UPCommons

Portal del coneixement obert de la UPC

<http://upcommons.upc.edu/e-prints>

Aquesta és una còpia de la versió *author's final draft* d'un article publicat a la revista *Chemistry and physics of lipids*.

URL d'aquest document a UPCommons E-prints:

<https://upcommons.upc.edu/handle/2117/335894>

Article publicat / Published paper:

Raju, R.; Torrent-Burgués, J.; Bryant, G. Interactions of cryoprotective agents with phospholipid membranes - A Langmuir monolayer study. "Chemistry and physics of lipids", Setembre 2020, vol. 231, art. 104949. DOI: [10.1016/j.chemphyslip.2020.104949](https://doi.org/10.1016/j.chemphyslip.2020.104949)

Journal Pre-proof

Interactions of cryoprotective agents with phospholipid membranes - a Langmuir monolayer study

Rekha Raju, Juan Torrent-Burgués, Gary Bryant

PII: S0009-3084(20)30080-3

DOI: <https://doi.org/10.1016/j.chemphyslip.2020.104949>

Reference: CPL 104949

To appear in: *Chemistry and Physics of Lipids*

Received Date: 28 February 2020

Revised Date: 2 July 2020

Accepted Date: 14 July 2020

Please cite this article as: Raju R, Torrent-Burgués J, Bryant G, Interactions of cryoprotective agents with phospholipid membranes - a Langmuir monolayer study, *Chemistry and Physics of Lipids* (2020), doi: <https://doi.org/10.1016/j.chemphyslip.2020.104949>

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 Published by Elsevier.

Interactions of cryoprotective agents with phospholipid membranes - a Langmuir monolayer study

Rekha Raju^{a,b}, Juan Torrent-Burgués^{b,*} and Gary Bryant^{a,*}

^a School of Science, RMIT University, Melbourne, Australia.

^b Universitat Politècnica de Catalunya (UPC), C/ Colom 1, E08222 Terrassa, Barcelona, Spain

*Corresponding authors: gary.bryant@rmit.edu.au ; juan.torrent@upc.edu

Graphic_Abstract

Highlights

- DMSO expands the area per lipid for DPPC, but causes compression for POPC, highlighting species dependent effects
- Ethylene glycol and glycerol expand the monolayers regardless of lipid species, implying that they insert into the headgroup region
- Dimethylformamide compresses the monolayer for all lipid species, implying it dehydrates the lipid head groups
- Under physiological conditions, the penetrating action of cryoprotectants may only occur for unsaturated phospholipids

Abstract

The influence of four common cryoprotectants (dimethyl sulfoxide, glycerol, ethylene glycol and dimethylformamide) on monolayers of four common phospholipids (DPPC, DOPC, POPC and POPE) have been studied using Langmuir isotherms and monolayer insertion experiments. The cryoprotectant concentrations were chosen to be directly relevant to cryoprotection. We show that DMSO causes an expansion of the DPPC area per lipid (in contrast to previous work

at higher concentrations). However, it caused compression for POPC, and had little effect for POPE or DOPC. As most previous studies have involved only DPPC, this highlights the importance of studying different lipid types as these may have a significant effect on the interactions. We show that both ethylene glycol and glycerol cause a small expansion of the monolayer at fixed pressure, implying that they insert into the headgroup regions, regardless of lipid species, and consistent with their ability to penetrate membranes. By contrast, dimethylformamide causes monolayer compression for all lipid species, implying it dehydrates the lipid head groups. Membrane insertion experiments at physiological values of lateral pressure highlight that DPPC is the most difficult lipid to penetrate, implying that the penetrating action of cryoprotectants may only occur for unsaturated phospholipids. Thus, extrapolations of results based solely on the DPPC need to be made with care.

Keywords: *Cryoprotectants, Langmuir monolayers, Compression isotherms, Insertion, Permeability*

1 Introduction

Cryopreservation is a viable option for the long-term storage of biological material. In cryopreservation, samples are stored in a vitrified state at liquid nitrogen temperatures (-196 °C). One requirement of a successful cryopreservation protocol is an effective cryoprotective agent (CPA). CPAs reduce cellular damage by limiting the formation of intra- and extra-cellular ice, minimizing cell dehydration, and inhibiting membrane phase changes¹⁻⁵.

When a cell suspension is frozen, ice tends to form external to the cells. Solutes are excluded from the ice structure, which leads to an increase in the extra-cellular solute concentration, resulting in cell shrinkage due to the efflux of water via osmosis⁵. Thus, the formation of ice removes liquid water – ie, it causes dehydration, which can damage cell membranes^{1,6}. By contrast, if a cell suspension is cooled rapidly, cells become supercooled, leading to the formation of intracellular ice, which is almost always lethal to cells⁷. Thus, optimal cryopreservation usually involves cooling at an intermediate rate, to avoid the formation of intracellular ice, and limit freeze-induced dehydration. If cells can be cooled below the glass transition, then further growth of ice is inhibited, and biochemical processes effectively cease.

The addition of penetrating CPAs is generally essential for successful cryopreservation. CPAs need to have the following properties: they should depress the equilibrium freezing point and encourage supercooling; they should have a relatively high glass transition temperature; and they must permeate cell membranes so that intracellular vitrification can be achieved^{3,5}. The proposed mechanisms of action and toxicity of a range of CPAs, are discussed in the literature^{8,9}. Several studies have been carried out to understand the membrane protection effects of natural CPAs such as sugars, especially disaccharides, and the mechanisms are becoming clearer^{6,10-13}. The mechanisms of action of penetrating cryoprotectants have been less widely studied, with the exception of dimethyl sulphoxide (DMSO) and glycerol¹⁴⁻²¹. However, these molecules are toxic to cells and cryopreservation involves balancing these two effects⁵. Other classes of potential penetrating CPAs include sulfoxides, alcohols, amides and imides^{22,23}, including dimethyl formamide (DMF) and ethylene glycol (EG). These compounds offer an alternative to more conventional cryoprotectants, such as DMSO and glycerol, and may have lower toxicity, so are also worth exploring.

