearly. As mentioned above, a solution of 25 mol % DMSO will cause a pure DOPC bilayer to be destroyed. In contrast, the bilayers containing \( \beta \)-sitosterol show greater resistance to the effects of DMSO. In the case of S10 the bilayer thins to such extent that a pore is able to form in the bilayer but the system is stable on the 300 ns timescale simulated. The S33 and S50 systems experience significant thinning and are accompanied by a 39 and 30 % lateral expansion, respectively, but no pores are formed in the bilayers (for comparison the relative increase in the lateral area of a pure DOPC bilayer in 12.5 mol % DMSO is 32 %). De Ménorval et al. found that 15.0 mol % DMSO was a sufficiently high concentration to cause the formation of pores in a 80/20% DOPC/cholesterol bilayer.\(^{33}\) Due to the different force-fields and temperatures used, it is difficult to compare that result with our results directly but the findings of both studies are consistent.

The density profiles of the membranes are shown in Figure 8, and snapshots of the S10 and S50 systems are shown in Figure 9. For the 2.5 mol % DMSO solution there is a build-up of DMSO at the surface of the bilayer. The density profiles of the lipids and sterols are also affected by the presence of DMSO, with the lipid density profiles becoming less well defined, indicating that the membrane is becoming less well ordered. The sterol density profile not only becomes less well defined, with peaks tending to merge together, but also show a greater asymmetry than the profiles of the same systems in water. The reason for the greater asymmetry of the profiles is due to the presence of DMSO increasing the tendency of sterol molecules to move from one leaflet to the other, resulting in the possibility that the number of sterol molecules in each leaflet may no longer be equal. While such behaviour is also seen in systems where no DMSO
is present, it occurs only rarely, as shown in Table 5, and does not occur at 303 K. The mechanism by which the sterol molecules move between leaflets involves the tail of the sterol molecule remaining in the centre of the bilayer while the head moves downwards so that the sterol molecule lies parallel to the plane of the bilayer, leading to the head diffusing into the other leaflet. Overall the molecule “flips” from one leaflet to the other, as shown in Figure 10. These flips are generally quite fast taking only a few nanoseconds; however, a \( \beta \)-sitosterol molecule occasionally becomes trapped for a while between the two leaflets and may remain there in a horizontal position for some time before moving into a leaflet. The majority of flips occur between the two leaflets but in a small minority of cases a sterol molecule will move from a leaflet to the centre of the bilayer before reinserting itself into the leaflet it came from but at a different place. This mechanism is in agreement with the results of both atomistic\(^{54,59}\) and course-grained simulations of PC-cholesterol bilayers.\(^{56–58}\)

However, it is inconsistent with the pathway for sterol flipping determined from a recent theoretical study which used an implicit description of the (DPPC) membrane,\(^{78}\) where it was proposed that the rotation and translation of the sterol molecules occurred in a two-stage process, and that the movement of a sterol molecule between the leaflets would be perpendicular to the plane of the bilayer. The explanation of the inconsistency between theory and simulation could be due to a number of factors, in particular the sterol concentration (the theoretical work was done for membranes at very low sterol concentrations) and the degree of unsaturation of the phospholipid. Another coarse-grained study showed that increasing unsaturation increased the residence time of cholesterol in the membrane interior during flip-flops.\(^{56}\) For the 25.0 mol % DMSO solution the weakening of the membrane becomes more apparent. For the S10 system the formation of a pore is clearly illustrated by the density profile of water becoming
non-zero at the centre of the bilayer. In the case of S50, while the membrane has become significantly thinned and disordered, the characteristic peak and trough in the lipid density profile is still present. In 25.0 mol % DMSO solution the rate of sterol flips increases significantly, and allows the observation that as the sterol concentration in the membrane increases the relative number of flips decreases. In the case of the S10 system the formation of the pore means that in addition to the flipping of sterol molecules between the leaflets the DOPC molecules are also able to flip, this behaviour is consistent with the flipping of phospholipids in bilayers containing no sterols in which a pore has formed.26

