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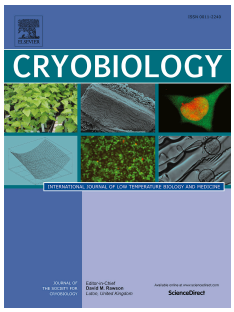

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Increased cryosurvival of osteosarcoma cells using an amphipathic pH-responsive polymer for trehalose uptake

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Abstract

Amphipathic pH-responsive polymers have shown to increase the permeability of cell membranes to trehalose hence improving the cryopreservation of mammalian cells. However, the trafficking of both the polymer and trehalose across the cell membrane has not yet been thoroughly analysed. The objective of this study was to investigate the effect on cryopreservation of the trafficking of the disaccharide trehalose along PP-50, an amphipathic polymer, through an osteosarcoma cell line (SAOS-2). Confocal microscopy analysis confirmed the presence of intracellular labelled trehalose only when incubated in the presence of PP-50. Further analysis confirmed that both trehalose and PP-50 localised in the cytoplasm, accumulated mainly in the perinuclear area. Quantitative analysis of the colocalisation between trehalose and PP-50 showed Pearson and Manders coefficients of 0.862 ± 0.008 and 0.766 ± 0.033, respectively, suggesting a high degree of intracellular colocalisation between these molecules. Cryopreserved cells pre-incubated with trehalose and PP-50 showed increased cryosurvival when compared with cells pre-incubated in the absence of the polymer. PP-50 showed to be directly involved in the uptake of trehalose, a critical characteristic towards use in cryopreservation and biomedical applications.

Key words

Amphipathic polymer, trehalose, cryopreservation, drug delivery, colocalisation

1. Introduction

Recent developments in tissue engineering, recombinant DNA technologies and biopharmaceutical production have increased the importance of living cells for clinical medical care. The establishment of these areas in the past several decades has made it possible for the development of numerous therapeutic proteins and treatments to address a corresponding variety of unmet medical needs [1].

Major advances in these areas are frequently limited by the lack of understanding of long-term cryostorage of cells [2]. Cryopreservation of cell lines is a technically challenging process leading to persistent failures in terms of poor revival rate and atypical cell properties. Currently, no single cryopreservation method has been universally applied with variations in techniques applied depending on the facility and aim of use [3,4].

Cells normally require cryoprotective agents (CPAs) to survive long-term storage at low temperatures [5]. Cryoprotectants are essential additives to cell concentrates able to inhibit the formation of intra and extracellular crystals and hence cell death [3,6,7]. Successful cryopreservation of mammalian cells involves high CPAs concentrations and their toxicity has been recognized as a critical barrier to further progress in the field [5,8].

Dimethyl sulfoxide (Me_2SO) is the standard cryoprotectant used to prevent freezing damage to living cells. Whilst the effectiveness of Me_2SO as a cryoprotectant is generally improved with increasing concentration, its toxicity to cells is both time and temperature dependent and has been thoroughly documented [9,10]. Serious adverse reactions have also been reported related to cardiovascular, respiratory and renal systems [3,11]. On the other hand, foetal bovine serum (FBS) is an essential component in cryopreservation media, although presents many challenges for the pharmaceutical industry since its usage involves both moral and scientific problems. In clinical applications the use of reagents of animal origin is undesirable due to potential transmission of animal pathogens and the risk of developing antibodies against FBS, leading to rejection of the transfused cells [12]. Because of the safety risks involved, regulatory authorities discourage the use of FBS for the production of biological products for human use [13]. Hence, significant interest exists in the development of non-toxic cryopreservation agents and cryopreservation techniques. Studies over the last 30 years have explored a variety of cryoprotective agents. Most attention has focused on the role of sugars in mammalian cells, predominantly the disaccharide trehalose [14].

Non-permeating sugars are important ingredients in a vast number of cryopreservation protocols. Sugars help in the dehydration of cells by increasing osmolarity, in addition to preserving structural integrity. They also function as an osmotic buffer, reducing osmotic shock by decreasing the speed and amount of cell swelling [15]. Although monosaccharides can transit across animal cell membranes, disaccharides cannot [16]. This issue is crucial when high intracellular concentrations of disaccharides such as trehalose are needed for viable cryopreservation [2,5].

