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The Effects of Cryosolvents on DOPC–β-Sitosterol Bilayers Determined from Molecular Dynamics Simulations

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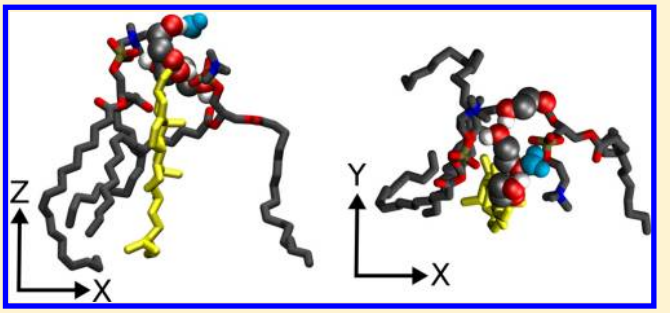
Supporting Information

ABSTRACT: Polyhydroxylated alcohols and DMSO are common cryosolvents that can damage cell membranes at sufficiently high concentrations. The interaction of representative plant cell membranes composed of mixed 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)-β-sitosterol bilayers, at a range of compositions, with a variety of cryosolvent molecules (DMSO, propylene glycol, ethylene glycol, glycerol, and methanol) has been investigated using molecular dynamics simulations. All the cryosolvents cause the bilayers to thin and become disordered; however, DMSO and propylene glycol have a greater disordering effect on the bilayer. Propylene glycol is shown to have the ability to cause the formation of pores in pure DOPC bilayers in a manner similar to that previously shown for DMSO. As the concentration of β-sitosterol within the bilayer increases, the membranes become more resistant to the deleterious effects of the cryosolvents. All three polyhydroxylated species are observed to form hydrogen bonds to multiple phospholipid molecules, effectively acting as cross-linkers, with glycerol being the most effective cross-linker. Increases in the concentration of β-sitosterol reduce overall hydrogen bonding of the bilayer with the cryosolvents as well as cross-linking, with glycerol and ethylene glycol being the most affected. The ability of all of these cryosolvents to affect the integrity of cell membranes appears to be the result of the balance of their ability to disorder lipid bilayers, to diffuse across them, and to interact with the lipid head groups.



INTRODUCTION

Cryopreservation is a process whereby biological samples are stored in liquid nitrogen (at 77 K), resulting in all biological, chemical, and physical processes occurring within the sample being effectively suspended.[1−7] This in principle enables cryopreserved samples to be stored for decades without any changes/decay occurring in the sample, and when the sample is needed again, it can be reheated and will still be viable. One difficulty in cryopreservation is that the supercooling and/or reheating of the biological samples can cause damage to cells and tissues through the formation of intra- and extra-cellular ice crystals, cell desiccation, and deleterious phase changes to the cell membranes. In an attempt to combat these problems, a number of cosolvents, so-called cryosolvents, are often added to the biological samples to reduce water content and promote the vitrification of water, thus avoiding the deleterious formation of ice.[8−12] However, these cryosolvents can themselves damage cell membranes at the high concentrations at which they are used.

Molecular dynamics (MD) simulations of penetrative cryosolvents such as DMSO, ethylene glycol, propylene glycol, and glycerol have shown both similarities and differences in the way such molecules interact with cell membrane model lipid bilayers.[13−23] All simulations show a buildup of cryosolvent molecules at the surface of the bilayer. In the majority of studies, this buildup occurs at the same time as an expansion of the bilayer parallel to the plane of the membrane and a thinning of the bilayer normal to the plane. The overall consequence of this is that the bilayer becomes disordered. Sufficiently high concentrations of DMSO have been observed to cause the formation of pores in the bilayer, and if the concentration is increased further, then the bilayer is destroyed.[16,20,21] The creation of these pores reduces the energy barrier to both lipid translocation and the diffusion of salt ions across the bilayer.[17,24,25] DMSO itself has been shown to readily diffuse across a variety of bilayers.[20,21,23] The effect of alcohols is not as clear with some contradictory results, but the degree of membrane expansion is lower,[18,19] except in the case of propylene glycol which exhibits an effect comparable to DMSO.[22] No formation of pores has been observed with ethylene glycol, propylene glycol, or glycerol at concentrations comparable to those which cause pores to form in bilayers solvated in DMSO.

The majority of the studies have investigated the interaction of cryosolvent molecules with phosphocholine (PC) bilayers, but such model membranes can differ significantly from the real

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cell membranes of plants and animals, which contain a variety of different phospholipid and sterol molecules, as well as proteins. Cell membranes in plants have phospholipids with a higher degree of unsaturation than in animal cells, with by far the most abundant sterols being β-sitosterol and stigmasterol (as opposed to cholesterol in animal cells).[26−30] The phospholipid−sterol ratio of plant membranes can vary considerably.[29] The effect of sterols on the properties of cell membranes has been investigated experimentally in some detail.[26,27,29,31−36] The presence of sterols makes a membrane more ordered and plays an important role in determining the fluidity and mechanical properties of a membrane. In bilayers containing certain sterol species (including cholesterol and β-sitosterol) at sufficiently high concentrations, the transition between the liquid-crystalline (liquid-disordered, l_d) phase and the gel (or solid-ordered, s_o) phase is eliminated, and instead, the bilayers are in a fluid phase over a wide temperature range (280−320 K).[36] There have been a considerable number of computational studies that have determined the effect of sterols upon fully hydrated lipid bilayers.[37−53] The general conclusions of these simulations have been that the incorporation of sterol molecules into a bilayer has a condensing effect on the phospholipids, causing the area per lipid to decrease. At the same time, the membrane is thickened and made more rigid.

Recent efforts have aimed at improving the representation of model plant cell membranes by simulating systems containing 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and β-sitosterol. As the ratio of sterols to lipids in plant cells is not well characterized and can vary considerably,[31] a number of different ratios have been simulated in order to determine how increasing the concentration of sterol in a hydrated DOPC bilayer would affect the damaging effects of DMSO. A recent study[23] has shown that bilayers containing both DOPC and β-sitosterol solvated in aqueous solutions of DMSO behave in a qualitatively similar manner to pure DOPC and DPPC bilayers.[20] The bilayer expands laterally to the plane of the membrane and thins normally to the plane of the membrane. At the same time, the bilayer becomes more disordered and irregular. However, the presence of β-sitosterol makes the bilayers more resistant to the deleterious effects of DMSO, with the bilayers being able to withstand DMSO concentrations that would otherwise destroy pure DOPC bilayers, as well as a reduction in the rate of diffusion of DMSO molecules across the bilayer.

The aim of the present work is to use MD simulations to study for the first time the interaction of various hydrated binary DOPC−β-sitosterol bilayers with DMSO, methanol, ethylene glycol, propylene glycol, and glycerol, all species that are used experimentally in the cryopreservation of plant tissues.[10] The actions of DMSO on lipid bilayers are beginning to be understood, but there has not been as much work studying the effects of polyalcohols, especially propylene glycol. Investigation of the interaction of these species with DPPC bilayers has shown that they behave in a similar manner to DMSO but to a lesser degree (except for propylene glycol, which thins and disorders the membrane to a level comparable to DMSO).[18,19,22,54] This work investigates how the incorporation of β-sitosterol into a bilayer can alter its behavior and determine how different cryosolvents interact with the bilayer in different ways, in particular looking at how the ability of alcohols to form hydrogen bonds affects the bilayers.

METHODS

As in our previous work,[20,23] we have used the GROMOS 54A7 force-field[55] for the lipids, sterols, alcohols, and DMSO. This force-field is known to reproduce a wide range of membrane properties accurately.[56−59] The water molecules were represented with the widely used SPC model.[60]

MD simulations were performed using GROMACS v.3.3.3.[61] Twin-range cutoffs were used to evaluate the nonbonded forces: interactions within 0.8 nm were calculated every time step, while interactions within 1.4 nm and the pair list were updated every five time steps. A 2 fs time step was used, and the equations of motion were integrated via the leapfrog algorithm.[62] The electrostatic interactions of the systems were calculated using the reaction-field method with a relative dielectric permittivity constant, ε_{RF}, of 62, suitable for SPC water.[60] The simulations were performed in the isothermal−isobaric ensemble with the Berendsen thermostat and barostat.[63] The bilayer (consisting of the phospholipid and β-sitosterol molecules) and the solution (consisting of the water and alcohols) were independently coupled to temperature baths with a coupling constant of 0.1 ps. The box dimensions parallel and perpendicular to the plane of the bilayer were allowed to vary independently, with the isothermal compressibility of both set to 4.6×10^{-5} bar^{-1} and a coupling constant, τ_P, of 1 ps used. The GROMOS 54A7 force-field was developed for use with a reaction-field method for computing the long-range electrostatic interactions. Moreover, it has been shown that the difference in results obtained using a reaction-field and an Ewald sum method are minor for bilayers solvated in water with the G54A7 force-field.[58] The addition of cryosolvent species is unlikely to make a significant difference, as the solution will retain a high dielectic constant.

