THE EFFECT OF DIMETHYLSULPHOXIDE ON THE PHASE BEHAVIOUR OF MODEL LIPID MEMBRANES USING SMALL ANGLE SCATTERING

A thesis submitted in fulfilment of the requirements for the degree of Master of Science

Uzma Malik
(MSc BioChem UAAR, Pakistan)
(B.Ed (Sci) AlOU, Pakistan)
(BSc (Sci) PU, Pakistan)
(Dip (ECEC) MCIE, Australia)

School of Science
College of Science Engineering and Health
RMIT University

April 2018
Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the choose an item is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Uzma Malik

April 2018
Acknowledgements

A profound gratitude to my life-coach, my late father, because I owe it all to you. Many Thanks!

I would like to express my special appreciation and thanks to my supervisor Professor Dr. Gary Bryant, for being a tremendous mentor for me. Your useful and brilliant comments, immense knowledge, suggestions, remarks, priceless advice and engagement throughout the learning process of this thesis is remarkable. I would like to thank you for making me grow as a research scientist. I would also like to thank my second supervisor, Assistant professor Christopher Garvey.

A very special gratitude goes out to School of Applied Science for Research Scholarship.

I am also grateful to my seniors, colleagues and friends who have supported me along the way. Dr Lisa Dias, Dr Matthew Taylor, Reece, Daniel, Dr Arwen Pagon directly or indirectly supported me during this time. Thanks for all your encouragement!

A special thanks to all my family. Words cannot express how grateful I am to them.

At the end, I would like express appreciation to my beloved husband, who always stood by me especially during the period of this study (a marathon), to strive towards my goal, in all the circumstances, whether its joyful moments of birth of our beautiful children or the sad time of loss of my astounding father.
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ABSTRACT AND PROJECT AIMS

Dimethyl sulfoxide (DMSO) is a universal solvent which has diverse applications in research, medicine and industry. It is a commonly used cryoprotectant in cellular systems, preventing the cellular damage caused by freezing. While the physical and chemical properties of DMSO are understood, its effects on lipid bilayers have only previously been studied under a limited range of conditions, and its mechanism of action in membrane protection remains unresolved. In particular, the location of DMSO within a membrane-water system at modest hydration is unclear. This thesis presents an experimental investigation into the effects of DMSO on the structure of phospholipid membranes as functions of DMSO/lipid ratio, temperature and hydration. Small angle X-ray scattering (SAXS) and Differential Scanning Calorimetry (DSC) are used to determine the phase behavior of this system at a range of hydrations in the temperature range 20°C – 90°C. Experiments are conducted on randomly oriented stacks of model lipid bilayers of DPPC. Membrane structure and phase behavior were studied as a function of the molar ratio of DMSO to lipid, and experiments were conducted using DSC and both laboratory based and synchrotron SAXS. Data from these complimentary techniques show that DMSO increases the gel-fluid transition temperature, effectively dehydrating the membranes. However, the dehydrating effect becomes less pronounced at lower hydrations, with virtually no effect for samples equilibrated to 57% relative humidity. DMSO also has significant effects on the average spacing between repeating layers, and on the average distance between lipid chains in both the fluid and gel phases. These results provide insight into the interaction between DMSO and lipid bilayers, and allow us to infer the location of DMSO within the lipid/water system. This is the first experimental study to demonstrate that DMSO must reside in the bilayer (rather than transiently passing through), and this insight will inform future research into understanding cryoprotectant action.
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Biological and model membranes

All living cells are surrounded by a cell membrane that has several roles including protecting cells, maintaining shape, acting as a semi-permeable barrier to create a distinction between the inside and outside of a cell, and allowing transportation of various substances between the cell and its environment. All of these functions are vital in maintaining the viability and function of a cell. The fundamental structural component of all biological membranes is the lipid bilayer. Lipids belong to a group of fat-soluble organic compounds and are amphiphilic. There are hundreds of species of lipid molecules present in biological membranes. To understand biological membranes in terms of functionality, the knowledge of the structure of lipid membranes is very important. Gorter and Grendel determined experimentally for the first time that lipids formed bilayers (Gorter & Grendel 1925). The lipid bilayer consists of two opposed layers of amphiphilic lipid molecules, with the hydrophilic head groups directed outwards and encountering the aqueous phase, and hydrophobic tails sequestered in the interior of the layers in contact with each other (Yeagle P.L 2011). Danielli & Davson proposed a model that describes a phospholipid bilayer that lies between two layers of globular proteins (Danielli & Davson 1935). A simple model of membranes called “the fluid mosaic model” developed by Singer and Nicholson is shown in Figure 1.1. According to this model the lipid has two layers (a bilayer) of phospholipids in which lipids and proteins are free to diffuse laterally within the plane of the membrane (Singer & Nicolson 1972).
Figure 1.1 shows the lipid-globular protein mosaic model. Small circles show the lipid head groups with the tails and the large protein molecules embedded in the lipid matrix (Singer and Nicolson 1972).

Lipid head groups are polar and can be zwitterionic or charged, and lipid tails can have different lengths and degrees of saturation, such that there are hundreds of lipid species that can be present in biological membranes. However, in order to better understand the structural characteristics of membranes, model membrane systems are commonly used which consist of a single component (or a small number of components) dispersed in water.

The main structural components of biological membranes for most biological species are the phospholipids, which is a class of lipids that are very well studied. Phospholipids
have the ability to exist in a number of different phases depending on temperature, pressure and hydration (Tardieu et al. 1973; Koynova & Caffrey 1998; Lenné et al. 2009; Garvey, et al. 2010; Kent et al. 2010). It is well known that biological membranes can have a different lipid composition in the inner leaflet (i.e. the lipid layer on the inside of the bilayer), in which phosphatidylserine and phosphatidylethanolamine are more abundant, compared with the outer leaflet, where phosphatidylcholine preferentially resides (Daleke 2003). Phospholipid bilayers, when fully hydrated, exist primarily in the fluid phase where the lipids are free to diffuse laterally within the bilayer, and the tails are free to move. Their movement within a monolayer is rapid but they flip-flop between bilayer leaflets extremely slowly (Contreras et al. 2010).

More generally, phospholipids, when dispersed in aqueous solutions, can spontaneously self-assemble into a range of structures determined by the properties of the individual head and tail groups, the amount of water present and the temperature. At constant temperature, bilayer forming phospholipids can assume structures ranging from spherical and cylindrical micelles (at high water content), through to lamellar phases (at low water content) (Oki & Aono 1970).

Some lipids can also form structures other than lipid bilayers - one of the most extensively investigated non-bilayer structures is the inverse hexagonal phase (H_{II} phase) (Tardieu et al. 1973; Kent et al. 2009; M. Rappolt et al. 2003). Some of the possible bilayer arrangements and lipid geometries are shown in Figure 1.2. Roberts & Gabriel suggest that in aqueous solutions, phospholipids form aggregates whose structure depends on the fatty acyl chain length. Long-chain phospholipids which are greater than 12 carbon fatty acyl chains form multilamellar structures, while 6-8 carbon fatty acyl chains that are short-chain lecithins form micelles (Roberts & Gabriel 1987).
Figure 1.2 shows possible bilayer arrangements of a lipid and lipid geometries. (Phillipps et al., 2009)

A widely studied model membrane lipid is Dipalmitoylphosphatidylcholine (DPPC), which is a phospholipid consisting of two palmitic acids that are attached to a phosphatidylcholine head-group and has a molecular formula C_{40}H_{80}NO_{8}P. Figure 1.3a shows a space filling model of a typical lipid, along with a schematic representation and a bilayer. The main structural features of the bilayer are also indicated. A detailed structure of phosphatidylcholine is shown in Figure 1.3b.
Figure 1.3 shows the (a) physical and (b) chemical structure of phosphatidylcholine. $R_1$, $R_2$ represent fatty acid chains, with chain lengths typically between 12-22 carbons. In the case of DPPC the chains $R_1$ and $R_2$ are identical saturated hydrocarbon chains with 16 carbons each.

In this project DPPC was selected because it is stable for long periods of time, and has a fluid-gel transition in an accessible temperature range (~41°C), allowing the study of the effects of DMSO in both the fluid and gel phases. The other lipid most commonly used for such studies is DOPC. This lipid was not chosen, as its gel-fluid transition is around -
20 °C, and for systems in excess water, the freezing of the excess water leads to dehydration, making interpretation of the data in the gel phase difficult.

Table 1 shows some of the properties of DPPC.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
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<tr>
<td>Molecular Weight</td>
<td>734.053 g/mol</td>
</tr>
<tr>
<td>Exact Mass</td>
<td>733.562 g/mol</td>
</tr>
<tr>
<td>Covalently-Bonded Unit Count</td>
<td>1</td>
</tr>
<tr>
<td>Phase transition temperature (referred to as the main transition) (Marsh 1991)</td>
<td>$T_m = 41.2$ °C</td>
</tr>
<tr>
<td>Phase Transition Enthalpy (Mabrey &amp; Sturtevant 1976)</td>
<td>$\Delta H = 36–38$ kJ/mol.</td>
</tr>
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Table 1.1 shows physical and chemical properties of DPPC.

1.2 Effects of temperature, dehydration and freezing on membranes

Changes in the local environment, such as changes in temperature, pH, salt and light conditions, can cause stress to organisms and many can respond, at the molecular level, to avoid harm. At a physical level, stress on lipid membranes can cause phase changes which are damaging to the cell. For cells to remain viable, the lamellar fluid phase, Lα, is required, and transformations into other phases often result in the protective bilayer
losing function. The phase behavior of membranes is discussed in the following sections.

1.2.1 Effects of temperature on membranes

Schematic representations of the common bilayer phases are shown in figure 1.4 (Kranenburg & Smit 2005). The low-temperature phase is the subgel \( L_c \), in which the hydrocarbon tails are highly ordered and show a tilt with respect to the bilayer in most common phospholipids (Kranenburg & Smit 2005).

When the temperature is increased the subgel \( L_c \), transforms to a lamellar gel \( L_\beta \) phase in phosphatidylethanolamines (PEs) or the \( L_\beta' \) phase in phosphatidylcholines (PCs). In these gel phases, the bilayer is more hydrated than in the \( L_c \) phase.

At higher temperatures, the gel phase undergoes a transition to the physiologically most important fluid phase \( L_\alpha \), also known as the liquid crystalline phase. A pre-transition from the gel phase to the ripple phase \( P_\beta' \) can occur before going into the fluid phase (Kranenburg & Smit 2005). For DPPC in excess water, the sub-transition \( T_s \) occurs at \( \sim 18^\circ C \), the pre-transition \( T_p \) occurs at \( \sim 33^\circ C \) and the main transition (chain melting) \( T_c \) occurs at \( \sim 41^\circ C \). The enthalpy changes accompanying these transitions are about 14.2, 5.6 and 32.2 kJ/mol lipid, respectively (Csiszár, Á.; Bóta, A.; Novák, C.; Klumpp, E.; Subklew 2001).
Figure 1.4  Schematics of the various bilayer phases: (a) subgel phase, (b) lamellar gel phase (in PEs), (c) lamellar gel phase (in PCs), (d) ripple phase, (e) fluid phase Lα or liquid crystalline phase. The filled circles representing hydrophilic headgroup and lines are representing the hydrophobic tails (Kranenburg & Smit 2005)

1.2.2 Effects of dehydration on membranes

Severe dehydration is very harmful for living organisms on a number of levels. In particular, dehydration can lead to phase changes within membranes. A fluid lamellar phase in which lipids are able to move freely is essential for the survival of a healthy living cell. On the other hand, if a gel phase or other phase is formed as a result of a stress (such as dehydration), this can disrupt cell function and be lethal to the cell. Dehydration can be caused by desiccation at temperatures above freezing, by the method, freeze-induced dehydration (see section 1.2.3). Figure 1.5 shows a schematic of the effects of moderate dehydration on a bilayer.

