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Effect of trehalose as an additive to dimethyl sulfoxide solutions on ice formation, cellular viability, and metabolism

Jason Solocinski [a], Quinn Osgood [a], Mian Wang [a, 1], Aaron Connolly [a], Michael A. Menze [b, **], Nilay Chakraborty [a, *]

[a] Department of Mechanical Engineering, University of Michigan-Dearborn, 4901 Evergreen Road, Dearborn, MI 48128, United States
[b] Department of Biology, University of Louisville, Louisville, KY 40292, United States

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ABSTRACT

Cryopreservation is the only established method for long-term preservation of cells and cellular material. This technique involves preservation of cells and cellular components in the presence of cryoprotective agents (CPAs) at liquid nitrogen temperatures (−196 °C). The organic solvent dimethyl sulfoxide (Me_2SO) is one of the most commonly utilized CPAs and has been used with various levels of success depending on the type of cells. In recent years, to improve cryogenic outcomes, the non-reducing disaccharide trehalose has been used as an additive to Me_2SO-based freezing solutions. Trehalose is a naturally occurring non-toxic compound found in bacteria, fungi, plants, and invertebrates which has been shown to provide cellular protection during water-limited states. The mechanism by which trehalose improves cryopreservation outcomes remains not fully understood. Raman microspectroscopy is a powerful tool to provide valuable insight into the nature of interactions among water, trehalose, and Me_2SO during cryopreservation. We found that the addition of trehalose to Me_2SO based CPA solutions dramatically reduces the area per ice crystals while increasing the number of ice crystals formed when cooled to −40 or −80 °C. Differences in ice-formation patterns were found to have a direct impact on cellular viability. Despite the osmotic stress caused by addition of 100 mM trehalose, improvement in cellular viability was observed. However, the substantial increase in osmotic pressure caused by trehalose concentrations above 100 mM may offset the beneficial effects of changing the morphology of the ice crystals achieved by addition of this sugar.

1. Introduction

Cryoprotective agents (CPAs), are traditionally used to ensure survival of cellular samples at cryogenic temperatures. Due to toxicity concerns of penetrating CPAs such as dimethyl sulfoxide (Me_2SO), several additives such as glycerol [1], disaccharides (e.g. trehalose, sucrose [1–3]), amino acids (e.g. proline [2,4,5]), and proteins (e.g. sericin [6]) have been used in recent years. Several organisms in nature are frequently exposed to subzero temperatures and a common strategy in these organisms is to accumulate biocompatible osmolytes such as trehalose before the onset of water loss due to freezing, drying, or both [7–9]. Trehalose has been found to improve the cryogenic outcome in a variety of biological materials including mammalian cells and cellular monolayers [4,5,10–12]. However, the actual mechanism for improvement of cellular viability in presence of trehalose following cryopreservation remains poorly understood [13,14]. Here, we present an in-depth analysis of the effect of trehalose addition to a Me_2SO-based freezing solution on ice-formation, cumulative osmotic stress, viability, and post-thaw metabolic activity of human hepatocellular carcinoma (HepG2) cells.

At low cooling rates (~1 °C/min) 'solution effects' injury stemming from exposure of cells to a hypertonic extracellular environment for an extended period of time is the primary cause of cellular damage [15–18]. During freezing, water crystalizes in the extracellular environment — a process that increases the solute concentration in the non-frozen water fraction

* Corresponding author.
** Corresponding author.
E-mail addresses: michael.menze@louisville.edu (M.A. Menze), nilay@umich.edu (N. Chakraborty).
[1] Current address: Department of Mechanical Engineering, University of Minnesota, Minneapolis, MN 55455, United States.

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surrounding the cells. In addition to osmotic stress, extracellular ice morphology can have a strong bearing on cellular viability [19]. However, most experimental techniques do not allow characterizing spatial differences in ice morphology and solute distribution in frozen systems. Spatially correlated Raman microspectroscopy techniques were used at −40 and −80 °C to characterize changes in the ice formation and solute distribution after addition of trehalose to Me_2SO based freezing solutions.

Vibrational Raman microspectroscopy is a highly sensitive technique that relies on detection of vibration in molecular moieties when excited with laser irradiation [20,21]. Since the vibrational information is specific to the chemical bonds and symmetry of molecules, Raman microspectroscopy provides a fingerprint by which a molecule can be identified [21,22]. This extends to different physical states in the same molecule such as the transition from water to ice [23,24]. Therefore, it is feasible to use Raman microspectroscopy to study cryoprotective formulations and investigate the distribution, state, and concentration of compounds at different sub-zero temperatures. While Me_2SO has been widely used as a penetrating cryoprotectants and is known to depress the freezing point of aqueous solutions [25,26], at relatively low concentration (<1 M) Me_2SO has little influence on the average water-water hydrogen bonding strength [27]. In contrast to Me_2SO, Raman microspectroscopic observations [28] and molecular dynamic simulations [29,30] have revealed that trehalose promotes a destructive effect on the tetrahedral hydrogen-bond network of pure water [30]. These studies suggest that in presence of trehalose, water binds stronger to the sugar than to other water molecules. Trehalose obstructs the water-crystallization process, thereby destroying the water network and forming a sugar-water network [31]. At low temperatures formation of ice creates a partially dehydrated environment and while the additive trehalose may be excluded from the immediate vicinity of the biomolecules of interest [32] in presence of the sugar ice formation occurs at lower temperatures but at more independent nucleation sites [33].

