The association of dimethylsulfoxide and model membranes studied by pulse-field gradient NMR ¹

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Abstract. The use of dimethylsulfoxide (DMSO) as a cryoprotectant to reduce cellular injury during freezing is well known, however the intermolecular interactions between this amphiphilic molecule and biological membranes that form the basis of this protection are unknown. DMSO–dipalmitoylphosphatidylcholine (DPPC) vesicle interactions were investigated in pulsed-field gradient NMR (PFGNMR) experiments and spectra analysis allowed for the determination of self-diffusion coefficients for each species present. The mole fraction of DMSO associated with the DPPC vesicles was then calculated from the diffusion coefficients: the mole fraction increased from 14% to 42% as the membrane was heated from below to above the main phase transition temperature.

1. Introduction

The study of the interactions of cryoprotectants with cell membranes has become increasingly important because of the expanding need for long-term cryopreservation of cells.

An important cryoprotectant widely used in cell cryopreservation is the amphiphilic molecule dimethylsulfoxide (DMSO). The ability of DMSO to protect cells during freezing and reduce cellular damage is well known [1–9]. DMSO appears to protect cells from freezing induced injury not only by influencing extracellular and intracellular ice crystal formation but also through exerting a protective effect on cell membrane integrity [6] as reflected by a change in the main phase transition temperature of the membrane. Purely colligative effects cannot explain this change in membrane transition temperature and it is suspected that DMSO must also interact with the membrane in a non-colligative manner [8]. Despite this basic knowledge the precise nature of how DMSO interacts with cell membranes remains poorly understood.

To simplify analysis, it is common in the study of membranes to use model systems, as these systems have well defined transition temperatures and their size can be easily controlled. For studies on

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model systems to be particularly useful it is necessary for the model membranes to mimic some features of biological cells, such as a single bilayer structure with composition consisting of naturally abundant phospholipids. For this work large unilamellar vesicles (LUVs) of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were studied.

Previous studies of DMSO–membrane interactions have used primarily calorimetry [10–13], infrared spectroscopy [12,14], and X-ray analysis [10–12]. Nuclear magnetic resonance, despite its relatively poor sensitivity requiring higher sample concentrations and larger sample volumes, can nevertheless prove particularly powerful for investigations of intermolecular interactions. The importance of pulse-field gradient NMR (PFGNMR) techniques in particular has already been demonstrated in the study of micelles [15–19] and vesicles [20–22]. In PFGNMR the magnetic field intensity varies along the sample length and as individual molecules diffuse throughout the sample there is a loss of phase coherence, which leads to a decrease in signal intensity. The loss in signal intensity can be directly related to the diffusion of the species under investigation and, subsequently, the mole fraction of solute or solvent molecules in the bound and free states. The use of PFGNMR techniques to simultaneously study self-diffusion of multiple species can also provide valuable information relating to the presence of intermolecular associations. The present study demonstrates the usefulness of PFGNMR techniques in the study of the association of DMSO for DPPC LUVs.

2. Experimental

Phospholipids obtained as lyophilised powders (>99%) from Avanti Polar Lipids (Alabaster, AL); deuterium oxide and dimethylsulfoxide (99.9%) obtained from Aldrich Chemical Co. (Milwaukee, WI) were used without further treatment.

2.1. Vesicle preparation

Large unilamellar vesicles were prepared by hydrating 50 mg of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine in 1 ml of 99.9% deuterium oxide at 55–60°C for 1 hour with gentle agitation every 10 to 15 mins. The suspension was subjected to five freeze thaw cycles before extrusion. Extrusions were done using an Avanti mini-extruder equipped with stacked polycarbonate membranes (two 0.1 μm pore size membranes stacked with one 1 μm pore size membrane) while maintaining the temperature at 55°C. Following extrusion the vesicles were allowed to equilibrate at 4°C for 12 hours. DMSO was added as needed in 0.5 μl aliquots, such that the final molar concentration was equal to that of the phospholipid (6.81 mmol l^{-1}).

2.2. Vesicle characterisation

Vesicles were characterised in the usual manner [23] using light scattering techniques to determine the particle size distribution and ^{31}P NMR techniques to establish lamellarity.

2.3. Pulse field-gradient NMR (PFGNMR)

All NMR experiments were performed on an Avance 600 spectrometer (Brüker, Billerica, MA). PFGNMR spectra were collected with 64 scans at varying z -gradient field strengths, from 1.41 to

21.12 G cm⁻¹, above and below the main phase transition temperature utilising a 5 mm triple gradient inverse broad band probe. The two temperature points chosen for the series of experiments were 48°C and 35°C, with spectra acquired initially at the higher temperature and then at the lower temperature after slow cooling and equilibration. A total of sixteen field gradient experiments were done at each temperature using a previously established protocol, the bipolar phase stimulated echo pulse sequence [24]. Data were processed using an exponential apodisation function prior to Fourier transform. Self-diffusion coefficients, those dependent upon Brownian motion, were determined using the relationship below [24,25],

$$I_i = I_0 \exp[-D\gamma^2\delta^2g^2(\Delta - \delta/3)], \quad (1)$$

where I_0 is the integral of the signal at the lowest gradient strength, I_i is the integral at successively greater gradient strengths, D is the self diffusion coefficient of the species being investigated, and γ is the magnetogyric ratio of the nucleus being observed ($^1\text{H} = 2.675 \times 10^8 \text{ T}^{-1} \text{ s}^{-1}$). The diffusion time Δ , the time between pulse pairs, was 1 sec and the duration of the gradient pulse, δ , was 1 msec. These values are user defined and chosen so as to obtain sufficient signal attenuation at the maximum gradient strength. The gradient field strength is represented by g .