Many organisms have become physiologically adapted to survive low temperatures and/or dehydration. One of the mechanisms is the accumulation of small solutes within the cells which play a role in protection. Solute interactions with bio-membranes are of major importance in terms of their structure and function under extreme stress. Solutes like small sugars for example are involved in reducing the damage caused by the process
of dehydration. Small carbohydrates (glucose, sucrose, fructose etc.) and sugars alcohols (like glycerol) have been found in many desiccation tolerant species (Crowe et al. 1984), (Koster & Leopold 1988). These systems have been studied in detail by a number of authors over the years (Polge et al. 1949; Crowe et al. 1987; Caffrey et al. 1988; Hincha 1989; Anchordoquy & Carpenter 1996; Oliver et al. 1998; Hincha & Hagemann 2004). One of the effects of sugars is to enable vitrification (glass formation) within the cells, and this has been explored in detail (Koster 1991; Koster & Lynch 1992; Koster et al. 1994; Koster et al. 1996; Koster et al. 2000).

Recent work has concentrated on understanding the role of sugars prior to vitrification, and the mechanisms of sugar protection are now largely understood (Wolfe & Bryant 1999; Bryant et al. 2001; Koster et al. 2003; Bryant & Koster 2004; Lenné et al. 2006; Bryant et al. 2007; Lenné et al. 2009; Lenné, Bryant, et al. 2010; Garvey et al. 2013). There are however other molecules which are important in reducing freezing and dehydration damage which have not been so widely studied.

In particular, artificial cryoprotectants such as dimethyl sulphoxide (DMSO), have received less attention, despite their relevance to cryopreservation.
Figure 1.5  Shows lipid water phases of model membranes exhibiting dehydration stress (Wolfe & Bryant 2001).

1.2.3  Effects of freezing on membranes

When biological tissue is cooled to sub-freezing temperatures, cell death can occur via two primary mechanisms: 1) when cooling is slow, ice forms first in the extracellular region. As the ice structure does not incorporate solutes very easily, most solutes (salts, sugars, etc) are excluded, and the remaining unfrozen fraction will become concentrated. This increases the osmotic pressure external to the cell, and causes the cell to expel water and shrink to try to restore osmotic balance. This results in freeze-induced dehydration to the cell which can lead to membrane damage and cell death (Wolfe & Bryant 1999); Alternatively, if cooling is fast, ice can form inside cells (called intracellular ice formation), which is almost universally lethal (Wolfe & Bryant 1999).
Thus, intracellular ice formation is primarily responsible for the destruction of cells at fast cooling rates. Where as, at slow cooling rates, where freeze-induced-dehydration predominates, osmotic injury (sometimes called solute effects) causes damage. This is known as the two-factor hypothesis (Mazur et al. 1972; Mazur & Cole 1989; Mazur 2004).

1.3 Cryopreservation and the glass transition

Cryopreservation of living cells and tissues has applications in various fields (Taylor & Fletcher 1998; Engelmann 2004; Woods et al. 2004; Meryman 2007; Stacey & Masters 2008; Zhou et al. 2010; Kaczmarczyk et al. 2011; Menon et al. 2012). The cryobiology field was established in the late nineteenth and early twentieth century and was focused on studying freezing, cold hardiness and frost resistance in plants (Fuller 2004). Polge and colleagues laid the foundations for using added cryoprotectants (glycerol in their study) as cryoprotective freezing agents to store the semen of fowl (Polge 1951). Various solutes like alcohols, sugars (sucrose), amides (dimethyl sulphoxide) and diols (1,2 propanediol) have been shown to have good CPA (cryoprotective agent) activity (Lovelock 1954).

One of the key factors in cryopreservation is the formation of a glass – a glass is an amorphous solid, where transport is virtually zero. Thus if the inter- and extra-cellular liquid can be converted to a glass, biochemistry stops and the cells can remain stable for very long periods of time. The glass to liquid transition (also known as glass transition) is a reversible process which transforms solutions from a hard and relatively brittle "glassy" state into a viscous or rubbery state with increasing temperature (British Standards Institution (BSI) 2014). The glass transition is always lower than the equilibrium melting temperature of the crystalline state of the material. While the glass transition is important in cryopreservation, it is not the focus of this thesis, which deals with temperatures above the glass transition.
1.4 Techniques used in this thesis

1.4.1 Scattering techniques

The use of scattering (light, neutrons, X-rays) to probe the structure of materials at the mesoscopic (1 nm to 1 µm) scale is very widespread (Spruijt et al. 2013). Small angle X-ray scattering (SAXS) is used to probe a wide variety of problems in structural biology and is an established method for low-resolution structural characterization of biological macromolecules in solution (e.g. macro-molecular solutions, detergents, nanocomposites, alloys, synthetic and bio-polymers, biomaterials, organic/inorganic films etc.) (Petoukhov & Svergun 2007; Clerici et al. 2009; Hilge 2009; Jehle et al. 2010; Guo et al. 2012; Bach et al. 2012; Petoukhov & Svergun 2013).

Of direct relevance to this thesis, structural information about lipid bilayers is most often probed using Small-angle X-ray Scattering (SAXS). This technique has been applied widely to study how different molecules (e.g., sugars, cholesterol) can affect phospholipid membrane structure (Mills et al. 2009; Ivankin et al. 2010; Garvey et al. 2013; Kynde et al. 2014). The structure can be tracked with a cycle time of less than a second using synchrotron techniques. SAXS has been extensively used to determine the structure and phase behavior of phospholipid bilayers in the presence of sugars (Caffrey et al. 1988; Tsvetkov et al. 1989; Nakagaki et al. 1992; Nagase et al. 1997; Nagase et al. 1998; Demé et al. 2002; Bryant & Koster 2004; Lenné et al. 2009; Kent et al. 2009) and has been used to understand the kinetics of phase changes (Caffrey 1985; Caffrey 1989; Caffrey & Cheng 1995; Koynova & Caffrey 1998; Erbes et al. 2000; Koynova & Caffrey 2002; Rappolt et al. 2003; Kraineva et al. 2005; Lenné, Garvey, et al. 2010). Researchers have also shown a considerable interest in the transitions of naturally occurring phospholipids from the lamellar phase to non-lamellar phases (Luzzati & Husson 1962; Luzzati et al. 1968; Tardieu et al. 1973; Tate & Gruner 1989; Tate et al. 1992; Gruner 2002), though this is not the focus of this study.
1.4.2 Differential Scanning Calorimetry

The most widely used technique to determine the phase transition temperatures of phospholipid bilayers is Differential Scanning Calorimetry (DSC). It has been extensively used to study the phase transition properties of many synthetic phospholipids like DPPC (Lis et al. 1982; Crowe et al. 1984; Crowe et al. 1984; Nakagaki et al 1992; Nagase et al. 1997; Nagase et al. 1998; Shashkov et al. 1999; Kiselev et al. 2005; Gardikis et al. 2006; Garvey et al. 2010; Bryant, et al. 2010; Bulavin et al. 2015). DSC is ideal for determining the temperature and enthalpy of lipid phase transitions.

1.5 Dimethyl Sulfoxide (DMSO)

Dimethyl sulfoxide (DMSO) is an organosulphur compound with the formula \((\text{CH}_3)_2\text{SO}\). Its cryoprotective effects were discovered by Lovelock and Bishop (Lovelock & Bishop 1959) and it has been shown to be of great importance as a cryoprotectant since then. DMSO is a colorless polar aprotic solvent, and the pure chemical is odorless. It is miscible in a wide variety of organic solvents as well as with water. It has the unusual property of giving the perception of a garlic-like taste in the mouth after contact with the skin. It also penetrates the skin very readily (Novak 2002).

DMSO is an intermediate product of the global Sulfur Cycle which distributes bioavailable sulfur to all organisms (Parcell 2002). It has anti-inflammatory properties and anesthetic properties (Jacob & Herschler 1986). It is also used as a membrane fusogen (Ahkong et al. 1975). It is involved in inducing cell differentiation (Lyman et al. 1976). It also has high permeability across membranes because of its hydrophobic groups (two methyl groups) (Anchordoguy et al. 1992). DMSO has a high glass transition temperature and it may interact electrostatically with phospholipid bilayers (Anchordoguy et al. 1987). When DMSO is co-administered with other drugs, it tends to enhance their effects (Kurihara-Bergstrom et al. 1987). Among the sulfur compounds, DMSO has the greatest number of therapeutic applications (Morton, 1993).
1.5.1 DMSO as cryoprotectant (CPA)

DMSO has proved to be very effective as a cryoprotective agent and has become one of the most widely used cryoprotectants (Rall & Fahy 1985; Karlsson et al. 1993; Sakai & Engelmann 2007; Lovelock & Bishop 1959). Although it is widely used as a cryoprotectant because it permeates membrane quickly (Wolfe & Bryant 2001), it can be toxic to cells, so small ratios are recommended to prevent ice formation and encourage vitrification, without causing too much toxicity (Menon et al. 2012).

1.5.2 Effects of DMSO on freezing and glass formation

Although the vitrification (the transformation of a substance into a glass) aspect of DMSO is well known (Isachenko et al. 2006; Tian et al. 2007; Kartberg et al. 2008; Mandumpal, et al. 2011) the interactions of DMSO with membranes is less well understood (Anchordoguy et al. 1991; Leekumjorn & Sum 2006). In particular the molecular mechanism of DMSO transporting through membranes, and the mechanism for its toxicity, are still not fully understood. There have been a number of studies looking at the structure of model membranes in the presence of DMSO (Yu & Quinn 1995; Yu & Quinn 1998; Yu & Quinn 2000; Kiselev et al. 2001; Sum & de Pablo 2003; Chen Yu, et al. 2004; Kiselev et al. 2005), however, these have been largely limited to systems in excess water, and covered a limited part of the phase diagram. The use of model membrane systems for studying the behavior and properties of biological membranes has proven very useful, as these are simple, well-studied and well-defined systems.