Bilayers containing 0, 10, 33, and 50 mol % β-sitosterol were simulated, with each bilayer having a total of 128 DOPC and β-sitosterol molecules (64 molecules per leaflet). The starting configurations for the bilayers were taken from previous work, where the DOPC−β-sitosterol bilayers had been equilibrated in water for 300 ns.[20,23,64] Water molecules were randomly replaced by cryosolvent molecules, with the total number of solvent molecules kept constant at 5841 (~45.6 solvent molecules per DOPC/β-sitosterol). To enable comparison with previous work, the simulations were performed at 350 K.[13,16,20,23] The bilayers were simulated in typical 10.0 mol % solutions of the different cryosolvents. In addition, pure DOPC bilayers were simulated at a range of propylene glycol and glycerol concentrations. Propylene glycol and glycerol were simulated at multiple concentrations, as they appeared to be two representative species.[22] Table 1 gives the details of the different simulation runs performed. Each system was simulated for a total of 300 ns, with the final 60 ns used for analysis. The area of the bilayers and the order parameters of the lipids were monitored to ensure that the systems had reached equilibrium.

RESULTS AND DISCUSSION

Bilayer Structure. The average lateral area and relative area of the bilayer (compared against the values of the same bilayer in water), for different bilayers in water and in 10.0 mol % solutions of the five different cryosolvents, are shown in Table 2. Typically, in simulations of bilayers, a common property reported is the area per lipid (APL); however, it is difficult to determine the APL for these systems. Usually, in the case of binary bilayers, the method described by Hofsass[40] can be used

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Table 1. Details of the Different Systems Simulated

system	no. of lipids	no. of sterols	solution	concentration(s) (mol %)
pure DOPC	128	0	water	
			DMSO	10.0
			methanol	10.0
			ethylene glycol	10.0
			propylene glycol	2.5, 10.0, 15.0, 20.0, 25.0
			glycerol	2.5, 10.0, 25.0
10% β-sitosterol	116	12	water	
			DMSO	10.0
			glycerol	10.0
33% β-sitosterol	86	42	water	
			DMSO	10.0
			methanol	10.0
			ethylene glycol	10.0
			propylene glycol	10.0, 20.0
			glycerol	10.0
50% β-sitosterol	64	64	water	
			DMSO	10.0
			methanol	10.0
			ethylene glycol	10.0
			propylene glycol	10.0, 20.0
			glycerol	10.0

to determine the APL; however, that requires knowledge of the volume occupied by the solvent molecules. In the case of bilayers solvated in water, this is relatively trivial to calculate, but for these solutions, it would be more difficult. Table 2 hence reports the total area of the bilayers. The bilayer thickness, D_{HH} (measured as the distance between the peak heights of the DOPC density profile), for the different systems is given in Table 3. For a pure DOPC bilayer solvated in water at 303 K, the GROMOS 54A7 force-field predicts an area per lipid (APL) of 0.653 nm^2.[23] The experimental and simulation ranges of APLs at 303 K are 0.674−0.725 and 0.651−0.660 nm^2, respectively.[57] Thus, the predicted APL is slightly lower than the experimental range but compares well with other simulation values. To the authors' knowledge, there is no experimental or simulation data for DOPC at 350 K to compare against.

When no cryosolvents are present, the incorporation of β-sitosterol into DOPC bilayers causes the phospholipid molecules to condense and the lateral area of the bilayer is decreased. At the same time, the bilayer is thickened and becomes more ordered. For a pure DOPC bilayer, the interaction of DMSO and the alcohols with the lipid bilayers causes the systems to expand laterally to the plane of the bilayer. The same type of behavior is seen in the case of the four alcohols regardless of the concentration of β-sitosterol in the bilayer. It is clear, however, that, for an equivalent concentration, DMSO and propylene glycol have a greater effect on the DOPC bilayer than the other three alcohols. This difference between DMSO/propylene glycol and methanol, ethylene glycol, and glycerol was previously observed for pure DPPC bilayers.[22] The comparatively greater effect of propylene glycol/DMSO on the lateral area is also observed for the DOPC−β-sitosterol binary bilayers with a β-sitosterol concentration of 33% or less but is less clear in the case of a 1:1 DOPC−β-sitosterol bilayer. It is nonetheless clear that for a bilayer containing 50% β-sitosterol the relative effect of the cryosolvents is reduced; for example, a 10 mol % propylene glycol solution causes an expansion of ∼24% in the case of bilayers with a β-sitosterol concentration of 33% or less, whereas when the amount of DOPC and β-sitosterol is equal an expansion of only 11% is observed.

Table 3. Bilayer thickness, D_{HH}, and Relative Thickness % of the Bilayer (Compared to the Bilayer in Water) of DOPC−β-sitosterol Bilayers in the Presence of Various Cryosolvents at 10.0 mol %

	D_{HH} (nm) (relative thickness)			
solution	pure DOPC	10% β-sitosterol	33% β-sitosterol	50% β-sitosterol
water	3.11 (100)	3.22 (100)	3.62 (100)	3.92 (100)
methanol	2.77 (89)		3.24 (90)	3.68 (94)
ethylene glycol	2.78 (89)		3.33 (92)	3.68 (97)
DMSO	2.39 (77)	2.51 (78)	3.09 (85)	3.53 (90)
propylene glycol	2.31 (74)		2.96 (82)	3.77 (96)
glycerol	2.54 (82)	2.72 (84)	3.22 (89)	3.81 (97)

Density Profiles. The density profiles of the different cryosolvents with the 1:1 DOPC/β-sitosterol bilayers are shown in Figure 1 (and in the Supporting Information). The density profiles of the other bilayers with lower β-sitosterol content are effectively equivalent. The β-sitosterol molecules are positioned below the DOPC phosphate groups, and the center of the bilayer shows a trough in the density profile of both the DOPC and the β-sitosterol. As the bilayer expands laterally, it thins normal to the plane of the bilayer, while at the same time the structure of the bilayer becomes more disordered with the density profiles of the DOPC and β-sitosterol broadening out and the peak height diminishing. Consistent

Table 2. Lateral Area and Relative Area % (Compared to the Bilayer in Water) of DOPC−β-sitosterol Bilayers in the Presence of Various Cryosolvents at 10.0 mol %

	area (nm^2) (relative area)			
solution	pure DOPC	10% β-sitosterol	33% β-sitosterol	50% β-sitosterol
water	43.73 ± 0.51 (100)	40.39 ± 0.66 (100)	32.96 ± 0.38 (100)	29.63 ± 0.29 (100)
methanol	50.20 ± 0.74 (115)		38.30 ± 0.65 (116)	32.18 ± 0.47 (109)
ethylene glycol	50.87 ± 0.91 (116)		36.60 ± 0.77 (111)	31.29 ± 0.55 (106)
DMSO	56.14 ± 0.82 (128)	52.39 ± 1.10 (130)	41.50 ± 0.82 (126)	34.19 ± 0.81 (115)
propylene glycol	56.58 ± 0.93 (129)		41.04 ± 1.20 (124)	32.94 ± 0.62 (111)
glycerol	51.12 ± 0.91 (117)	47.19 ± 0.73 (117)	37.11 ± 0.72 (113)	31.04 ± 0.44 (105)

