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Viscosities encountered during the cryopreservation of dimethyl sulphoxide systems

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ABSTRACT

This study determined the viscous conditions experienced by cells in the unfrozen freeze concentrated channels between ice crystals in slow cooling protocols. This was examined for both the binary Me_2SO-water and the ternary Me_2SO-NaCl-water systems.

Viscosity increases from 6.9 ± 0.1 mPa s at −14.4 ± 0.3 °C to 958 ± 27 mPa s at −64.3 ± 0.4 °C in the binary system, and up to 55387 ± 1068 mPa s at −75 ± 0.5 °C in the ternary (10% Me_2SO, 0.9% NaCl by weight) solution were seen. This increase in viscosity limits molecular diffusion, reducing adsorption onto the crystal plane. These viscosities are significantly lower than observed in glycerol based systems and so cells in freeze concentrated channels cooled to between −60 °C and −75 °C will reside in a thick fluid not a near-solid state as is often assumed.

In addition, the viscosities experienced during cooling of various Me_2SO based vitrification solutions is determined to below −70 °C, as is the impact which additional solutes exert on viscosity. These data show that additional solutes in a cryopreservation system cause disproportionate increases in viscosity. This in turn impacts diffusion rates and mixing abilities of high concentrations of cryoprotectants, and have applications to understanding the fundamental cooling responses of cells to Me_2SO based cryopreservation solutions.

1. Introduction

The major physical stresses that are important in determining the cellular response during conventional cryopreservation, i.e. slow cooling, are well documented [4,5]. Following ice formation all solutes and suspended materials, including cells, become localised into freeze concentrated compartments [10,14]. During the subsequent reduction in temperature more ice forms and cells are exposed to increasingly concentrated solutions. This process continues until the freeze concentrated solution crystallises as a eutectic or becomes a glass. The combination of the increase in concentration and the reduction in temperature results in an increase in the viscosity of the unfrozen compartment and this has been determined for aqueous solutions of glycerol [14]. Ice growth during cooling occurs by the diffusion of water from the bulk aqueous phase to the ice crystals and in the case of aqueous solutions of glycerol diffusion limited ice crystallization occurs at rapid cooling rates [14]. The hypertonic conditions that cells encounter during slow cooling lead to an osmotic loss of water, the extent of which is dependent both on the rate of cooling and on the viscosity of the freeze concentrated compartment [12–14].

The toxicity of cryoprotectants is reduced at lower temperatures and methods such as liquidus tracking (also known as progressive lowering of temperature) have been examined as an alternative approach to cryopreservation [1,3,8,21]. Liquidus tracking involves adding a cryoprotectant to a sample to reduce its freezing point. The sample is then cooled to just above the new freezing point and more cryoprotectant is added to further lower the freezing point [3,16]. This stepwise process is continued until enough cryoprotectant is present to vitrify the sample. It has been speculated that the limiting factor in this process is the increase in viscosity encountered at lower temperatures, which results in limited diffusion of cryoprotectant into tissues. However, with the most common cryoprotectant dimethylsulphoxide (Me_2SO) used in liquidus tracking procedures the viscosity of the solutions at low temperatures has been little studied [7,16,21,22].

This study examined the increase in viscosity experienced by cells cooled in Me_2SO solutions under conditions which result in equilibrium freezing (i.e. the composition of ice and freeze

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concentrated materials follows the equilibrium phase diagram) down to −75 °C, and calculates the diffusivity of water molecules at various temperatures. Finally, the effect of the addition of sugars on the viscosity and diffusivity of water in Me_2SO solutions is measured at low sub-zero temperatures.

2. Materials and methods

2.1. Reagent preparation

All reagents were sourced from Sigma Aldrich (Gillingham, UK) and concentrations given as percentage by weight unless otherwise stated.

To measure the viscosity of the freeze-concentrated compartment, aqueous solutions with various Me_2SO (Sigma #D4540) concentrations were prepared according to the phase diagram [19]. In addition the system Me_2SO-NaCl-Water of unfrozen composition 10% Me_2SO in 0.15M NaCl was studied.

Two binary vitrification solutions were also prepared, containing 60% and 70% Me_2SO in 0.15M NaCl. In addition, two solutions 60% Me_2SO were mixed with either 10% by weight glucose (Sigma #G8270) or 10% by weight raffinose (Sigma #R0250) to study the impact of additional cryoprotectants.

2.2. Viscometer measurements

All viscosity measurements were carried out using an Anton Paar (St. Albans, UK), RheolabQC Rheometer with a DG42 concentric cylinder attachment.

To measure viscosity, 12 ml of each sample solution was separately added to the rheometer measuring attachment. K-type thermocouples were added to both the inner and outer wall of the attachment. These thermocouples were attached to a picologger unit (Picotechnology, St. Neots, UK), and temperatures recorded using picologger software (Picotechnology, St. Neots, UK).

