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Molecular Mechanism of the Synergistic Effects of Vitrification Solutions on the Stability of Phospholipid Bilayers

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Abstract

The vitrification solutions used in the cryopreservation of biological samples aim to minimize the deleterious formation of ice by dehydrating cells and promoting the formation of the glassy state of water. They contain a mixture of different cryoprotective agents (CPAs) in water, typically polyhydroxylated alcohols and/or DMSO, which can damage cell membranes. Molecular dynamics simulations have been used to investigate the behavior of pure DPPC, pure DOPC, and DOPC-β-sitosterol bilayers solvated in a vitrification solution containing glycerol, ethylene glycol and DMSO at concentrations that approximate the widely used Plant Vitrification Solution 2 (PVS2). As in the case of solutions containing a single CPA, the vitrification solution causes the bilayer to thin and become disordered, and pores form in the case of some bilayers. Importantly, the degree of thinning is, however, substantially reduced compared to solutions of DMSO containing the same total CPA concentration. The reduction in the damage done to the bilayers is a result of the ability of the polyhydroxylated species (especially glycerol) to form hydrogen bonds to the lipid and sterol molecules of the bilayer. A decrease in the amount of DMSO of the vitrification solution with a corresponding increase in the amount of glycerol or ethylene glycol diminishes further its damaging effect due to increased hydrogen bonding of the polyol species to the bilayer headgroups. These findings rationalize for the first time the synergistic effects of combining different CPAs and form the basis for the optimization of vitrification solutions.

Key words: bilayer; cryopreservation; DMSO; plant vitrification solution; glycerol; ethylene glycol

Introduction

Cryopreservation aims to ensure the long term storage of cells and tissues. In the required liquid nitrogen storage conditions all physical, chemical and biological processes are halted, allowing biological material to be stored for decades without any change in its viability.(1–5) However, the process of cryopreservation can itself cause damage to biological materials. To reduce this damage and ensure the highest survival rates, samples are treated with so-called vitrification solutions containing cryoprotective agents (CPAs).

Commonly used CPAs such as DMSO, polyols and sugars reduce the damage to cells during cryopreservation by reducing colligatively the melting temperature of water and promoting the formation of its glassy state (vitrification), avoiding the deleterious formation of ice.(6–12) Unfortunately a number of these species are toxic, partly because they can damage cell membranes at sufficiently high concentration.(8) Consequently developing suitable cryopreservation protocols for biological samples requires optimization (often by trial-and-error) of the amount of CPAs used to balance their cryoprotective and toxic properties. A number of experimental(13–20) and theoretical(21–30) studies have attempted to determine how individual CPAs interact with model cell membranes.

Molecular dynamics (MD) simulations of lipid bilayers in the presence of cell "penetrating" CPAs (e.g. DMSO, ethylene glycol, propylene glycol and glycerol) have shown that their individual action is similar, causing the bilayer to expand parallel to the plane of the bilayer while thinning normal to that plane.(21–24, 27) However, there are also important differences. Firstly, DMSO has a much greater ability to diffuse across phospholipid (PL) bilayers than the alcoholic species. Secondly, DMSO and propylene glycol cause thinning of bilayers to a greater extent than ethylene glycol and glycerol,(31, 32) resulting in the spontaneous formation of pores at sufficiently high concentration.(23, 28, 31) Even higher concentrations of DMSO will completely destroy bilayers.(31, 32) Thirdly, polyols are able to form hydrogen bonds to the bilayer and act as cross linkers between multiple PL molecules. This cross-linking ability counteracts to some extent the expansion/thinning effects of polyols, making them less damaging than DMSO.(31, 32)

While the majority of these studies have been performed on bilayers containing a single phosphocholine lipid species, recently the inclusion of sterols has been shown to increase the resistance of bilayers to the damaging lateral expansion and thinning effects of CPAs.(28, 30) As a consequence, the concentrations of DMSO and propylene glycol needed to induce pore formation are higher and the free energy barrier for these CPAs to diffuse across the bilayer increases with the amount of sterol within the bilayer.(28, 30) This is important since the cold acclimation of plants has been demonstrated to modify the relative proportion of sterols in cell membranes and influence survival after cryopreservation.(33)

Previously MD simulations have characterised the effect of binary solutions (water and one CPA species) on bilayers. However, the vitrification solutions used in practice nearly always contain two, and often more, species of CPAs.(9, 34) This is believed to be due to the synergistic vitrification effect of combining several CPAs, requiring lower concentrations of each species and reducing their combined toxicity.(2) However, optimization of vitrification solutions is often an empirical process involving trial-and-error during cryopreservation.(5)

This work investigates for the first time the interaction of various single and double-component bilayers with vitrification solutions containing three of the most commonly used CPAs: DMSO, ethylene glycol and glycerol. The composition of one of the vitrification solution is similar to that of Plant Vitrification Solution 2 (PVS2), a solution widely used in the cryopreservation of plant material.(9, 34, 35) For PL bilayers containing a realistic amount of β-sitosterol it is found that this vitrification solution has a substantially lower damaging effect than what would be expected on the basis of the effect of single-component CPA solutions **(the structure of β-sitosterol is shown in Figure S1)**. The synergistic effects of the vitrification solutions are then further investigated by simulating DPPC bilayers in vitrification solutions where the amount of DMSO present in the solution has been reduced and the amounts of glycerol or ethylene glycol increased. These findings provide a molecular rationale for the use of vitrification solutions containing several CPAs and the basis for strategies aimed at optimizing their composition.