To overcome this barrier, different methods have been used to increase intracellular disaccharides levels, such as the use of ATP receptor channels or engineered membrane pores [2,17,18]. Disadvantages of these alternatives include the induction of cell death by either necrosis or apoptosis, amongst others [19].

One successful method to induce disaccharide transport into cells includes the use of amphipathic biodegradable pH-responsive polymers given their ability to permeate cell membranes and biocompatibility [20–22]. These polymers were developed to mimic the membrane permeabilising activity of viral and bacterial peptides [23]. Typically, they go through a conformational change from extended charged chains to aggregated hydrophobic structures as the environmental pH drops below their pKa, thus interacting with the hydrophobic interior of phospholipid bilayers. This facilitates increased membrane permeability by pore formation or by increasing membrane solubilisation [24,25].

Recent studies have shown the impact of PP-50, a biopolymer consisting of poly(L-lysine iso-phthalamide) grafted with the hydrophobic amino acid L-phenylalanine, on increased post-thaw viabilities of cryopreserved red blood cells and osteosarcoma cells [26,27]. Despite its successful use in cryopreservation in previous studies, trehalose uptake mediated by PP-50 has not been studied in depth. In this work, the effect of PP-50 on trehalose transport was evaluated in osteosarcoma cells, a model for adherent cells, to provide key information regarding this amphipathic biopolymer towards use in cryopreservation and other clinical applications.

2. Materials and methods

2.1. Materials

The SAOS-2 cells were obtained from the European Collection of Cell Cultures. Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), L-glutamine, penicillin, trypan

blue, and streptomycin were purchased from Invitrogen (UK). Me_2SO and sodium hydroxide (NaOH) were purchased from ThermoFisher (UK). Phosphate-Buffered Saline (PBS), trypsin–EDTA, Alexa Fluor 647 cadaverin were purchased from Life Technologies™ (UK). N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (sulfo-NHS), Hoechst H33342, propidium iodide, and trehalose were obtained from Sigma-Aldrich (UK). The CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was obtained from Promega (UK). Visking tubing was purchased from Medicell Membranes Ltd. (UK). FITC-labelled trehalose was trehalose was produced by the Davis Group, University of Oxford (UK) [28]. All chemicals and biochemicals used were of analytical grade.

2.2. PP-50 synthesis

The synthesis and characterisation of the PP-50 polymer were as previously described by Lynch *et al*. [26]. PP-50 consists of a poly(L-lysine iso-phthalamide) backbone grafted with L-phenylalanine at a degree of grafting of 46.2% (Mn = 23.0 kDa).

2.2.1. PP-50/AF647 synthesis

The conjugation of AF647 cadaverine to PP-50 was achieved by standard EDC/sulfo-NHS amide reaction [29]. The product was purified by dialysis against water in Visking tubing (MW cut-off 3,500 Da) and lyophilised using a Fisher Scientific Heto LyoLab3000 (UK) to yield a blue solid powder.

PP-50/AF647 was analysed by using attenuated total reflectance FTIR (ATR-FTIR) spectroscopy to determine whether PP-50 were successfully conjugated with Alexa Fluor 647 cadaverine. Spectra were collected using a Thermo Nicolet Nexus 870 spectrometer (Waltham, MA, USA) as the average of 32 scans with a wavenumber resolution of 4 cm^{-1} in the 600-4000 cm^{-1} range.

2.3. Cell culture

SAOS-2 osteosarcoma cells were cultured in growth media consisting of DMEM with FBS (10% v/v), L-glutamine (2 mM), penicillin (100 IU/ml), and streptomycin (100 µg/ml) in 75 cm^2 flasks supplied by Corning (UK). Cells were incubated at 37 ℃ with 5 % of CO_2. At 70% confluence, cells were washed twice with PBS, then subcultured with trypsin (0.05% w/v) and EDTA (0.02% w/v) and subsequently replated for further expansion or experiments.