As in the case of pure DOPC bilayers, the lateral expansion of the membrane reduces the ordering of the lipid tails, as shown in Figure 11. The presence of the sterol molecules again counteracts the effect of DMSO to some extent. This is more apparent at 25.0 mol % DMSO where, although the ordering has been reduced substantially, the sterol molecules still manage to keep the lipid tails ordered to some extent. Part of the reason for this decrease in the order of the acyl tails is that the increased undulations of a bilayer solvated in a solution of DMSO result in the close interactions of the sterol molecules with the DOPC molecules being disturbed. As discussed above, in water DOPC molecules can act as umbrellas to the sterol molecules, which imposes an ordering effect on the acyl tails of the lipids. As DMSO disorders the bilayer, the interaction of the DOPC with the \( \beta \)-sitosterol is hindered and the umbrella structure is weakened, which in turn weakens the ability of the \( \beta \)-sitosterol molecules to order the acyl chains. The disordering of the membrane also affects the sterols. Figure 12 shows the distribution of the tilt angles of the sterols for the bilayers solvated in DMSO. The distributions of the tilt angles have become noticeably broader due to the fact that the sterol molecules tend to lie at more of an angle to the bilayer.
In pure DOPC bilayers DMSO molecules are able to diffuse across the bilayer relatively easily. In 2.5 mol % DMSO solution the free energy barrier for a molecule to diffuse across the bilayer is \( \approx 14 \text{ kJ mol}^{-1} \). For the bilayers containing \( \beta \)-sitosterol DMSO molecules are still observed crossing the bilayer (even for a bilayer containing 50% sterol), but the frequency of such crossings is reduced significantly for sterol concentrations greater than 10%, as shown in Table 6. In addition to the sterols making it more difficult for DMSO to cross the bilayer, they also change the mechanism of diffusion of DMSO through the membrane.

For a pure DOPC bilayer the maximum of the free energy barrier for a DMSO molecule to cross through the bilayer is located at the centre of the bilayer, where the hydrophobic tails of the lipids are. This is also true for bilayers with only a small amount of sterol; however, for bilayers with a high sterol concentration, DMSO molecules that cross the bilayer can be seen to spend some time in the centre of the membrane, (as shown in Figure 13) indicating that the centre of the bilayer can no longer be at the maximum in the free energy profile. The reason for this is that, as the sterol concentration increases, the interdigitation between the tails of the lipid and the sterol molecules in the two leaflets is reduced substantially. This means that the centre of the bilayer becomes a local minimum between two maxima where the density of the hydrophobic tails is highest.

In addition to the simulations of single bilayers in solutions of DMSO, simulations of systems containing two bilayers were performed. In these simulations a DMSO concentration gradient was established by having one of the chambers (A) separating the bilayers contain a solution of 2.5 mol % DMSO and the other chamber (B) contain only water. The full details of the setup and findings have been described in detail previously for pure DOPC bilayers. \( \text{Double bilayer} \)}
Simulations of 2:1 DOPC-β-sitosterol and 1:1 DOPC-β-sitosterol systems have been performed.

Figure 14 shows the number of DMSO and water molecules present in chamber A over 300 ns. On the timescale simulated none of the systems reached equilibrium but the diffusion of molecules across the bilayers was observed. For all three systems, pure DOPC, 33% and 50% β-sitosterol, there is a flow of DMSO molecules from chamber A to chamber B and the flow of water molecules occurs in the opposite direction. The presence of sterol molecules in the bilayer strongly reduces the rate of the flow of both DMSO and water molecules: the greater the sterol concentration in the bilayer the greater the reduction in the rate of flow.

These simulations show that DOPC bilayers containing sterols are better able to withstand the deleterious effects of DMSO. Experimentally it has been shown that subjecting plants to cold temperatures can increase the sterol content of their cell membranes. Thus, our work helps to explain how cold-acclimation may assist in reducing the damage to the tissues caused by cryosolutions during cryopreservation through the changes that occur to the composition of cell membranes.