Methods

The interaction of a vitrification solution with five different PL bilayers, DPPC, DOPC, 9:1 DOPC-β-sitosterol, 2:1 DOPC-β-sitosterol and 1:1 DOPC-β-sitosterol, was investigated. Mixed DOPC-β-sitosterol bilayers were chosen as experimental studies have shown that the most common sterol species in plant membranes are β-sitosterol and stigmasterol, and that unsaturated lipids are more common in plants than animals.(33, 36–38) As the sterol/PL ratio of plant bilayers can vary considerably,(39) a range of different compositions were simulated. The DPPC bilayer was chosen to act as a reference point as it is a very widely studied system, both experimentally and theoretically.

The initial configurations of the bilayers were taken from systems previously equilibrated in water (a total of 5841 water molecules, ∼46 water molecules per lipid). Each bilayer consisted of 128 PL/sterol molecules. Initially 64 molecules were placed in each leaflet and the number of β-sitosterol molecules in each leaflet was equal. The total number of solvent molecules was kept constant but water molecules were replaced by CPA molecules.

As previously mentioned, this study attempted to model a solution containing the CPAs used in PVS2; however, a few changes were made. In practice PVS2 is an aqueous solution of 15.0 % w/v DMSO, 15.0 % w/v glycerol and 30.0 % w/v ethylene glycol, with 0.2 M sucrose added to it.(2, 9) This was approximated in the simulations as a solution, VS1, containing 78.21 mol % water, 9.35 mol % glycerol, 6.93 mol % ethylene glycol and 5.51 mol % DMSO. Sucrose was not included in the vitrification solution as the interaction of sugars with lipid bilayers is a highly debated topic: while it is generally accepted that sugar molecules prevent damage to bilayers, a number of different mechanisms for this effect have been proposed.(18–20) The main point of disagreement is whether the sugar molecules directly interact with the bilayer surface, with computer simulations and some experimental work indicating that they do, while other experimental work indicating that they remain in solution.(18–20) As the systems investigated are quite complex already (binary bilayers and four different solvent species), it was decided not to complicate things further by including sucrose. Thus the vitrification solution used in this study consisted only of the penetrating CPAs. Each of the five bilayers were solvated in VS1. The simulations indicated that pure DOPC bilayers solvated in VS1 were close to the pore forming regime, and as such two independent simulations of these systems were performed. The DPPC bilayer was simulated solvated in two further solutions containing a mixture of CPAs. In these solutions, VS2 and VS3, the total mol % of CPAs was the same as that in VS1 but the percentage of DMSO in the solution was reduced by half and the percentage of the either ethylene glycol or glycerol increased by an equivalent amount. The composition of the three vitrification solutions is given in Table 1.

A number of solutions containing a single CPA were simulated, and these systems, as well as others previously simulated under the same conditions,(27, 30, 31) allowed the effect of VS1 on bilayers to be compared against that of single component solutions. The binary systems simulated were DPPC in 2.5, 7.0, 10.0, 15.0, 20.0 and 25.0 mol % ethylene glycol, DPPC in 2.5, 10.0,

Solution	mol % of component **(No. of molecules)**			
	Water	Glycerol	Ethylene glycol	DMSO
VS1	78.21 **(4568)**	9.35 **(546)**	6.93 **(405)**	5.51 **(322)**
VS2	78.21 **(4568)**	9.35 **(546)**	9.69 **(566)**	2.76 **(161)**
VS3	78.21 **(4568)**	12.10 **(707)**	6.93 **(405)**	2.76 **(161)**

Table 1: The composition of the three vitrification solutions

15.0 and 20.0 mol % glycerol, and the pure DOPC and and 2:1 DOPC-β-sitosterol bilayers in 7.0 and 20.0 mol % ethylene glycol. The full list of the systems simulated is given in Table 2

The structure and dynamics of lipid bilayers can take hundreds of nanoseconds to fully equilibrate. As such the GROMOS 54A7 united atom force-field was used for the lipids and cryosolvent molecules.(40–42) Recent studies have confirmed that this force-field reproduces a wide range of lipid parameters well.(43, 44) The SPC model was used to represent water molecules.(45)

The GROMACS v3.3.3 code was used to perform the MD simulations.(46) The simulation parameters used matched those for the development of the GROMOS 54A7 force-field,(40, 41) namely, twin-range cutoffs for the non-bonded forces and 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. Consistently with the development of the GROMOS 54A7 force-field, the reaction-field method was used to evaluate electrostatic interactions ($\varepsilon_{\mathrm{RF}} = 62$). Studies have shown that for lipid systems the difference between simulations performed with a reaction and those performed with an Ewald sum method are minor.(40, 47) Moreover the GROMOS 54A7 force-field was developed using the reaction-field method. The simulations were performed in the isothermal-isobaric ensemble with the temperature and pressure maintained using the Berendsen thermostat and barostat with coupling constants of 0.1 and 1.0 ps, respectively.(48) The simulation box dimensions parallel and perpendicular to the plane of the bilayer were allowed to vary independently. Each system was simulated for 300 ns with the last 60 ns of simulation time being used for analysis.

