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MEASUREMENT OF MOLECULAR MOBILITY IN MAMMALIAN CELLS

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ABSTRACT

Preservation of mammalian cells requires establishing a reversible stasis condition by reducing the intra/extracellular molecular mobility ensuring reduced chemical reaction and deterioration rates. Molecular mobility may be reduced by various techniques. For example, in cryopreservation, mobility within and surrounding the cell is reduced through freezing the free water that constitutes 70-90% of the cell's composition. In dried-state preservation applied successfully to preserve seeds, pharmacological materials and foodstuff (mimicking the anhydrobiosis phenomenon seen in nature), reduction in molecular mobility is established by removing intra/extracellular water. Certain carbohydrates (such as trehalose and sucrose) can be artificially uploaded into mammalian cells to replace the removed water and to form an intra/extracellular glass. In this research, a fluorescent rotor is utilized to determine the changes in intracellular molecular mobility during carbohydrate uploading of mammalian cells. It was shown that using this technique, it is feasible to make real-time mobility measurements at a single cell level.

INTRODUCTION

Mammalian cells require very strict environmental conditions (chemical, thermal and mechanical) to perform their activities. Preservation of cells requires minimizing the cell's response to an otherwise lethal environment by establishing a reversible stasis condition. Stasis is established by reducing the intra/extracellular molecular mobility. Water is the primary solvent in biomaterials enabling vital (or during storage causing detrimental) chemical processes to occur. Physical and chemical properties of water change depending on its interaction with its surroundings. The amount of solvent (free) water in a mammalian cell governs its osmotic response. Almost all of the free water in a cell can be removed without any permanent damage. The vicinal and bound (unfreezable) water on the other hand, are tightly associated with the ions, proteins, membranes, organelles and macromolecules in the cytoplasm and upon uncontrolled removal may cause irreversible denaturation of these structures.

In nature, some organisms such as yeast, brine shrimp, tartigrade and plant seeds can survive extreme draught conditions when they lose all of their water and *resurrect* when conditions are fair. The common behavior seen in all of these *anhydrobiotic organisms*, is that they produce high concentrations of disaccharides (such as trehalose), which play a major role in dried preservation of seeds (for a review see [1]). Trehalose is known to be effective in cryopreservation [2] of mammalian cells, protection against dehydration induced fusion of lipid membranes [3,4] and protein denaturation [5]. In two contradicting hypotheses, the protection mechanism is offered as preferential exclusion from or replacing the water close to [6] macromolecule surfaces in the cytoplasm. No matter what the mechanism is, the consensus is that during desiccation carbohydrates form a cytosolic "glass" and therefore reduce the molecular mobility significantly.

In this research, the change in intracellular mobility of a mammalian cell (rat hepatocyte) in the presence of intra-/extracellular carbohydrate solutions was measured using a fluorescent molecular rotor. When it is excited, the rotor deactivates either by internal rotation or (if the viscosity of the environment is very high hindering mechanical rotation) by photonic emission and can therefore be used as an indicator of the viscosity of its local environment [7].

MATERIALS AND METHODS

Hepatocytes were isolated from adult female Lewis rats (Charles River Laboratories, Boston, MA) weighing 150-200 g, according to the two-step collagenase perfusion technique previously described [8], as modified by Dunn et al. [9]. The procedures on animals and animal care were carried out in accordance with National Research Council guidelines and approved by the Subcommittee on Research Animal Care at the Massachusetts General Hospital. Each isolation yielded 200-350x10^6 hepatocytes. Viability ranged from 90% to 98% as determined by trypan blue exclusion. Type 1 collagen was prepared from by extracting acid-soluble collagen from Lewis rat tail tendons as described elsewhere [9]. A collagen gelling solution was prepared by mixing 9 parts of collagen (at a concentration of 1.1 mg/ml) with 1 part of concentrated 10X DMEM. 35 mm diameter culture dishes (Becton Dickinson, Franklin Lakes, NJ) were coated with 1 ml of the gelling solution and incubated at 37 ^oC for 1 hour to promote gel formation. Standard hepatocyte culture medium contained DMEM supplemented with 10% heat-inactivated FBS, 200 U/mL penicillin, 200 μg/mL streptomycin, 7.5 μg/mL hydrocortisone (Pharmacia and Upjohn, Kalamazoo, MI), 20 ng/mL EGF, 14 ng/mL glucagon (Eli-Lilly, Indianapols, IN) and 0.5 U/mL insulin (Eli-Lilly). Isolated hepatocytes were suspended in hepatocyte culture medium at a concentration of 1x10^6 cells/ml and seeded on the collagen-coated dishes at a density of 0.75-1 million cells/dish. A collagen sandwich configuration was formed by subsequent deposition of a second layer of collagen 24 hours after seeding the hepatocytes. The cultures were placed at 37 ^oC for up to 1 hour to begin the gelation process and the cells were subsequently maintained in hepatocyte culture medium. The cultured hepatocytes were maintained at 37 ^oC under a humidified gas mixture of 90% air/10% CO_2 and the medium was replaced every 24 hours.