143 2.4. Trehalose uptake mediated by PP-50
144
145 Cells were seeded on 1.8 cm^2 chambered cell culture cover glasses (Nunc, UK) at a density
146 of 3 x 10^4 cells/well and grown for 24-48 hours. Cells were then incubated with 50 mM FITC-
147 trehalose in the presence or absence of 250 μg/ml PP-50 for 2, 4, and 6 hours. FITC-
148 trehalose and PP-50 solutions were prepared in DMEM. Cells were washed twice with PBS
149 and extracellular labelled molecules were quenched using 0.4 % trypan blue [30]. Cells were
150 washed again with PBS and left in growth media. Cells were incubated for 15 minutes with 5
151 μg/ml PI and 5 μg/ml H33342 then incubated in growth media and analysed using a TCS
152 SP5 inverted laser scanning microscope (Leica, Germany). An argon laser was used to
153 visualise FITC-trehalose (emission at 488 nm and an emission filter set at 505-555 nm). To
154 visualise propidium iodide, a helium neon laser was used (emission at 633 nm and emission
155 filter set at 650-700 nm). H33342 stained nuclei were excited using a diode laser emitting at
156 405 nm. Images were taken sequentially.
157
158 2.5. Colocalisation of labelled PP-50 with labelled trehalose
159
160 Cells were assessed using 250 μg/ml PP-50/AF647 and 50 mM FITC-trehalose after 4 hours
161 of incubation. In addition, the permeable dye Hoechst 33342 (5 μg/ml) was used to evaluate
162 the nuclear morphology of cells. Cells were placed on 1.8 cm^2 chambered cell culture cover
163 glasses (Nunc, UK) at a density of 3 x 10^4 cells and imaged using a TCS SP5 inverted laser
164 scanning microscope (Leica, Germany). To visualise PP-50/AF647, a helium neon laser was
165 used (emission at 633 nm and emission filter set at 650-700 nm). Images were taken
166 sequentially.
167
168 2.6. Image analysis
169
170 Fluorescent images of cells incubated with labelled trehalose in the presence of labelled PP-
171 50 were acquired using the appropriate wavelengths for each molecule, as explained above.
172 Images were then merged and converted to 8 bit RGB images using ImageJ software.
173 Analysis with the JACoP plugin provided Pearson's and Manders' overlap coefficient.
174
175 2.7. Cryopreservation using PP-50
176
177 SAOS-2 cells were seeded into 6-well plate culture dishes at 3 x 10^5 cells/well. Once cells
178 reached 75% confluence, they were washed twice with PBS and incubated in one of the

following solutions: 1) 100 % (v/v) DMEM containing 200 mM trehalose with and without 250 µg/ml PP-50; 2) 90 % (v/v) DMEM and 10 % (v/v) FBS containing 200 mM trehalose with and without 250 µg/ml PP-50. All DMEM solutions were supplemented with L-glutamine, penicillin, and streptomycin at a pH of 7.05. After 4 hours of incubation, treated cells were washed twice with PBS and 200 µl/well of trypsin/EDTA was added. Cells were then incubated for 5 minutes and centrifuged at 350 g for 5 minutes. Each pellet was then resuspended with 1 ml of a solution of 90 % (v/v) FBS and 10 % (v/v) DMEM. Control cells were resuspended in two different solutions; 100 % (v/v) DMEM was used as a negative control C(-) for cryosurvival, whereas 90 % (v/v) FBS with 10 % (v/v) Me_2SO was used as a positive control C(+). All samples were transferred to cryovials and placed into freezing containers at -80 ℃ for 48 hours and then stored i n liquid nitrogen for 72 hours.

Individual vials were thawed in a 37 ℃ water bath with continuous and mild agitation for 5 min. Viability was firstly determined by the trypan blue exclusion method using a cell counter [31]. Cells negative for trypan blue were considered live and those stained blue were considered dead. Viable cells were also determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay according to the manufacturer's instructions. Briefly, the MTS assay was performed in 96-well plates by adding 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt to the cells [32,33]. Cells were then incubated for 1 hour at 37 ℃. Absorbance was then measured at 490 nm using a 96-well plate reader (BMG Labtech, UK) to determine the formazan concentration, which is proportional to the number of living cells. Absorbance was corrected by subtraction of background absorbance. Data was normalised to the positive freezing control C(+).