DMSO is known to promote the formation of the gel-phase in pure PC lipid bilayers,(13, 14) causing the gel-liquid crystal phase transition temperature to increase. Consequently many previous simulations of DPPC and DOPC bilayers in DMSO have been performed at 350 K. It is likely that PVS2 behaves in a similar manner, also raising the transition temperature. Therefore, both to ensure that the system is in the liquid-crystalline phase

Bilayer	Solution	Area / nm^2	D_{HH} / nm
DPPC	2.5 mol % ethylene glycol	43.86 ± 1.09	2.95
	7.0 mol % ethylene glycol	47.02 ± 1.09	2.69
	10.0 mol % ethylene glycol	48.79 ± 1.40	1.50
	15.0 mol % ethylene glycol	51.18 ± 1.38	2.40
	20.0 mol % ethylene glycol	53.27 ± 0.98	2.13
	25.0 mol % ethylene glycol	54.82 ± 1.10	2.05
	2.5 mol % glycerol	44.17 ± 1.00	2.88
	10.0 mol % glycerol	49.47 ± 1.46	2.33
	15.0 mol % glycerol	52.60 ± 0.84	2.27
	20.0 mol % glycerol	53.92 ± 0.78	2.21
	VS1[a]	pore formed	
	VS2[b]	55.72 ± 1.19	2.04
	VS3[c]	56.01 ± 1.13	2.13
DOPC	7.0 mol % ethylene glycol	48.93 ± 0.65	2.71
	20.0 mol % ethylene glycol	54.81 ± 1.06	2.20
	VS1[a] run 1	58.69 ± 1.15	1.64
	VS1[a] run 2	58.88 ± 1.02	1.74
9:1 DOPC-β-sitosterol	VS1[a]	55.49 ± 1.06	2.10
2:1 DOPC-β-sitosterol	7.0 mol % ethylene glycol	35.54 ± 0.70	3.56
	20.0 mol % ethylene glycol	39.30 ± 0.83	3.15
	VS1[a]	43.00 ± 0.84	3.65
1:1 DOPC-β-sitosterol	VS1[a]	34.32 ± 0.90	3.65

Table 2: Area and thickness, D_{HH}, of the different bilayers solvated in the different cryosolvent and vitrification solutions.
Composition of vitrification solutions (/ mol %): water, glycerol, ethylene glycol, DMSO. [a] VS1: 78.21, 9.35, 6.93, 5.51 [b] VS2: 78.21, 9.35, 9.69, 2.76 [c] VS3: 78.21, 12.10, 6.93, 2.76

and to allow comparison with previous studies the systems were simulated at 350 K.

For the analysis of the hydrogen bonding of difference species we define a hydrogen bond present when the Acceptor-Hydrogen distance is $\leq$ 2.5 Å and the Acceptor-Donor-Hydrogen angle is $\leq 30°$.

Results and Discussion

Table 2 lists the area of the bilayers in the *xy*-plane and the bilayer thickness (defined as the distance between the peaks in the PL density profiles) for the different systems simulated in this study. For comparison Tables S1-S5 show data previously reported(27, 30, 31) for these bilayers solvated in other solutions but with the simulations performed under the same conditions. In all systems there is an increase in the area of the bilayer parallel to the plane and a concurrent thinning normal to the plane when compared against the same bilayers solvated in pure water. In the case of the pure DPPC bilayer in VS1 the bilayer area expands and thins to such a degree that a pore forms in the bilayer (Fig. 1(a)). The pore is filled with all four of the solvent species and the PLs adjacent to the pore rearrange themselves such that any contact between the solvent molecules and the hydrophobic backbone of the lipids is kept at a minimum, just as has been reported in the case of pores formed by binary DMSO-aqueous solutions.(23, 27) **Such pore formation did not occur for either of the simulation runs of the pure DOPC bilayers nor DOPC-β-sitosterol bilayers during the 300 ns simulation time**. Interestingly, for DPPC bilayers the reduction in the amount of DMSO with a corresponding increase in either ethylene glycol or glycerol did not lead to the formation of a pore for VS2 and VS3, suggesting that DMSO is the most damaging species in vitrification solutions.