**Conclusion**

Simulations of mixed DOPC-sterol bilayers have been performed at different sterol concentrations, different temperatures and a variety of DMSO concentrations. Comparison of bilayers containing β-sitosterol with those containing cholesterol showed that the differences between such systems were minor. In both cases the findings of previous phospholipid-sterol simulations - that sterols cause the condensing of the phospholipids - were confirmed. The APL of DOPC-
$\beta$-sitosterol decreases with increasing sterol concentration, up to the point where one third of the bilayer is $\beta$-sitosterol, with the APL remaining constant above this point. The presence of sterols in the bilayer also causes the membrane to thicken and leads to an increase in the ordering of the lipid acyl-chains. While increasing the temperature caused the APL to increase, $D_{HH}$ to decrease and $S_{CD}$ to decrease, no phase change was observed for any of the systems simulated. The diffusion of sterol molecules from one leaflet to the other was affected when the bilayers were simulated at 350 K. At 303 K no sterol molecules were observed to “flip” between leaflets on the timescales simulated. At 350 K, while such flipping was still a rare event (~1 flip in 100 ns), it was observed.

At low DMSO concentrations the mixed bilayers behaved in a similar manner to pure DOPC bilayers, with the bilayer expanding laterally while concurrently thinning transversely. The relative increase in bilayer area was approximately the same for bilayers with $\geq 33\%$ $\beta$-sitosterol as for pure DOPC bilayers. Above this point the relative increase in APL was reduced for the sterol-containing bilayers compared with the pure DOPC bilayers. The build up of DMSO at the surface of the bilayer caused the membrane structure to become less well defined. At high DMSO concentrations (25.0 mol %) the pure DOPC membrane are destroyed. By contrast, even a small amount of $\beta$-sitosterol (10%) provided enough rigidity such that the bilayer was able to maintain its integrity, although a pore forms in the bilayer. Increasing the amount of $\beta$-sitosterol stopped the formation of pores (on the 300 ns timescale simulated) and significantly reduced the deleterious effects of DMSO upon the bilayers.

The lateral expansion of the bilayer causes the acyl-tails of the lipids to become more disordered and the $\beta$-sitosterol molecules to lie at more of an angle to the normal of the bilayer. The presence of DMSO also caused an increase in the number of sterol flips. As in the case of pure DOPC bilayers, DMSO
molecules are observed to diffuse across the bilayers, but at a much lower rate, indicating that the energy barrier to diffusion increases with the amount of sterol present within the bilayer. Despite the same relative increase in the APL for 2:1 DOPC-β-sitosterol bilayers and pure DOPC bilayers, the rate of diffusion of DMSO was significantly reduced in the former compared to the latter, suggesting that “looseness” of the bilayer does not play a major role in determining the height of the energy barrier to the diffusion of molecules. The presence of β-sitosterol not only appeared to increase the energy barrier for DMSO molecules to cross the bilayer but also modifies the mechanism of diffusion of DMSO across the bilayer. In pure DOPC or bilayers with 10% β-sitosterol the DMSO molecules cross the hydrophobic tail region of the bilayer (where the maximum of the energy barrier is) in a single jump. At β-sitosterol concentrations of ≥ 33% the DMSO molecules diffuse across each leaflet in a separate jump. This change in behaviour is due to the fact that as the concentration of β-sitosterol increases the interdigitation of the two leaflets is reduced. Double bilayer simulations confirmed that the rate of diffusion of both DMSO and water molecules is not only reduced at equilibrium but also when a concentration gradient is present in the system.

The findings of this work also reveal how the cold-acclimation of plants may reduce the damage that DMSO causes to cells during cryopreservation, by increasing the sterol concentration of cell membranes and thus making these lipid bilayers more resistant to the effects of DMSO.

Acknowledgements

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References


[34] McKersie, B. D.; Thompson, J. E. Plant Physiol. 1979, 63, 802–805.