2.8. Statistical analysis

All measurements were carried out in three independent replicates. Results were analysed using a one-way ANOVA followed by a Tukey's test for multiple comparisons. The tests were analysed using GraphPad Prism (GraphPad Software, US). The difference was considered statistically significant when $p < 0.05$.

3. Results

3.1. Trehalose uptake mediated by PP-50

The effect on trehalose uptake when incubated with PP-50 was investigated using confocal microscopy (Fig. 1). Cells were incubated with FITC-trehalose in the presence and absence

of PP-50 for 2, 4 and 6 hours. As seen in Fig. 1, intracellular labelled trehalose (green fluorescence) was seen only when incubated in the presence of PP-50. In addition, a direct correlation was found between intracellular labelled trehalose and incubation time. Cells displayed normal nuclei features, shown by the staining with Hoechst H33342 (blue fluorescence), and high viability, shown by the lack of propidium staining (red fluorescence).

3.2. Colocalisation of labelled PP-50 with labelled trehalose

The colocalisation of PP-50 with trehalose was analysed by confocal microscopy (Fig. 2). Cells were incubated with PP-50/AF647 (red fluorescence) and FITC-trehalose (green fluorescence) for a period of 4 hours. The colocalisation of these molecules is shown in yellow. As seen in the figure, osteosarcoma cells incorporated intracellularly both labelled PP-50 and trehalose. Both molecules appeared in the cytoplasm, located mainly in the perinuclear space. A high degree of colocalisation was observed between the polymer and trehalose. Cells also displayed normal nuclei features, shown by the staining with Hoechst H33342.

3.3. Image analysis of PP-50 colocalisation with trehalose

The analysis of the colocalisation of labelled PP-50 with labelled trehalose was assessed using ImageJ software and at least 5 independent images. The Pearson (PC) and Manders (M1) overlap coefficients were derived with the JaCoP ImageJ tool. The PC and M1 coefficients were 0.862 ± 0.008 and 0.766 ± 0.033, respectively.

3.4. Cryopreservation using PP-50

To evaluate the effect of trehalose uptake mediated by PP-50 on cryopreservation, osteosarcoma cells were pre-incubated with trehalose, FBS or a combination of both molecules in the presence and absence of PP-50, and then cryopreserved (Fig. 3). Trypan blue staining and the MTS assay were used to measure viability immediately post-thaw, i.e. cryosurvival. As shown by both assays, a higher cryosurvival was observed when cells were incubated in the presence of PP-50 for all pre-freezing incubation media. The largest cryosurvival was observed when cells were pre-incubated with trehalose and serum in the presence of PP-50 (64 ± 3 %), as measured by trypan blue staining. A significant disparity in cryosurvival was observed between both methods used.

4. Discussion

In this study, the effect of PP-50 on trehalose uptake was analysed in SAOS-2 cells. Analysis by confocal microscopy, quantification of colocalisation, and cryopreservation, all showed that incubation with PP-50 induced the transport of trehalose across the cell membrane resulting in increased cryosurvival of osteosarcoma cells.

Confocal microscopy was used to investigate labelled trehalose uptake mediated by PP-50 on osteosarcoma cells (Fig. 1). Labelled trehalose diffused across cell membranes only in the presence of PP-50, demonstrating the polymer's ability to transport hydrophilic molecules across the cell membrane. Propidium iodide, a non-permeable dye, was used to assess cell viability as it is not taken up by living cells [34,35]. This dye was utilised after incubation with PP-50 and trehalose to confirm that trehalose uptake was due to polymer transport and not cell permeability caused by polymer cytotoxicity. Lack of propidium iodide staining confirmed PP-50 mediated transport and suggested the absence of noticeable apoptosis and necrosis. This is in agreement with previous studies regarding the biocompability of PP-50, where higher concentrations of the polymer showed no negative effect on osteosarcoma cells [22].