We hypothesized that the destructuring effect of trehalose on water-water hydrogen bonding will be maintained in presence of Me_2SO. Therefore, by reducing the availability of water molecules to join a tetrahedral hydrogen network that plays a formative role in creating ice crystals during freezing, an overall smaller ice crystal size may be observed in presence of water, trehalose, and M_2SO compared to the binary water Me_2SO system. In this study a highly sensitive confocal Raman microspectroscopic (CRM) system was used to generate spatially correlated chemical maps of the distribution of ice, Me_2SO, and trehalose in the frozen systems. Special attention was paid to the effect of trehalose concentration on the formation and distribution of ice crystals and the recovery of metabolic functions after cryopreservation of HepG2 cells.

2. Materials and methods

2.1. Sample preparation

Low endotoxin α, α-trehalose dihydrate was obtained from Pfanstiehl Inc. (Waukegan, IL) and dimethyl sulfoxide (Me_2SO) was procured from Sigma Aldrich (St. Louis, MO). Solutions of 10% (v/v) Me_2SO were made by mixing 10% pure Me_2SO with 90% phosphate buffered saline (PBS) solution from Sigma Aldrich (St. Louis, MO) volume by volume and then dissolving trehalose to reach final concentrations of 0 mM, 100 mM, and 300 mM trehalose. These solutions were used in the confocal Raman microspectroscopy studies and in the cell freezing studies.

2.2. Low temperature confocal Raman microspectroscopy

Low temperature Raman measurements were conducted using a customized confocal microscope and Raman spectrometer combination (UHTS 300, WITec Instruments Corp., Germany). Raman spectra were collected using a highly sensitive EMCCD camera (Andor Technology, UK). A 532 nm solid-state laser was used for excitation and images were captured using a 10X objective (Carl Zeiss, Germany). A liquid nitrogen cooled freezing stage (FDCS 196, Linkam Scientific Instrument, UK) was integrated with the microscopy setup and was used to cool the samples at a predetermined rate. For each experiment, the freezing stage with 20 μl of solution was mounted on the Raman microscope stage with a custom-made stage adaptor. Samples were cooled to −40 and −80 °C at a rate of 1 °C/min, and then held for approximately 10 min at each temperature before the spectral information was collected. Spatially correlated hyperspectral Raman images were created using the Raman signals collected from a window of 50 × 50 μm. Each array of Raman scans was collected using a low integration time (0.3s) to minimize impact of laser irradiation on the ice crystals formed. Each experiment was repeated 3 times and the confocal Raman images presented here are representative for all 3 repetitions.

2.3. Image processing

Images were processed using the open source software Image J [34]. All images were processed for identification and quantification of ice crystals using a standard bandpass filter for particle analysis. A threshold was applied to convert raw hyperspectral images to a binary image. The Watershed segmentation algorithm [35] in Image J was used to prevent the individual ice crystals from merging to one another and the Particle Analysis tool was used to quantify both the number and area of the ice crystals.

2.4. Cell culture and cryopreservation

Human hepatocellular carcinoma (HepG2) cells were obtained from the American Type Culture Collection (Manassas, VA), and grown in 75 cm^2 cell culture flasks (Corning Incorporated, Corning, NY). Standard culture medium for HepG2 cells was composed of Opti-MEM I (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Gibco) and penicillin-streptomycin solution to yield final concentrations of 100 units/mL penicillin G and 100 μg/mL streptomycin sulfate (HyClone-Thermo Scientific, Logan, UT). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Upon reaching 80–90% confluency, cells were dissociated using 0.25% trypsin plus 1 mM EDTA in a balanced salt solution for 10 min, and trypsin activity was stopped by adding fully supplemented medium to the flask followed by centrifugation for 5 min at 200 × g. The cells were washed once with fully supplemented medium and the final cell pellet was resuspended in one of the three different solutions containing CPAs previously mentioned. Cell samples were diluted in the CPA solutions to a concentration of 1×10^6 cells/mL. A volume of 1 mL of the samples was transferred into type D micro tubes (Sarstedt, Radnor, PA), and placed into a passive freezing device (Cool Cell LX, Biocision, Menlo Park, CA), which provides a cooling rate of 1 °C/min. After loading with samples, the freezing device was quickly transferred to a −80 °C commercial freezer for 24 h. The following day, the tubes were quickly collected and transferred to a LN2 storage container.

2.5. Mathematical modeling of cumulative osmotic stress

The progressive loss of osmotically active intracellular water with the increase of extracellular osmolality during freezing at 1 °C/