The various factors affecting membrane permeability of cells during preservation are reviewed in a recent study by Wolkers et al.²⁴ The interaction of DMSO with membranes has been studied using a range of techniques summarized elsewhere²⁵. DMSO can diffuse across a lipid bilayer²¹ and can partition within an intermembrane space to limit membrane compression by forming a physical barrier between lipid headgroups⁴. However, DMSO has also been reported to dehydrate lipid membranes^{26,27}. Molecular Dynamics simulations show that DMSO induces membrane thinning and increased membrane fluidity at low concentrations and causes the formation of pores across the bilayer at higher concentrations^{4,15-17}. Schrader and co-workers²⁸ found that DMSO reduces the repulsive forces between bilayers, whereas glycerol increases them, and proposed that the differences in hydrogen bonding capability of the two solutes leads DMSO to dehydrate the lipid head groups, while glycerol affects surface hydration only as much as it affects the bulk water properties.

One approach to studying the interactions of molecules with biological membranes is to use model systems of monolayers. Lipid monolayers have been widely accepted as ideal model systems to mimic biological membranes (reviewed for example in²⁹⁻³⁴) as they represent exactly half a bilayer³⁵. They have particular advantages in examining the interactions of biomolecules with lipid membranes, and have been used to study such diverse systems as sugars³⁶, cryoprotectants^{37,38}, proteins²⁹, amphiphilic drugs^{30,31} and antimicrobial peptides³². The conditions under which monolayers and bilayers may be directly compared has also been examined³⁹. The key advantage over bilayer (or multilamellar) systems is that in monolayer studies temperature is constant and the lateral pressure is varied – this enables us to directly probe interactions with water soluble molecules. By contrast the lateral pressure in lamellar systems can only be changed by dehydration (eg.^{40,41}). The effects of solutes on lipid organization can be assessed in terms of the changes in the area per lipid molecule, phase transition pressures and inverse compressibility modulus values^{34,42-44}. These can be directly related to the properties of bilayers: for example, the gel and fluid phases of a bilayer are analogous to the liquid condensed and liquid expanded phases, respectively, of a monolayer^{35,45-47}.

Despite the relevance of monolayer techniques, the interaction between penetrating cryoprotectants and lipid monolayers has not been widely studied except for some early studies using DMSO and glycerol. Williams and Harris⁴⁸ found that DMSO and glycerol are excluded from the lipid-water interface. Bianco et al.⁴⁹ observed that the presence of glycerol modifies the molecular area, surface potential and thermodynamic properties of monolayers of various lipids. Dabkowska et al.³⁷ found that the presence of DMSO dehydrates the phosphatidylcholine headgroup. Chen and co-workers found similar effects using Vibrational Sum Frequency Generation (VSFG)⁴⁵ and Brewster angle microscopy (BAM)²⁵ and found that the effect of DMSO is concentration dependent. These authors suggest that DMSO-induced condensing and caging is the molecular mechanism that may account for the enhanced permeability of membranes upon exposure to DMSO.

In this paper we present a comprehensive study of the effects of four common CPAs (DMSO, glycerol, ethylene glycol and dimethylformamide) on monolayers of four common phospholipids (DPPC, POPC, DOPC and POPE). We study four different phospholipids to determine if the headgroup or level of chain saturation influences the interaction. The CPA concentrations were chosen to be directly relevant to cryoprotection. To the best of the authors' knowledge, there has no previous studies of the effects of glycerol, EG or DMF on phospholipid monolayers although they are widely used as membrane penetrating

cryoprotectants. These are also the first membrane insertion experiments reported for these molecules.

2 Materials and methods

2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) from Sigma-Aldrich Chemistry (Madrid, Spain), 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) from Avanti Polar Lipids (Alabaster, AL, USA) were used without further purification. Both of the lipids were dissolved in chloroform, PA-ACS-ISO grade, purchased from PANREAC (Barcelona, Spain) to a final concentration of 1mg.mL^{-1} and kept in the refrigerator. The subphase used for preparing the Langmuir films was mainly Ultrapure water (Milli Q®, $18.3\text{ M}\Omega\text{cm}$ resistivity). Other than water, various sub-phases such as dimethyl sulphoxide (DMSO), glycerol, ethylene glycol and dimethylformamide (DMF) (all 5% v/v in ultrapure water) were used. All of the above chemicals, PA-ACS-ISO grade, were purchased from PANREAC.

2.2 Techniques and equipment

2.2.1 Experiment 1 – Compression isotherms using standard Langmuir trough

Langmuir compression isotherms were carried out on a NIMA teflon trough (Nima Technology, Coventry, UK), model 1232D1D2 (area 1200 cm^2), equipped with two movable barriers, and the surface pressure was measured using a pressure sensor with a Wilhelmy plate ($10\text{ mm} \times 50\text{ mm}$ filter paper; Whatman International, Maidstone, UK). The linear velocity of the barriers was 2.5 cm.min^{-1} , which corresponds to an area change of $50\text{ cm}^2.\text{min}^{-1}$ in the trough used.

The trough was placed on a vibration-isolated table (Newport, Irvine, CA, USA) and enclosed in an environmental chamber. The resolution of surface pressure measurement was $\pm 0.1\text{ mN m}^{-1}$. In all experiments, the temperature was controlled at $22.0 \pm 1^\circ\text{C}$. In each experiment, the trough and barriers were cleaned twice with chloroform, allowing complete chloroform evaporation, and once with MilliQ® quality water before the subphase addition. In order to ensure the cleanliness of the subphase, the following procedure was implemented prior to each run: the trough area was compressed to the minimum surface area, and any surface contaminants were removed by aspiration until zero surface pressure was measured. This procedure was repeated at least 3 times prior to each measurement. Other than MilliQ® quality water, several different CPA sub-phases were used at 5% v/v (dimethyl sulphoxide (DMSO), glycerol, ethylene glycol (EG) and dimethylformamide (DMF)). 5% v/v was chosen as it is a common concentration used in cryoprotective solutions.