<table>
<thead>
<tr>
<th>System</th>
<th>Sterol</th>
<th>% sterol</th>
<th># Lipids</th>
<th># Sterols</th>
<th>mol. % DMSO</th>
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<tbody>
<tr>
<td>DOPC</td>
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<td>128</td>
<td>0</td>
<td>0, 2.5, 25.0</td>
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<tr>
<td>S10</td>
<td>β-sitosterol</td>
<td>10</td>
<td>116</td>
<td>12</td>
<td>0, 2.5, 25.0</td>
</tr>
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<td>S33</td>
<td>β-sitosterol</td>
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<td>42</td>
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<td>β-sitosterol</td>
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<td>78</td>
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<td>0, 2.5</td>
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<td>β-sitosterol</td>
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<td>64</td>
<td>64</td>
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<tr>
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<td>cholesterol</td>
<td>50</td>
<td>64</td>
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Table 1: Details of the different systems simulated.
<table>
<thead>
<tr>
<th>System</th>
<th>Area per lipid / nm²</th>
<th>303K</th>
<th>350K</th>
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<tbody>
<tr>
<td>DOPC</td>
<td>0.653 ± 0.010</td>
<td>0.683 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>S10</td>
<td>0.622 ± 0.008</td>
<td>0.659 ± 0.011</td>
<td></td>
</tr>
<tr>
<td>S33</td>
<td>0.587 ± 0.008</td>
<td>0.603 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>S40</td>
<td>0.594 ± 0.007</td>
<td>0.596 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>S50</td>
<td>0.565 ± 0.007</td>
<td>0.594 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>S50R</td>
<td>0.566 ± 0.007</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>C10</td>
<td>0.619 ± 0.014</td>
<td>0.683 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>C50</td>
<td>0.554 ± 0.005</td>
<td>0.588 ± 0.011</td>
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Table 2: Area per lipid for the different systems at 303K and 350K.
<table>
<thead>
<tr>
<th>System</th>
<th>$D_{HH}$ / nm</th>
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</tr>
<tr>
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<td>3.37</td>
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<td>S33</td>
<td>3.83</td>
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<td>S40</td>
<td>3.97</td>
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<td>S50</td>
<td>3.98</td>
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<td>4.00</td>
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<tr>
<td>C10</td>
<td>3.38</td>
</tr>
<tr>
<td>C50</td>
<td>3.99</td>
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Table 3: Membrane thickness, $D_{HH}$, determined from the distance between the peaks of the lipid density profile.
Table 4: Lateral area of and the relative area and thickness of DOPC-β-sitosterol bilayers in the presence of DMSO compared against the same bilayers in water.

<table>
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<th>System</th>
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<th>25.0 mol % DMSO</th>
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<td></td>
<td>Area /nm²</td>
<td>% Area</td>
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<tr>
<td>DOPC</td>
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</tr>
<tr>
<td>S10</td>
<td>44.2 ± 0.8</td>
<td>109</td>
</tr>
<tr>
<td>S33</td>
<td>36.0 ± 0.6</td>
<td>109</td>
</tr>
<tr>
<td>S40</td>
<td>33.5 ± 0.4</td>
<td>106</td>
</tr>
<tr>
<td>S50</td>
<td>31.1 ± 0.4</td>
<td>105</td>
</tr>
<tr>
<td>System</td>
<td>Number of sterol flips</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Water (303 K)</td>
<td>Water (350 K)</td>
</tr>
<tr>
<td>S10</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>S33</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>S40</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>S50</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>C10</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>C50</td>
<td>0</td>
<td>3</td>
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Table 5: Number of sterol flips for the different bilayers under the different simulation conditions.
<table>
<thead>
<tr>
<th>System</th>
<th>DMSO concentration /mol %</th>
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<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
</tr>
<tr>
<td>DOPC</td>
<td>14 Destroyed</td>
</tr>
<tr>
<td>S10</td>
<td>14 Pore</td>
</tr>
<tr>
<td>S33</td>
<td>7 93</td>
</tr>
<tr>
<td>S40</td>
<td>2 -</td>
</tr>
<tr>
<td>S50</td>
<td>3 34</td>
</tr>
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</table>

Table 6: Number of DMSO molecules that cross the bilayer in a 60 ns period.
Figure Captions

Figure 1: The molecular structure of (a) \(\beta\)-sitosterol and (b) cholesterol.