To investigate the trafficking of trehalose and PP-50 across the cell membrane, colocalisation of labelled trehalose and polymer was accomplished using confocal microscopy (Fig. 2). As observed, the majority of labelled molecules localised jointly within the perinuclear area. This area is located next to the cell nucleus and contiguous with the lumen of the endoplasmic reticulum, where most delivery system agents are targeted [36]. As PP-50 was developed to mimic viral peptides that penetrate cell membranes, this is in agreement with the fate of most efficient DNA viruses that localise in this area to deposit their genomic DNA near the host nucleus [21,37,38]. A similar accumulation has been shown also for polyethylenimine (PEI), one of the most efficient non-viral gene transfer agents known, when used along DNA complexes [39]. This result highlights the use of PP-50 as a drug delivery system. It is worth noticing that the labelling of trehalose and PP-50 may have affected their physical properties and cell trafficking. The impact of this labelling will be the topic of future studies

The trafficking of trehalose through the endolysosomal pathway was not investigated in this study. However, previous studies have shown that escaping the endolysosomal route is a process preceding the accumulation in the perinuclear area [39]. The fate of both the polymer and trehalose after being accumulated in the perinuclear area should be investigated in future studies.

Next, trehalose and PP-50 colocalisation was quantitatively analysed by using the Pearson and Manders coefficients. These coefficients show the fraction of trehalose colocalised with the polymer, and vary from 0 for non-overlapping images to 1 for complete colocalisation [40,41]. Both coefficients were close to 0.8, suggesting a high degree of colocalisation between trehalose and PP-50. Confocal visualisation and quantitative analysis of the colocalisation were in agreement, strongly indicating the direct involvement of PP-50 in the trafficking of trehalose across the cell membrane.

A trehalose concentration of 200 mM was chosen for cryopreservation experiments, as previous studies have shown intracellular trehalose concentrations higher than 200 mM when cells were pre-incubated in the presence of PP-50 [26]. This concentration was higher than the one used in confocal microscopy, and was selected to ensure an intracellular concentration sufficient to provide freezing protection [2,42,43]. A labelled trehalose concentration of 50 mM was used for confocal analysis due to material limitation.

In order to further analyse the impact of trehalose transport mediated by PP-50, cells were pre-incubated with trehalose, FBS or a combination of both molecules in the presence and absence of PP-50, and then cryopreserved (Fig. 3). The cytotoxicity of these molecules was previously investigated and no negative effect on cellular viability was observed [22]. As seen in the figure, a large disparity was observed between cryosurvival as measured by trypan blue staining and metabolic activity. A vast majority of cells alive according to the trypan blue staining were not confirmed with the MTS assay. Trypan blue is excluded from intact cells and the MTS assay depends on the activity of cell cytoplasmic esterases that are capable of degrading permeable MTS into formazan [44,45]. Since both methods rely exclusively on the permeability of lipid bilayers immediately after thawing, these assays may not be the most suitable options when cell membranes are transiently more stable due to the trehalose, proven to stabilise membrane components [46].

Other factors possibly involved in this discrepancy of cell cryosurvival include the limitations of both methods used to quantify cryosurvival. Although the trypan blue staining method is widely used to measure cell viability, it has been described as highly subjective and possessing significant accuracy errors in numerous studies [31,47]. Additionally, cell metabolism may have been undermined immediately after thawing in cells incubated with trehalose or FBS. True cryosurvival, i.e., cell growth, was not taken into account using either trypan blue staining or the MTS assay [48]. Cells with intact membranes that have initiated apoptotic pathways not manifested yet with the achievement of cell death were also not

considered. Normally, cell death is manifested hours after the cells are thawed through apoptosis or necrosis. Assessments conducted at different time points could give insights on the delayed decline in cell viability, a process often not detected immediately post-thawing [49]. Future work should aim to use an assay that considers both the capability of cells to duplicate and their ability to maintain cell properties after cryopreservation.