Combining data obtained in previous studies(27, 30, 31) (with the simulations performed under the same conditions) with the results of the present study, Fig. 2 shows the % increase in the area of the DPPC, DOPC and 2:1 DOPC-β-sitosterol bilayers in a variety of different solutions. As previously noted,(31, 32) DMSO increases the **area of the bilayer** to a greater extent than an equivalent concentration of ethylene glycol or glycerol (which give approximately equal increase in **the area of the bilayer**). The increase in the lateral areas of the bilayers solvated in VS1 are similar to the values observed for bilayers solvated in 12-15 mol % DMSO solution.(27, 28, 30) As with VS1, a 12.5 mol % solution of DMSO causes pores to form in a

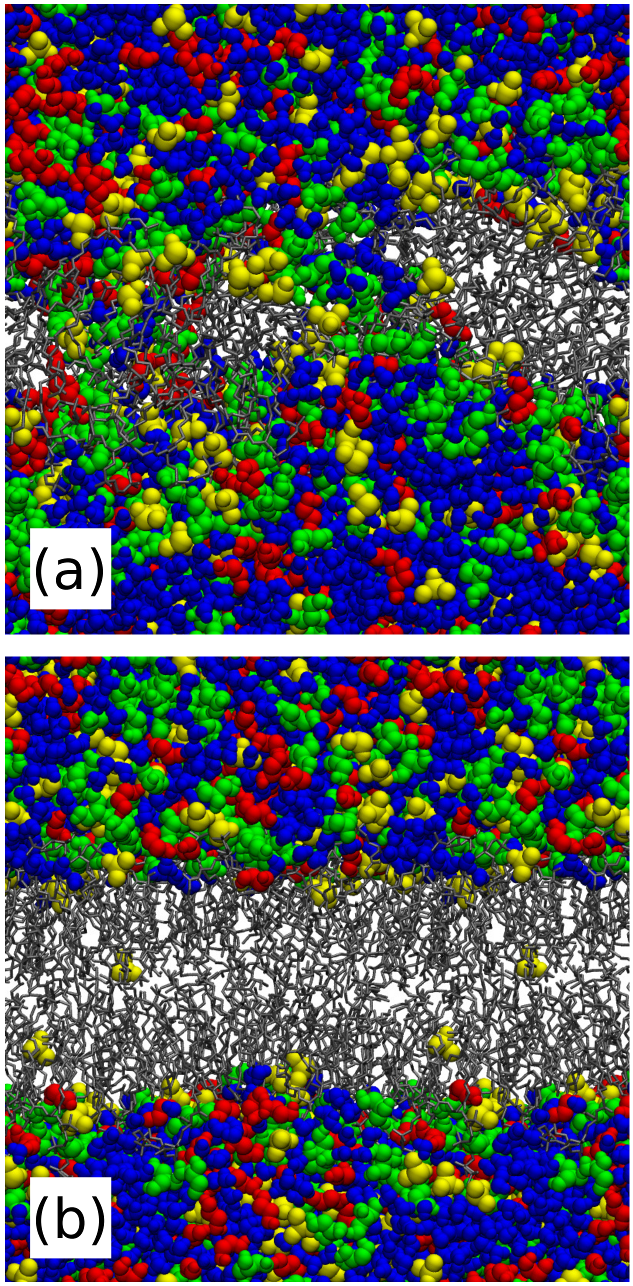

Figure 1: Snapshots of (a) the DPPC and (b) the 1:1 DOPC-β-sitosterol bilayers. The DPP/DOPC/β-sitosterol, water, DMSO, glycerol and ethylene glycol molecules are shown in grey, blue, yellow, green and red, respectively. In (a) the formation of a pore containing all four solvent species is observed

