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Hydration forces and membrane stresses: cryobiological implications and a new technique for measurement

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Abstract

Very large, repulsive forces are measured between various surfaces in water at separations of about a nanometer or less. These forces are important in cryobiology because extracellular freezing usually causes extreme osmotic dehydration of cells. This brings membranes and macromolecules into close approach, and imposes large, anisotropic stresses on them. It is therefore important to study these forces at freezing temperatures. We have studied the freezing and thawing behaviour of lamellar phases of egg yolk lecithin and D_2O. Force-hydration and force-separation relations are obtained from the deuterium nuclear magnetic resonance signal as a function of temperature. From these measurements we estimate the magnitude of freezing-induced membrane stresses and discuss their effect on the response of cells and organelles to freezing and thawing.

Keywords: Hydration forces; Membrane hydration; Cryobiology; Phosphatidylcholine; Nuclear magnetic resonance; Lamellar phases

1. Introduction

Large repulsive forces have been measured between surfaces separated by about one nanometer of water or aqueous solution. Although such forces have been described for a variety of surfaces [1], lipid bilayers, either in pairs or in lamellar phases, are the system that has been most often studied [2–9]. The forces decrease approximately exponentially with distance with a characteristic length of about 0.2 nm and the extrapolated force per unit area at zero separation may be hundreds of MPa. They dominate the low hydration behaviour of a variety of systems formed from biomolecules including amphiphile-water phases with a range of geometries and the hexagonal phase of DNA [10]. Many of the articles in this issue deal with properties of such systems.

Lipid bilayers and lipid lamellar phases are often studied as a model of biological membranes of which lipids are an important component – the lipids are a two-dimensional solvent in which membrane proteins are dissolved. Nevertheless, the state of very low hydration – the state in which hydration forces are appreciable – is only rarely of interest to biologists. In most cells and tissues, water represents a large volume fraction and surfaces are separated by many nm. Biologi-

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cal membranes have a thickness of several nm, biological macromolecules have dimensions of order one to several nm. If membranes and/or macromolecules are brought into the range in which hydration forces dominate – i.e. average aqueous separations of one nanometer or less, then the aqueous solution must form about 20% by volume, or even less. Further, pressures of the order associated with hydration forces (tens or hundreds of MPa) are not usually encountered in most living tissues. Even the turgor pressure of plant cells – the pressure that pushes the plasma membrane against the matrix of the cell wall – is usually small in comparison.

Hydration forces are important, however, in at least two areas of biology: anhydrobiology and cryobiology [11]. In unsaturated atmospheres, seeds and spores of many species are capable of dehydration to low water contents of several percent, and some entire organisms (including some invertebrates and the resurrection plants) are capable of surviving desiccation and rehydration [12].

Freezing of whole organisms or suspensions of cells usually involves cellular dehydration: the extracellular solution usually freezes first, concentrating the extracellular solution and thus causing osmotic dehydration of the cell [13,14]. Freezing to several degrees below zero causes cells to dehydrate to a level where all non-aqueous cellular components are brought to separations of order of nm. Further dehydration at lower temperatures is opposed by the large repulsive forces at close proximity (see fig. 1).

It has been argued that the anisotropic stresses produced in membranes by these large forces may be responsible for one type of freezing damage [15]. Because of the agronomic importance of freezing damage and the medical interest in cryopreservation, not to mention scientific curiosity, it is interesting to study the forces between lipid bilayers at sub-freezing temperatures.

Two different techniques are commonly used to study hydration forces. In the osmotic stress technique [3], a known (negative) pressure is produced between membranes in a lamellar phase by equilibration of the interlamellar water with an unsaturated atmosphere, and the separation is inferred from the results of X-ray diffraction measurements. Membranes may also be deposited on the molecularly smooth mica surfaces of the surface forces apparatus [16] in which the force is measured by deflection of a calibrated spring and the separation by an optical technique using fringes of equal chromatic order. Neither technique can be used in its present form to measure hydration forces at freezing temperatures.