A $100\text{ }\mu\text{L}$ aliquot of lipid dissolved in chloroform (concentration 0.5 mg.mL^{-1}) was spread on the surface of the subphase solution with a Hamilton microsyringe (Bonaduz, Switzerland). An equilibration time of 15 min was allowed before starting the experiment, to allow lipid spreading and solvent evaporation. Every Π -A isotherm was repeated at least twice, and the

isotherms showed satisfactory reproducibility. As the concentration and trough area are known, the area per lipid molecule can be calculated.

2.2.2 Experiment 2 - Insertion experiments

In these experiments, the effect of inserting the CPA directly into the subphase was measured by the change in surface pressure of the lipid monolayer. The experiments were carried out using a NIMA Langmuir Film Balance equipped with a Wilhelmy plate and a small teflon trough that was rinsed with chloroform and ultrapure water before use. All experiments were performed at $22 \pm 1^\circ\text{C}$.

For these experiments, a lipid stock solution was prepared and added dropwise on the water subphase [$\sim 70\text{mL}$] until the desired surface pressure was achieved. After 15 min, when spreading equilibrium was reached, the desired cryoprotectant in the specific volume [$\sim 4\text{mL}$] to attain a concentration of 5% (v/v) was injected into the subphase. At moderate to high surface pressures there was sometimes a small initial reduction in surface pressure of between 1-5% following removal of the syringe, presumably due a small amount of lipid being removed by the insertion needle, as per Langmuir-Blodgett deposition. The value of surface pressure following insertion was therefore used as the initial surface pressure in the calculations. Following insertion, changes were monitored as a function of time for at least 15 min, until the surface pressure remained constant. For the control experiment the same procedure was followed but with a lipid-free interface. For each lipid, three lipid concentrations were measured (corresponding to different initial surface pressures). The change in surface pressure upon cryoprotectant addition is a direct measure of its effect on the interfacial properties of the monolayer.

3 Results and Discussion

3.1 Effect of cryoprotectants on water surface tension

The surface activity of the cryoprotectants is measured in this section. Initially the surface tension, γ , of the water subphase was measured and found to be 72.6 mN.m^{-1} . CPAs were then added to give a final concentration of 5% by volume, and the surface tension remeasured. Table 1 shows the measured surface tensions, and the CPA concentration in both mol.L^{-1} , and mass fraction X_{CPA} . It is seen that DMF has significant surface activity, reducing the surface tension by 5.6 mN.m^{-1} compared to pure water, while the other molecules have minimal activity.

Sub-phase solutions (5% v/v)	γ (mN.m^{-1})	C_{CPA} (mol/L)	X_{CPA}
Water	72.6	--	--
Glycerol	74.8	0.683	0.0128
EG	72.8	0.894	0.0167
DMF	67.0	0.646	0.0121
DMSO	72.6	0.706	0.0132

Table 1: Surface tension of water and CPA subphases

3.2 Influence of cryoprotectants on lipid monolayers - Compression Isotherms

The phase behavior can be characterized by surface pressure-area isotherms. Figure 1(a-d) shows the surface pressure-area isotherms of monolayers of the 4 lipids DOPC, POPE, DPPC and POPC on subphases consisting of water, or aqueous solutions with 5% CPA (v/v). The isotherms were reproducible on successive runs. The following parameters are extracted from these isotherms: the lift off area (where the surface pressure rises above zero); the inflection points (where there is a phase change); and the collapse pressure (where pressure reaches a maximum, and further compression leads to collapse of the monolayer). These parameters are provided in Table 2.

The first thing to note from these isotherms is that each pure lipid has slightly different behavior, with DOPC and POPC showing continuous isotherms up to the collapse pressure, whereas both DPPC and POPE show inflections corresponding to a phase change. In all cases, the behavior is not qualitatively changed by the presence of the CPAs, though there are quantitative changes in the values of key parameters.

For all lipids studied, DMF causes a significant compression, shifting the isotherms to the left, whereas glycerol and EG both cause an expansion. This means that at the same surface pressure, DMF causes a reduction in area per molecule, whereas glycerol and EG cause an increase in the area per molecule. A compression of the monolayer implies that the presence of DMF is effectively dehydrating the headgroups, forcing them closer together. By contrast, the behavior of glycerol and EG implies that they are inserting between the lipids, thus increasing the average area per lipid molecule at fixed surface pressure. This effect of glycerol agrees with that reported in literature²⁸. These different behaviors have important implications for the mechanisms of cryoprotection. The behavior of glycerol and EG are consistent with their behavior as membrane penetrating cryoprotectants - these must reside between lipids during the passage across the membrane, so the observed expansion is consistent with this behavior. The fact that this effect is fairly similar for all four lipids implies that the effect is not strongly dependent on lipid headgroup or chain configuration.

DMSO shows different behavior. For DPPC (saturated) it causes a clear expansion, whereas for POPC (singly unsaturated) it induces a small compression. For POPE and DOPC, the presence of DMSO has little effect. How do we interpret these conflicting results? First, the clear expansion in the presence of DPPC implies significant DMSO penetration into the monolayer. For POPC on the other hand there appears to be some dehydration. For DOPC, there is little effect. So, it appears that DMSO has both a membrane penetration effect, and a headgroup dehydration effect, which leads to subtle differences depending on the packing of the headgroups. We will return to this point later. Finally, we note that there is only a small difference between POPC and POPE, which implies only a small headgroup dependence in the interaction with DMSO.

It is known that these molecules are cell permeable, and so must interact with and pass through the bilayer. The permeability of cells to a particular cryoprotectant is generally termed the cryoprotectant permeability⁵⁰, and has been observed in previous work for glycerol, EG, and DMSO⁵⁰⁻⁵². These papers found that glycerol has much lower permeability than DMSO and EG. This is interesting in the light of the isotherm results – glycerol expands the monolayer, and so must enter into the headgroup region. The low measured permeability implies that it may take a long time to cross the hydrocarbon region of the bilayer. To the best of the authors'

knowledge, there are no measurements of DMF cell permeability. The monolayer studies suggest that DMF dehydrates the headgroups as it causes a net compression. However, DMF has been successfully applied as a cryoprotectant (eg²²).

Figure 1: Effect of cryoprotectant on the surface properties of lipids. Surface pressure vs. area per molecule for (a) DOPC, (b) POPC, (c) DPPC, (d) POPE with subphases as shown in the legend to (a). Note that the scales are different for each lipid.