As the ratio of DMSO is increased, the overall glass transition of the solution broadens, which corresponds to a glass transition that occurs over a wider temperature range and a thermodynamically stronger glassy state forms which has less chance of ice formation. Consequently, the addition of DMSO to liquid water improves its vitrification properties (Mandumpal et al. 2011).
1.5.3 Computational studies on DMSO

Molecular dynamics simulations have been shown to be a powerful tool to elucidate the molecular mechanisms of membranes and solutes. Properties of DMSO/water mixtures have been modeled extensively using molecular dynamics techniques (Rao & Singh 1990; Luzar & Chandler 1993; Liu et al. 1995; Vaisman & Berkowitz 1992). Early results of molecular dynamics (MD) simulation studies of DMSO with lipid bilayers by (Smondyrev & Berkowitz 1999) reported that DMSO does not penetrate extensively into the hydrophobic region of the lipid bilayer. They also suggested that the addition of DMSO to water decreases the distance between the surfaces of membrane by expelling extra solvent. On the whole, they found that the bilayer structure remains stable and is unaffected by DMSO. Paci and Marchi studied the transport of a single DMSO molecule through a lipid bilayer, and studied a wide range of concentrations (1994). Zheng and Ornstein studied the effects of DMSO on the structure of the enzyme subtilisin (Zheng & Ornstein 1996) and the effects of DMSO on the enzyme Leu-Enkephalin (Van der Spoel & Berendsen 1997). However, many of these computational studies are inconsistent with experimental data.

More recently a comprehensive molecular dynamics study was done by Sum and de Pablo. Using various concentrations of DMSO at several temperatures, they found that favorable binding of water molecules to DMSO induces a dehydration of a lipid bilayer (Sum & de Pablo 2003). They also showed that DMSO has the ability to penetrate and diffuse across the lipid bilayer. Although their findings disagreed with previous simulation studies, they are more consistent with experimental data. Lee and coworkers found, from their molecular dynamics simulations on the interactions of DMSO with DPPC bilayers in the liquid crystalline phase, that the bilayer density of DPPC remained relatively unchanged, but the area per molecule increased, leading to a thinning of the bilayer (Lee et al. 2004; Lee et al. 2005).

A study in 2007 using atomic-scale MD simulations, Gurtovenko and Anwar provided further evidence that addition of 10 to 20 mol % concentrations of DMSO can lead to
formation of transient pores (Figure 1.6) and water defects in addition to progressive thinning of membranes, which explains the permeability enhancement of membranes to hydrophilic molecules by DMSO, as well as its cryoprotective activity (Gurtovenko & Anwar 2007).
Figure 1.6  Pore formation in the bilayer system with 10 mol % of DMSO. Water is shown in red, DMSO in cyan, choline and phosphate groups of lipid headgroups in green and yellow, respectively. Lipid acyl chains are not shown (A. a Gurtovenko & Anwar 2007).
More recently, Hughes et al., (2012) conducted molecular dynamic simulations of the interactions of DMSO with DPPC and DOPC. Their results suggested that progressive thinning of the membrane is due to increased concentrations of DMSO which is followed by pore formation and then ultimately loss of membrane integrity. (Hughes et al. 2012; Malajczuk et al. 2013; Hughes & Mancera 2013; Hughes et al. 2013; Hughes & Mancera 2014).

1.5.4 Experimental studies on DMSO

There are several experimental investigations in the literature, where the structure of phospholipid bilayers (as a model cell membrane system) have been investigated in the presence of DMSO (Gordeliy et al. 1998; Tristram-Nagle et al. 1998; Shashkov et al. 1999; Yamashita et al. 2000; Yu & Quinn 1995; Yu & Quinn 1998; Yu & Quinn 2000; Krasteva et al. 2001; Chang & Dea 2001; Long et al. 2003; Kiselev et al. 2001; Kiselev et al. 2005; Lee et al. 2005). X-ray diffraction and differential scanning calorimetry techniques were used in these studies for the analysis of the structure and phase behavior of model lipid bilayers over a wide range of DMSO concentrations. Gordeliy et al. concluded that there is a strong interaction between DMSO and the bilayer membrane surface from X-ray diffraction measurement studies on lipid bilayers in the presence of a wide range of different concentrations of (DMSO/water) (Gordeliy et al. 1998).

Tristam-Nagle and coworkers concluded that DMSO produces a new subgel phase in dipalmitoylphosphatidylcholine (DPPC), and also highlighted the dehydrating effect caused by DMSO (Tristram-Nagle et al. 1998). Yu and Quinn performed X-ray diffraction on bilayers and found the dramatic effect of DMSO on the lamellar repeat spacing of DPPC. They showed that the thickness of liquid-crystal bilayers decreases as DMSO molecules penetrate between the polar head groups, and results in an increase of the area per headgroup. The presence of DMSO in the solvent induced an increase in the temperature of the lamellar-gel to lamellar-liquid-crystal phase transition and a decrease
in the temperature of the lamellar-liquid-crystal to inverted-hexagonal phase transition, and caused a decrease in the repeat spacings of all the phases in particular study. They also showed that DMSO modifies hydration forces and affects the stability of phospholipids. (Yu & Quinn 1995; Yu & Quinn 1998; Yu & Quinn 2000; Chen, Xie, et al. 2004).

By contrast, Kiselev and coworkers conducted small angle neutron scattering (SANS) experiments, and concluded that DMSO molecules do not penetrate into the region of the polar head group of DPPC membrane and thus, do not influence membranes thickness. Their DSC results suggested that DMSO molecules do not penetrate to the region of bound water. They highlighted the strong interactions of DMSO with water molecules, and stated that the new phase was only detected in the presence of excess water (M. a. Kiselev et al. 2005). Similarly, Shashkov and coworkers used infrared spectroscopy in addition to X-ray diffraction and calorimetry and reported that the dehydration of the lipid bilayer because of the strong interaction between DMSO and water (Shashkov et al. 1999).

However, it is known that DMSO can make hydrogen bonds with water (Wong et al. 2012; Luzar & Chandler 1993; Shashkov et al. 1999) and it has the ability to replace water and solvate lipid headgroups (Gordeliy et al. 1998; Tristram-Nagle et al. 1998). Chang and Dea used calorimetry to investigate the effect of DMSO on the phase transitions of lipid bilayers, and found that the presence of DMSO affects the solvation of the lipid bilayer (Chang & Dea 2001). Yamashita et al. (2000) performed experiments with low DMSO concentrations to probe the stability of bilayers and concluded that the transition temperature from a gel to a liquid-crystalline phase increases with increasing DMSO concentration. Long and coworkers used infrared spectroscopy and differential scanning calorimetry and concluded that the enthalpy and the thermogram peak shape of the main transition were not significantly influenced by solute/solvent substitutions or increasing concentrations of DMSO/d6 –DMSO (Long et al. 2003).
Clearly there is a lot of confusion in the literature, with different groups using similar techniques but reaching different conclusions. This thesis aims to address some of these issues.

1.6 Aims and Thesis structure

The aim of this thesis is to investigate the effects of DMSO on model lipid membranes as functions of hydration, temperature and molar ratio – in particular to perform the first systematic studies under a wide range of conditions to better understand their effects on membrane.

Chapter 2 outlines basic scattering theory, the experimental apparatus used, and the materials, sample preparation and experimental methods used in the project. Chapter 3 presents the DSC and SAXS/WAXS results for fully hydrated systems as a function of DMSO:DPPC ratio. Chapter 4 examines the effects of DMSO on DPPC samples at reduced hydration, again using DSC and SAXS/WAXS. Chapter 5 summarizes the main conclusions from this project and outlines future work aligned with the aims of this thesis.
Chapter 2: EXPERIMENTAL TECHNIQUES AND THEORY

2.1 Introduction

Small and wide angle X-ray scattering (SAXS/WAXS) and Differential Scanning Calorimetry (DSC) were used to determine the effects of DMSO on model DPPC (dipalmitoylphosphatidylcholine) membranes at a range of hydrations and DMSO:DPPC ratios. The theory and experimental techniques are outlined in this chapter.

2.2 Scattering theory

The scattering vector q is the difference between the incident beam and scattered beam as measured at the detector, as shown in figure 2.1.

![Scattering vector diagram](image)

Figure 2.1 shows the scattering vector q, which is the vector difference between the incident beam and scattered beam (Amemiya & Shinohara 2010).

Scattering vector (q) is given by:
In a typical diffraction experiment, a monochromatic and collimated X-ray beam is scattered from a crystal lattice and peaks are observed when the angle of incidence is equal to angle of scattering and the path length difference is equal to an integral number of wavelengths. The condition for maximum intensity is given by Bragg's law. It allows the calculation of details about the crystal structure, or to determine the wavelength of the x-rays incident upon the crystal (Thornton & Rex, 1993). Bragg’s law is shown schematically in Figure 2.2. (The left incident radiation is green and right reflected radiation is red).

\[ q = \frac{4\pi}{\lambda} \sin\left(\frac{\theta}{2}\right) = \frac{4\pi}{\lambda} \sin(\theta_B) \]

**Figure 2.2** shows x-rays scattered from a crystal lattice. The incident waves and scattered waves are shown as green and red line respectively. \( d \) is the interplaner distance and \( \theta \) is the scattering angle.
When layers are aligned in a highly ordered crystalline arrangement, constructive interference leads to a distinct scattering peak (a Bragg peak). The position of the peak ($q$) and the distance ($d$) between the aligned particles can be represented by Bragg’s law;

$$2d \sin \left(\frac{\theta}{2}\right) = 2d \sin(\theta_B) = m\lambda$$

As $\theta$ is defined relative to the incident beam, so that $\theta = 2\theta_B$, so after eliminating $\theta$ the equation will become;

$$q = \frac{4\pi}{\lambda} \sin \left(\frac{\theta}{2}\right) = 2\pi \frac{m}{d}$$

The above equation shows that that the scattering vector $q$ is inversely related to $d$ (distance). In the above equation $m$ is an integer representing the order of the diffraction maxima, $\lambda$ is the wavelength of the incident X-rays, $d$ is the distance and $\theta$ is the angle between the incident ray and the scattering plane of the crystal lattice. Figure 2.3 shows a schematic of basic SAXS setup.

**Figure 2.3**  *shows a schematic of a typical scattering set up.*
In SAXS, scattering data is generally obtained in the form of a two dimensional scattering image, due to the elastic scattering of X-rays from differences in electron density in the sample. If there are periodic structures present, this will manifest as rings in the scattered intensity on the detector (Figure 2.4). To obtain diffraction data from these images they are radially averaged to form a one dimensional plot of intensity as a function of scattering vector q (Figure 2.5).

**Figure 2.4** shows two dimensional scattering image of elastic scattering of X-rays from a synchrotron X-ray source (at Australian synchrotron).

A typical SAXS diffraction intensity versus scattering vector plot is shown in Figure 2.5. The peaks within q values between 0 and 1 Å⁻¹ (SAXS) are from scattering from the stacks of bilayers within the sample (the repeat spacing, d_{repeat}), and the peaks between
1.4 and 1.6 Å⁻¹ are from the smaller internal distance between the chains of the lipid molecules (d\text{chain}).

**Figure 2.5** shows intensity versus scattering vector q for DMSO DPPC sample.

The repeat spacing and chain spacing in a lipid bilayer structure can be calculated directly from the peak positions in the SAXS/WAXS data (Figure 2.6). Lamellar repeat spacing (d\text{repeat}) is the combined thickness of the lipid bilayer and the water between the bilayers. The average distance between the lipid chains is the chain spacing (d\text{chain}) which is obtained by the WAXS data.
Figure 2.6 shows repeat spacing, chain spacing and water spacing in a lipid bilayer structure.