The use of FBS in the pre-incubation media significantly increased the cryosurvival of osteosarcoma cells compared to pre-incubation media without it. This trend was even more pronounced when PP-50 was also present in the pre-incubation media. This highlights the role of FBS as an important source of proteins that may play a major role in cell cryopreservation when used along permeating agents such as PP-50. However, since FBS presents several disadvantages when used in biomedical applications, future work should aim at identifying effective substitutes for cryopreservation-promoting factors present in FBS.

In this work, PP-50 has been shown to mediate the transport of trehalose, highlighting its use in existing cell cryopreservation protocols and therapeutic applications.

5. Conclusions

In this study, the cellular trafficking of labelled trehalose when using an amphipathic pH-responsive biopolymer was studied. Intracellular trehalose loading in the presence of PP-50 was shown by confocal microscopy. Quantitative analysis of colocalisation resulted in a high degree of colocalisation between the trehalose and polymer. Both molecules were shown to accumulate in the perinuclear area of cells, highlighting the use of PP-50 as a drug delivery system. Cryopreservation of cells incubated in the presence of trehalose and PP-50 showed increased cryosurvival rates as measured by trypan blue staining and metabolic activity. Further work is needed to determine the intracellular fate of trehalose and PP-50. This study provides an important step toward the use of amphipathic biopolymers for cryopreservation and biomedical applications.

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7. References

[1] W.R. Strohl, D.M. Knight, Discovery and development of biopharmaceuticals: current issues, Curr. Opin. Biotechnol. 20 (2009) 668–672. doi:10.1016/j.copbio.2009.10.012.

[2] A. Eroglu, M.J. Russo, R. Bieganski, A. Fowler, S. Cheley, H. Bayley, et al., Intracellular trehalose improves the survival of cryopreserved mammalian cells, Nat. Biotechnol. 18 (2000) 163–167. doi:10.1038/72608.

[3] D. Berz, E.M. Mccormack, E.S. Winer, G.A. Colvin, P.J. Quesenberry, Cryopreservation of Hematopoietic Stem Cells, Am J Hematol. 82 (2007) 463–472.

[4] S. Zhang, H. Qian, Z. Wang, J. Fan, Q. Zhou, G. Chen, et al., Preliminary study on the freeze-drying of human bone marrow-derived mesenchymal stem cells, J Zhejiang Univ-Sci B. 11 (2010) 889–894. doi:10.1631/jzus.B1000184.

[5] T. Chen, J.P. Acker, A. Eroglu, S. Cheley, H. Bayley, A. Fowler, et al., Beneficial effect of intracellular trehalose on the membrane integrity of dried mammalian cells, Cryobiology. 43 (2001) 168–181. doi:10.1006/cryo.2001.2360.

[6] D.E. Pegg, M.P. Diaper, The effect of initial tonicity on freeze/thaw injury to human red cells suspended in solutions of sodium chloride, Cryobiology. 28 (1991) 18–35. http://www.ncbi.nlm.nih.gov/pubmed/2015759.

[7] J. Acker, The Use of Intracellular Protectants in Cell Biopreservation, in: Adv. Biopreservation, CRC Press, 2006: pp. 299–320. doi:10.1201/9781420004229.ch11.

[8] E. Szurek, A. Eroglu, Comparison and avoidance of toxicity of penetrating cryoprotectants, PLoS One. 6 (2011) e27604. doi:10.1371/journal.pone.0027604.

[9] K. Liseth, J.F. Abrahamsen, S. Bjørsvik, K. Grøttebø, Ø. Bruserud, The viability of cryopreserved PBPC depends on the DMSO concentration and the concentration of nucleated cells in the graft, Cytotherapy. 7 (2005) 328–333. http://www.ncbi.nlm.nih.gov/pubmed/16162454.

[10] A. Zambelli, G. Poggi, G. Da Prada, P. Pedrazzoli, A. Cuomo, D. Miotti, et al., Clinical toxicity of cryopreserved circulating progenitor cells infusion, Anticancer Res. 18 (1998) 4705–4708. http://www.ncbi.nlm.nih.gov/pubmed/9891544.