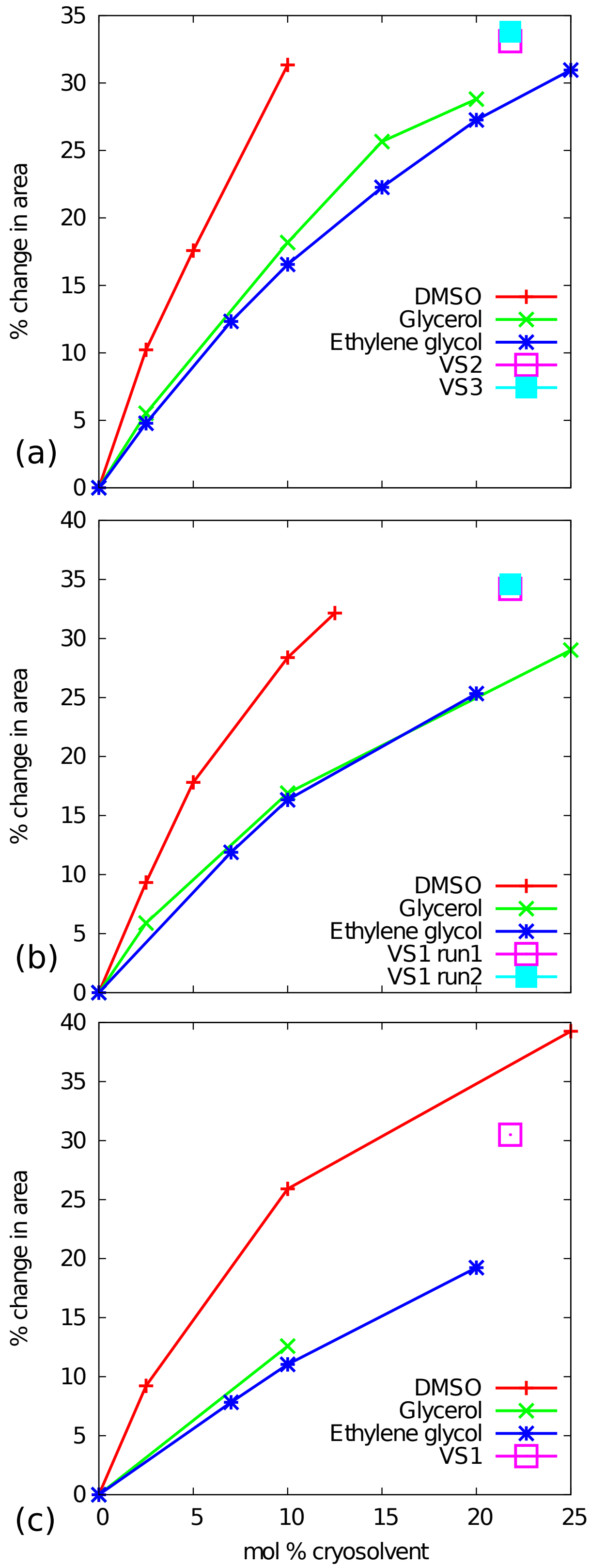

Figure 2: Plots of the % change in the area of the (a) DPPC, (b) DOPC and (c) 2:1 DOPC-β-sitosterol bilayers solvated in a variety of different solutions.

DPPC bilayer, while a DOPC bilayer solvated in a 12.5 mol % solution DMSO shows an increase in the lateral area of 32 % compared with 34 % for VS1.(27) From Fig. 2(c) and extrapolating from data points that we have for the 2:1 DOPC-β-sitosterol bilayer, it also appears that VS1 would give a similar increase in the **area of the bilayer** to that of 15 mol % DMSO. In contrast, it is clear that, despite the fact that VS1 has approximately the same total CPA concentration, it is less damaging than a solution of 20 or 25 mol % DMSO. In the case of VS2 and VS3 with the DPPC bilayer the solutions are less damaging than both VS1 and a 12.5 mol % solution of DMSO as they do not cause the formation of pores **on the timescales simulated**. Nonetheless VS2 and VS3cause expansion of the membrane than 10.0 mol % DMSO or 25.0 mol % ethylene glycol, indicating that they must be close to the pore formation regime. **While the formation of pores on a simulation timescale is a stochastic process the fact that neither simulation run of the pure DOPC bilayers in VS1 showed any signs of pore formation allows us to be reasonably confident that our results are reproducible.**

The density profiles of the pure DOPC and 1:1 DOPC-β-sitosterol bilayers solvated in the VS1 are shown in Fig. 3 (the density profiles of the other systems are shown in Figure S2 in the Supporting Information). The structure of the pure DOPC bilayer is significantly distorted but still retains some order, with the characteristic trough in the DOPC density profile at the centre of the bilayer. On the other hand, the density profile of the 1:1 DOPC-β-sitosterol bilayer is not significantly different from that observed in pure water. The asymmetry in the β-sitosterol profiles of the 1:1 bilayer is due to the ability of cryosolvents to promote the "flipping" of sterol molecules between leaflets.(30, 31) A snapshot of this system (Fig. 1(b)) shows a bilayer that is still highly ordered and which does not experience any of the disorder that affects bilayers solvated in high concentrations of DMSO.(23, 27, 30)

In the single component solutions a build up of the CPA at the surface of the bilayer around the phosphate head groups has been observed.(21, 23, 27, 30–32) In the case of the multi-component vitrification solutions, however, the different species compete against each other. Fig. 4 shows the number density profiles of the CPAs in the pure DOPC and 1:1 DOPC-β-sitosterol bilayers solvated in VS1. Glycerol exhibits the strongest propensity to interact with the bilayer surface, as evidenced by a significant peak in the number density profile at the bilayer surface compared with the bulk value. This agrees with simulations of DPPC bilayers solvated in single-component solutions, which have shown that glycerol has the strongest preference of all

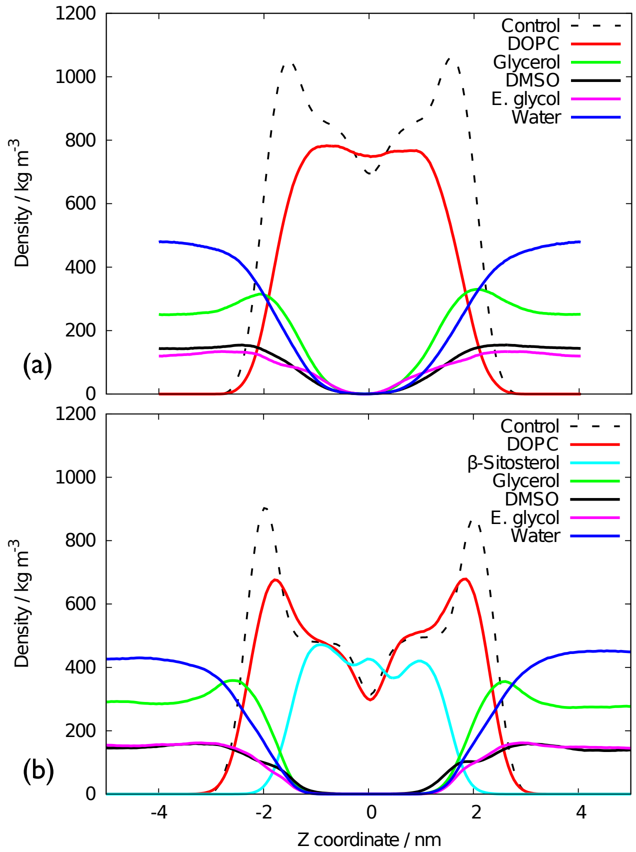

Figure 3: Density profile of (a) the pure DOPC and (b) the 1:1 DOPC-β-sitosterol bilayers solvated in the vitrification solution.