2.3 Instrumentation and Experimental Techniques

The instrumentation and experimental techniques used in this study are discussed in the following section.

2.3.1 BRUKER Microcalix (SAXS/WAXS AND DSC)

Small angle X-ray scattering and differential scanning calorimetry of the lipid/solvent samples were performed on a BRUKER Microcalix SAXS instrument with \textit{in-situ} DSC capabilities (Figure 2.7). The Microcalix utilizes a copper Kα X-ray source that is monochromated and collimated through Montel optics and a Kratky collimator. 2D X-ray scattering patterns over a $q$ range of 0.004 to 0.56 Å$^{-1}$ were collected on a Pilatus 100 K camera and wide angle scattering data was collected on a Vantec-1 wire-gas detector over
a q range of 1.2 to 1.95 Å⁻¹. Full specifications of the Bruker Microcalix are provided in table 2.1. An example of a 2D scattering pattern measured on this system for a lipid system is shown in Figure 2.8.

Figure 2.7  *Bruker Microcalix (Applied physics, RMIT).*

**Technical Specifications for Bruker SAXS**

Table 2.1 shows the technical specifications for Bruker SAXS.
<table>
<thead>
<tr>
<th>Beam</th>
<th>Wavelength</th>
<th>1.54 Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flux</td>
<td></td>
<td>$10^8$ photons/s</td>
</tr>
<tr>
<td>Beam size</td>
<td></td>
<td>250 μm point (FWHM)</td>
</tr>
<tr>
<td>q-range</td>
<td></td>
<td>0.05567 - 0.5833 Å</td>
</tr>
</tbody>
</table>

**SAXS**

- **Model:** Dectris - Pilatus 100K-S
- **Sensor:** Si diode array
- **Size:** 981 x 1043 pixels
- **Pixel size:** 172 x 172 μm

**WAXS**

- **Model:** Vantec-1
- **Sensor:** MicrogapTMgas detector
- **Size:** 981 x 195 pixels
- **Pixel size:** 172 x 172 μm

**Table 2.2**  *Bruker Microcalix specifications.*
Figure 2.8  shows two dimensional scattering image of elastic scattering of X-rays from the Bruker X-ray source. The circular rings represent \( d_{\text{repeat}} \).

### 2.3.2 Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) can be run on the same instrument simultaneously with the SAXS. The Bruker Microcalix DSC stage is shown in Figure 2.5, and controls temperature to 0.01 °C over the range from -30 to 200 °C. DSC was performed in capillary tubes that are hermetically sealed with araldite, and are stable to evaporation for months.
Figure 2.9  Bruker Microcalix DSC sample stage unit showing the reference and sample holder.

Figure 2.10 shows a typical differential scanning calorimetry (DSC) scan of DPPC in excess water with heat flow (heat input) on the y axis, and temperature on the x axis. In the example shown, the temperature was scanned from 20°C to 70°C at a rate of 1°C/minute, scanned back to 20°C at the same rate, and then the cycle was repeated several times. Data was analyzed only when at least two consecutive experimental runs coincided. The heat input (y axis) represents the heat required to maintain the sample at the same temperature as the reference capillary while scanning the temperature. When the temperature increases the heat flow also increases in order to maintain the sample at that particular temperature. The Calisto software calculation tool is used to determine the peak position, peak area, enthalpy, onset and offset temperatures. This is done on both
warming and cooling scans. Exothermic transitions show an upwards peak, and endothermic transitions show a downwards peak. Gel to fluid onset and peak temperatures as well as the fluid to gel onset and peak temperatures were measured. The average of these four results is presented as average transition temperatures to have the consistency in data analysis and to estimate the uncertainties.

Figure 2.10  DSC scan of fully hydrated DPPC. The data is shown for three consecutive experimental runs.
2.3.3 Australian Synchrotron Source

The SAXS/WAXS beamline at the Australian Synchrotron uses a 1M Pilatus detector for SAXS and a 200k Pilatus detector for WAXS that provide great range and fast time resolution. The beamline utilizes an undulator source with a very high flux to moderate scattering angles and a good flux at the minimum q limit (0.0012 Å⁻¹) (http://www.synchrotron.org.au/ausyncbeamlines/SAXSWAXS).

The Synchrotron SAXS results were recorded and analyzed with the inbuilt Scatterbrain software, with post processing done in Igor Pro.

The samples were weighed and sealed before transportation to the Australian synchrotron and reweighed at the Australian synchrotron. After reweighing, the samples were mounted onto the in-house temperature controlled sample stage. For the temperature controlled experiments, a thermocouple was placed next to the capillaries. The setup is shown in Figure 2.11.
Figure 2.11  End station of the small and wide angle X-Ray scattering (SAXS / WAXS) beam line instrument at Australian Synchrotron. (Image retrieved from http://www.synchrotron.org.au/aussyncbeamlines/SAXSWAXS)

Specifications for the Australian synchrotron SAXS/WAXS beamline are shown in table 2.2.
Table 2.2  *Australian synchrotron SAXS/WAXS beamline specifications*  
(http://www.synchrotron.org.au/aussyncbeamlines/saxswaxs/saxspecificaton)

<table>
<thead>
<tr>
<th>Source</th>
<th>In-vacuum undulator, 22mm period, 3m length, Kmax 1.56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy range</td>
<td>5 - 21 KeV. Optimized for 8.15 KeV and 11.00 KeV.</td>
</tr>
<tr>
<td>Beam size at sample (sample position focus)</td>
<td>250 µm horizontal × 150 µm vertical (FWHM)</td>
</tr>
<tr>
<td>Instrument background</td>
<td>Minimum &lt; 0.02 cm⁻¹ @0.01 Å⁻¹</td>
</tr>
<tr>
<td>Camera length (mm)</td>
<td>Q-range (Å⁻¹)</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>500</td>
<td>0.02 - 1.1</td>
</tr>
<tr>
<td>650</td>
<td>0.015 - 0.95</td>
</tr>
</tbody>
</table>

**Table 2.3** shows Q-range & SAXS Camera Lengths of Australian synchrotron SAXS/WAXS beamline (http://www.synchrotron.org.au/aussyncbeamlines/saxswaxs/saxs specifications)

### 2.3.4 Instrument Calibration

The small angle X-Ray scattering instrument is calibrated with standard Silver Behenate (AgC₂₂H₄₃O₂) as it contains many well defined peaks in the small angle region (Huang et al, 1993). The sample to detector distance was calibrated by fitting the diffraction pattern of the Silver Behenate sample to known values. The center of the beam on the detector was calculated by fitting a 2-dimensional Gaussian function to the center of the scattering intensity through a semi-transparent nickel filter placed in the beam.
2.4 Sample preparation

2.4.1 Mixing DPPC and Water

The lipid sample used for this project is 1,2-Palmitoyl-\textit{sn}-Glycero-3-Phosphocholine (DPPC), which was obtained from Avanti Polar-Lipids, Inc. and used without further purification (Figure 2.12). DPPC was chosen as it has been widely studied, has a transition in an accessible temperature range, is stable for long periods of time and is readily available.

![Image of DPPC bottle]

Figure 2.12 1,2-Palmitoyl-\textit{sn}-Glycero-3-Phosphocholine (supplied by Avanti Polar Lipids) was used for this project.

Plastic Eppendorf tubes (volume 2 mL) were used in preparation and for storage of all samples. Eppendorf tubes were cleaned manually with brush and washing detergent
before use. The cells were then rinsed with tap water and then Milli-Q filtered water. Finally, the cells were rinsed with ethanol. Dry phospholipid powder was weighed (~5mg) and added in an Eppendorf tube with the help of a metal spatula. Two techniques were used to achieve known hydrations. Method 1: direct addition of the desired amount of Millipore water using a Hamilton 10μl syringe, followed by final mass determination; and Method 2: Hydrating samples to excess (50% water by mass), followed by equilibration with saturated salts solutions (described below).

In either case, following hydration the samples were mixed using a Chilter MT 19 auto vortex mixer for 10 min. To ensure good mixing, samples with lower hydrations were subjected to heat/cold cycles, to induce a phase change in the lipids from gel phase to fluid phase. To achieve this, sealed tubes were submerged in a hot water bath at 65°C for 8 min. To achieve homogeneous mixing the tubes was placed in a Beckman GPR centrifuge for 40 min at 2500 rpm, leading to the final homogeneous gel. A prepared lipid sample in an Eppendorf tube is shown in Figure 2.13
Samples were also prepared at fixed vapour pressures, by equilibrating full hydration samples over saturated salts of known fixed relative humidities (RH): sodium chloride (NaCl) and sodium bromide (NaBr), which have RH of 75% and 57.5% (at 23°C) respectively (Rockland, 1960). As they are at fixed relative humidity, the osmotic pressure \( \Pi \) can be determined using the following equation (Nobel 1983).

\[
\Pi = -\frac{RT}{V_w} \ln \left( \frac{RH}{100} \right)
\]

where \( R \) is the Universal Gas Constant, \( T \) is the temperature in K, and \( V_w \) is the molar volume of water. This can be related to the equivalent freezing point depression (to first order) by (Wolfe & Bryant 2001).

\[
\Pi = 1.2 \times 10^6 \Delta T
\]

These results are summarized in table 2.4 below. These RH are generated by saturated solutions of the salts, and the osmotic pressure and freezing points of solutions in equilibrium with those RH.

<table>
<thead>
<tr>
<th>Saturated Salt</th>
<th>RH (%)</th>
<th>Osmotic Pressure (MPa)</th>
<th>Freezing Point (°C) (approximate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>75</td>
<td>39</td>
<td>-33</td>
</tr>
<tr>
<td>NaBr</td>
<td>57</td>
<td>75</td>
<td>-62</td>
</tr>
</tbody>
</table>
Table 2.4 shows the RH%, osmotic pressure and freezing point of NaCl and NaBr.

In this technique, the salts are placed in a Petri dish and Milli-Q water added until the salts become fully saturated. The Petri dish is then placed in a sealed plastic container with a piece of circular plastic mesh placed over the Petri dish. Samples in open Eppendorf tubes were placed on the mesh, above the saturated salt solution, and the container sealed. The set-up for sample equilibration with saturated salts is shown in Figure 2.14. Samples were prepared with excess water, and then equilibrated in the chambers for at least 7 days, whilst monitoring the relative humidity.

The mass of the samples decreased until they reached equilibrium. Sample masses were weighed before and after, so that water content (by mass) could be determined after equilibration. As the relative humidity and osmotic pressure of saturated salts are dependent on temperature, the equilibration chambers were stored in a room at a constant temperature of 23°C.

The relative humidity was periodically confirmed using a Tinytag RH datalogger (Hastings, Port Macquarie, Australia) as shown in figure 2.14.
2.4.2 Preparing DMSO Mixtures

DMSO was added to fully hydrated DPPC/Water samples by using a Hamilton syringe. Samples were prepared and stored at room temperature until further use. The DMSO:DPPC ratios were: 1:1, 2:1, 3:1, 4:1, 5:1, 7:1, 7.5:1 and 10:1. A single sample was used for each combination of DMSO/DPPC/water and temperature. Each sample was scanned multiple times to ensure reproducibility. Error bars were calculated by excel. While there is the possibility of some DMSO evaporation during equilibration, it would be exceedingly small compared to the water evaporation, as the vapour pressure of water if 55 times the vapour pressure of DMSO.