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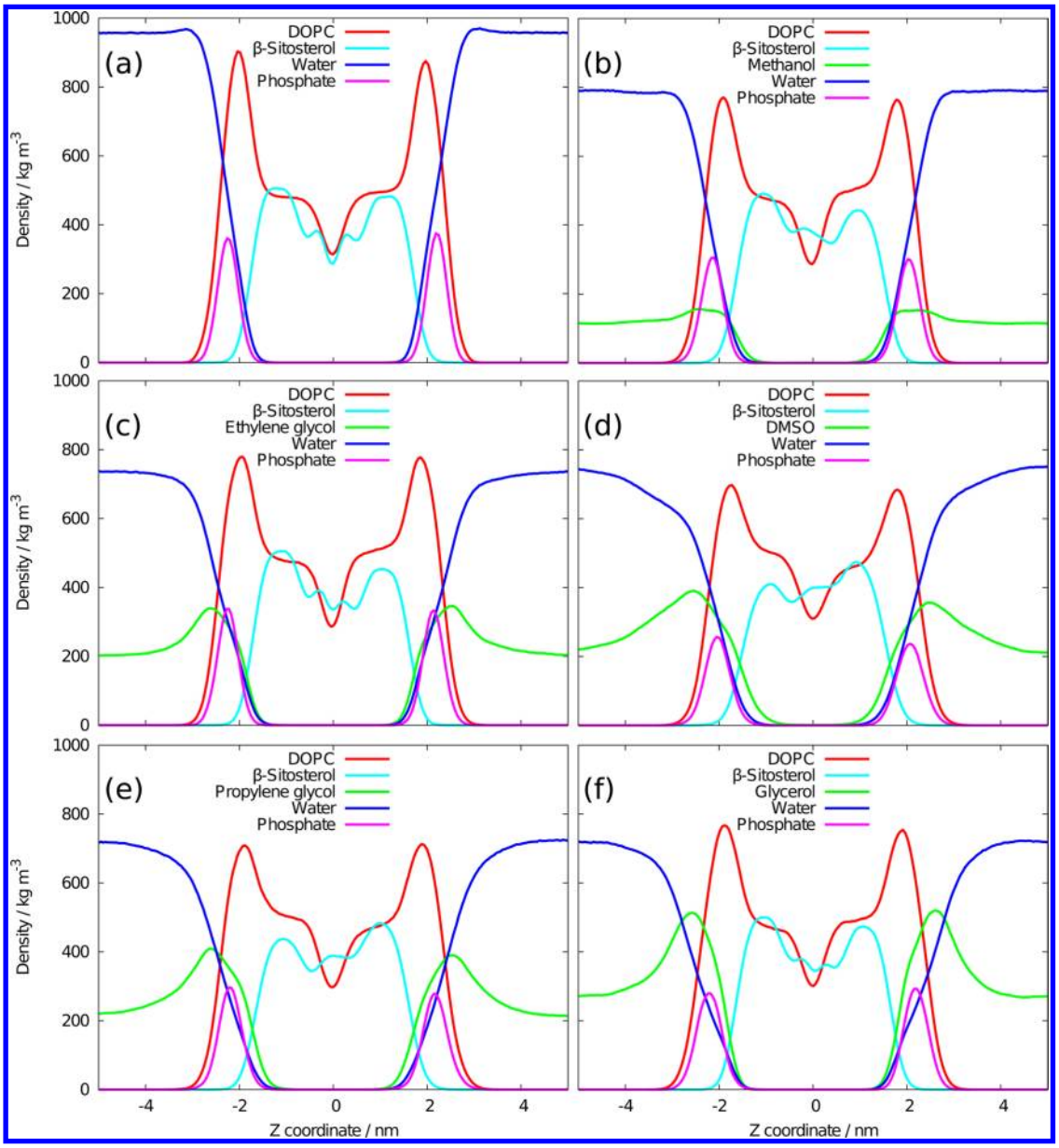

Figure 1. The density profiles of the 50% DOPC–β-sitosterol bilayer in (a) water, (b) 10.0 mol % methanol, (c) 10.0 mol % ethylene glycol, (d) 10.0 mol % DMSO, (e) 10.0 mol % propylene glycol, and (f) 10.0 mol % glycerol.

with the change in the lateral area of the bilayer, DMSO and propylene glycol have a greater effect than the other three alcohols. While a concentration of 10% β-sitosterol in the bilayer does not make much difference to the degree of thinning caused by the cryosolvents, for systems with 33 and 50% β-sitosterol, the relative change in the membrane thickness is appreciably smaller, especially for DMSO and propylene glycol.

All the cryosolvents build up at the interface of the bilayer, though to different degrees, and penetrate different depths into the bilayer. Methanol shows only a small peak in the density profile around the position of the DOPC phosphate groups and penetrates further into the bilayer than the water molecules. DMSO and propylene glycol show a significant buildup of cryosolvent molecules around the lipid head groups, with the peak of their density profiles being located just above the position of the phosphate groups. The same behavior is seen in simulations of pure DPPC and DOPC bilayers solvated in aqueous DMSO solutions, with DMSO building up at the surface of the bilayer and disordering the membrane.[20] In addition, both propylene glycol and DMSO penetrate further into the lipid bilayer than both water and methanol. Ethylene glycol and glycerol do not penetrate as far into the bilayer as methanol but show a greater buildup of molecules at the interface (glycerol to a greater degree than ethylene glycol).

The density profiles of pure DOPC bilayers in different concentrations of glycerol and propylene glycol are shown in Figure 2, and the area and bilayer thickness are given in Table 4. As expected, as the concentration of the alcohol increases, the lateral expansion of the bilayer also increases and the membrane becomes progressively thinner and more disordered. In the case of DMSO, if the membrane expands to a certain dimension, then a pore, filled with DMSO and water, forms within the bilayer. If the concentration is increased further, then the bilayer structure is destroyed.[16,20,21] At the same time, increasing the concentration of β-sitosterol within the

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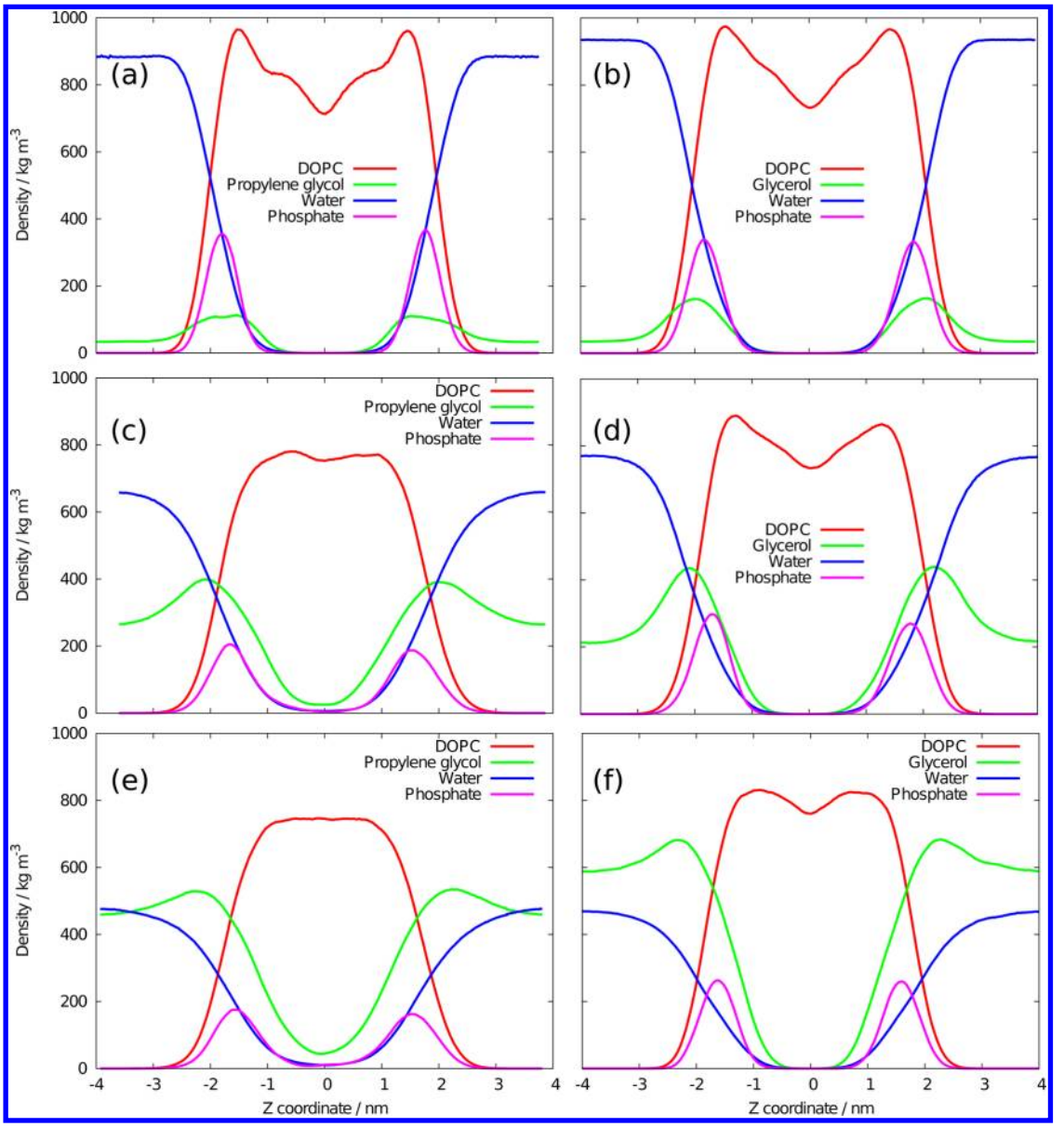

Figure 2. The density profiles of the DOPC bilayer in (a) 2.5 mol % propylene glycol, (b) 2.5 mol % glycerol, (c) 15.0 mol % propylene glycol, (d) 10.0 mol % glycerol, (e) 25.0 mol % proylene glycol, and (f) 25.0 mol % glycerol.

membrane reinforces its structure, making the formation of pores and the destruction of the membrane less likely. In the case of methanol, ethylene glycol, and glycerol at the concentrations simulated, no pore formation is observed for any of the bilayers. In the case of propylene glycol interacting with a pure DOPC bilayer, the formation of a pore is observed at a concentration of 15.0 mol %, the same concentration at which DMSO starts to cause the formation of pores. This can be seen in Figure 2c, where the density of propylene glycol at the center of the bilayer is significant, and in Figure 3 where a snapshot of the systems during the simulations is shown.