In order to further understand these data, Figure 2 shows the calculated inverse compressibility modulus (C_s^{-1})⁴⁴:

$$C_s^{-1} = -A \left(\frac{d\Pi}{dA} \right)_T \quad \text{Eq. (1)}$$

for the data in figure 1. The values of C_s^{-1} allows one to assign the monolayer physical state during compression: C_s^{-1} values between $12.5\text{--}100 \text{ mN.m}^{-1}$ correspond to the liquid expanded (LE) state, $100 < C_s^{-1} < 250 \text{ mN.m}^{-1}$ corresponds to the liquid condensed (LC) state, and $C_s^{-1} > 250 \text{ mN.m}^{-1}$ corresponds to the solid (S) state^{53,54}.

Figure 2: Inverse compressibility modulus vs. surface pressure for the data in figure 1.

DOPC and POPC exhibit the LE state over the whole range, with collapse occurring as the value of C_s^{-1} approaches that of the LC phase. POPE shows a clear inflection corresponding to a phase change, but collapse occurs immediately afterwards, so again a stable LC phase is not seen. POPE has its transition temperature at 25 °C, above the working temperature of 22 °C. A result of this is the phase transition around 40 mN/m in the isotherm. Similarly, DPPC exhibits a clear inflection corresponding to a phase change, with C_s^{-1} value rising to above 200 mN.m^{-1} , corresponding to the LC phase. This is due to the fact that the transition temperature is 41 °C, above the working temperature of 22 °C. By contrast POPC (transition temperature of -2 °C) and DOPC (transition temperature of -17 °C) do not show phase transition as the corresponding transition temperatures are below 22 °C.

For DOPC and POPC (Figures 2a, b) the C_s^{-1} values for DMSO, EG and glycerol subphase are practically the same as that of water subphase until the collapse, with DMF inducing collapse at a slightly lower surface pressure. Interestingly, for DOPC glycerol exhibits slightly lower C_s^{-1} values at lower surface pressures (Figure 2a).

For DPPC (Figure 2c) the C_s^{-1} values of the CPA and water subphases show a similar value until the inflection ($\sim 8 \text{ mN.m}^{-1}$). Above the inflection, there is a notable influence of the CPA

subphases, with C_s^{-1} values for DMSO and glycerol significantly lower than that of the water subphase, whereas the EG subphase shows higher values of C_s^{-1} than water subphase.

Finally, for POPE (Figure 2d), DMSO shows similar C_s^{-1} values to that of water. Glycerol exhibits lower values at low surface pressures, whereas DMF shows a significantly lower transition.

The key parameters extracted from Figures 1 and 2 are summarized in table 2 and discussed here. DOPC (Fig 1a, 2a) shows a lift off area of $118.7 \text{ \AA}^2/\text{molecule}$, with a liquid expanded (LE) state until the collapse (area $\sim 58.7 \text{ \AA}^2/\text{molecule}$, $\Pi \sim 45.4 \text{ mN.m}^{-1}$, $C_s^{-1}_{\text{max}} = 108 \text{ mN.m}^{-1}$). Similar behavior was observed for POPC (Fig 1b, 2b) where the lift-off is at $102.5 \text{ \AA}^2/\text{molecule}$, with a LE state until 50.1 mN.m^{-1} ($C_s^{-1}_{\text{max}} = 118.2 \text{ mN.m}^{-1}$).

The DPPC isotherm (Fig 1c, 2c) behaves very differently. After the lift-off at $\sim 95.5 \text{ \AA}^2/\text{molecule}$, it exhibits LE state until 6.4 mN.m^{-1} , then there is a marked inflection followed by a regular increase in surface pressure corresponding to a Liquid condensed state (LC), and finally a collapse at $\sim 59.9 \text{ mN.m}^{-1}$. Similarly, the POPE monolayer on pure water (Fig 1d, 2d) shows several phases such as the liquid expanded (LE_1) state but with a maximum C_s^{-1} value around 118 mN.m^{-1} at $\Pi \sim 32.3 \text{ mN.m}^{-1}$, which is followed by a phase transition to what is either another liquid expanded (LE_2) state, or to a liquid condensed state (LC) till the collapse at $\Pi \sim 51.1 \text{ mN.m}^{-1}$ - the proximity between the inflection and the collapse do not permit a unique determination of monolayer state in this region.

We now turn to the effects of the CPAs on the lipid monolayer behavior. Looking first at the different headgroups (POPC vs POPE) it can be observed that each of CPAs induces qualitatively similar changes in the isotherms and compressibility moduli for the two lipids, even though small quantitative changes can be observed – in other words, there are no notable effects which are specific to the lipid headgroup. As most previous studies have only been carried out on PC headgroups, this is an important finding, and will be discussed further below.

Considering now the individual CPAs, it is observed that the compounds with hydroxyl groups (glycerol and EG) cause an expansion of the monolayer across the whole pressure range for all four lipids, but with no significant effect on the collapse or transition surface pressures, as evidenced by the similarity of the C_s^{-1} plots. Glycerol, with 3 hydroxyl groups, causes a slightly greater expansion of the monolayer than EG, with 2 hydroxyl groups.

The CPA that shows the largest effect is DMF, which induces a compression for all four lipids and also causes significant reductions in the collapse surface pressures and transition surface pressures - this decrease in the π_c may be related in part to the small surface-active character of DMF, as it competes with the lipid at the interface.