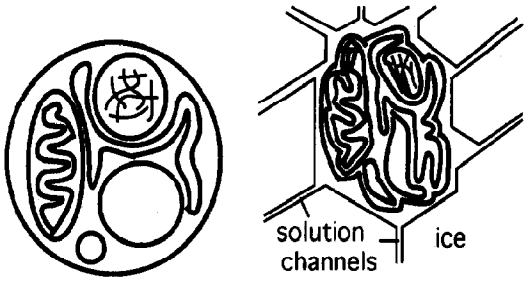


Fig. 1. A highly idealised sketch shows a cell before and after the freezing of its tissue or supporting medium. The elevated osmotic pressure causes a large reduction in the aqueous volume of the cell. Consequently, all of the non-aqueous components are brought into close proximity. In this state, stacks of membranes may begin to resemble lamellar phases.

We have developed a technique in which the temperature of a water–ice phase equilibrium controls the pressure and thus the inter-membrane force in a lamellar phase, and in which the hydration is determined from the nuclear magnetic resonance signal of deuteriated water [17]. In this paper we describe the production of membrane stresses by freezing, the hydration behaviour of egg-lecithin at freezing temperatures, and we discuss the consequences for cryobiology.

2. Freezing damage and membrane stress

A range of different physical and physicochemical processes may prevent the survival by cells of a cycle of freezing and thawing. At least two of these processes are the result of osmotic excursions which result from the slow cooling encountered in the natural [#1] world [13,15].

Ice formation is to some extent probabilistic so, because the external solution is larger than

the internal solution, extracellular freezing usually occurs before intracellular freezing. The extracellular ice is almost pure H_2O, so the external solutes are concentrated in the remaining unfrozen extracellular solution. Water then diffuses out from the cytoplasm to the hypertonic external medium: the cell contracts osmotically and its membranes become flaccid. If the membranes remain intact and semipermeable, then the process is sometimes reversible: warming melts the external ice, this dilutes the external solution, water diffuses back into the cytoplasm and the cell re-expands osmotically.

Cellular membranes are the principal site for damage. Under natural conditions where cooling is relatively slow, the plasma membrane is of particular importance. If it remains intact, the cells contract osmotically, concentrating the internal solution and thus hindering intracellular ice formation. The membranes may be damaged during the process of freezing, they may lose their property of semipermeability while in the osmotically contracted state, or they may rupture during osmotic expansion. Only one type of freezing induced injury will be considered here: the loss of osmotic response in the dehydrated state. Membrane rupture during the process of freezing *per se* [18] or during the phase of osmotic re-expansion [19] are attributed to different physical causes. It has been suggested that hydration forces are involved with membrane damage and loss of osmotic response in the dehydrated state [11,20].

The dehydration of a lamellar phase of lipids produces anisotropic stresses and strains in the membranes. If the phase remains lamellar, the aqueous volume can be reduced by reducing the separation between membranes, or by reducing the area per lipid molecule in the plane of the membrane. The lateral compression of the membranes can be regarded as the result of a lateral pressure Π (a force per unit length acting in the plane of the membrane). This lateral pressure can be readily calculated. The pressure P in the aqueous, inter-lamellar layers is negative [#2] and has a magnitude equal to the repulsive force per unit area between the membranes. (In the literature of interfacial forces, the symbol F is used for interfacial force per unit area, with the convention $F > 0$ for repulsive forces.) If the interlamellar separation is y, mechanical equilibrium requires:

$$\Pi = -Py = Fy. \tag{1}$$

This stress is considerable at low hydration: the area elastic modulus k_a of a membrane is defined by

$$k_a = \frac{d\gamma}{d \ln a} = -\frac{d\Pi}{d \ln a},$$

where γ is the tension in the plane of the membrane and a is any measure of area. For a bilayer of egg yolk lecithin, Kwok and Evans [21] give a value of 140 mN m^{-1}. An interlamellar repulsion of say 30 MPa at a separation of 0.5 nm would give a lateral pressure of 15 mN m^{-1}, and thus a contraction of order 10% if the linear elastic approximation holds.

The anisotropy of this stress is important. The bulk moduli of condensed phases are measured