Figure 2: The area of the bilayers at 303K over the last 100 ns of the simulations.

Figure 3: Density profiles for the different bilayer systems (a) S10, (b) S33, (c) S50, (d) S50R, (e) C10 and (f) C50.

Figure 4: Snapshot of two DOPC molecules forming an “umbrella” over a \(\beta\)-sitosterol molecule, taken from the simulation of the S33 bilayer at 350K.

Figure 5: Order parameters of DOPC molecules in the bilayers (a) \(sn\)-1 chain at 303K, (b) \(sn\)-2 chain at 303K, (c) \(sn\)-1 chain at 350K and (d) \(sn\)-2 chain at 350K.

Figure 6: Tilt angles of the sterol molecules with the plane of the bilayer. (a) sterol head at 303K, (b) sterol head at 350K, (c) sterol tail at 303K and (d) sterol tail at 350K.

Figure 7: The probability distribution function of the angle between the bilayer normal, directed away from the middle of the bilayer, and the vector between the \(P\rightarrow N\) atoms in the lipid headgroup for simulations of the DOPC molecules at (a) 303K and (b) 350K in water.

Figure 8: Density profiles for the bilayer systems (a) DOPC, (b) S10, (c) S33, (d) S50 at 350K and 2.5 mol % DMSO, (e) S10 and (f) S50 at 25.0 % mol DMSO.

Figure 9: Snapshots of mixed lipid-sterol bilayers in DMSO, (a) S10 in 2.5 mol % DMSO, (b) S10 in 25.0 mol % DMSO, (c) S50 in 2.5 mol % DMSO and (d) S50 in 25.0 mol % DMSO. The DOPC phosphate headgroups, DOPC tails, \(\beta\)-sitosterol, water and DMSO are coloured red, grey, green, blue and yellow, respectively.

Figure 10: Snapshots from the S10 system at 350K showing how sterol molecules flip from one leaflet to the opposite, taken at (a) 91.6, (b) 94.6, (c) 95.3, (d) 96.1, (e) 96.6 and (f) 100.0 ns. The DOPC/\(\beta\)-sitosterol are coloured grey, the water molecules, phosphate headgroups and \(\beta\)-sitosterol molecule undergoing the “flip” are coloured blue, purple/red and yellow, respectively.

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**Figure 12:** Tilt angles of the sterol molecules with the plane of the bilayer (a) sterol head in 2.5 mol % DMSO, (b) sterol head in 25.0 mol % DMSO, (c) sterol tail in 2.5 mol % DMSO and (d) sterol tail in 25.0 mol % DMSO.

**Figure 13:** The trajectories along the z-axis (normal to the bilayers) of DMSO molecules that diffuse through the S50 bilayer (shown by the blue and green lines), taken from 40 ns simulation of a system at a DMSO concentration of 2.5 mol % at 350K. The red lines indicate the positions of the phosphate groups.

**Figure 14:** The number of (a) DMSO and (b) water molecules in chamber A (the high DMSO concentration chamber) of the double bilayer simulations as a function of simulation time.
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Table of Contents Figure: Snapshot of two DOPC molecules forming an “umbrella” over a \( \beta \)-sitosterol molecule (left). A snapshot of a 1:1 DOPC-\( \beta \)-sitosterol bilayer solvated in 2.5 mol. \% DMSO solution (right). The DOPC, \( \beta \)-sitosterol, DMSO and water molecules are coloured grey, cyan, yellow and blue, respectively.