Lipids/ Subphase	A_0 ($\text{\AA}^2/\text{molecule}$)	A_c ($\text{\AA}^2/\text{molecule}$)	Π_c (mN.m^{-1})	$C_s^{-1}_{\text{max}}$ (mN.m^{-1})	Π_{max} (mN.m^{-1})
DOPC	118.7	58.7	45.4	108	39.5
+Glycerol	142	63.3	45.9	108	43.1
+EG	121.2	63.3	45.9	113	41.8
+DMF	108.5	58.2	38.3	103	33.8
+DMSO	117.7	60.5	43.4	106	41.4

POPC	102.5	50.1	49.0	118.2	42.4
+Glycerol	114.7	54.8	47.7	119	36.7
+EG	108.0	55.7	44.9	116	40.2
+DMF	85.7	46.2	40.9	117	36.7
+DMSO	97.5	50.5	45.6	121	42.0
DPPC	95.5	36.1	59.9	29.3/233	6.4/41.1
+Glycerol	107.5	39.3	62.9	32.4/211	7.2/44.1
+EG	100.5	39.9	60.7	29.2/247	6.3/41.8
+DMF	72.4	30.7	57.3	20.5/216	2.2/35.9
+DMSO	102.5	38.6	59.8	29.3/174	4.6/41.3
POPE	92.0	38.2	51.1	118/106	32.3/45.9
+Glycerol	114.3	36.5	53.5	112/86.7	32.2/46.7
+EG	98.5	41.3	48.9	124/84.1	31.6/44.3
+DMF	78.6	35.8	46.1	106/127	25.6/38.1
+DMSO	90.4	39.2	49.6	116/106	31.3/43.5

Table 2: Parameters extracted from figures 1 & 2: lift off area (A_0), collapse area (A_c), collapse pressure (Π_c), C_s^{-1} and Π in the maximum of C_s^{-1} ($C_{s^{-1}}^{-1} \max$, $\Pi_{\max}$)

Finally, DMSO has almost no effect on the position of the isotherms for DOPC, POPC or POPE except for a slightly lower collapse surface pressure and/or transition surface pressure. Similarly, there is little effect on the C_s^{-1} plots for these lipids. By contrast, DMSO has a significant effect on the fully saturated lipid DPPC (Fig 1c & 2c) – the presence of DMSO shifts the isotherm to the right *ie* it provokes an area increase over the whole range. Both the lift off area and the area of the transition from LE to LC are significantly higher with DMSO than for the pure DPPC lipid. However, the cryoprotectant effects follow the same trends (figure 1C) both below and above the transition from liquid expanded to liquid condensed states (analogous to the fluid and gel states in a bilayer). This implies that headgroup interactions dominate over chain packing effects.

3.3 Insertion experiments

The insertion ability of CPAs into the lipid monolayers was monitored by measuring the variation in surface pressure, $\Delta\Pi$, as a function of the surface pressure immediately following insertion (see section 2.2.2). The results are shown in Figure 3 (a-d). For all lipids and all CPAs, it is observed that the change in pressure following insertion, $\Delta\Pi$, decreases linearly as the initial surface pressure Π_i is increased. This is not surprising - higher initial surface pressure means lower area per lipid, and therefore less room for the CPAs to insert between the lipids. However, although the CPAs follow the same trend, there are significant quantitative differences, with the slope and $\Delta\Pi$ values generally higher for DMF, and to a lesser extent glycerol.

In order to examine these differences, linear fits to the data yield two parameters, the slope and the extrapolation to $\Delta\Pi = 0$, called the Maximum insertion pressure (MIP). According to the treatment of Calvez et al.,⁵⁵ (eq 2), the slope is $(a-1)$ where a is called the “synergy factor”. Values of a and MIP are given in Table 3.

Figure 3: Variation in surface pressure vs. initial surface pressure in insertion experiments. The lines are linear regressions with the fit parameters presented in Table 3.

In all cases $a > 0$, which corresponds to favorable conditions for molecule monolayer binding, with higher values indicating more favorable binding of the CPA into the phospholipid monolayer. A number of trends can be identified from the table. First, DMF generally has the highest MIP, and the lowest value of a . The MIP is the maximum surface pressure beyond which the molecules cannot insert into the monolayer (ie they would be squeezed out at surface pressures higher than this). Clearly DMF can remain within the monolayer to higher surface pressures. Conversely, the fact that DMF has the highest slope means that changes in surface pressure have a larger effect on the insertion.

LIPIDS	DPPC		POPC		DOPC		POPE	
CPAs	MIP	a	MIP	a	MIP	a	MIP	a
Glycerol	17.5	0.81	26.4	0.85	33.5	0.78	33.5	0.80
EG	23.9	0.91	27.7	0.92	32.1	0.96	22.6	0.91
DMF	23.6	0.26	43.5	0.48	71.9	0.79	40.6	0.62

DMSO	18.0	0.93	28.7	0.91	29.5	0.93	20.8	0.90
-------------	------	------	------	------	------	------	------	------

Table 3: Values of the Maximum Insertion Pressure (MIP) and synergy factor (a) for the data in figure 3.

The results shown in Table 3 are instructive for comparing the different lipids. Comparing the MIP and values of a for POPC and POPE, which only differ in the headgroup, shows qualitatively small differences, as also seen in figures 3 b, d, but some effects can be observed for DMF and glycerol. The largest difference is for DMF, where the slope is significantly larger for POPC (ie the values of a are lower) – this means small increases in surface pressure excludes DMF more strongly for POPC than POPE. For glycerol the insertion is slightly higher for POPE than for POPC, probably due to the ability of glycerol to interact stronger with POPE headgroups through H-bonding. Nevertheless, this influence is lower than that one might expect. Schrader et al.²⁸ points out that glycerol affects surface hydration only as much as it affects bulk water - ie glycerol doesn't show a preferential interaction with the water surface relative to the bulk, which might explain why the influence of a PE headgroup weaker than might be expected. Most studies have been done with PC headgroups; thus, more studies should be done with PE headgroups in order to understand better the headgroup influence.

Clearly the MIP is lower for DPPC than the other lipids – this makes sense given that DPPC has saturated chains, and a smaller inherent area per molecule, attaining an LC state at moderate surface pressures. The slopes (and hence a values) are comparable between DPPC and the other lipids with the exception of DMF, which has the lowest a value of all the systems studied – here small increases in surface pressure lead to significant exclusion of DMF from the monolayer.