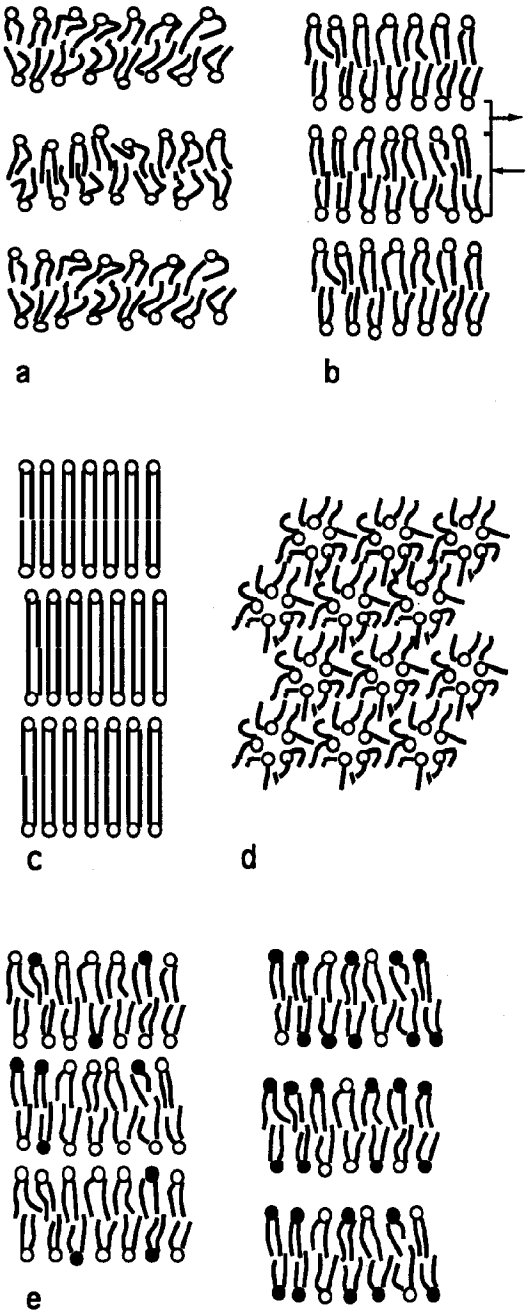
[#1] In cryopreservation laboratories, on the other hand, high cooling rates and permeating solutes are used to achieve rapid vitrification of the cytoplasm at the much higher levels of hydration which may be maintained by avoiding osmotic equilibrium. The dangers that await cells in this regime – including the elevated probability of intra-cellular ice formation – seem unlikely to involve hydration forces in a simple way and are not addressed in this paper.

[#2] Let us deal with two possible objections. First, for water volumes with dimensions of nanometers; different definitions of pressure are in principle possible. In the study of hydration forces, the interlamellar water is treated as a sub-phase with a finite pressure. In an alternative accounting, one could define the pressure of the entire lamellar phase to be zero and introduce an energy of hydration of the lipids which would decrease with increasing hydration and fall to zero at a mole ratio of order 20 or 30:1. The two systems of accounting are physically equivalent: the pressure in the former equals the derivative of the energy of hydration with respect to partial molar volume of water. The former system is adopted because it gives greater insight into the interaction. Second, it is worth pointing out that cavitation of the interlamellar water is unlikely even at very large suctions: the water is between two highly hydrophilic surfaces and its thickness is smaller than the critical radius for cavitation.

in GPa; for instance that of water is 2 GPa. Thus an isotropic stress of tens of MPa would be expected to produce only a small volumetric strain. Changes in density of the water or the lipids in a lamellar phase are therefore usually neglected. The contraction in the plane of the membrane is accompanied by a thickening in the normal direction. These strains are not negligible.

Fig. 2 includes a sketch of a lamellar phase of bilayer membranes, and shows several different resultant strains. The first is the deformation described above. Also shown are three other responses which, in certain cases, can relax the internal stresses. These are liquid crystal–gel phase transition, fluid–fluid lateral phase separations and lamellar-inverted hexagonal phase transitions. All of these responses have been observed in model systems, and there is evidence that they occur in cells dehydrated by freezing.

The elevation of the chain melting point by lateral pressure is the analogue in two dimensions of the elevation of melting point by pressure in three dimensions [#3]. The explicit relation between lateral stress and melting temperature has been described for bilayer membranes [22] and in greater detail for monolayers. Although the lateral stress in lamellar phases is rarely determined, the elevated melting temperature which is a consequence of dehydration-induced lateral



[#3] For the small number of three dimensional cases where the density decreases upon melting – water is an example – the melting point increases with applied pressure.