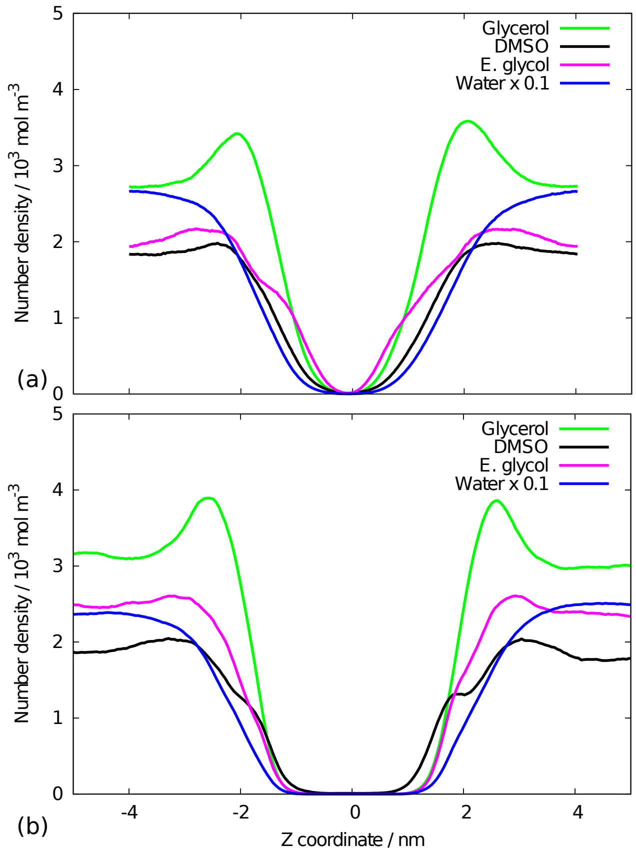

Figure 4: Number density profiles of (a) the pure DOPC and (b) the 1:1 DOPC-β-sitosterol bilayers.

these CPAs for the bilayer surface.(32) There are small peaks in the density profiles of DMSO and ethylene glycol near the bilayer surface, but these are much reduced compared with the peaks observed for the corresponding single-component solutions. DMSO is able to penetrate deeper into the bilayer structure than either of the alcohols, due to the greater hydrophobicity of the DMSO molecule,(49, 50) and there is a second peak in the density profile of DMSO just below the position of the phosphate groups. While the height of the PL peaks is reduced, the density profile of the 1:1 DOPC-β-sitosterol bilayer interacting with VS1 retains the characteristic shape of a PL bilayer profile (Fig. 3), considerably more so than in an aqueous DMSO solution containing the same percentage of water. The density profile of the pure DOPC bilayer is considerably more distorted than that of the 1:1 bilayer, again indicating the ability of β-sitosterol to increase the stability of the bilayer and its resistance to the deleterious effects of the CPAs. Interestingly, despite the formation of a pore in the bilayer, the density profiles of the PLs in the 9:1 DOPC-β-sitosterol and DPPC bilayers (Figure S2) are not as flat as is usually the case in systems where a pore has formed in the presence of aqueous DMSO.(27, 30) As previously mentioned, glycerol and ethylene glycol are known to distort the membrane to a lesser degree than DMSO, and it appears that this behaviour is shared by VS1, even when a pore is formed in the bilayer.

Table 3 shows the average number of hydrogen bonds formed between each PL and sterol group and the solvent molecules for all bilayers solvated by VS1. In the case of the phosphate group the number of hydrogen bonds formed to glycerol molecules is approximately equal to that formed to water molecules, while far fewer hydrogen bonds are formed with ethylene glycol. This again indicates that the preference of glycerol for the bilayer surface rather than the bulk solution is greater than that of ethylene glycol. This relates to the greater ability of glycerol to cross-link PL headgroups.(31, 32) The PL glycerol group also forms a greater number of hydrogen bonds to the glycerol molecules than ethylene glycol molecules, but more hydrogen bonds are formed with water. The most likely reason for this is that the greater steric hindrance experienced by glycerol compared with water prevents it from forming as many hydrogen bonds to the lipid glycerol groups. The percentages of hydrogen bonds formed with glycerol molecules (present at a concentration of 9.35 mol % in VS1) are approximately 42 % and 35 % for the phosphate and glycerol groups of the lipids, respectively. This compares with values of $\sim$51 % and $\sim$48 % for lipids solvated in a 10.0 mol % glycerol solution. Thus, ethylene glycol does compete with glycerol to a certain extent for the formation of hydrogen bonds to the phospho-