4 General discussion

There have been relatively few studies of the effects of artificial cryoprotectants on monolayer isotherms, and most studies that exist have concentrated on DMSO and DPPC. Both Krasteva et al.³⁸ and Chen et al.^{25,45} found that DMSO compresses DPPC isotherms. This effect is the opposite of that found in the current study, where DMSO was observed to expand the DPPC monolayer slightly. Those previous studies used higher concentrations than here, and Chen et al., noted a significant concentration dependence, which may explain the differences observed in the present study. Both studies concluded that DMSO leads to lipid dehydration. Recently, Dabkowska et al.³⁷ performed Neutron reflectivity on DPPC monolayers in the presence of DMSO, and did not find a significant effect on the surface pressure, but found strong evidence to support the idea of that at high concentrations, DMSO dehydrates the headgroups by displacing water molecules bound to the choline group. Clearly concentration is a key factor – the concentrations chosen for the present study are relevant for cryopreservation.

The effects of DMSO are strongly dependent on the lipid species too – while DMSO caused expansion for DPPC (in both the liquid expanded and condensed phases), it caused compression for POPC, and had little effect for POPE or DOPC. So, it is clear that the effects of DMSO are subtle and concentration dependent – the variability seen for the effect of DMSO on the different lipids highlights this. Clearly further work is needed to understand these effects.

The authors are aware of no previous studies of the effects of glycerol, EG or DMF on phospholipid monolayers. However, while there are no monolayer studies, computer

simulations studying the interactions of CPAs on bilayers can provide useful information, as the technique allows for the detailed study of the location of the molecules near the headgroups. Sum et al.⁵⁶ found that DMSO penetrates more deeply into the bilayer than water – indeed they found that the DMSO sequesters beneath the headgroup, rather than interacting directly with the polar groups, leading to an increase in area per headgroup. Due to the strong DMSO-water interaction they also found that DMSO dehydrates the headgroups.

Malajczuk et al.⁴ examined the effects of DMSO, glycerol and ethylene glycol on DPPC in the fluid phase. They found that each solvent induced lateral expansion of the membrane, consistent with the results found here. These simulations found that EG and glycerol induced less expansion than DMSO, contrary to what was found in this study. Again, the simulation trends were observed for much higher CPA concentrations than are used here, so the differences may be due to the concentration.

The insertion studies showed that the phospholipid species has a notable impact on the interactions with the CPAs. Considering physiological values of lateral pressure (around 33 mN.m⁻¹), and looking at the MIP values, it is seen that EG and DMSO do not penetrate the phospholipid monolayers of DOPC, glycerol is at the limit, and only DMF clearly penetrates. For POPC and POPE only DMF penetrates at the physiological values of lateral pressure, and for DPPC none of the studied CPAs penetrate at the physiological values of lateral pressure. These results agree with Williams and Harris⁴⁸ who reported that DMSO and glycerol are excluded from the lipid-water interface. From these results it is seen that, in general, DPPC is the most difficult to penetrate due to its rigidity and compactness (tighter chain packing due to no unsaturation in the acyl chains), while DOPC is, in general, the most facile to penetrate due to its lower rigidity and compactness (two double bonds in the acyl chains). Consequently, penetration of CPAs at physiological values of lateral pressure needs the presence of phospholipids with unsaturation, as is commonly the case in natural membranes. Thus, extrapolations of results based solely on the saturated lipid DPPC need to be made with care.

5 Conclusions

Langmuir monolayers have long been used to obtain the information about the forces operating within the film by measuring the relationship between the surface pressure and area and then extending the results to bio membranes with similar lipid molecules. The results of this work clearly highlight the different interaction between various cryoprotectants and phospholipid molecules. This knowledge is very important in understanding the cryoprotection mechanism as well as toxicity of various molecules and this knowledge will aid in the development of novel non-toxic cryoprotectants.

At the concentration studied here (of relevance to cryopreservation), DMSO caused an expansion of the DPPC area per lipid. This is in contrast to previous studies^{25,38,45}, which reported compression. However, those studies used higher CPA concentrations, so this highlights the importance of understanding concentration dependent effects, and of studying concentrations relevant to the application (in this case cryopreservation). The results of this study reveal that the effect of DMSO varies with the lipid species. Since many of the previous studies have analyzed the effect of DMSO on DPPC membranes, this study highlights the importance of studying different lipid types as these may have a significant effect on the interactions.

This study showed that both EG and glycerol cause a small expansion of the monolayer at fixed pressure, implying that they insert into the headgroup regions. This effect is similar for all lipid species studied and is consistent with their ability to penetrate membranes. By contrast, DMF causes monolayer compression for all lipid species, implying it dehydrates the lipid head groups. This is perhaps related to the small inherent surface activity of DMF. This surface activity may also be responsible for the fact that DMF had the highest maximum insertion pressure (ie showed the strongest affinity to stay in the monolayer). DMF has been used as a cryoprotectant, but there have been very few studies of its lipid interactions – clearly more work needs to be done here.

All four CPAs are known to penetrate into cells. Insertion studies done here also reveals the importance of phospholipid species on cryoprotectant penetration and interaction with the lipid molecules. Understanding the mechanisms of this penetration in more detail is the goal of future work using neutron membrane diffraction in lamellar systems^{2,11,12}.

While previous studies have been conducted overwhelmingly with model membranes (often DPPC) it is clear that the lipid species has a significant effect – more studies are needed with membrane models which more closely mimic biological membranes. In particular, almost all previous studies have been conducted with zwitterionic (neutral) lipids. However, one might expect charged lipids (such as phosphatidylinositol) to play a significant role in interactions with polarizable molecules such as CPAs, and effects of salts and pH may also play a significant role. Further studies of such systems are clearly needed.

Conflicts of Interest: The authors declare no conflicts of interest.

Acknowledgements

RR thanks the European Union and UPC for an Erasmus-K7 grant and acknowledges the support of an Australian Government Research Training Program Scholarship from RMIT. GB acknowledges funding from the Australian Research Council under Linkage Grant (Nos. LP140100993 and LP160101496). The authors would like to thank an anonymous reviewer who highlighted anomalies in the data that led us to notice the slight drop in pressure following insertion (see section 2.2.2) leading to a more robust analysis.

6 References

- 1 Bryant, G., Koster, K. L. & Wolfe, J. Membrane behaviour in seeds and other systems at low water content: the various effects of solutes. *Seed Science Research* **11**, 17-25 (2001).
- 2 Garvey, C. J., Lenne, T., Koster, K. L., Kent, B. & Bryant, G. Phospholipid membrane protection by sugar molecules during dehydration-insights into molecular mechanisms using scattering techniques. *Int J Mol Sci* **14**, 8148-8163, doi:10.3390/ijms14048148 (2013).