For a pure DOPC bilayer at 350 K, 25.0 mol % DMSO will cause the bilayer to fall apart. In contrast, for a pure DOPC bilayer in 25.0 mol % propylene glycol, a pore is observed to form but the bilayer is stable (on the 300 ns time scale simulated), as can be seen in Figure 3c. The density profiles also illustrate the lesser effect of glycerol in comparison with propylene glycol, as the bilayer solvated in 25.0 mol % glycerol, though considerably disordered, still possesses the characteristic trough in the center of the DOPC density profile. In contrast, with 15.0 mol % propylene glycol, any trough in the density profile has been virtually eliminated. The ability of DMSO and propylene glycol, but not the other alcohols, to cause the formation of pores in DOPC bilayers, at least at the concentrations simulated, is linked to the fact that the level of bilayer expansion and thinning that they cause is greater. Thus, they are able to cause thinning of the membrane to a point at which solvent molecules are able to form a "chain" spanning the bilayer and a pore spontaneously forms. Their ability to penetrate more deeply into the bilayer structure also enhances their ability to form pores as they are better able to interconnect across the bilayer than the other molecules simulated. The reason for the greater thinning and penetration properties of DMSO and propylene glycol is due to their greater hydrophobic character compared to methanol, ethylene glycol, and glycerol, and their peculiar hydration properties.[65−67] While still hydrophilic enough to be fully miscible in water, they are nevertheless more able to mix with the

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Table 4. Bilayer Lateral Area (and Relative Area %), Area per Lipid (APL), and Thickness, D_{HH}, of the DOPC Bilayers at Different Concentrations of Propylene Glycol and Glycerol[a]

concentration (mol %)	solution	area (nm^2) (relative area)	APL (nm^2)	D_{HH} (nm)
0	water	43.73 ± 0.51 (100)	0.683 ± 0.008	3.11
2.50	DMSO	47.81 ± 0.56 (109)	0.747 ± 0.015	2.94
	propylene glycol	47.54 ± 0.71 (109)	0.743 ± 0.011	2.99
	glycerol	46.31 ± 0.74 (106)	0.724 ± 0.012	2.90
10.00	DMSO	56.14 ± 1.02 (128)	0.877 ± 0.013	2.39
	propylene glycol	56.58 ± 0.93 (129)	0.884 ± 0.015	2.31
	glycerol	51.12 ± 0.91 (117)	0.799 ± 0.014	2.54
15.00	DMSO	pore formed		1.41
	propylene glycol	pore formed		1.44
20.00	DMSO	pore formed		0.89
	propylene glycol	pore formed		0.89
25.00	DMSO	bilayer destroyed		
	propylene glycol	pore formed		
	glycerol	56.42 ± 1.17 (129)	0.882 ± 0.018	1.58

[a]The data for the same system in DMSO was taken from ref 20.

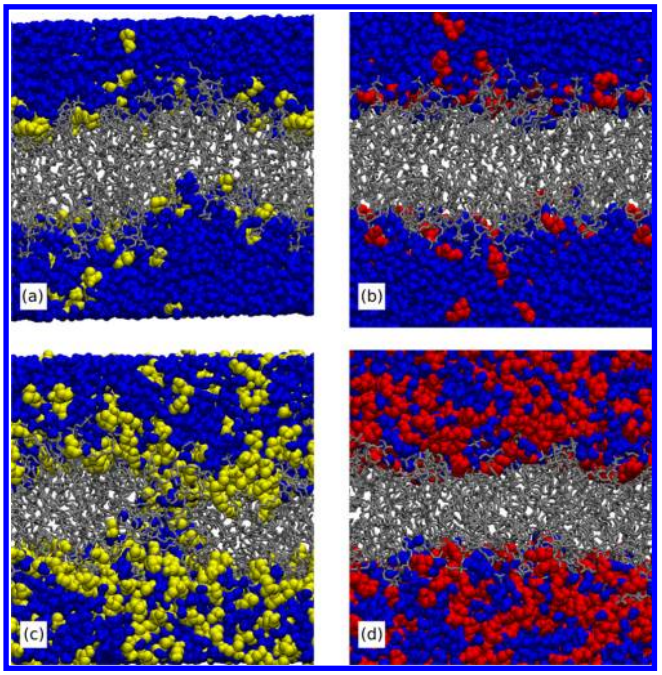

Figure 3. Snapshots of the DOPC bilayers in different solutions, (a) 2.5 mol % propylene glycol, (b) 2.5 mol % glycerol, (c) 15.0 mol % propylene glycol, and (d) 25.0 mol % glycerol.

hydrophobic parts of the lipids than the other three alcohols. These findings suggest that DMSO and propylene glycol can induce membrane damage at lower concentrations than other cryosolvents.

Order Parameters. The deuterium order parameter, S_{CD}, measures the relative orientation of C−D bonds with respect to the bilayer normal and provides a way of quantifying the ordering of the phopholipids acyl tails. S_{CD} is calculated from

$$S_{CD} = \frac{1}{2}\langle 3\cos^2\theta - 1\rangle \quad (1)$$

where θ 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 neighboring carbons and assuming a tetrahedral geometry of the methylenes (except in the case of the unsaturated carbons, where a planar geometry is assumed). Figure 4 shows the values of the order parameters of the *sn*-1 chain of the DOPC molecules in each of the four bilayer systems in pure water and in the different 10.0 mol % solutions of cryosolvents. The behavior of the *sn*-2 chain (not shown) is equivalent. It can be seen that the acyl tails of the DOPC molecules become more ordered as the amount of β-sitosterol in the bilayer increases. The effect on membrane expansion of the cryosolvents causes a decrease in the order parameters of the oleoyl chains. As DMSO and propylene glycol each cause the membrane to expand to a greater extent than the other three alcohols, they also induce a greater reduction in the ordering of the acyl tails. Figure 5 shows the order parameters for the pure DOPC bilayers solvated in different concentrations of propylene glycol and glycerol. The acyl tails of the bilayers solvated in propylene glycol show greater disorder than the bilayers solvated in the same concentration of glycerol. As the concentration of propylene glycol/glycerol is increased, the tails become progressively more disordered.

Bilayer−Water Interactions. The predicted water coordination numbers (the average number of water molecules in the first solvation shells) for the choline group and the sum of the two glycerol groups of DOPC, as well as the hydroxyl group of β-sitosterol, are reported in Table 5. The cutoff distance of the first solvation shell was the location of the first minimum in the radial distribution functions: 0.62 nm for the choline group and 0.34 nm for both the glycerol and hydroxyl groups. For the bilayers solvated in pure water, as the amount of β-sitosterol in the bilayer is increased, there is a small but significant increase in the water coordination number of the choline group. This is probably due to the fact that, as the β-sitosterol molecules lie below the head groups of the phospholipids, the choline groups become more exposed to the solvent. When the bilayers are solvated in aqueous cryosolvent solutions, DMSO and the alcohols build up at the surface of the bilayer, as discussed above, displacing water molecules and thus lowering the number of water molecules in the first solvation shell of the choline and glycerol groups and, to a lesser extent, the hydroxyl group. For the glycerol groups, the reduction in the coordination number is approximately the same for all the alcohol species (~1.4 water molecules), but DMSO displaces fewer water molecules (~0.4). The reduced ability of DMSO to