Figure 2.14 The sample chamber for equilibrating samples over saturated salts.
2.4.3 Techniques developed for loading samples into the X-ray capillaries

The samples have to be loaded into the bottom of the capillaries to ensure accurate SAXS/DSC measurements. In order to do this, samples were removed from the eppendorf tubes using a pasteur pipette, which was then placed inside the capillary. With the help of a compressed air regulator, air at higher pressure is pushed into the pipette as shown in Figure 2.15.

![Setup for collecting sample into a capillary](image)

**Figure 2.15** shows setup for collecting sample into a capillary.
The compressed air forces the sample down in the capillary, and in this way the sample is collected in the bottom of the capillary as shown in Figure 2.16. The process is repeated several times until an appropriate amount of sample is loaded into the capillaries.

![Image of sample collection](image)

**Figure 2.16** shows sample collected in a capillary after compressed air technique.

While transferring the samples into the capillaries, some of the samples can stick in the top or middle of the capillary, especially samples with lower water content. To position the sample at the bottom of the capillary, a metallic wire cleaned with ethanol is used to push the samples down at the bottom as shown in Figure 2.17.
After adding samples into the capillaries, they are sealed with Selleys 5 minute araldyte for further analysis. The magnified image of capillary with the sample is shown in Figure 2.18.
2.5 Data Processing and Analysis

The data processing in this thesis includes the use of following softwares;

- FIT2D - used for integration of the 2D SAXS data to obtain intensity versus scattering vector plots.

- Calisto - control software for SAXS based DSC, and used for analysis of peak positions and enthalpies.

- ASA3 - control software for SAXS/WAXS data acquisition

- Scatterbrain - control and analyze software used on the synchrotron SAXS/WAXS beamline.

- IGOR software package – used for data visualisation and graphing. The analysis details are described in the following sections.

**Figure 2.16**  *Magnified image of capillary with the sample is shown.*
2.5.1 Analysis of SAXS results and Peak Fitting

2.5.1.1 Lab based SAXS (Bruker Microcalix)

The BRUKER Microcalix SAXS output is shown on the monitor in Figure 2.19.

![SAXS Output](image)

**Figure 2.17** shows pattern of a SAXS image observed by ASA3 software showing the characteristic rings of the lamellar phase.
The procedure for integrating data is as follows. The 2D SAXS patterns collected on the instrument are saved as tif files, and imported into Fit2D. The beam center, input, z-scaling, mask, integrations and output are set in fit 2D, and a radial average performed. A typical display from fit2D is shown in Figure 2.20.

Figure 2.20 shows the output from fit2D.
The output by fit2D in the form of intensity versus scattering vector is shown in Figure 2.21.

Figure 2.218  shows peaks output from Fit2D.
This data is imported into Igor Pro, and fit using the Mfit function (assuming Gaussian distributions), as shown in Figure 2.22.

Figure 2.22 shows peaks created by FIT2D software after MFIT.

2.5.1.2 Australian Synchrotron

2D scattering patterns collected on the Pilatus 1M detector (Figure 2.23) which are radially integrated using Scatterbrain.
Figure 2.23 shows scatterbrain software output for DMSO/DPPC sample. The Figure shows both SAXS and WAXS diffraction. The black lines are the gaps between the detector modules.

The 2D patterns are processed using the in built software, and a typical plot of intensity versus scattering vector is shown in Figure 2.24.
Figure 2.24 shows intensity versus $q$ plots for DMSO DPPC sample. The SAXS and WAXS range is also shown in Figure.

Different order peaks are shown in the Figure and the $q$ values are obtained from these orders by using the following equation (1.4).

$$d_m = \frac{m2\pi}{q}$$

Peak fitting is done in Igor Pro, and an example is presented in figure 2.25.
Figure 2.25 shows SAXS peak fitting done by IGOR.

The peak type is Gaussian in this case (Figure 2.26) and fit results yield the peak position and peak area.
Figure 2.19 shows various parameters (smooth factor) for WAXS peak fitting.

WAXS peak fitting is shown in Figure 2.27. The WAXS region shows the overlapping of two peaks, which need to be fit separately as shown. Note that only peak maxima are extracted from this process – the peak areas are often poorly defined, and are not used.
Figure 2.20  shows WAXS peak fitting done by IGOR.

2.5.2  Analysis of DSC results and Peak Fitting

DSC was used to measure the phase transition onset temperatures of lipid samples as functions of humidity and DMSO and DPPC molar ratio.

Technical Specifications for DSC

The detailed technical specifications for DSC are given in table 2.4.
**Stage (Microcalorimeter)**

<table>
<thead>
<tr>
<th></th>
<th>Microcalorimeter for scanning or isothermal calorimetry.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range</td>
<td>-30 to 200°C.</td>
</tr>
<tr>
<td>Max heating rate</td>
<td>5°/min</td>
</tr>
<tr>
<td>Max cooling rate</td>
<td>5°/min (200-50°C), 1°/min (50-0°C) 0.5°/min (0 to-30°C).</td>
</tr>
<tr>
<td>Sample volume</td>
<td>20µl for liquids</td>
</tr>
<tr>
<td>F-32 Micro-calorimeter Chiller</td>
<td>Closed, stand-alone liquid tempering system</td>
</tr>
<tr>
<td>Temperature stability</td>
<td>± 0.01 °C in the tempering circuit</td>
</tr>
</tbody>
</table>

**Table 2.4** shows technical Specifications for DSC in the Microcalix Bruker instrument.

The experimental runs were scanned from 20°C to 75°C at 1°C per minute, then back to the starting temperature at the same rate. An example of the sequence used for a typical DSC scan is shown in Figure 2.28.
Figure 2.21  An example of the sequence used for a typical DSC (SAXS running simultaneously in the last cycle) scan is shown.
This cycle is repeated several times to ensure consistency in the results. The first run is often different due to equilibration issues. Only reproducible results were used for analysis as shown in Figure 2.29.

**Figure 2.29** shows DSC cycle is repeated several times (seven times in this case) to ensure consistency in the results before they were used for final analysis. There is the difference in the first run and the next runs shows the mixture is well mixed after the first run.
CHAPTER 3: Effect of DMSO on the structure and phase behavior of fully hydrated DPPC

In this chapter, the effects of DMSO on DPPC at full hydration are presented.

3.1 Differential Scanning Calorimetry (DSC)

A typical differential scanning calorimetry (DSC) scan of DPPC in excess water with heat flow (heat input) on the y axis, and temperature on the x axis is shown in Figure 3.1.

Figure 3.1 DSC scan of fully hydrated DPPC. The data is shown for two consecutive experimental runs. Heating scans are shown at the bottom, cooling scans at the top.
3.1.1 DSC measurements

DSC scans were run for all the DMSO:DPPC ratios. Figure 3.2 highlights the phase transition peaks for three DMSO ratios. The effect of increasing amounts of DMSO is to shift the peak to higher temperatures, as well as to reduce the height and to broaden the peak. DSC was performed in capillary tubes that are hermetically sealed with araldite, and are stable to evaporation for months.

Figure 3.2 DSC heating scans, magnified to highlight the main peak. The three curves shown are: Pure DPPC (red), 0.7:1 DMSO:DPPC (green) and 1:1 DMSO:DPPC (blue).
The transition temperatures determined as a function of the mole fraction of DMSO are shown in Figure 3.3. The transition temperatures increase significantly, from 41.26°C without DMSO, to 49.20°C at 7.5:1, before decreasing slightly to 48.61°C at 10:1. There is a maximum increase in the phase transition temperature of ~8°C. The maximum effect (where there is little further change) occurs around 5:1 (DMSO:DPPC) - beyond this ratio there is almost no change in phase transition temperatures. Within the errors there is a plateau from 5:1 to 10:1. Clearly the increase in the transition temperature indicates that the DMSO stabilizes the gel phase. It should also be noted that there is no significant increase in the transition temperature until about a 1:1 DMSO ratio.

Figure 3.3  The dependence of the main phase transition temperatures on the mole fraction of increasing amount of DMSO. Each data point is the average of the peak maximum and onset temperatures for both warming and cooling scans for at least three cycles of the same sample. Errors are the standard deviations.
3.1.2 Enthalpy of the Transition

The measurement of the peak area, combined with the mass of the sample, allows the calculation of the transition enthalpy in Joules per gram (total sample). Knowing the mass of lipid in the sample allows this to be converted to Joules per gram lipid. The dynamics of the lamellar to H\textsubscript{II} phase transition are well described (Yu & Quinn, 2000). The effect of DMSO on the transition enthalpy is shown in Figure 3.4 (small DMSO ratios) and 3.5 (large DMSO ratios).

![Figure 3.4](image)

**Figure 3.4** Enthalpy of transition of small molar ratios (0:1 to 0.9:1) of DMSO:DPPC. Both gel to fluid (heating) and fluid to gel (cooling) enthalpies are shown. (The trends are consistent for both cooling and warming scans, small quantitative differences are due to super cooling effects and proximity of pre-transition, and are not analyzed further here).
The enthalpy of the transition has increased with increasing DMSO content. For example, the enthalpy increases from 37.5 J/g to 49.7 J/g for the gel to fluid transition over this range, which is a 20% increase. However, this effect plateaus at higher DMSO ratios, as seen in Figure 3.5 which shows the enthalpy at ratios up to 10:1. The effect has peaked by 2.5:1, and then the enthalpy reduces again. The enthalpy increases to a maximum of 54.7 J/g for the gel to fluid transition followed by a decrease in enthalpy to 45.8 J/g at 10:1 DMSO:DPPC. The same trend of enthalpy change is seen in the fluid to gel transition.

![Figure 3.5 Enthalpy of transition of large molar ratios (0:1 to 10:1) of DMSO:DPPC. Both gel to fluid and fluid to gel enthalpies are shown. (The trends are consistent for both cooling and warming scans - small quantitative differences are due to super cooling effects and the proximity of the pre-transition, and are not analyzed further here).](image)

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3.2 Small angle X-ray scattering measurements

SAXS/WAXS measurements were carried out at DMSO:DPPC ratios from 0.1 – 10, at both room temperature, where all samples are in the gel phase, and at 70ºC, where all samples are in the fluid phase. In this chapter the full hydration samples are presented, and samples at lower hydrations are discussed in chapter 4. The details of the experimental set-up for measurements carried out with the Bruker SAXS/WAXS instrument in RMIT, and the SAXS/WAXS beamline at the Australian Synchrotron, are described in chapter 2.

Repeat spacing ($d_{\text{repeat}}$, $d_r$) is one of the structural parameters that can be obtained directly from the data, which is calculated by the location of peaks from SAXS data and the chain-chain repeat spacing ($d_{\text{chain}}$, $d_c$) can be calculated by the WAXS measurements. These are illustrated schematically in Figure 3.6.