[11] R. Zenhäusern, A. Tobler, L. Leoncini, O.M. Hess, P. Ferrari, Fatal cardiac arrhythmia after infusion of dimethyl sulfoxide-cryopreserved hematopoietic stem cells in a patient with severe primary cardiac amyloidosis and end-stage renal failure, Ann. Hematol. 79 (2000) 523–526. http://www.ncbi.nlm.nih.gov/pubmed/11043425.

[12] D.G. Halme, D.A. Kessler, FDA regulation of stem-cell-based therapies, N. Engl. J. Med. 355 (2006) 1730–1735. http://www.ncbi.nlm.nih.gov/pubmed/17050899.

[13] J. van der Valk, D. Mellor, R. Brands, R. Fischer, F. Gruber, G. Gstraunthaler, et al., The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture, Toxicol. Vitr. 18 (2004) 1–12. doi:10.1016/j.tiv.2003.08.009.

[14] A. Tunnacliffe, A. García de Castro, M. Manzanera, Anhydrobiotic engineering of bacterial and mammalian cells: is intracellular trehalose sufficient?, Cryobiology. 43 (2001) 124–132. doi:10.1006/cryo.2001.2356.

[15] J. Liebermann, J. Dietl, P. Vanderzwalmen, M.J. Tucker, Recent developments in human oocyte, embryo and blastocyst vitrification: where are we now?, Reprod. Biomed. Online. 7 (2003) 623–633. http://www.ncbi.nlm.nih.gov/pubmed/14748959.

[16] H. Meyer, O. Vitavska, H. Wieczorek, Identification of an animal sucrose transporter, J. Cell Sci. 124 (2011) 1984–1991. doi:10.1242/jcs.082024.

[17] P.E.R. Tatham, M. Lindau, ATP-induced pore formation in the plasma membrane of

rat peritoneal mast cells, J. Gen. Physiol. 95 (1990) 459–476. doi:10.1085/jgp.95.3.459.

[18] G.D. Elliott, X.-H. Liu, J.L. Cusick, M. Menze, J. Vincent, T. Witt, et al., Trehalose uptake through P2X7 purinergic channels provides dehydration protection, Cryobiology. 52 (2006) 114–127. doi:10.1016/j.cryobiol.2005.10.009.

[19] E. Adinolfi, C. Pizzirani, M. Idzko, E. Panther, J. Norgauer, F. Di Virgilio, et al., P2X(7) receptor: Death or life?, Purinergic Signal. 1 (2005) 219–227. doi:10.1007/s11302-005-6322-x.

[20] A.L. Lynch, N.K.H. Slater, Influence of intracellular trehalose concentration and pre-freeze cell volume on the cryosurvival of rapidly frozen human erythrocytes, Cryobiology. 63 (2011) 26–31. doi:10.1016/j.cryobiol.2011.04.005.

[21] A.L. Lynch, R. Chen, P.J. Dominowski, E.Y. Shalaev, R.J. Yancey, N.K.H. Slater, Biopolymer mediated trehalose uptake for enhanced erythrocyte cryosurvival, Biomaterials. 31 (2010) 6096–103. doi:10.1016/j.biomaterials.2010.04.020.

[22] S.A. Mercado, N.K.H. Slater, The functional and structural effects of an amphipathic pH responsive biopolymer: A comprehensive study in osteosarcoma cells, Eur. Polym. J. 74 (2016) 158–167. doi:10.1016/j.eurpolymj.2015.11.026.

[23] P.S. Stayton, A.S. Hoffman, N. Murthy, C. Lackey, C. Cheung, P. Tan, et al., Molecular engineering of proteins and polymers for targeting and intracellular delivery of therapeutics, J. Control. Release. 65 (2000) 203–220. doi:10.1016/S0168-3659(99)00236-9.

[24] J.C. Chung, D.J. Gross, J.L. Thomas, D.A. Tirrell, L.R. Opsahl-Ong, pH-Sensitive, Cation-Selective Channels Formed by a Simple Synthetic Polyelectrolyte in Artificial Bilayer Membranes, Macromolecules. 29 (1996) 4636–4641. doi:10.1021/ma9600522.