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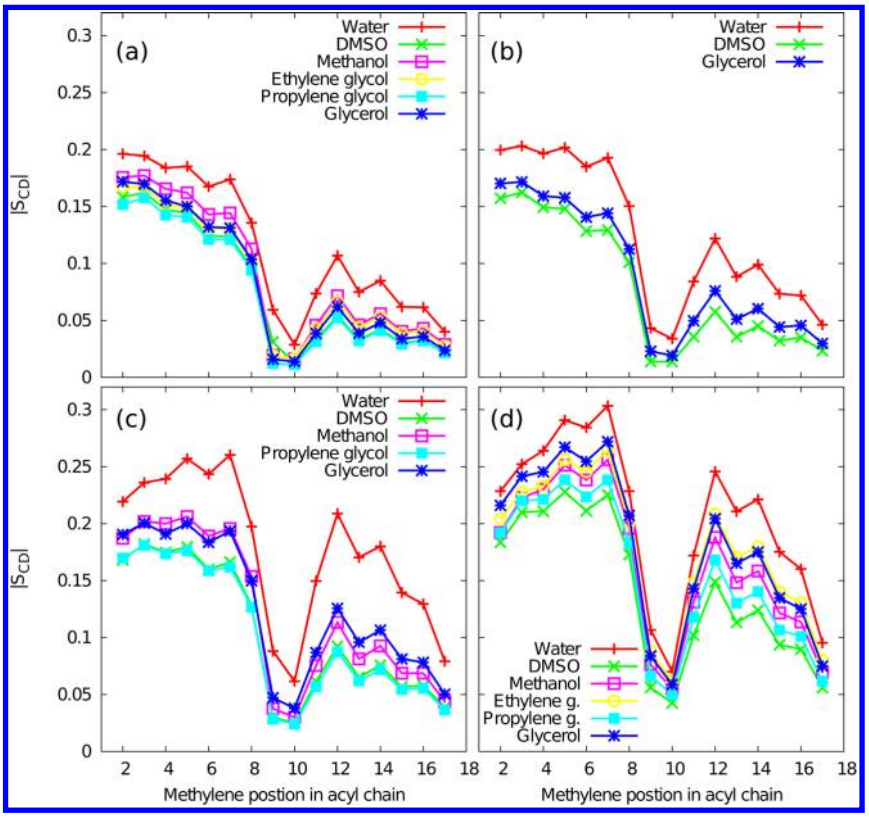

Figure 4. Order parameters of the *sn*-1 chain of the DOPC molecules in the bilayers of (a) pure DOPC, (b) 10% *β*-sitosterol, (c) 33% *β*-sitosterol, and (d) 50% *β*-sitosterol.

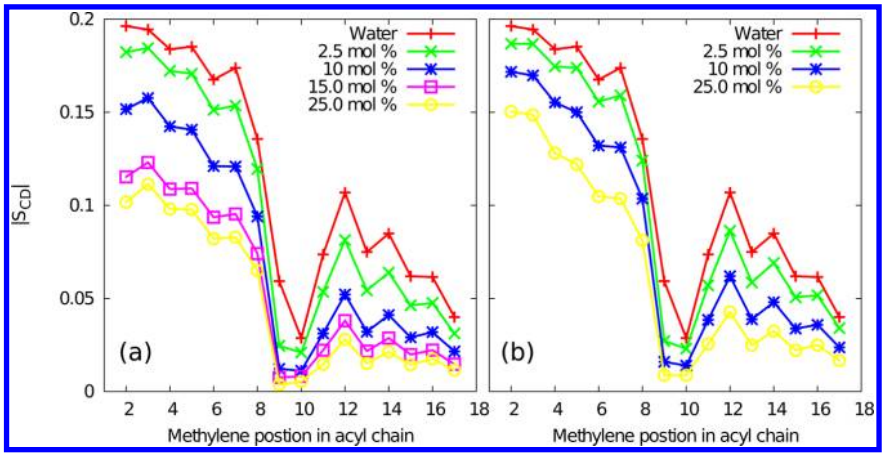

Figure 5. Order parameters of the *sn*-1 chain of the DOPC molecules in the DOPC bilayers in solutions of (a) propylene glycol and (b) glycerol at a number of different concentrations.

replace water molecules compared to the alcohols is likely to be a consequence of the fact that DMSO cannot act as a hydrogen bond donor (see below), resulting in a relatively lower number of DMSO molecules in the first hydration shell. The reduction in the coordination number of the glycerol groups of the lipids is largely the same regardless of the *β*-sitosterol concentration (indicating that the dehydration is not caused by the reduction in the lateral area of the bilayer but the buildup of cryosolvent molecules). In the case of the choline group, different alcohols dehydrate this group to different degrees. In general, the degree of dehydration is glycerol > propylene glycol > DMSO/ethylene glycol > methanol, roughly suggesting that the larger the cryosolvent molecule the more water molecules are displaced from the solvation shell of the choline group. The above order does not vary for the alcohols as the amount of *β*-sitosterol in the bilayer increases. In the case of DMSO, there might be a greater dehydrating effect of the choline group in bilayers with a high *β*-sitosterol concentration (≥33%) than in bilayers with a low *β*-sitosterol concentration; however, because of the level of the fluctuations, it is difficult to determine whether this is a real effect or simply statistical variation.

Hydrogen Bonding Analysis. The average numbers of hydrogen bonds formed between the different groups of each DOPC and *β*-sitosterol molecule are given in Table 6. The buildup of the cryosolvents at the bilayer interface translates into the number of hydrogen bonds between the DOPC/*β*-sitosterol groups and water molecules being reduced. The average number of hydrogen bonds formed between each

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Table 5. Coordination Number of Water Molecules to the Choline and Glycerol Groups (Sum of Both Glycerol Groups) of DOPC and the Hydroxyl Group of β-Sitosterol[a]

group	solution	pure DOPC	10% β-sitosterol	33% β-sitosterol	50% β-sitosterol
choline	water	16.2	16.2	16.8	17.5
	methanol	13.9		13.3	13.7
	ethylene glycol	11.6		11.2	11.8
	DMSO	12.3	12.3	11.9	11.9
	propylene glycol	11.1		10.5	10.3
	glycerol	9.6	9.7	9.4	9.6
glycerol	water	2.5	2.4	2.5	2.7
	methanol	1.3		1.2	1.3
	ethylene glycol	1.6		1.5	1.6
	DMSO	2.2	2.2	2.1	2.1
	propylene glycol	1.5		1.4	1.4
	glycerol	1.3	1.3	1.3	1.3
hydroxyl	water		1.0	1.1	1.4
	methanol			0.9	1.0
	ethylene glycol			0.8	1.0
	DMSO		0.9	0.9	1.0
	propylene glycol			0.8	0.8
	glycerol		0.7	0.8	0.8

[a]All systems have a cryosolvent concentration of 10.0 mol %.

group in the bilayer and the cryosolvent molecules (Table 7) shows that the overall number of hydrogen bonds between the DOPC/β-sitosterol groups and the cryosolvent molecule remains approximately constant (except in the case of DMSO) but that hydrogen bonds to water molecules are replaced by hydrogen bonds to the alcohols.

Due to the fact that DMSO is not able to be a hydrogen bond donor, it is unable to form hydrogen bonds with the phosphate or glycerol groups of DOPC (although it can form a hydrogen bond with the β-sitosterol hydroxyl group). However, it still causes a decrease in the number of hydrogen bonds formed between water and the lipid/sterol groups by displacing water molecules from the vicinity of the phosphate/glycerol/hydroxyl groups. Thus, it differs from the alcohols in making the DOPC/β-sitosterol molecules in the bilayer "hydrogen bond deficient" with respect to the bilayers solvated in water and aqueous alcohol solutions. For the alcohols, the ability to replace water is greatest in the case of glycerol, followed by propylene glycol, ethylene glycol, and methanol, in that order.

As previously shown in the case of a pure DPPC bilayer, the polyhydroxylated alcohols (ethylene glycol, propylene glycol, and glycerol) are able to form multiple hydrogen bonds to the lipid headgroups.[22] This allows them to bond to two lipid/sterol molecules, effectively cross-linking adjacent components of the bilayer. Table 8 reports the number of cryosolvent molecules forming two (and three in the case of glycerol) hydrogen bonds with the DOPC molecules and with the DOPC/β-sitosterol molecules, as well as the total number of hydrogen bonds between cryosolvent and DOPC−β-sitosterol molecules and the percentage of alcohol molecules that form two (or more) hydrogen bonds to the bilayer from the cryosolvent molecules that are hydrogen bonded to the bilayer, all for bilayers solvated in 10 mol % solutions. In the case of the pure DOPC bilayers, the number of molecules that form one and two hydrogen bonds to the DOPC molecules is higher for propylene glycol than for ethylene glycol. However, the percentage of molecules that form two hydrogen bonds to the bilayer is approximately the same for both cryosolvents, indicating that although propylene glycol is more likely than ethylene glycol to form a hydrogen bond to a DOPC molecule it does not display any greater tendency to cross-link DOPC molecules than ethylene glycol. In the case of glycerol, the total number of cryosolvent molecules that form one or more hydrogen bonds to the bilayer is lower than in the case of propylene glycol. Despite this, the number of molecules that form two hydrogen bonds to the bilayer is higher and, in addition, a number of molecules can form three hydrogen bonds to DOPC molecules. Thus, glycerol shows a much