- 3 Wolfe, J. & Bryant, G. Cellular cryobiology: thermodynamic and mechanical effects. *International Journal of Refrigeration-Revue Internationale Du Froid* **24**, 438-450, doi:10.1016/s0140-7007(00)00027-x (2001).
- 4 Malajczuk, C. J., Hughes, Z. E. & Mancera, R. L. Molecular dynamics simulations of the interactions of DMSO, mono- and polyhydroxylated cryosolvents with a hydrated phospholipid bilayer. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1828**, 2041-2055 (2013).
- 5 Wolfe, J. & Bryant, G. Freezing, drying, and/or vitrification of membrane-solute-water systems. *Cryobiology* **39**, 103-129, doi:10.1006/cryo.1999.2195 (1999).
- 6 Lenne, T., Garvey, C. J., Koster, K. L. & Bryant, G. Kinetics of the lamellar gel-fluid transition in phosphatidylcholine membranes in the presence of sugars. *Chem Phys Lipids* **163**, 236-242, doi:10.1016/j.chemphyslip.2009.12.001 (2010).
- 7 Mazur, P. Freezing of living cells: mechanisms and implications. *American Journal of Physiology-Cell Physiology* **247**, C125-C142 (1984).
- 8 Fahy, G. M. Cryoprotectant toxicity neutralization. *Cryobiology* **60**, S45-S53, doi:10.1016/j.cryobiol.2009.05.005 (2010).
- 9 Fuller, B. J. Cryoprotectants: the essential antifreezes to protect life in the frozen state. *CryoLetters* **25**, 375-388 (2004).
- 10 Kent, B. *et al.* Measurement of glucose exclusion from the fully hydrated DOPE inverse hexagonal phase. *Soft Matter* **6**, 1197, doi:10.1039/b919086d (2010).
- 11 Kent, B. *et al.* Direct comparison of disaccharide interaction with lipid membranes at reduced hydrations. *Langmuir* **31**, 9134-9141, doi:10.1021/acs.langmuir.5b02127 (2015).
- 12 Kent, B. *et al.* Localization of trehalose in partially hydrated DOPC bilayers: insights into cryoprotective mechanisms. *J R Soc Interface* **11**, 20140069, doi:10.1098/rsif.2014.0069 (2014).
- 13 Stewart, S. & He, X. Intracellular Delivery of Trehalose for Cell Banking. *Langmuir* (2018).
- 14 De Leeuw, F., De Leeuw, A., Den Daas, J., Colenbrander, B. & Verkleij, A. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology* **30**, 32-44 (1993).
- 15 Gurtovenko, A. A. & Anwar, J. Modulating the structure and properties of cell membranes: The molecular mechanism of action of dimethyl sulfoxide. *The Journal of Physical Chemistry B* **111**, 10453-10460 (2007).
- 16 Hughes, Z. E., Mark, A. E. & Mancera, R. L. Molecular dynamics simulations of the interactions of DMSO with DPPC and DOPC phospholipid membranes. *The Journal of Physical Chemistry B* **116**, 11911-11923 (2012).
- 17 Notman, R., Noro, M., O'Malley, B. & Anwar, J. Molecular basis for dimethylsulfoxide (DMSO) action on lipid membranes. *Journal of the American Chemical Society* **128**, 13982-13983 (2006).
- 18 Simonin, H., Winckler, P., Gros, A., Perrier-Cornet, J.-M. & Gervais, P. How dehydration with glycerol protects bacteria cell membranes against deleterious effects of supercooling. *Cryobiology* **71**, 549, doi:10.1016/j.cryobiol.2015.10.051 (2015).
- 19 Yu, Z.-W. & Quinn, P. J. The modulation of membrane structure and stability by dimethyl sulphoxide. *Molecular Membrane Biology* **15**, 59-68 (1998).

- 20 Anchordoguy, T. J., Cecchini, C. A., Crowe, J. H. & Crowe, L. M. Insights into the cryoprotective mechanism of dimethyl sulfoxide for phospholipid bilayers. *Cryobiology* **28**, 467-473 (1991).
- 21 Anchordoguy, T. J., Rudolph, A. S., Carpenter, J. F. & Crowe, J. H. Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology* **24**, 324-331 (1987).
- 22 Alvarenga, M. A., Papa, F. O., Landim-Alvarenga, F. & Medeiros, A. Amides as cryoprotectants for freezing stallion semen: a review. *Animal Reproduction Science* **89**, 105-113 (2005).
- 23 Hubálek, Z. Protectants used in the cryopreservation of microorganisms. *Cryobiology* **46**, 205-229, doi:10.1016/s0011-2240(03)00046-4 (2003).
- 24 Wolkers, W. F. *et al.* Factors affecting the membrane permeability barrier function of cells during preservation technologies. *Langmuir* **35**, 7520-7528 (2018).
- 25 Chen, X., Huang, Z., Hua, W., Castada, H. & Allen, H. C. Reorganization and caging of DPPC, DPPE, DPPG, and DPPS monolayers caused by dimethylsulfoxide observed using Brewster angle microscopy. *Langmuir* **26**, 18902-18908 (2010).
- 26 Kiselev, M., Lesieur, P., Kiselev, A., Grabielle-Madmond, C. & Ollivon, M. DMSO-induced dehydration of DPPC membranes studied by X-ray diffraction, small-angle neutron scattering, and calorimetry. *Journal of Alloys and Compounds* **286**, 195-202 (1999).
- 27 Cheng, C.-Y., Song, J., Pas, J., Meijer, L. H. & Han, S. DMSO induces dehydration near lipid membrane surfaces. *Biophysical Journal* **109**, 330-339 (2015).
- 28 Schrader, A. M., Cheng, C.-Y., Israelachvili, J. N. & Han, S. Communication: Contrasting effects of glycerol and DMSO on lipid membrane surface hydration dynamics and forces. *The Journal of chemical physics* **145**, 041101-041101, doi:10.1063/1.4959904 (2016).
- 29 Brockman, H. Lipid monolayers: why use half a membrane to characterize protein-membrane interactions? *Current opinion in structural biology* **9**, 438-443 (1999).
- 30 Eeman, M. & Deleu, M. From biological membranes to biomimetic model membranes. *Biotechnologie, Agronomie, Société et Environnement* **14**, 719 (2010).
- 31 Hąc-Wydro, K. & Dynarowicz-Łątka, P. Biomedical applications of the Langmuir monolayer technique. *Annales UMCS, Chemistry* **63**, 47-60 (2008).
- 32 Maget-Dana, R. The monolayer technique: a potent tool for studying the interfacial properties of antimicrobial and membrane-lytic peptides and their interactions with lipid membranes. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1462**, 109-140 (1999).
- 33 Siontorou, C. G., Nikoleli, G.-P., Nikolelis, D. P. & Karapetis, S. K. Artificial Lipid Membranes: Past, Present, and Future. *Membranes* **7**, 38, doi:10.3390/membranes7030038 (2017).
- 34 Giner-Casares, J. J., Brezesinski, G. & Möhwald, H. Langmuir monolayers as unique physical models. *Current Opinion in Colloid & Interface Science* **19**, 176-182 (2014).
- 35 Pinto, O. A. & Disalvo, E. A. A new model for lipid monolayer and bilayers based on thermodynamics of irreversible processes. *PLOS ONE* **14**, e0212269, doi:10.1371/journal.pone.0212269 (2019).
- 36 Arnett, E. M., Harvey, N., Johnson, E., Johnston, D. S. & Chapman, D. No phospholipid monolayer-sugar interactions. *Biochemistry* **25**, 5239-5242 (1986).