Fig. 2. (a) shows a lipid lamellar phase at relatively high hydration. In this sketch the circles represent the hydrophilic portions of lipids and the dark lines their alkane chains. As the water content is reduced, both the separation between membranes and the membrane area decrease (b). The membrane volume is approximately conserved by an increase in its thickness. In the normal direction, the large intermembrane repulsion is balanced by a negative pressure or suction in the interlamellar water. In the direction parallel to the membrane surface, the water suction is balanced by a compression stress or lateral pressure in the membranes, whence the reduction in area. These stresses can result in several different deformations. The membrane may undergo liquid-crystal to gel transition at elevated temperatures (c). The lipids may undergo the transition to the inverted hexagonal phase (d). If molecules with sufficiently different hydration repulsion are present, the membrane may undergo a hydration-induced lateral phase separation (e). In this sketch, the shaded circles represent the species with greater hydration.

stress has also been reported for dehydrated lamellar phases of a number of different lipids. In cells, this response is widely believed to be a cause of membrane damage [23,24]. A loss of permeability has been associated with the phase coexistence regime, and the increased lateral compressibility (decreased k_a) is a possible mechanism.

At sufficiently low hydrations, the phase diagram of some lipid–water mixtures includes an inverse hexagonal phase (Hex_{II}). The lamellar–Hex_{II} phase boundary has positive slope in a temperature–water content phase diagram: in other words the Hex_{II} phase appears at sufficiently low hydration and high temperature. Thus, at a given (local) composition, the Hex_{II} phase is produced by increasing temperature. It can also be produced by freezing – or more precisely by the dehydration associated with freezing. This is no paradox: over a temperature range of only several degrees below freezing, the degree of hydration falls dramatically [15,25]. The consequences of hexagonal phases for membrane damage are obvious: the topology of such phases is unsuited for the role of molecular semipermeability. Do Hex_{II} phases occur in frozen cells? The answer depends on definition. Gordon-Kamm and Steponkus [26] published electron-micrographs of freeze-dehydrated rye protoplasts which show regions of membrane interrupted by regions of stacks of cylinders in approximately hexagonal arrays. They interpreted these as stacks of cylindrical inverted micelles which thus formed a Hex_{II} phase. An X-ray crystallographer might object that the lack of long-range order (and consequent lack of diffraction pattern) violates some strict operational definition of a hexagonal phase. The two lipid geometries are nevertheless very similar.

In a bilayer of more than one component, hydration interactions with different magnitudes are expected. Because the hydration repulsion is exponential, the separation into phases of differing hydration repulsion results in a lower potential energy. If the repulsion is sufficiently different, this effect overcomes the mixing entropy and a phase separation results. This separation was predicted on theoretical grounds [27], and then reported for mixtures of phosphatidylcholine and phosphatidylethanolamine [28,29]. Membrane proteins, which are rather larger than lipid molecules, are expected to have a large energy of hydration. Dehydration of membranes including proteins and lipids is therefore expected to induce separations into lipid-rich and protein-rich phases. Such separations have been observed in the membranes of freeze-dehydrated rye protoplasts [26]. The significance of such lateral phase separations for the loss of semipermeability is that the lipids which form inverted hexagonal phases – such as phosphatidylethanolamine – have weak hydration repulsions. The concentration of such lipids in a protein-free phase of low hydration may allow the formation of hexagonal phases in membranes which, in their homogeneous state, would not form such phases.

3. Materials and methods

In previous studies we have used deuterium nuclear magnetic resonance (NMR) as one of a variety of techniques to study the phase behaviour of mixtures of water and one or two lipids [28,30]. This technique gives information about the geometry of the aqueous interface, so it gives information about the lamellar-Hex_{II} transition. It can also indicate whether the lipids are in the liquid crystal or gel lamellar phase. Here we describe the use of a new technique using NMR to determine force–hydration and force–distance relations at freezing temperatures. The hydration is determined from the intensity of the NMR signal from the water in the lamellar phase, and the interlamellar force is determined from the chemical potential of the interlamellar water, which is a known function of temperature.

Egg yolk lecithin (EYL; in chloroform solution, nominal purity 99%) dimyristoylphosphatidylcholine (DMPC; as a powder, nominal purity 99%) and D_2O (nominal purity 99.9 at.%) were bought from Sigma, (St. Louis, Mo.) and used without further purification. The EYL contained 0.1 wt% butylated hydroxytoluenes in the solvent as an anti-oxidant. The lipids were always handled in a dry nitrogen atmosphere, and the

chloroform was removed under a pressure of ≈ 0.1 Pa over P_2O_5. We prepared mixtures of several tens of mg of lipids with quantities of D_2O chosen to achieve mole ratios between 1:9 and 1:50. The mixtures were sealed in thin-walled glass NMR sample tubes. Spectra were obtained from the quadrupolar echo sequence using a Bruker MSL-300 spectrometer operating at 46.062 MHz with a 11 μs pulse. The temperature of the sample was measured with a thermocouple and controlled by feedback using a stream of pre-cooled air.