Table 6. Average Number of Hydrogen Bonds between Each Phospholipid or Sterol Group and the Water Molecules[a]

group	solution	pure DOPC	10% β-sitosterol	33% β-sitosterol	50% β-sitosterol
phosphate	water	3.19 ± 0.09	3.20 ± 0.10	3.26 ± 0.12	3.31 ± 0.14
	methanol	2.70 ± 0.10		2.65 ± 0.13	2.66 ± 0.16
	ethylene glycol	2.06 ± 0.11		1.97 ± 0.14	1.97 ± 0.16
	DMSO	2.67 ± 0.09	2.66 ± 0.10	2.63 ± 0.13	2.55 ± 0.14
	propylene glycol	1.98 ± 0.11		1.86 ± 0.14	1.80 ± 0.16
	glycerol	1.54 ± 0.10	1.53 ± 0.10	1.45 ± 0.13	1.48 ± 0.14
glycerol	water	2.34 ± 0.09	2.36 ± 0.09	2.42 ± 0.12	2.56 ± 0.13
	methanol	1.93 ± 0.09		1.83 ± 0.12	1.91 ± 0.14
	ethylene glycol	1.55 ± 0.09		1.52 ± 0.12	1.59 ± 0.13
	DMSO	2.12 ± 0.09	2.09 ± 0.09	2.05 ± 0.12	2.03 ± 0.13
	propylene glycol	1.44 ± 0.09		1.43 ± 0.13	1.42 ± 0.14
	glycerol	1.27 ± 0.09	1.28 ± 0.10	1.27 ± 0.10	1.33 ± 0.13
sterol	water		0.82 ± 0.22	0.93 ± 0.11	1.14 ± 0.10
	methanol			0.78 ± 0.11	0.87 ± 0.10
	ethylene glycol			1.68 ± 0.12	0.80 ± 0.10
	DMSO		0.75 ± 0.21	0.75 ± 0.12	0.82 ± 0.10
	propylene glycol			0.66 ± 0.11	0.71 ± 0.09
	glycerol		0.59 ± 0.19	0.64 ± 0.10	0.67 ± 0.10

[a]All systems have a cryosolvent concentration of 10.0 mol %.

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Table 7. Average Number of Hydrogen Bonds between Each Phospholipid or Sterol Group and the Alcohols/DMSO[a]

group	solution	pure DOPC	10% β-sitosterol	33% β-sitosterol	50% β-sitosterol
phosphate	methanol	0.49 ± 0.06		0.58 ± 0.08	0.60 ± 0.16
	ethylene glycol	1.06 ± 0.108		1.15 ± 0.10	1.19 ± 0.12
	propylene glycol	1.25 ± 0.08		1.31 ± 0.10	1.34 ± 0.12
	glycerol	1.57 ± 0.08	1.59 ± 0.09	1.61 ± 0.10	0.68 ± 0.12
glycerol	methanol	0.50 ± 0.05		0.65 ± 0.07	0.57 ± 0.08
	ethylene glycol	0.82 ± 0.06		0.80 ± 0.08	0.82 ± 0.09
	propylene glycol	1.05 ± 0.07		0.96 ± 0.09	0.92 ± 0.11
	glycerol	1.08 ± 0.08	1.10 ± 0.08	1.08 ± 0.80	1.07 ± 0.10
sterol	methanol			0.24 ± 0.07	0.24 ± 0.06
	ethylene glycol			0.33 ± 0.08	0.41 ± 0.08
	DMSO		0.12 ± 0.09	0.12 ± 0.05	0.13 ± 0.04
	propylene glycol			0.37 ± 0.09	0.42 ± 0.07
	glycerol		0.38 ± 0.17	0.41 ± 0.08	0.48 ± 0.07

[a]All systems have a cryosolvent concentration of 10.0 mol %.

Table 8. Analysis of How Polyhydroxylated Alcohol Molecules Hydrogen Bond to the DOPC Molecules and to Both the DOPC and β-Sitosterol in the Bilayers[a]

		33% β-sitosterol		50% β-sitosterol	
solution	pure DOPC	DOPC	bilayer	DOPC	bilayer
ethylene glycol					
no. of molecules forming 1 H-bond	132 ± 8	97 ± 4	100 ± 5	86 ± 13	99 ± 12
no. of molecules forming 2 H-bonds	57 ± 6	35 ± 4	41 ± 5	20 ± 3	27 ± 3
total no. of molecules	188 ± 4	131 ± 7	140 ± 7	106 ± 12	126 ± 12
% of molecules forming 2 H-bonds	30 ± 3	26 ± 3	29 ± 4	19 ± 4	22 ± 3
propylene glycol					
no. of molecules forming 1 H-bond	164 ± 9	112 ± 4	117 ± 4	97 ± 8	108 ± 7
no. of molecules forming 2 H-bonds	68 ± 4	41 ± 4	45 ± 5	30 ± 4	38 ± 3
total no. of molecules	232 ± 6	153 ± 5	162 ± 4	127 ± 10	146 ± 9
% of molecules forming 2 H-bonds	29 ± 2	27 ± 3	28 ± 3	24 ± 3	26 ± 2
glycerol					
no. of molecules forming 1 H-bond	123 ± 7	93 ± 9	101 ± 9	80 ± 9	90 ± 9
no. of molecules forming 2 H-bonds	72 ± 3	49 ± 5	48 ± 6	35 ± 4	36 ± 3
no. of molecules forming 3 H-bonds	21 ± 1	12 ± 3	15 ± 3	6 ± 1	11 ± 2
total no. of molecules	216 ± 5	154 ± 7	165 ± 6	121 ± 9	137 ± 10
% of molecules forming 2 H-bonds	33 ± 2	23 ± 2	22 ± 3	16 ± 2	17 ± 2
% of molecules forming 3 H-bonds	10 ± 1	6 ± 1	7 ± 1	3 ± 1	5 ± 1

[a]All systems have a cryosolvent concentration of 10.0 mol %.

greater propensity to cross-link DOPC molecules than the other two alcohols due to both the additional hydroxyl group present in the molecule and the greater spacing of its 1- and 3-hydroxyl groups, allowing cross-linking to molecules at a range of distances.

In the case of bilayers containing both DOPC and β-sitosterol, the hydrogen bonding of the cryosolvent molecules to the DOPC molecules and to both the DOPC and β-sitosterol molecules has been considered. As expected, as the number of DOPC molecules present in the bilayer is reduced (i.e., the amount of β-sitosterol in the bilayer increases), the total number of cryosolvent molecules hydrogen bonded to DOPC molecules also decreases. The number of alcohol molecules bonded to one or more bilayer molecules is always greater than the number of alcohol molecules bonded to DOPC, indicating that a small but significant number of hydrogen bonds are formed between the cryosolvent and the β-sitosterol molecules. Despite this, the overall number of cryosolvent molecules hydrogen bonded to molecules in the bilayer decreases as the β-sitosterol concentration within the bilayer is increased.

The main reason for the reduction in the number of alcohol molecules hydrogen bonded to the bilayer is that, for each DOPC molecule that is replaced by a β-sitosterol, the bilayer loses seven hydrogen bond acceptors and gains just a single hydrogen bond acceptor. This reduction in the number of sites in the bilayer that are capable of forming hydrogen bonds with the cryosolvent molecules makes it more difficult for the alcohol molecules to hydrogen bond to the bilayer. The reduction in the number of molecules forming hydrogen bonds may also be in part due to the condensing effect that β-sitosterol has on the DOPC molecules and their rigidifying and compacting effect on the bilayer.

Bilayers which are less disordered will have their hydrogen bond donor/acceptor sites less accessible than bilayers which undergo a large increase in area and are significantly distorted. Not only does the incorporation of β-sitosterol into the bilayer reduce the number of alcohol molecules that hydrogen bond to the bilayer, but the percentage of alcohol molecules that form two (and three) hydrogen bonds to DOPC and β-sitosterol molecules is also reduced. This means that, as the amount of β-sitosterol in the membranes increases, the ability of cryosolvent

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molecules to cross-link lipid/sterol molecules in the bilayer decreases. Again, this effect is probably due to the combination in the reduction of the number of hydrogen bonding sites and the more rigid/compacted nature of imposing a steric barrier on hydrogen bonds formed between the DOPC and β-sitosterol molecules and the cryosolvents. As discussed above, in pure DOPC bilayers, the percentage of ethylene glycol molecules that form two hydrogen bonds to the bilayer (30 ± 3%) is approximately the same as for propylene glycol molecules (29 ± 2%), and less than in the case of glycerol (33 ± 2%, 43 ± 3% if triple bonds are also included). All of these percentages decrease as the β-sitosterol concentration increases; however, they do so at different rates. In the case of propylene glycol, the percentage decreases only slightly, dropping to 28 ± 3% for a bilayer consisting of one-third of β-sitosterol molecules and to 26 ± 2% for a 1:1 DOPC−β-sitosterol bilayer. The decrease in the number of cross-linking cryosolvent molecules is greater in the case of ethylene glycol. The reduction in cross-linking is largest for glycerol: for a 1:1 DOPC−β-sitosterol bilayer, the number of multiple bonding glycerol molecules (22 ± 3%) has decreased to such an extent that it is lower than the number of multiple bonding propylene glycol molecules.