Before the experiment, each sample was incubated in 1X DPBS (Invitrogen Corporation, Carlsbad, CA) solution containing 40 nM of the fluorescent rotor, CCVJ (9-(2-carboxy-2-cyanovinyl)julolidine, Helix Research, Springfield, OR) for 10 minutes at 37 ^oC. The sample was then transferred to an environmental chamber connected to a micrsocope (Zeiss Axiovert 200M, Carl Zeiss Inc., Thornwood, NY) and was kept at 37 ^oC, 100 %RH for the duration of the experiment. Time-lapsed phase contrast and fluorescence images ($\lambda_{excitation}$=425/40 nm, $\lambda_{emission}$=528/50 nm) were taken at predetermined time intervals (30-150 s). At certain time points, the solution in the dish was aspirated and was replaced with: **1)** Isotonic (~300 mOsm) 0.2M 3-O-Methyl-D-glucopyranose (3-OMG) (Sigma-Aldrich Inc., St. Louis, MO) in DPBS, **2)** Hypertonic (~450 mOsm) 0.2M 3-OMG in DPBS or, **3)** Isotonic (~300 mOsm) 0.2M trehalose (Ferro Pfanstiehl Laboratories Inc., Waukegan, IL) in DPBS containing the fluorescent rotor at the same concentration as the incubation solution.

After the experiment was completed, collected time-lapsed images were analysed using an image analysis software (Metamorph, Universal Imaging Corporation, Downingtown, PA) (Figure 1). From each experimental group 3-7 cells were randomly chosen and the variation of cytosolic fluorescence intensity was measured using cell

tracking and area averaging. All of the images were corrected for background and the intensity measurements were normalized with respect to that measured during the initial 1X DPBS exposure. Dye concentration effects due to volume change were also accounted for.

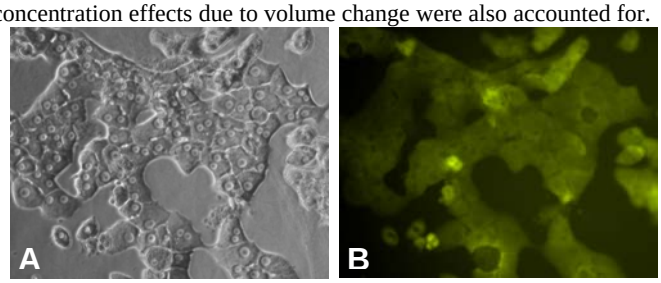

Figure 1: A) Phase Contrast Image, B) CCVJ Fluorescence Image of Rat Hepatocytes in 1X DPBS, Plated in a Collagen Double Gel (200x).

RESULTS

The non-metabolizable carbohydrate 3-OMG is transported into the cells through a glucose transporter and equilibrates with the extracellular concentration [10] within 30 minutes. When artifical membrane breaching techniques are not utilized, the membrane impermeable disaccharide, trehalose, on the other hand is mostly transported through the membrane by the very slow endocytosis process (for example, for mesenchymal stem sells an internal concentration of 20 mM can be reached after 24 hours exposure to an external trehalose concentration of 100 mM [11]). During continuous 1X DPBS exposure, there was an initial increase in cytosolic viscosity followed by a time dependent gradual decrease (Figure 2: diamond symbols).

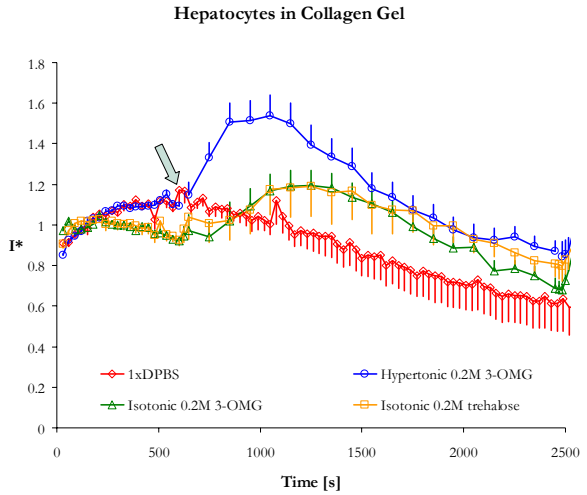

Figure 2: Real-time Measurement of Molecular Mobility in Rat Hepatocytes During Continuous Exposure to Membrane Permeable and Impermeable Carbohydrates *(the arrow indicates the time point where the 1X DPBS solution is replaced with the experimental solution)*

This was attributed to photobleaching and also re-organization of the cytoskeleton (results not reported here), resulting in cell lysis after 60-90 minutes of exposure. The cell response to extracellular isotonic trehalose (squares) was almost identical to 3-OMG response (triangles). When the osmolality of the extracellular 3-OMG solution was increased, however, a significant initial increase in the cytosolic viscosity was observed. At the end of the equilibration period (when the 3-OMG equilibrated with its outside concentration at t=2400s), there was not a significant difference among the values measured for the three different solutions (but all of them were higher than the viscosity values measured for 1X DPBS indicating uptake).

CONCLUSIONS

It was observed that real-time measurement of molecular mobility inside live cells during exposure to various carbohydrate solutions is feasible. Much work is needed to quantify all of the cellular mechanisms responsible for the changes observed.

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