A low temperature bath was prepared in a vacuum flask consisting of dry ice (Dioxice, Leicester, UK) in industrial methylated spirits, IMS (No Nonsense Ltd. Yeovil, UK, 13028). Control over the temperature could be achieved through adding/removing dry ice, with the temperature of the bath measured using a thermocouple.

Viscometric readings were taken at room temperature (measured by the thermocouples), and recorded using Rheocompass software (Anton Paar, St. Albans, UK). The measuring cylinder was lowered into the low temperature bath. Temperature and viscometric readings were taken as the cylinder cooled until the target temperature was reached. This target temperature depended on the freezing point of the solution being tested. On this target temperature being achieved, the low temperature bath was removed, with the sample being allowed to warm whilst temperature and viscosity continued to be recorded.

To prevent the formation of ice in the cylinder which would gave erroneous readings, care was taken not to lower any solution below its melting point during the experiment. In addition, data were monitored for temperature discontinuities related to ice formation, and erratic viscosity readings which would occur if crystals formed in the system.

2.3. Diffusivity calculations

The mean distance, x, travelled by a molecule in a solution diffusing from a point source of concentration in time, t, can be approximated by the equation:

$$x = 2\sqrt{\frac{Dt}{\pi}}$$

where D is the diffusion coefficient [2]. The Stokes–Einstein equation can be used to relate D to the dynamic viscosity, μ:

$$D = \frac{kT}{6\pi\mu a}$$

where k is the Boltzmann constant and a is the Van der Waals radius of a water molecule (0.282 nm) [14].

2.4. Rate of increase of viscosity

The average rate of increase in viscosity per °C can be calculated by Ref. [20]:

$$= \mu_{increase}^{\left(\frac{1}{\Delta T}\right)}$$

Where $\mu_{increase} = \frac{Final\ Viscosity}{Start\ Viscosity}$ and $\Delta T = End\ Temperature - Start\ Temperature$. Insertion of experimental values can be used to determine increase in viscosity of a fixed solute solution when the start and final viscosity and temperature values are for that system are inserted. Alternatively it is valid to determine average increase over a specific temperature range for an equilibrium freezing system where viscosity and temperature values for the system of interest are inserted.

3. Results

The viscosity of the unfrozen fraction formed during the equilibrium cooling of an aqueous solution of Me_2SO is shown in Fig. 1. The black line displays the viscosity in a Me_2SO-water binary system, showing that viscosity increases exponentially from 6.9 ± 0.1 mPa s at −14.4 ± 0.3 °C to 958 ± 27 mPa s at −64.3 ± 0.4 °C. With a ternary Me_2SO-NaCl-water system (Fig. 1 in grey) that in the liquid state is composed of 10% Me_2SO and 0.15M NaCl in water, the viscosity increases more rapidly with decreasing temperature, reaching 53387 ± 1068 mPa s at −75 ± 0.5 °C.

Fig. 2 plots the viscosity of 60% and 70% aqueous Me_2SO solution as a function of sub-zero temperature. For a 70% solution the viscosity increases from 4.3 ± 0.1 mPa s at 23 ± 1 °C to 3685 ± 15 mPa s at −73.6 ± 1 °C. and for a 60% solution the viscosity increases from 3.7 ± 0.2 mPa s at 23 ± 1 °C to 1973 ± 54 mPa s at −66.5 ± 1 °C.

Adding either glucose or raffinose to the 60% Me_2SO solution increases its viscosity at all measured temperatures. The Me_2SO-water-glucose system has a viscosity increase from 3.9 ± 0.3 mPa s at 24.9 ± 1 °C to 12662 ± 408 mPa s at −73.6 ± 1 °C, and Me_2SO-water-raffinose system's viscosity increase from 4.7 ± 0.1 mPa s at 23 ± 1 °C to 13296 ± 129 mPa s at −74 ± 1 °C.

This equates to a viscosity increase of 10.38% and 16.25% per °C cooled for the Me_2SO-water and the Me_2SO-NaCl-water equilibrium freezing systems respectively over the tested ranges. For the 60% and 70% Me_2SO binary non-freezing systems, this increase was 7.27% and 7.23% per °C cooled respectively. On average, the viscosity increased by 8.87% and 8.54% per °C cooled for the Me_2SO-glucose and Me_2SO-raffinose systems respectively.

The diffusion coefficients for water molecules within Me_2SO (60% and 70%), as well as diffusion distances in freeze concentrated channels during equilibrium freezing in Me_2SO with and without NaCl has been calculated (Fig. 3). At high sub-zero temperatures (approximately −10 °C), the diffusion coefficient of water molecules is typically 10^{-10} m^2/s, being lower in concentrations with