To determine the peak positions (and therefore $d_r$ and $d_c$), the diffraction peaks are fitted using Igor Pro software.
3.2.1 SAXS and WAXS measurements

Figure 3.7 shows the intensity versus scattering vector (q) plot of pure DPPC at 20°C in gel phase and at 70°C in fluid phase, with the 2-dimensional scattering pattern shown in the inset (see chapter 2 for details). In Figure 3.7 the distinction between the two phases is evident. The wide angle scattering data shows a characteristic sharp peak for the gel phase (in red) and broad peak representing the fluid phase (in blue), both located between 1.3 and 1.5 Å⁻¹. The lamellar peaks are at smaller q values. For the gel phase (in red) the peaks are sharper with a larger number of higher order reflections. On the other hand, for the fluid phase (blue), the peaks are broad with a small number of higher order reflections.
Figure 3.7  Intensity versus scattering vector (q) plots for pure fully hydrated DPPC in the gel phase (red) at 20°C and the fluid phase (blue) at 70°C. The y-axis values of each trace are offset for clarity. The inset shows the diffraction pattern in the SAXS/WAXS region.

Hydration was maintained throughout the experiments as the capillaries were glue sealed and there was no evaporation. The two temperatures were chosen so that they would be in the gel (20°C) or fluid (70°C) phase regardless of the DMSO ratio, enabling a direct comparison of the membrane structure.
Figures 3.8 and 3.9 show examples of the effects of DMSO on intensity versus scattering vector ($q$) plots in the gel phase and fluid phase respectively. It is clear from these figures that the presence of DMSO significantly affects the location and shape of peaks in both the gel and fluid phase. Figure 3.8 shows that the addition of DMSO to DPPC in the gel phase leads to a reduction in the number of peaks, implying reduced ordering of the multilayers. In addition, there is a significant increase in the peak position, corresponding to a reduction in the repeat spacing.

Figure 3.8  Intensity versus scattering vector ($q$) plots of SAXS for 0, 0.2, 0.5, 0.8 (bottom to top) molar DMSO:DPPC in the gel phase at 20°C. The y-axis values of each
trace are offset for clarity. Note that the broad peak around $q=0.39 \, \text{Å}^{-1}$ is due to the kapton windows of the DSC cell. The curves are vertically offset for clarity.

Figure 3.9 shows that in the fluid phase the behavior is similar to the gel phase, with the addition of DMSO leading to a reduction in the repeat spacing and a general reduction in ordering of the multilayers.

**Figure 3.9** Intensity versus scattering vector ($q$) plots of SAXS for 0, 0.2, 0.5, 0.8 (bottom to top) molar DMSO:DPPC in the fluid phase at 70°C. The y-axis values of each trace are offset for clarity. Note that the broad peak around $q=0.39 \, \text{Å}^{-1}$ is due to the windows of the DSC cell. The curves are vertically offset for clarity.
Figures 3.10 and 3.11 show the WAXS data for the samples shown in Figures 3.8 and 3.9 respectively. A visual inspection of the shape of the wide angle reflections in Figure 3.10 shows that the samples with low DMSO ratio exhibit the sharp peak characteristic of the gel phase, corresponding to the ordered chain structure shown in the inset. When the ratio of DMSO increases it appears to disrupt this structure, implying that the DMSO is penetrating the bilayer, leading to less order.

**Figure 3.10** Intensity versus scattering vector (q) plots of WAXS for 0, 0.2, 0.5, 0.8 molar DMSO:DPPC in the gel phase at 20°C. The inset shows the compact arrangement of the lipid molecules in the gel phase.
Figure 3.11 shows the effect of DMSO in fluid phase, where the characteristic fluid peak becomes broadened by the presence of DMSO. This suggests that for small ratios, DMSO enters the bilayers, disrupting the ordering. However, when the ratio of DMSO is increased beyond 0.4:1, it starts saturating in the membranes and excess DMSO goes into water phase.

**Figure 3.11**  *Intensity versus scattering vector (q) plots of WAXS for 0, 0.2, 0.5, 0.8 (from top to bottom) molar DMSO:DPPC in fluid phase at 70°C. The inset shows the fluid arrangement of the lipid molecules in fluid phase.*
Turning now to higher DMSO:DPPC ratios, Figure 3.12 shows intensity verses scattering plots at 20°C in the gel phase with the 0:1, 1:1, 2.5:1, 5:1 and 7.5:1 DMSO:DPPC molar ratios.

**Figure 3.12**  Intensity verses scattering plots at 20°C. The red plot shows pure hydrated DPPC without DMSO. The next four plots from bottom to top represents 1:1, 2.5:1, 5:1 and 7.5:1 DMSO:DPPC molar ratios. The y-axis values of each trace are offset for clarity.

The first thing to note is that all of these data show multiple reflections, indicating substantial multilamellar ordering. As more DMSO is added, the 5th order peak disappears, and the wide angle gel peak becomes less clearly defined. There is also a
slight shift in the peak positions with an increase in the DMSO ratio in the gel phase. The peaks become more well defined as DMSO is increased, due to a reduction in the diffuse scattering in the region between Bragg peaks (especially noticeable between the first and second peaks).

By contrast, the effect of DMSO in the fluid phase is to significantly increase the multilamellar ordering, as shown in Figure 3.13. Both the number and sharpness of the reflections increases as DMSO is added. Despite this, the fluid phase is retained as evidenced by the broad WAXS peak.

Figure 3.13  *Intensity verses scattering plots at 60°C. The red plot shows pure hydrated DPPC without DMSO. The next four plots from bottom to top represents 1:1, 2.5:1, 5:1*
and 7.5:1 DMSO:DPPC molar ratios. The y-axis values of each trace are offset for clarity.

To try to understand these effects, we need to look at the measured repeat spacings, which are presented for low DMSO ratios in Figures 3.14 and 3.15, and for high DMSO ratios in Figures 3.16 and 3.17. As seen in Figure 3.14, the repeat spacing is observed to decrease with the addition of small amounts of DMSO, but beyond a ratio of 0.2 the effect diminishes. The effect of DMSO is larger for the fluid phase where the repeat spacing reduces from 58Å to 43Å, than for the gel phase where it changes from 66Å to 56Å at the highest ratio.
Figure 3.14  *Membrane repeat spacings at 20°C (gel phase) and 70°C (fluid phase) as a function of increasing DMSO ratio.*

3.15 shows that addition of DMSO has a much smaller effect on chain-chain spacing ($d_{\text{chain}}$), with almost no change in the spacing in the gel, representing minimal change in the chain-chain packing. There is however an increase in the wide angle spacing from 4.5 Å to 4.6 Å in the fluid phase upon the addition of 0.1 ratio DMSO, but it then stays approximately constant up to a DMSO:DPPC molar ratio of 0.8.

Figure 3.15  *Wide angle spacings at 20°C (gel phase) shown in blue and 70°C (fluid phase) shown in pink, as a function of increasing DMSO ratio.*
Returning to the higher DMSO ratios, Figures 3.16 and 3.17 show the same data for the larger DMSO:DPPC ratios. For both phases there is a significant decrease in the repeat spacing from 0:1 to 2.5:1 DMSO:DPPC from 66Å to 57Å in the gel phase from 60Å to 45.70Å in the fluid phase. The repeat spacing then plateaus in both the gel and fluid phases, but increases again at the highest ratio of 10:1 DMSO:DPPC.

![Figure 3.16](image)

**Figure 3.16** Membrane repeat spacings at 20°C (gel phase) shown in blue and 70°C (fluid phase) shown in pink, as a function of increasing DMSO:DPPC ratio.
The wide angle spacing is presented in Figure 3.17. There is a minor reduction in the chain spacing as observed in the gel phase but then it remains constant from 1:1 up. By contrast an increase is observed in the fluid phase up to 2.5:1, then after adding more DMSO, it remains almost constant, though there is some scatter.

Figure 3.17  Wide angle spacing at 20°C (gel phase) shown in blue, and at 70°C (fluid phase) shown in pink, as a function of increasing DMSO ratio.
3.2.2 SAXS/WAXS Discussion

The effect of DMSO on the phase behaviour of DPPC has been examined by DSC and SAXS in detail. Before summarizing the results and comparing with the literature, it is important to note that almost all previous studies have been conducted keeping the solvent: lipid ratio constant, while varying the DMSO ratio (with some studies going as high as 100% DMSO solvent). From the viewpoint of DMSO as a cryoprotectant, it has two key roles: i) to permeate membranes so it is in both the intra- and extra-cellular spaces; and ii) to enable the resulting water:DMSO solution to vitrify (form a glass), thus protecting the membranes from further dehydration and damage. Thus, its cryoprotective properties are only relevant when there is a significant amount of water present. The novelty of the work presented here is that water:lipid ratios have been maintained well above the excess hydration level (> 40 water molecules per lipid), while varying the DMSO:DPPC ratio, making this study more relevant to the cryobiological applications of DMSO.

The effects of moderate dehydration are explored in the next chapter. For this reason, rather than framing the discussion in terms of the DMSO:water mole fraction, in this study it is discussed in terms of the DMSO:lipid mole ratio, which is a more direct indicator of its effect on membrane properties. The overall results are summarized below:

1) At low DMSO:lipid ratios up to 1:1 there is a ~20% increase in the enthalpy of the lipid transition – i.e. more energy is needed to melt the gel phase in the presence of the DMSO. This happens although there is no significant change in transition temperature over this range. The enthalpy reaches its largest value at around 2.5:1 (while the transition temperature is still rising). (The enthalpy values were obtained after averaging gel-fluid and fluid-gel transition temperatures. There was no significant hysteresis).
2) Increasing DMSO:l lipid ratio beyond 1:1 leads to an increase in the phase transition temperature by up to 8 °C. In other words, the DMSO stabilizes the gel phase. This effect is similar to the effect of moderate dehydration. An 8 °C transition temperature rise is seen for a water content of 5.7 water molecules per lipid, which would be the equivalent of an osmotic pressure of ~22 MPa (or equilibration to a relative humidity of ~86%) (Bryant & Koster 2004). Thus the effect of adding moderate amounts of DMSO is to dehydrate the membranes. This is in agreement with previous results [Tristram-Nagle et al 1998, Shahkov et al., 1999; Kiselev et al 1999; Chen, Xie, et al. 2004; Shrader et al, 2016]. However, note that in the current study the water content is always above 40 waters per lipid, showing conclusively that the effect is due to DMSO (rather than DMSO simply replacing water as water is removed).

3) Turning to the effect of DMSO on the structure of the gel phase at fixed temperature (well below the transition), it is found that ordering within the lipid phase is reduced (as evidenced by the weakening and eventual disappearance of the sharp wide angle peak). Note that the WAXS data show some differences between samples measured after different incubation periods, consistent with behavior observed previously by Tristram-Nagel et al. (Tristram-Nagle et al. 1998). They have found evidence that DMSO induces a new sub gel phase which develops with incubation time. This was not explored further in the current study.