The reason for these changes in comparative hydrogen bonding effectiveness is linked to the ability of the cryosolvents to thin/expand the bilayers. As discussed above, β-sitosterol makes the bilayer more rigid and condensed, inhibiting the formation of hydrogen bonds between the DOPC/β-sitosterol molecules and the alcohols. Propylene glycol causes a greater increase in the lateral area of the bilayer and can penetrate further into the structure of the membrane than the other two alcohols. Thus, the ability of propylene glycol to cross-link DOPC/β-sitosterol molecules is only reduced slightly as the concentration of β-sitosterol is increased. Ethylene glycol and glycerol do not cause the bilayer to expand to the same degree, meaning that as the level of β-sitosterol within the bilayer increases their ability to cross-link decreases more rapidly than in the case of propylene glycol. This is particularly evident in the case of glycerol, which is the larger molecule with a greater steric barrier than ethylene glycol, causing it to go from the most to the least successful cross-linking molecule.

Translocation of DOPC/β-Sitosterol between Leaflets. The interaction of DMSO with DOPC−β-sitosterol bilayers has been shown to promote the movement of β-sitosterol molecules from one leaflet to the other.[23] This sterol "flipping" has been shown to occur via the β-sitosterol molecule moving such that it lies horizontally in the center of the bilayer before the head of the β-sitosterol molecules reinserts itself into the opposite leaflet. Other simulation studies, both atomistic and coarse-grained, have shown that cholesterol flips between leaflets using the same mechanism.[44,46−48,50] The alcohol cryosolvents affect the DOPC−β-sitosterol bilayers in the same way, increasing the likelihood of sterol flips, as shown in Table 9. One might expect that the ability of a cryosolvent to promote flipping would be coincident with its ability to cause membrane expansion, as a "looser" membrane movement between the leaflets would be easier. Interestingly, however, this does not appear to be the case: the movement of β-sitosterol molecules between leaflets, even on a 300 ns time scale, is still a relatively rare event, and as such, any conclusions can only be drawn cautiously. DMSO causes a large expansion of the membrane and shows the greatest ability to promote sterol flipping, but bilayers solvated in propylene glycol, which undergo an approximately equal expansion, do not show a much greater incidence of sterol flipping than those solvated in glycerol, and both show fewer flipping events than the bilayers solvated in methanol, both of which cause a smaller increase in bilayer areas. As the amount of sterol in the bilayer increases, the likelihood of sterol flipping decreases due to the fact that the bilayers become more rigid.

Table 9. Number of β-Sitosterol Molecules That Flip between Leaflets during the 300 ns Simulation Time in the Different Systems at a Cryosolvent Concentration of 10.0 mol %

solution	10% β-sitosterol	33% β-sitosterol	50% β-sitosterol
water	2	3	3
methanol		10	15
ethylene glycol		6	1
DMSO	9	23	12
propylene glycol		8	7
glycerol	2	8	3

Gurtovenko and co-workers[25] showed that the formation of pores in PC bilayers caused by DMSO also induced the translocation of phospholipid molecules across the bilayer by means of a flip-flop event. Normally, such events do not occur on a time scale accessible by simulation, but the formation of the pore enabled tens of such events to be observed in atomistic simulations of a number of different PC lipid bilayers. The formation of pores caused by interaction of the DOPC bilayer with propylene glycol also induces phospholipid flip-flops. Table 10 shows the number of occurrences of such events at the different propylene glycol concentrations simulated (see also the Supporting Information). The incidence of flip-flop events is significantly lower than that observed by Gurtovenko, but there are a number of factors that differ between that work and the present study (such as different phospholipids, different force-fields, and different solvation levels), which make direct comparison of the ability of DMSO and propylene glycol to induce PC lipid translocation impossible. However, it is clear that propylene glycol does share the ability of DMSO to cause pores to form in PC bilayers, which in turn induces the movement of PC lipids between bilayer leaflets.

Table 10. Number of DOPC Molecules That Flip between Leaflets during the 300 ns Simulation Time in Different Concentrations of Propylene Glycol

concentration (mol %)	no. of lipid translocations
2.50	0
10.0	0
15.0	3
20.0	14
25.0	15

Diffusion across the Bilayers. DMSO diffuses across PC lipid bilayers readily enough that such bilayer crossing events can be observed in MD simulations.[13,14,16,20,68] The incorporation of β-sitosterol into a DOPC bilayer increases the energy barrier for a DMSO molecule to diffuse across the bilayer, but such events are still observed.[23] In the case of the systems and time scales simulated here, not only DMSO but also methanol and, to a much lesser degree, propylene glycol are observed to cross the bilayer. Table 11 shows the number of crossing events observed in a 60 ns time period. Neither ethylene glycol nor

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Table 11. The Number of Cryosolvent Molecules That Diffuse across the DOPC−β-Sitosterol Bilayers in the Final 60 ns of the Simulations[a]

solution	pure DOPC	10% β-sitosterol	33% β-sitosterol	50% β-sitosterol
methanol	29		29	13
DMSO	85	69	41	8
propylene glycol	1		0	0

[a]All systems have a cryosolvent concentration of 10.0 mol %.

glycerol are observed to diffuse across any of the bilayers at any concentration simulated. It is clear that DMSO is the molecule that most readily diffuses across the bilayer, followed by methanol and then propylene glycol. It can be seen that the incorporation of β-sitosterol into the bilayer reduced the number of methanol molecules that diffuse across the bilayer, as in the case of DMSO. This is also likely to be true in the case of propylene glycol; however, the fact that trans-bilayer diffusion of propylene glycol is such a rare event makes it difficult to draw any conclusions for that species. Once the concentration of propylene glycol is great enough to cause the formation of a pore in the bilayer, there is a significant increase in the number of molecules that diffuse across the bilayer, but that is a different process than the diffusion of cryosolvent molecules across a nonporous bilayer. Interestingly, while the presence of β-sitosterol within the bilayer makes the membrane more resistant to the trans-bilayer diffusion of both DMSO and methanol, the effect seems to be much greater in the case of DMSO than methanol. For a 2:1 DOPC−β-sitosterol bilayer, the number of DMSO molecules crossing in a 60 ns time period is approximately half that for a pure DOPC bilayer. However, the number of methanol molecules observed to diffuse through the bilayer is unchanged. While increasing the β-sitosterol concentration further to 50% does cause the number of methanol molecules crossing the bilayer to decrease, the relative decrease is again lower than in the case of DMSO. Indeed, for a 1:1 bilayer, the ability of methanol to cross the bilayer is at least as great as that of DMSO. The greater ability of methanol and DMSO to diffuse across the bilayers compared to the polyhydroxylated alcohols is due to a combination of factors: size, the balance between the hydrophobicity and hydrophilicity of the cryosolvent, and the fact that the polyhydroxylated alcohols are able to hydrogen bond to the bilayer more effectively.

The free energy barrier for the diffusion of a molecule of DMSO or methanol across the bilayer can be determined from the probability distribution of the species using

$$\Delta G(z) = k_B T \ln(\rho_{eq}/\rho(z)) \quad (2)$$

where $\Delta G(z)$ is the change in free energy as a function of position along the direction normal to the bilayer (the z-axis), ρ_{eq} is the density of DMSO or methanol in the bulk solution, and $\rho(z)$ is the density of DMSO/methanol along the direction normal to the bilayer. The free energy profiles calculated using eq 2 are shown in Figure 6. Previous simulations[15,20] have shown that at low concentrations the diffusion of a DMSO molecule across a DPPC or DOPC bilayer is a three-stage process. The DMSO molecule first diffuses across the phosphate head groups of the lipids, then it diffuses across the acyl tails to a position just below the phosphate head groups of the lipids in the opposite leaflet, and finally the molecule

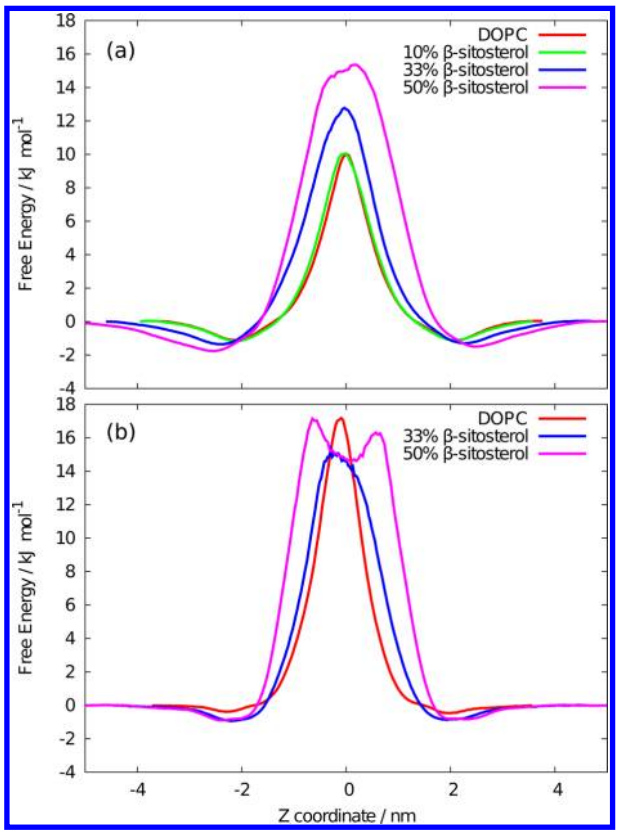

Figure 6. Free energy profiles for the diffusion of a DMSO or methanol molecule across the DOPC−β-sitosterol bilayers in (a) 10.0 mol % DMSO and (b) 10.0 mol % methanol solutions.