- 37 Dabkowska, A. P. *et al.* Modulation of dipalmitoylphosphatidylcholine monolayers by dimethyl sulfoxide. *Langmuir* **30**, 8803-8811 (2014).
- 38 Krasteva, N., Vollhardt, D., Brezesinski, G. & Möhwald, H. Effect of sugars and dimethyl sulfoxide on the structure and phase behavior of DPPC monolayers. *Langmuir* **17**, 1209-1214 (2001).
- 39 Marsh, D. Lateral pressure in membranes. *Biochimica et Biophysica Acta (BBA) - Reviews on Biomembranes* **1286**, 183-223, doi:10.1016/S0304-4157(96)00009-3 (1996).
- 40 Bryant, G. & Koster, K. L. Dehydration of solute-lipid systems: hydration forces analysis. *Colloids and Surfaces B: Biointerfaces* **35**, 73-79 (2004).
- 41 Lenné, T., Bryant, G., Holcomb, R. & Koster, K. L. How much solute is needed to inhibit the fluid to gel membrane phase transition at low hydration? *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1768**, 1019-1022 (2007).
- 42 Nowotarska, S. W., Nowotarski, K. J., Friedman, M. & Situ, C. Effect of structure on the interactions between five natural antimicrobial compounds and phospholipids of bacterial cell membrane on model monolayers. *Molecules* **19**, 7497-7515 (2014).
- 43 Torrent-Burgués, J. Phase separation in mixed monolayers of arachidic acid and a phthalocyanine of zinc. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **396**, 137-143 (2012).
- 44 Torrent-Burgués, J. Langmuir films study on lipid-containing artificial tears. *Colloids and Surfaces B: Biointerfaces* **140**, 185-188 (2016).
- 45 Chen, X. & Allen, H. C. Interactions of dimethylsulfoxide with a dipalmitoylphosphatidylcholine monolayer studied by vibrational sum frequency generation. *The Journal of Physical Chemistry A* **113**, 12655-12662 (2009).
- 46 Nagle, J. F. Chain model theory of lipid monolayer transitions. *The Journal of Chemical Physics* **63**, 1255-1261 (1975).
- 47 Nagle, J. Theory of lipid monolayer and bilayer phase transitions: effect of headgroup interactions. *The Journal of membrane biology* **27**, 233-250 (1976).
- 48 Williams, R. J. & Harris, D. The distribution of cryoprotective agents into lipid interfaces. *Cryobiology* **14**, 670-680 (1977).
- 49 Bianco, I. D., Fidelio, G. D. & Maggio, B. Effect of glycerol on the molecular properties of cerebrosides, sulphatides and gangliosides in monolayers. *Biochemical Journal* **251**, 613 (1988).
- 50 Shu, Z. Q. *et al.* A study of the osmotic characteristics, water permeability, and cryoprotectant permeability of human vaginal immune cells. *Cryobiology* **72**, 93-99, doi:10.1016/j.cryobiol.2016.03.003 (2016).
- 51 Pedro, P. B. *et al.* Permeability of Mouse Oocytes and Embryos at Various Developmental Stages to Five Cryoprotectants. *Journal of Reproduction and Development* **51**, 235-246, doi:10.1262/jrd.16079 (2005).
- 52 Vian, A. M. & Higgins, A. Z. Membrane permeability of the human granulocyte to water, dimethyl sulfoxide, glycerol, propylene glycol and ethylene glycol. *Cryobiology* **68**, 35-42, doi:10.1016/j.cryobiol.2013.11.004 (2014).
- 53 Patterson, M., Vogel, H. J. & Prenner, E. J. Biophysical characterization of monofilm model systems composed of selected tear film phospholipids. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1858**, 403-414 (2016).

- 54 Vitovič, P., Nikolelis, D. & Hianik, T. Study of calix [4] resorcinarene–dopamine complexation in mixed phospholipid monolayers formed at the air–water interface. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1758**, 1852-1861 (2006).
- 55 Calvez, P., Demers, E. r., Boisselier, E. l. & Salesse, C. Analysis of the contribution of saturated and polyunsaturated phospholipid monolayers to the binding of proteins. *Langmuir* **27**, 1373-1379 (2011).
- 56 Sum, A. K. & de Pablo, J. J. Molecular simulation study on the influence of dimethylsulfoxide on the structure of phospholipid bilayers. *Biophysical journal* **85**, 3636-3645, doi:10.1016/S0006-3495(03)74781-X (2003).