4) In contrast to the effect on the ordering of the lipids within the plane of the membrane, the ordering between multilayers shows two regimes. For DMSO:l lipid ratios < 1, the number and sharpness of reflections reduces (Figure 3.8, implying less long range ordering of the multilamellar stacks. However, for higher DMSO:l lipid ratios, the (Figure 3.12) strong multilamellar ordering is again observed.

5) In the fluid phase, well above the transition, the WAXS data shows a similar trend, with the amount of order (determined by the area of the broad fluid WAXS peak) reducing with increasing DMSO. Again for low DMSO ratios, there is a
reduction in the multilamellar stacking (fewer, less well defined peaks). However for high DMSO ratios the effect is to significantly increase the ordering, with the highest DMSO ratio inducing strong ordering, with 4 clear reflections, as well as a significantly reduced baseline between peaks, consistent with previous observations (Tristram-Nagle et al. 1998).

6) Finally, for both the fluid and gel phases, the repeat spacing drops significantly (by ~9Å in the gel phase and 14 Å in the fluid phase), up to about 2 DMSO:lipid, after which it remains roughly constant until 10 DMSO:lipid, where it begins to rise.

The interpretation of these data is complicated by the fact that this is a three component system with 3 phases, as shown schematically in Figure 3.18. The measured repeat distance is given by: \(d_{\text{repeat}} = d_{\text{water}} + d_{\text{lipid}}\). The observed reduction in \(d_{\text{repeat}}\) as a function of increasing DMSO can be due to a reduction in either \(d_{\text{water}}\) or \(d_{\text{lipid}}\), or a combination of the two. In the gel phase there is only a very small decrease in \(d_{\text{chain}}\), so as the lipid volume is approximately fixed, there can only be a very small increase in \(d_{\text{lipid}}\). In the fluid phase on the other hand, \(d_{\text{chain}}\) increases by a maximum of ~3%, corresponding to an increase in area per lipid of ~6%. To maintain constant volume, \(d_{\text{lipid}}\) will need to decrease by about 6%. However, \(d_{\text{repeat}}\) reduces by about 9%, so this implies that there must be a thinner water layer between the membranes – i.e. the presence of DMSO leads to a larger excess phase, as the DMSO dehydrates the membranes slightly.
Figure 3.18  Schematic of a region within a lipid/water/DMSO sample. DMSO molecules are represented as blue circles. The relative volumes of lipid, water and DMSO are approximately to scale for the system studied here at 2:1 DMSO: lipid ratio. Note that the excess water region would not be contiguous with the lipid tails as shown in the schematic, but would be in small pockets within a multi-lamellar region surrounded by polar head groups. This is one possible representation - others, including those with the DMSO also distributed within the lipid phase, are possible.

It has been shown previously that the thickness of the solvent layer separating the bilayers is significantly reduced in the presence of DMSO (Tristram et al, 1998; Gordeliy et al 1998; Zhi-Wu Yu & P.J Quinn, 2000) consistent with the results shown here. DMSO exhibits unique properties because of its small size and dual character (hydrophilic and hydrophobic). The decrease in the solvation of the polar headgroups shows a dehydration of the lipid bilayer because of favorable binding of water molecules to DMSO (Sum & de Pablo 2003). The dehydration induced by DMSO can be explained as a result of the strong interaction between DMSO and water molecules (Shashkov et al. 1999). These conclusions are also supported by recent work which also suggests that DMSO dehydrates surface water from phospholipid bilayers (Cheng et al. 2015; Lee et al. 2016).
According to molecular dynamics simulations (Notman et al. 2006), at high ratios, DMSO has been observed to induce water pores in the lipid membrane, which could possibly provide a mechanism of action for the enhancement of membrane permeability. The study performed by (Gurtovenko & Anwar, 2007) provides further evidence that pore formation is a key aspect of DMSO’s mechanism of action. This also explains that due to hydrophilic molecules of DMSO, there is a significant enhancement in permeability of membranes.

It is also known that DMSO molecules do not penetrate into the polar head group region, their location is either directly below the head group in the hydrocarbon area or in the aqueous phase (Gordeliy et al. 1998; Shashkov et al. 1999; Sum & de Pablo 2003; Kiselev et al. 1999). Water molecules on the other hand penetrate into the region of the hydrocarbon chains as was shown thorough calculations of water distribution function across the bilayer (Sum & de Pablo 2003).

The results presented in this chapter are consistent with the previous studies. However, quantitative interpretation is limited by the multicomponent nature of the system. To obtain a stronger understanding on the effect of DMSO on the lipid bilayer we need to look at samples that are not in an excess of water – i.e. where there is no excess phase (at least for the pure water system). These low hydration experiments are discussed in detail in the next chapter.
CHAPTER 4: Effect of DMSO on the structure and phase behavior of DPPC at moderate dehydration

This chapter explores the effects of moderate dehydration on the phase behavior of DPPC in the presence of DMSO. The relationships between Relative Humidity (RH), osmotic pressure and equivalent freezing point are discussed in chapter 2.

4.1 Differential Scanning Calorimetry (DSC)

4.1.1 DSC measurements

Figure 4.1 shows DSC scans of four different DPPC:DMSO molar ratios at 57% RH. The transition temperature increases from 53°C without DMSO, to 54°C at 5:1. There is an increase in the phase transition temperature of less than ~2°C, so the DMSO appears to stabilize the gel phase slightly, though the effect on the transition temperature is modest – much less than for the fully hydrated system.
Figure 4.1  The DSC scan showing dependence of the main phase transition temperatures on the mole fraction of increasing amount of DMSO (at 57% RH). Note that these are raw data – the change in relative peak heights are mostly due to differences in the mass of the samples. The transition enthalpies are discussed below.

Figure 4.2 plots the phase transition temperature as a function of DMSO ratio for all three hydrations studied: full hydration, 75% RH and 57% RH. There are two clear conclusions from this graph. First, there is a continuous increase in phase transition temperature with dehydration (as expected); and second, for the moderately dehydrated samples, the DMSO has little effect on the transition temperature: for full hydration, the transition temperature increases significantly from 41 °C to 48 °C, whereas there is only a very
small change for the moderately dehydrated samples - from 51°C to 53°C at 75% RH and 53°C to 54°C at 57% RH. Thus the dehydrating effect of DMSO that is observed at full hydration (as discussed in chapter 3) is much less pronounced in the low RH samples.

**Figure 4.2** The dependence of the main phase transition temperatures on the mole fraction of increasing amount of DMSO at full hydration, 75% RH and 57% RH. Data shows error bars.
4.1.2 **Enthalpy of Transition**

The effect of DMSO on the transition enthalpy is shown in Figure 4.3 for all three hydration conditions.

![Figure 4.3](image)

**Figure 4.3** *Enthalpy of transition of molar ratios for 0, 1, 2.5 and 5 DMSO:DPPC at full hydration, 75% RH and 57% RH warming scan (gel to fluid). Note that these are the enthalpies per g of lipid.*

A clear trend can be observed here, with the increase in enthalpy with increasing DMSO being most significant for the full hydration (~46% increase), smaller for 75% RH (~10%
increase), and very small for the 57 (~5% increase). Again, as the lipid becomes more dehydrated, the effects of DMSO become smaller. The effects on transition enthalpy and transition temperature for the moderately dehydrated samples appear to reach a maximum at a DMSO:DPPC ratio of 2.5:1 – increasing to 5:1 causes little additional change. Therefore, the SAXS results presented below will concentrate on DMSO:DPPC ratios up to 2.5:1.

4.2 Small angle X-ray scattering measurements

Results are presented here for SAXS/WAXS at 75% and 57% RH at two temperatures: 20°C where the lipids are in the gel phase, and 90°C where the lipids are in the fluid phase. These measurements were carried out on the Bruker SAXS/WAXS instrument in RMIT as detailed in chapter 2.

4.2.1 SAXS/WAXS results for 75% RH

Figure 4.4 shows intensity versus scattering vector (q) plots of SAXS and WAXS for DPPC at 75% RH in the gel phase at 20°C and the fluid phase at 90°C. As expected, the gel phase is characterized by a large number of sharp SAXS reflections, while in the fluid phase only the 1st order reflections are prominent (note that the broad peak at ~3.9 Å⁻¹ is due to the kapton windows of the DSC cell). The gel phase also has the distinctive sharp reflection in the WAXS region, while the fluid phase shows the characteristic broad reflection. At the higher temperature the reflections shift to higher q, corresponding to a reduction in d_repeat.
Figure 4.22  Intensity versus scattering vector (q) plots of SAXS and WAXS for DPPC in gel phase at 20°C and fluid phase at 90°C at 75% RH. Inset shows WAXS data for the same sample. Note that the broad peak around q=0.39 Å⁻¹ is due to the kapton windows of the DSC cell.

Figure 4.5 shows the effect of adding DMSO to DPPC in the gel phase. The addition of DMSO leads to a slight increase in the q value of the reflections, corresponding to a slight reduction in d-repeat. There is also a slight reduction in the strength of the 2nd and 3rd order reflections. The WAXS data is shown in the inset, and shows a slight increase in the q value of the reflection, indicating a reduction in the packing distance of the chains.
Figure 4.5 shows intensity versus scattering vector (q) plots for DMSO:DPPC molar ratios 0, 1 and 2.5 (bottom to top) at 75% RH in gel phase. Inset shows WAXS data.

Figure 4.6 shows the same samples in the fluid phase – the position of the peak is shifted significantly towards higher q with increasing DMSO, corresponding to a lower d\textsubscript{repeat}\(^1\). Interestingly, the addition of DMSO also causes the appearance of a second order reflection (absent in the sample without DMSO). This implies that the DMSO is increasing the ordering of the lamellar bilayers within the sample. The WAXS peak again

\(^1\) There is a slight asymmetry in the primary peak which implies there might be a small amount of a different phase present. This represents only a few % of the area of the peak, and remains after temperature cycling. This is likely due to a small amount of sample stuck to the wall of the capillary separate to the main bulk of the sample. Please see chapter 2 for discussion of the difficulties involved in sample loading. This does not affect the overall conclusions of the study.
shows a small increase in q value of the chain packing reflection peak, although this is difficult to see due to the broad nature of the WAXS peak.

**Figure 4.6** shows example of intensity versus scattering vector (q) plots for DMSO DPPC molar ratios 0, 1 and 2.5 (bottom to top) at 75% RH in fluid phase for SAXS data and inset shows WAXS data.
4.2.2 SAXS/WAXS Results for 57% RH

Figures 4.7 and 4.8 show intensity versus scattering vector (q) plots of SAXS and WAXS for moderately dehydrated DPPC at 57% RH in the gel phase at 20°C and the fluid phase at 90°C respectively.

It is clear from these figures that the presence of DMSO significantly affects the location and shape of peaks in both the gel and fluid phase. Figure 4.7 shows that the addition of DMSO to DPPC in the gel phase again leads to an increase in the reflection q value. The effect on the higher order reflections is more complicated – there appears to be a reduction in intensity of the 2nd and 3rd reflections with 1 DMSO:DPPC, but these peaks are again larger with 2.5 DMSO:DPPC. These differences may represent subtle differences in sample preparation, as they were reproducible on repeat runs.²

² The strength of higher order reflections is a function of ordering within multilayers. This can be affected (for example) by the amount of shear applied to the sample during sample loading.
Figure 4.7  Intensity versus scattering vector (q) plots of SAXS for 0, 1 and 2.5 (bottom to top) molar DMSO:DPPC in gel phase at 20°C for 57% RH. The y-axis values of each trace are offset to enable a clearer comparison. Inset shows WAXS data.