Group	Solvent	DPPC	DOPC	9:1 DOPC	2:1 DOPC	1:1 DOPC
PL Phosphate	Water	1.28 ± 0.09	1.28 ± 0.10	1.27 ± 0.10	1.21 ± 0.11	1.22 ± 0.13
	Glycerol	1.25 ± 0.08	1.19 ± 0.07	1.23 ± 0.09	1.24 ± 0.10	1.20 ± 0.13
	Ethylene glycol	0.47 ± 0.06	0.46 ± 0.05	0.48 ± 0.06	0.45 ± 0.07	0.44 ± 0.08
PL Glycerol	Water	1.17 ± 0.10	1.09 ± 0.08	1.16 ± 0.10	1.10 ± 0.11	1.16 ± 0.12
	Glycerol	0.84 ± 0.06	0.83 ± 0.07	0.83 ± 0.07	0.83 ± 0.09	0.76 ± 0.09
	Ethylene glycol	0.35 ± 0.05	0.33 ± 0.05	0.35 ± 0.05	0.32 ± 0.06	0.33 ± 0.07
β-sitosterol	Water			0.28 ± 0.11	0.56 ± 0.10	0.61 ± 0.09
Hydroxyl	Glycerol			0.05 ± 0.06	0.34 ± 0.08	0.32 ± 0.09
	Ethylene glycol			0.05 ± 0.06	0.16 ± 0.06	0.17 ± 0.05
	DMSO			0.01 ± 0.03	0.06 ± 0.04	0.05 ± 0.03

Table 3: Average number of hydrogen bonds formed by the different PL/sterol groups to the solvent molecules in VS1. Data normalised according to the number phospholipid/sterol molecules present

Group	Solvent	VS1	VS2	VS3
Phosphate	Water	1.28 ± 0.09	1.22 ± 0.10	1.17 ± 0.10
	Glycerol	1.25 ± 0.08	1.26 ± 0.08	1.60 ± 0.09
	Ethylene glycol	0.47 ± 0.06	0.71 ± 0.07	0.42 ± 0.05
Glycerol	Water	1.17 ± 0.10	1.13 ± 0.10	1.06 ± 0.10
	Glycerol	0.84 ± 0.06	0.87 ± 0.08	1.05 ± 0.08
	Ethylene glycol	0.35 ± 0.05	0.50 ± 0.06	0.33 ± 0.04

Table 4: Average number of hydrogen bonds formed by the different phospholipid groups of the DPPC bilayers to the solvent molecules in the different vitrification solutions. Data normalised according to the number phospholipid molecules present

lipid headgroups. All average numbers of hydrogen bonds for PL phosphate and glycerol groups remain largely unchanged upon addition of β-sitosterol, whose hydroxyl group interacts primarily with water, followed by glycerol and, finally, ethylene glycol.

Table 4 shows the average number of hydrogen bonds formed between the PL headgroups of the pure DPPC bilayer with the three different vitrification solutions. For VS2 and VS3 the increase in the number of ethylene glycol or glycerol molecules causes a reduction of the number of hydrogen bonds formed by the phospholipids with the water molecules. Instead the number of hydrogen bonds formed with either ethylene glycol (VS2) or glycerol (VS3) increases due to the increased number of polyol molecules. The greater propensity of glycerol to form hydrogen bonds compared with ethylene glycol is also seen in the case of VS2. In VS2 the number of glycerol and ethylene glycol molecules is approximately equal but there are more PL-glycerol hydrogen bonds than PL-ethylene glycol hydrogen bonds. Even taking into account the additional hydroxyl present in glycerol, i.e. multiplying the ethylene glycol values by 1.5, glycerol still forms a greater number of hydrogen bonds. Moreover, the total number of hydrogen bonds formed between poly-ol molecules and the PL headgroups is greater in VS3 than in VS2, again illustrating the greater tendency of glycerol to form hydrogen bonds compared with ethylene glycol.

DMSO has been determined to easily cross lipid bilayers interacting with DMSO-water binary solutions. In contrast, in recent simulations of DPPC and mixed DOPC-β-sitosterol bilayers in aqueous solutions of glycerol and ethylene glycol neither alcohol was observed to diffuse across the bilayer in the lifetime of the simulations.(31) Table 5 gives the number of CPA molecules that are observed to diffuse across the bilayers during the last 60 ns of the simulations. There appear to be two regimes: one where the membrane remains intact and one where a pore has formed. In the case of bilayers that remain intact (the DOPC and DOPC-β-sitosterol bilayers), DMSO is observed to diffuse across the bilayer but as the amount of β-sitosterol in the bilayer increases the number of DMSO molecules observed to diffuse across the bilayer decreases. This behaviour is expected; however, the number of DMSO molecules that are observed crossing is equal to, or less than, that of the same bilayers solvated in 2.5 mol% DMSO solution.(27, 30) This is somewhat surprising considering the greater thinning experienced by the bilayers solvated in VS1 compared with a 2.5 mol % DMSO solution. Indeed, as VS1 contains 5.51 mol % DMSO, it is only possible to conclude that the glycerol and ethylene glycol molecules are actually impeding the diffusion of DMSO across the bilayer. The relatively small number of DMSO molecules that are observed to diffuse across the bilayer makes it difficult to determine the reason for this behaviour or how large the effect is, but the build up of glycerol (and, to a much lesser extent, ethylene glycol) at the surface of the bilayer (Fig. 3) is likely to make it more difficult for DMSO molecules to pass across the bilayers.