diffuses across the phosphate head groups back into solution. As the concentration of DMSO increases, the diffusion mechanism transitions into a single-stage process.[20] It has been shown[23] that for low DMSO concentrations (2.5 mol %) the presence of β-sitosterol at concentrations of ∼33% caused a change in the diffusion mechanism of DMSO. Due to the presence of β-sitosterol within the bilayer, the interdigitation of the two bilayer leaflets is reduced, which turns the center of the bilayer from the maximum of the free energy profile to a local minimum.[23] For DMSO concentrations of 10.0 mol % (Figure 6a), the replacement of some of the DOPC molecules by β-sitosterol does not change the shape of the free energy profile (a single peak with slight energy minima at the position of the lipid head groups) but does increase the height of the free energy barrier to diffusion. In contrast, in the case of methanol (10.0 mol %), the presence of β-sitosterol has only a minor effect on the height of the free energy barrier, but for 50% β-sitosterol bilayers it causes a change in the shape of the profile (Figure 6b). For pure DOPC bilayers, the free energy profile has a single maximum at the bilayer center and two very slight local minima at the headgroup positions. For a bilayer with 33% β-sitosterol, the profile widens slightly but has the same general shape. If the concentration of β-sitosterol within the bilayer is increased further to 50%, then the peak splits into two, with a shallow free energy minima forming at the bilayer center (where the bilayer density is low).

The free energy profiles for the diffusion of water across the bilayers in the different solutions have also been calculated (see Figure S4 in the Supporting Information). As the proportion of water molecules that diffuse through the bilayer is smaller than that of the cryosolvent molecules, there is a greater error

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associated with the profiles and they are not as smooth. Even so, it is apparent that the incorporation of sterol molecules into the bilayer causes the profiles to widen. It is also clear that the presence of cryosolvent molecules causes the height of the free energy barriers to be reduced, but the larger the percentage of β-sitosterol in the bilayer the lower this reduction is. The reduction in the free energy barrier is especially significant for bilayers containing 10% or less of β-sitosterol solvated in DMSO and propylene glycol.

CONCLUSIONS

DMSO, ethylene glycol, propylene glycol, and glycerol are all used in the cryopreservation of samples to try to reduce the damage that the sample suffers as a consequence of ice formation during the cryopreservation protocol. However, their presence can also compromise the integrity of cell membranes. MD simulations of each of these solvents, and methanol, reveal that their interaction with binary DOPC−β-sitosterol bilayers causes the bilayer to thin normal to the plane of the bilayer, while at the same time the bilayers expand laterally. The increase in the lateral area of the bilayer causes it to become more disordered, and a buildup of the cryosolvents is observed at the interface. However, while the general picture of the effect that all five of the solvents simulated have on DOPC−β-sitosterol bilayers is the same, there are some important differences. First, propylene glycol and DMSO cause the bilayers to thin, expand, and become disordered to a greater degree than the other three alcohols. The greater effect of propylene glycol and DMSO is due to the fact that they have a slightly greater hydrophobic character than the other three species, allowing them to penetrate more deeply into the structure of the membrane. The incorporation of β-sitosterol makes the bilayer more structured and counteracts the membrane thinning to some degree but does not change the overall pattern of behavior of the cryosolvents. Previous simulations have shown that cholesterol and β-sitosterol have a similar effect on phospholipid bilayers;[23] therefore, it is reasonable to assume that while there will be some minor quantitative differences in behavior the general effect of the cryosolvents on the bilayers containing cholesterol would be the same.

All four of the cryosolvents are generally characterized as penetrating cryosolvents, meaning that they are able to diffuse across cell membranes. On the time-scales simulated, DMSO and methanol have been observed to diffuse through bilayers containing up to 50% β-sitosterol. Propylene glycol was observed to diffuse across bilayers not containing any β-sitosterol, while glycerol and ethylene glycol were not observed to diffuse any type of bilayer in the 300 ns simulations. As the amount of sterol present in the bilayer is increased, the free energy barrier to the diffusion of molecules across the bilayers is increased, especially in the case of DMSO. In addition, for a bilayer containing an equal amount of DOPC and β-sitosterol, the shape of the free energy profile changes, with a local energy minimum appearing at the center of the bilayer. The creation of this local minimum is due to the reduced interdigitation of the two leaflets, leading to reduction in density at the center of the bilayer.

The presence of the cryosolvents at the bilayer interface causes the headgroups of the lipids to become partially dehydrated. The level of dehydration is linked to the size of the cryosolvent molecule: the larger the molecule, the greater the dehydration. As the cryosolvent molecules replace water molecules, the hydrogen bonds formed between water molecules and DOPC/β-sitosterol are replaced with hydrogen bonds formed between the alcohol molecules and DOPC/β-sitosterol (as DMSO is not a hydrogen bond donor, it behaves differently from the alcohols). The ability of the alcohol molecules to form hydrogen bonds with the bilayer is again determined by the size of the molecule (and thus its number of hydroxyl groups), with glycerol being the most hydrogen bonding species at all concentrations of β-sitosterol. Ethylene glycol, propylene glycol, and glycerol hydrogen bond to two or more molecules in the bilayer. The amount of β-sitosterol plays a significant role in determining the percentage of alcohol molecules that form two (or three) hydrogen bonds rather than a single hydrogen bond. Glycerol and ethylene glycol both experience a drop in the number of cross-linking alcohol molecules as the β-sitosterol concentration increases. The reason for this change is that β-sitosterol makes the bilayer more rigid and condensed, inhibiting the formation of hydrogen bonds between the DOPC/β-sitosterol molecules and the alcohols. In contrast, the number of propylene glycol molecules cross-linking only decreases slightly as the β-sitosterol concentration increases due to its greater ability to expand/thin the bilayer and penetrate more deeply into the bilayer structure.

The ability of propylene glycol to hydrogen bond to two bilayer molecules is one of the reasons why it does not destroy bilayers, which would have been otherwise destroyed at a similar concentration of DMSO. Like with DMSO, propylene glycol is able to cause the formation of pores in pure DOPC bilayers at a concentration of 15 mol %. These pores facilitate the diffusion of water and solvent molecules through the bilayer as well as the translocation of phospholipid from one leaflet to the other. However, whereas a DOPC bilayer solvated in 25 mol % DMSO is unstable, a DOPC bilayer in 25 mol % propylene glycol remains intact (over 300 ns).

The cryosolvents simulated in this study primarily reduce the damage to cells during the cryopreservation process by dehydrating cells and preventing the formation of ice crystals. This is done by their promotion of the glass transition, the formation of a vitrified state in water within cells. Thus, a desirable cryosolvent is one that is able to cross the bilayer relatively easily, favors the formation of a glassy state, and promotes the diffusion of water across the bilayer (by thinning the bilayer) while, importantly, not causing significant damage to the bilayer. There is a balance of factors at play here, as the cryosolvents that are most able to cross the bilayer and have the greatest ability to promote the formation of the glassy state are DMSO and propylene glycol; however, these two solvents are also the most damaging to the bilayer. The simulations performed in this work would indicate that ethylene glycol and glycerol have more balanced properties. The ability of methanol to diffuse across bilayers would, in principle, make it a better cryosolvent, but its toxicity means that in practice it is not used widely in cryopreservation. A comparison of DMSO and propylene glycol is interesting as, while DOPC−β-sitosterol bilayers are able to withstand higher concentrations of propylene glycol, DMSO is more able to diffuse across bilayers.

In practice, most cryopreservation protocols use vitrification solutions containing a number of different cryosolvents to try to achieve an appropriate balance between their glass-promoting ability and their toxicity. However, the overall effectiveness of a species as a cryosolvent will depend not only on its effect on

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cell membranes, which has been investigated here with model bilayers, but also on the interaction of the cryosolvent species with other structural and biochemical components of the cell.

■ ASSOCIATED CONTENT

Supporting Information

Density profiles of the 9:1 and 2:1 DOPC−β-sitosterol bilayers in the different solutions, the coordinates along the z-axis as a function of time of atoms in the head groups of β-sitosterol and DOPC molecules translocating between leaflets and the free energy profiles associated with the diffusion of water across the four different bilayers in the different solutions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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