At freezing temperatures, the spectrum showed signals of two different types. A very broad signal (many tens of MHz) indicated D_2O with negligible rotational mobility and was interpreted as the signal from macroscopic phases of ice. A signal width of one to two hundred Hz indicated water with a small non-zero order parameter and was interpreted as interlamellar water. The former very broad signal was used as a baseline, and subtracted from the narrow signal (hereafter referred to as the lamellar water signal) which was integrated over frequency to obtain the corresponding signal intensity.

At a given temperature, the intensity is proportional to the number of molecules contributing to the signal. The temperature dependence of this proportionality (due to both the Curie law and the sensitivity of the coil) was calibrated using a sample of perdeuteriated methanol.

When macroscopic ice (at atmospheric pressure) equilibrates with interlamellar water at temperatures below zero, the pressure P in the water phase is determined from the freezing point depression:

$$T - T_c = \frac{P v_w T_c}{L}, \qquad (2)$$

where T is the temperature, T_c is the melting temperature at atmospheric pressure, v_w is the molar volume of water and L its latent heat of fusion. Taking the values [31] for D_2O gives the interlamellar repulsion $F = -P = (1.26\ \text{MPa}\ \text{K}^{-1})(T_c - T)$. Further experimental details and details of the derivation are given elsewhere [17].

4. Results and discussion

Fig. 3 shows the NMR deuterium spectra lamellar phases of D_2O with the lipids DMPC (3a) and EYL (3b). The temperature is 273 K for both. The DMPC is in the gel phase and the EYL in the liquid crystal phase. This temperature is nearly 4 K below that at which D_2O melts, so most of the D_2O is in the form of ice.

First consider fig. 3a. The very narrow signal corresponds to water molecules in rapid isotropic motion and is attributed to macroscopic domains of unfrozen water. The signal of width about 100 Hz corresponds to water molecules in slightly anisotropic motion and is attributed to the un-

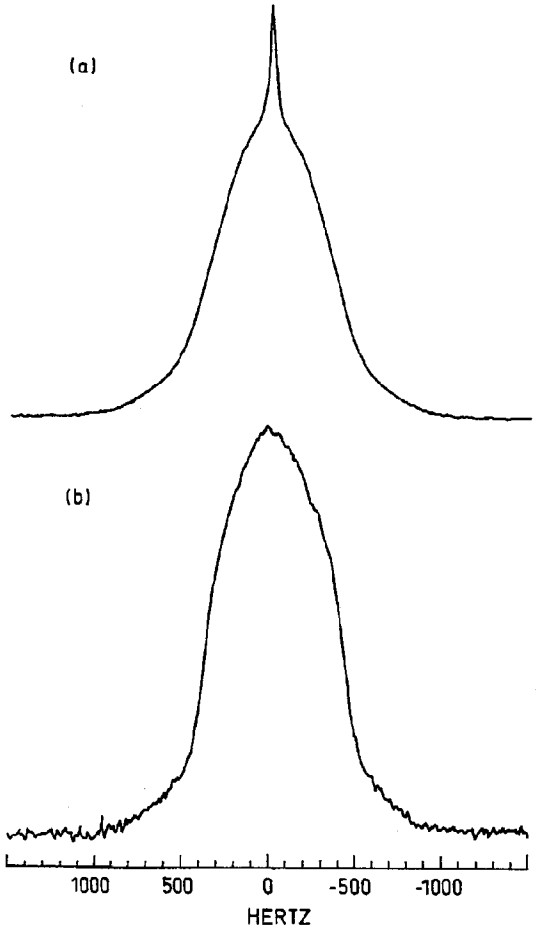


Fig. 3. Deuterium NMR spectra of lamellar phases of DMPC (a) and EYL (b) at 273 K.

frozen water between the lipid bilayers in the lamellar phase. There is also a much broader signal with width about 240 kHz which is not shown on this scale – indeed it represents the baseline for these spectra. This corresponds to the molecules of ice whose motion is much slower than those of the liquid water.