PFGNMR experiments were carried out on several different samples. Initially the experiments were performed on a sample containing 68.1 μmoles of DMSO in 1 ml of deuterium oxide to determine the self-diffusion coefficient of ‘free’ DMSO at 35°C and 48°C. The self-diffusion coefficients of DMSO and DPPC vesicles were then determined in samples of DPPC LUVs that also contained 68.1 μmoles of DMSO at both 35°C and 48°C.

3. Results and discussion

Light scattering, using a 90° scattering angle, revealed a homogeneous population distribution of vesicles with an average diameter of 100 nm and a narrow size distribution. ^{31}P NMR experiments demonstrated that the vesicles contained a single bilayer and were therefore unilamellar [26].

All spectra were processed in the same manner and as the gradient strength of the applied gradient pulses was increased it was observed that the NMR signals were attenuated. To determine I_i , at each gradient strength, spectra were integrated for all species present and normalized to a reference signal. The reference integral was that of the signal of interest at the lowest strength gradient, which was taken as the value of I_0 . The self-diffusion coefficients, D , were determined using Eq. (1).

The observed diffusion coefficient of DMSO was determined both in the presence of and the absence of vesicles. The self-diffusion coefficient in the absence of vesicles is referred to as that of the ‘free’ species (D_{free}) of DMSO, whereas that obtained in the presence of vesicles must take into account the fraction of DMSO bound to the vesicle (D_{bound}) as well as the effect of obstruction caused by the presence of the vesicles. D_{free} was calculated by determining the diffusion coefficient of the species in aqueous solution as described above.

The value of D_{bound} is assumed to be equal to the self-diffusion coefficient for DPPC vesicles (D_{vesicles}). Therefore the observed self-diffusion coefficient of DMSO (D_{obs}) obtained in the presence of vesicles is the average (D_{avg}) of the diffusion coefficient of both the free and bound species of DMSO. The mole fraction of DMSO in the bound state at equilibrium may be determined using Eq. (2) [18]:

Table 1
Self-diffusion coefficients obtained by PFGNMR methods

Species ^a	<i>T</i> (°C)	<i>D</i> _{obs} (×10 ^{−9} m ² s ^{−1})	<i>X</i> _{bound}
6.81 mM DMSO + 1 ml D ₂ O	35	1.39	
6.81 mM DMSO + 1 ml D ₂ O + 6.81mM DPPC	35	1.22	
6.81 mM DPPC + 1 ml D ₂ O + 6.81 mM DMSO	35	0.18	0.14
6.81 mM DMSO + 1 ml D ₂ O	48	2.54	
6.81 mM DMSO + 1 ml D ₂ O + 6.81 mM DPPC	48	1.62	
6.81 mM DPPC + 1 ml D ₂ O + 6.81 mM DMSO	48	0.36	0.42

^aObserved diffusion coefficient of the bold faced component.

$$D_{\text{avg}} = D_{\text{obs}} = (X_{\text{free}}D_{\text{free}}) + (X_{\text{bound}}D_{\text{bound}}) \quad (2)$$

or

$$D_{\text{avg}} = D_{\text{obs}} = (1 - X_{\text{bound}})D_{\text{free}} + (X_{\text{bound}}D_{\text{bound}}), \quad (3)$$

where X_{free} and X_{bound} are the mole fractions of DMSO in the bound and free states at equilibrium.

The value obtained for D_{avg} must also account for the obstruction of movement of free DMSO by the vesicles. Assuming spherical vesicle geometry, Eq. (4) [27] accounts for the obstruction and yields a corrected self-diffusion coefficient (D_{corr}) for free DMSO

$$D_{\text{corr}} = \frac{D_{\text{free}}}{1} - \frac{\Phi}{2}, \quad (4)$$

where Φ is the estimated ‘spherical volume’ of the vesicles. The value for Φ in the present experimental conditions is negligible [28–31] and D_{corr} effectively equals D_{free} .

From the variables determined above and assuming D_{vesicles} is equal to D_{bound} , rearranging Eq. (3) and substitution yields Eq. (5) and the mole fraction of DMSO in the bound state [18].

$$X_{\text{bound}} = \frac{D_{\text{free}} - D_{\text{avg}}}{D_{\text{free}} - D_{\text{vesicles}}}. \quad (5)$$

The self-diffusion coefficients obtained and the mole fraction of DMSO in the bound state are shown in Table 1. There is a threefold increase in the mole fraction of DMSO in the bound state at equilibrium above the transition temperature ($X = 0.42$) compared to below the transition temperature ($X = 0.14$). This clearly indicates that as the membrane is cooled DMSO is expelled from the vesicles: presumably from both the membrane (as the rigidity increases) as well the interior of the vesicles. These observations are in agreement with those of Anchordoguy et al. [7], who noted that the hydrophilicity of DMSO decreased at higher temperatures. Their observations were based on the partitioning of DMSO in a water/octanol system and they conclude that the association of DMSO with bilayers is enhanced at higher temperatures.

4. Conclusions

Pulse-field gradient NMR has been demonstrated as a useful tool to evaluate the association between DMSO and model membranes. It was determined that DMSO is most likely expelled from the vesicles

as they are cooled below the main phase transition temperature of the membrane. The fraction of DMSO associated with the model membrane may be easily quantified using this technique. Pulse-field gradient NMR could easily be applied to similar systems as well as intact cells.

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