The diffusion of glycerol and ethylene glycol molecules across the DPPC bilayer where a pore was formed is appreciable. However, the number of alcohol molecules passing through the bilayer is still smaller than the number of DMSO molecules. It has been shown previously that pores formed in PL bilayers due to the presence of DMSO will allow Na^+ and Cl^- ions to diffuse across bilayers and it is apparent that glycerol and ethylene glycol can also

System	Pore formed	DMSO	Ethylene glycol	Glycerol
DPPC VS1	Yes	64	38	54
DPPC VS2	No	12	0	0
DPPC VS3	No	13	0	0
DOPC VS1 Run 1	No	14	1	0
DOPC VS1 Run 2	No	17	1	0
9:1 DOPC-β-sitosterol VS1	No	19	0	0
2:1 DOPC-β-sitosterol VS1	No	4	0	0
1:1 DOPC-β-sitosterol VS1	No	3	0	0

Table 5: Number of different CPA molecules that diffuse across the bilayers during a 60 ns period

exploit pores for trans-bilayer diffusion.(24)

Pores formed through the interaction of the bilayer with DMSO (and propylene glycol) are also known to facilitate the trans-location of PLs between leaflets.(30, 51) This is also observed in this study where pores have formed in the presence of the VS1. In the case of the DPPC bilayer, PL molecules are observed moving between leaflets. The mechanism by which the trans-location of the lipid molecules occurs appears to be the same as described for bilayers solvated in DMSO.(51) In the bilayers where no pores form, no movement of DOPC molecules between leaflets is observed. However, in all bilayers with β-sitosterol the sterol molecules are observed to move between leaflets. This was also observed in simulations with a single CPA.(30, 31)

Conclusions

MD simulations of the interactions of vitrification solutions containing penetrating CPAs with DPPC and DOPC-β-sitosterol bilayers have been conducted to investigate the effects of a mixture of CPAs on the stability of these bilayers compared with the effect of a single CPA. The vitrification solutions cause the bilayer to expand laterally while thinning normal to the plane of the bilayer, as has been observed for solutions containing a single CPA. In the case of VS1 (a solution containing CPAs in a similar proportion to the experimentally used PVS2), the solution induces the formation of pores in DPPC bilayers, as in the case of single-component aqueous solutions of DMSO (which occurs at a concentration of 12.5 mol % for DPPC and 15 mol % for DOPC). For pure DOPC bilayers and DOPC bilayers containing only a small amount (10 %) of β-sitosterol, the bilayers remain very close to the pore formation "regime". The bilayers containing 33 and 50 % β-sitosterol not only showed no evidence of pore formation but their bilayer structure remained remarkably undamaged. If the amount of DMSO in the vitrification solution is reduced by half and that of either ethylene glycol or glycerol increased by an equivalent amount, then the resultant solutions are less damaging and do not induce pore formation in DPPC bilayers.

Comparison of these findings with those of bilayers solvated in single-component aqueous DMSO solutions show that, in terms of bilayer expansion/thinning and pore formation, VS1 is less damaging than an aqueous 20.0 mol % DMSO solution (which contains an approximately equivalent total CPA concentration). VS1 is also less damaging to the pure DOPC bilayer than a 20.0 mol % solution of propylene glycol (which causes a pore to form). Overall, VS1 is approximately equivalent, in terms of damage to the bilayer, to a 12.5 mol % DMSO solution. However, the diffusion of DMSO across the bilayers is markedly lower than in bilayers solvated in 12.5 mol % DMSO. The build up of glycerol at the bilayer surface appears to be responsible for this effect, as it makes it more difficult for DMSO molecules to diffuse across the bilayer. Experimental evidence shows that the addition of DMSO to solutions increases the diffusion of other species across membranes. There is no evidence of this on the systems where pores do not form, but it may be that longer timescales are needed to observe the diffusion of ethylene glycol and glycerol.

The greater build up of glycerol at the bilayer surface means that the PL/sterol molecules form a much greater number of hydrogen bonds to glycerol than ethylene glycol. Indeed almost as many hydrogen bonds are formed to glycerol as they are to water. This level of hydrogen bonding between the PL/sterol molecules of the bilayer and glycerol (and, to a lesser extent, ethylene glycol) molecules explains the less destructive nature of the vitrification solution in comparison with equivalent mol % DMSO solutions. PVS2 is only one of the different vitrification solutions used in cryopreservation. This work reveals for the first time the synergistic effect of combining several CPAs in a vitrification solution to reduce the damaging effect of DMSO while maintaining a sufficiently high concentration of CPAs to retain their ability to induce the vitrification of water.

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Supporting Information

An online supplement to this article can be found by visiting BJ Online at http://www.biophysj.org. The Supporting Information provides **the molecular structure of β-sitosterol**, the density profiles of the DPPC, 9:1 and 2:1 DOPC-β-sitosterol bilayers in VS1 as well as the area and thickness of pure DPPC, pure DOPC, 9:1-β-sitosterol, 2:1 DOPC-β-sitosterol and 1:1 DOPC-β-sitosterol bilayers in a variety of solutions.

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