Figure 4.8 shows that in the fluid phase the behavior is similar to the gel phase. Again there are slight differences in the higher order reflections between samples. At 2.5 molar ratio the position of the peak shifts to significantly higher q. The WAXS data is shown in the inset, and shows small differences between samples.
4.2.3 Comparison of all three hydrations

To try to understand the effects of hydration, we need to look at the measured $d_{\text{repeat}}$ of all the hydrations, which are presented in Figure 4.9. As seen, $d_{\text{repeat}}$ is observed to decrease at every RH in the gel phase with the addition of a small amount of DMSO. At full hydration it decreases from 57Å to 51Å; at 75% RH from 60Å to 57Å; and at 57% RH from 56Å to 51Å.
Figure 4.9 Membrane $d_{\text{repeat}}$ at 20°C (gel phase) shown as a function of increasing DMSO:DPPC molar ratios at full hydration (blue circles), 75% RH (green cubes) and 57% RH (red diamonds). The error bars are smaller than the symbols.

Figure 4.10 shows that there is a similar trend in the fluid phase, though the effect is most pronounced for the full hydration sample – again the dehydrating effects of DMSO are strongest at full hydration.
Figure 4.10  *Membrane* $d_{\text{repeat}}$ at 90°C (fluid phase) shown as a function of increasing DMSO:DPPC molar ratios at full hydration (blue circles), 75% RH (green cubes) and 57% RH (red diamonds). The error bars are smaller than the symbols.

Figure 4.11 compares the effects of humidity on $d_{\text{chain}}$ in both the gel and fluid phases. In the gel phase, there is a very small reduction observed, however there is also considerable scatter, so it is difficult to assign any trend here. By contrast in the fluid phase samples at 100%RH, 75%RH and 57%RH showed a significant increase in $d_{\text{chain}}$ (4.6 – 4.7 Å), (4.5 - 4.6 Å) and (4.5 - 4.6 Å) respectively, as a function of added DMSO.
Figure 4.11  Wide angle spacings ($d_{\text{chain}}$) at 20°C (gel phase – filled symbols) and 90°C (fluid phase – open symbols) as a function of DMSO:DPPC molar ratio. The error bars are smaller than the symbols.

4.3 Discussion

The effect of DMSO on the phase behavior of moderately dehydrated DPPC has been examined by DSC and SAXS in detail. Before summarizing the results, it is important to
note that no previous studies have been conducted at these conditions i.e. 75% RH and 57% RH and therefore there is no literature to compare with. The results are summarized below:

1) More energy is needed to melt the gel phase in the presence of DMSO as the enthalpy per lipid increases for all three hydrations (Figure 4.3). The enthalpy increase is more prominent in the fully hydrated lipid (~46% increase), than for 75% RH (~10% increase), or 57% RH (~5% increase). This implies that the DMSO is stabilizing the gel phase in all three cases, but the effect decreases with dehydration.

2) The transition temperature for the full hydration sample increases by 7 °C with increasing DMSO. However, the moderately dehydrated samples show only a slight increase which is within the error margins (Figure 4.2). Again, for the full hydration samples this implies that the DMSO stabilizes the gel phase, but this effect is much less clear in the moderately dehydrated case. This is consistent with previous work, e.g. (Yamashita et al. 2000), but in order to understand this, we need to look at the membrane structure.

3) For the gel phase, the trend of reduced $d_{\text{repeat}}$ with the increase in DMSO can be seen for all hydrations, though the reduction is largest for full hydration (Figure 4.9). As illustrated in figure 3.18, $d_{\text{repeat}} = d_{\text{water}} + d_{\text{lipid}}$. Thus, a reduction in $d_{\text{repeat}}$ can occur either by reducing $d_{\text{water}}$, $d_{\text{lipid}}$ or both. However, if $d_{\text{lipid}}$ is reduced, the area per lipid must increase (as the lipid volume is approximately constant). This implies that $d_{\text{chain}}$ must increase. However, if anything there is a slight reduction in $d_{\text{chain}}$ (Figure 4.1). Therefore, the reduction in $d_{\text{repeat}}$ must correspond to a reduction in $d_{\text{water}}$ (and a slight increase in $d_{\text{lipid}}$). Thus, the DMSO is dehydrating the membrane.

4) For the fluid phase, $d_{\text{repeat}}$ decreases with added DMSO at all hydrations (Figure 4.10), though the reduction is more significant at full hydration. The WAXS data shows a
similar trend at all three hydrations (three treatments), with $d_{\text{chain}}$ increasing by 1 Å. The increase in $d_{\text{chain}}$ must lead to a slight decrease in $d_{\text{lipid}}$. Therefore, the inter-lamellar water thickness $d_{\text{water}}$ must decrease.

As highlighted in chapter 3, the full hydration system is a three-component system with three phases (Figure 3.18). However, the moderately dehydrated systems do not have an excess fluid phase, which potentially simplifies the interpretation of the results. Thus, under these moderately dehydrated conditions, all of the components are contained within the multi-lamellar system (although at higher DMSO ratios than are studied in this chapter, it is possible that DMSO is excluded into an excess phase).

The results in both the gel and fluid phases are consistent with the observations of Yu and Quinn on samples in excess water (Yu & Quinn 1995; Yu et al. 1996; Yu & Quinn 1998). They found that both the lipid thickness and area per lipid increased with increasing DMSO ratio, and their calculations suggest that the DMSO molecules penetrate between the polar head groups. They also inferred from electron density profiles that there was little evidence of the DMSO penetrating the hydrocarbon region of the bilayer.

By contrast, Shashkov and coworkers used infrared spectroscopy, X-ray diffraction and calorimetry and inferred that there is a strong interaction between DMSO and water, and the primary effect of DMSO was to dehydrate the membranes by removing water from the inter-lamellar region (Shashkov et al. 1999). Molecular Dynamics simulations and experiments have shown that DMSO molecules do not penetrate into the polar head group region and locate either directly below the head group in the hydrocarbon area or in the aqueous phase (Chang & Dea 2001; Gordeliy et al. 1998; Shashkov et al. 1999; Sum et al. 2003; Kiselev et al. 1999). Recent simulations show that for moderate ratios DMSO diffusion across the bilayer is a multistage process, with a free energy minimum just below the lipid headgroup. However, at higher ratios DMSO diffuses readily across the bilayer with no favored position (Hughes et al. 2012).
Thus, this work has found that the dehydrating effects of DMSO which are observed in excess water continue to be observed in moderately dehydrated membrane systems. However, these effects are much less pronounced, as the systems are already moderately dehydrated. The specific location of the DMSO molecules within the membranes phases under these conditions cannot directly be determined by these experiments. However, these results, along with previous experiments and simulations allow the following speculation about the effects of DMSO in the fluid phase:

(i) DMSO has a small dehydrating effect even for moderately dehydrated systems. The DMSO which is in the aqueous layer must be hydrated, so there is less water available for the lipids.

(ii) However, if there is not sufficient water to hydrate the DMSO, the lowest energy state may be for the DMSO to move into the bilayer just below the lipid headgroup, as suggested by simulations (Hughes et al. 2012).

(iii) From this we infer that in moderately dehydrated systems, as there is not enough water to hydrate the DMSO, as the concentration increases, the DMSO must move freely into the hydrocarbon region, as suggested by the simulations (Hughes et al. 2012).

The schematic shown in Figure 4.12 illustrates these ideas (figure 4.11). At sufficiently low DMSO:lipid ratios, the DMSO will likely hydrate within the aqueous phase (left). However, as the concentration rises, some of the DMSO must enter the bilayer. Finally, for sufficiently high concentrations the DMSO will permeate the whole bilayer, and would not have a preferred location.
Figure 4.12  Schematic of the effect of increasing DMSO:lipid ratio (from left to right) in a moderately dehydrated lipid system. The blue circles represent DMSO molecules.
CHAPTER 5: CONCLUSION

5.1 Summary

Two experimental techniques (DSC and SAXS) have been used to conduct a comprehensive study on the effects of DMSO on the DPPC water systems at a range of hydrations.

In case of fully hydrated DPPC, the gel-fluid transition temperature increases significantly (by 8°C), and the transition enthalpy also increases significantly (~20%). The SAXS results showed that the presence of DMSO alters the bilayer structure of DPPC at full hydration. The ordering within the gel phase is reduced as evidenced by the weakening and eventual disappearance of the sharp wide angle peak. In the fluid phase, the WAXS data shows a similar trend, with the amount of order (determined by the area of the broad fluid WAXS peak) reducing with increasing DMSO. For low DMSO ratios, there is a reduction in the ordering of the multilamellar stacking, which is evidenced by fewer and less well-defined peaks. However, for high DMSO ratios the effect is to significantly increase the ordering, with the highest DMSO ratio inducing strong ordering. Finally, for both the fluid and gel phases, the repeat spacing is reduced significantly in the presence of DMSO. Interpretation of this data is complicated by the fact that there are three regions within the samples (bilayer, interbilayer water and excess water), as described in chapter 3 – so DMSO can in principle exist in any or all of these regions. Despite this, the results showed that d_repeat reduces significantly, which implies that there must be a thinner water layer between the bilayers, which suggests that the presence of DMSO leads to a larger excess phase, as the DMSO dehydrates the membranes slightly. Whether the DMSO enters the bilayers is difficult to judge from these results.
In the case of DPPC dehydrated to 75% Relative Humidity and 57% Relative Humidity, the transition temperatures and enthalpies per lipid both increase with added DMSO, which implies that the DMSO is stabilizing the gel phase, but the effect is much smaller than for the fully hydrated system.

Most interestingly, in the fluid phase, the SAXS results show that $d_{\text{repeat}}$ decreases and $d_{\text{chain}}$ increases as DMSO is added. One of the possible interpretation of this is that DMSO is present in both the aqueous layer (increasing $d_{\text{repeat}}$) and the lipid layer (increasing $d_{\text{chain}}$). The implications are that there is concentration dependence, with a trend in behavior as illustrated in figure 4.13. This behavior may well explain some of the discrepancies seen in the literature for the effects of DOPC on membranes.

5.2 Suggestion for further work

- While this thesis has covered a broad range of conditions relevant to freeze-induced dehydration, it would be interesting to explore very dry systems using similar techniques (RH < 57%).
- In order to take the next step and determine definitively where the DMSO resides in the membrane system, high resolution diffraction measurements on multi-lamellar stacks would seem to be an ideal choice. This technique has recently been applied with great success to the interactions between sugars and lipids (Kent et al. 2015).
- Similar experiments with the same methodologies and techniques should now be done with other cryoprotective molecules such as glycerol and ethylene glycol, to compare how their cryoprotection abilities are related to their effects on membrane structure.
- Experiments done in thesis are on simple model systems, further experiments on biological membranes (or membrane extracts) would be interesting and fruitful.
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