The spectrum shown in fig. 3a does not represent equilibrium: over a period of several hours, the very narrow signal gradually disappears, and the broader signal becomes slightly larger. We interpret this as the diffusion of supercooled water from isolated, macroscopic domains and into the lamellar phase. Because of the relatively long time to reach equilibrium, we do not report force–hydration curves for lipids in the gel phase.

When EYL samples were studied, equilibration was much more rapid. In response to step changes in temperature, the spectra varied over a period of minutes. After a period of 10–40 min (depending on the temperature range) the spectra varied only in the noise level, and the integral of the spectra – the quantity of interest in this study – did not vary measurably over several hours. Fig. 3b is such a spectrum. There is no very narrow signal and hence, we infer, no supercooled water. The broader signal we interpret as unfrozen, interlamellar water in equilibrium with ice. The freezing point of the interlamellar water is depressed by the negative pressure.

Fig. 4 shows the repulsive force per unit area between membranes as a function of the hydration. The abscissa is the number of water molecules per lipid in the lamellar phase. As this semi-log plot shows, the data are well fit by an exponential law over this range. The extrapolated intercept is 90 MPa and the force decreases by a factor e for each additive increase of 3.1 in the mole ratio of unfrozen water to lipid.

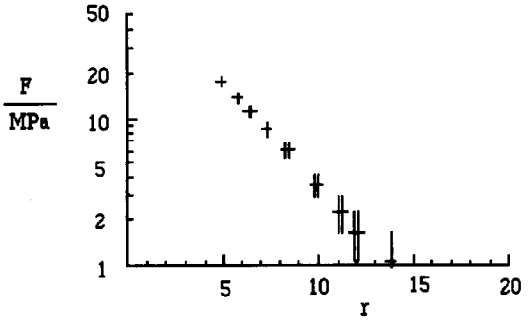


Fig. 4. The repulsive force per unit area between EYL bilayers as a function of their hydration. The abscissa is the number of water molecules per lipid in the lamellar phase. The temperatures for these data range from −12°C (at high F) to 3°C.

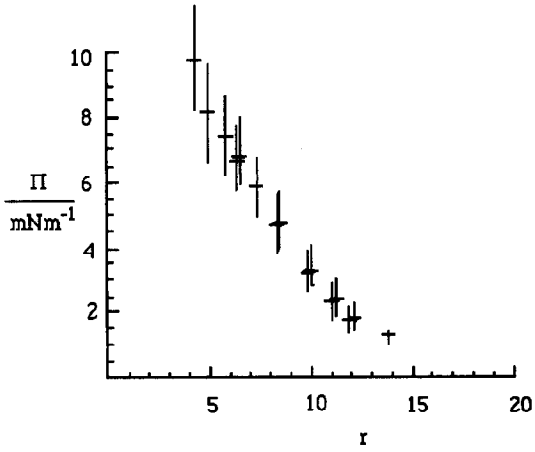


Fig. 5. The calculated lateral stress in membranes as a function of the hydration. The abscissa is the number of water molecules per lipid in the lamellar phase. There is considerable uncertainty in the area per molecule at freezing temperatures, hence the large error range. The temperatures for these data range from −15°C (at high Π) to 3°C.

If one assumes a value of the area a_0 per lipid molecule in the plane of the membrane and of the area elastic modulus, these data may be converted into a plot of force as a function of separation [17]. The lateral stress Π in the membranes, calculated from (1), is shown in fig. 5. The values used are $a_0 = 0.67 \pm 0.05$ nm^2 and $k_a = 140$ mN m^{-2}. The relatively large errors are the result of this uncertainty in a_0.

In this study, the only response to the lateral stress in the membrane is a change in membrane area. Indeed this was one of the reasons for choosing EYL for the first study: over the region of interest (the temperatures just below freezing), the interpretation is not complicated by phase transitions. Other lipid model systems can be chosen to produce, in a controlled way, the more exotic responses exhibited by living membranes and discussed in the introduction. These are in principle available to study with this method.

NMR signals give information not only about the degree of hydration (as used here), but also about the geometry of the water surface and the degree of ordering imposed on the lipids near that surface. Thus it can be used as one tool in the analysis of phase behaviour [28,30].

The technique described above operates in just the temperature range that is of greatest interest in environmental cryobiology – the range immediately below freezing. It is therefore suitable for the investigation of several of the physical phenomena produced by freezing induced dehydration. A further advantage of this technique is that it does not require long-range order in the sample and thus, in principle, it may be applied to quite complicated systems, even biological ones.

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