[25] J.L. Thomas, S.W. Barton, D.A. Tirrell, Membrane solubilization by a hydrophobic polyelectrolyte: surface activity and membrane binding, Biophys. J. 67 (1994) 1101–1106. doi:10.1016/S0006-3495(94)80575-2.

[26] A.L. Lynch, R. Chen, N.K.H. Slater, pH-responsive polymers for trehalose loading and desiccation protection of human red blood cells, Biomaterials. 32 (2011) 4443–4449. doi:10.1016/j.biomaterials.2011.02.062.

[27] D.M.C. Sharp, A. Picken, T.J. Morris, C.J. Hewitt, K. Coopman, N.K.H. Slater, Amphipathic polymer-mediated uptake of trehalose for dimethyl sulfoxide-free human cell cryopreservation, Cryobiology. 67 (2013) 305–311. doi:10.1016/j.cryobiol.2013.09.002.

[28] K.M. Backus, H.I. Boshoff, C.S. Barry, O. Boutureira, M.K. Patel, F. D'Hooge, et al., Uptake of unnatural trehalose analogs as a reporter for Mycobacterium tuberculosis, Nat. Chem. Biol. 7 (2011) 228–235. doi:10.1038/nchembio.539.

[29] Z. Grabarek, J. Gergely, Zero-Length Crosslinking Procedure with the Use of Active Esters, Anal. Biochem. 185 (1990) 131–135.

[30] J. Rejman, A. Bragonzi, M. Conese, Role of clathrin- and caveolae-mediated endocytosis in gene transfer mediated by lipo- and polyplexes, Mol. Ther. 12 (2005) 468–474. doi:10.1016/j.ymthe.2005.03.038.

[31] V. Katsares, A. Petsa, A. Felesakis, Z. Paparidis, E. Nikolaidou, S. Gargani, et al., A Rapid and Accurate Method for the Stem Cell Viability Evaluation: The Case of the Thawed Umbilical Cord Blood, Lab. Med. 40 (2009) 557–560. doi:10.1309/LMLE8BVHYWCT82CL.

[32] N.A. Odintsova, N. V. Ageenko, Y.O. Kipryushina, M.A. Maiorova, A. V. Boroda, Freezing tolerance of sea urchin embryonic cells: Differentiation commitment and

cytoskeletal disturbances in culture, Cryobiology. 71 (2015) 54–63. doi:10.1016/j.cryobiol.2015.06.002.

[33] P. Kilbride, S. Lamb, S. Milne, S. Gibbons, E. Erro, J. Bundy, et al., Spatial considerations during cryopreservation of a large volume sample, Cryobiology. (2016) 1–8. doi:10.1016/j.cryobiol.2016.05.013.

[34] A.M. Rieger, K.L. Nelson, J.D. Konowalchuk, D.R. Barreda, Modified annexin V/propidium iodide apoptosis assay for accurate assessment of cell death, J. Vis. Exp. (2011) 37–40. doi:10.3791/2597.

[35] J. Fried, A.G. Perez, B.D. Clarkson, Flow cytometry analysis of cell cycle distributions using propidium iodide, J. Cell Biol. 71 (1976) 172–181.

[36] T.E. Shaiken, A.R. Opekun, Dissecting the cell to nucleus, perinucleus and cytosol., Sci. Rep. 4 (2014) 4923. doi:10.1038/srep04923.

[37] S. Boyapalle, T. Wong, J. Garay, M. Teng, H. San Juan-Vergara, S. Mohapatra, et al., Respiratory syncytial virus NS1 protein colocalizes with mitochondrial antiviral signaling protein MAVS following infection., PLoS One. 7 (2012) e29386. doi:10.1371/journal.pone.0029386.

[38] P.-J. Xiao, R.J. Samulski, Cytoplasmic Trafficking, Endosomal Escape, and Perinuclear Accumulation of Adeno-Associated Virus Type 2 Particles Are Facilitated by Microtubule Network, J. Virol. 86 (2012) 10462–10473. doi:10.1128/JVI.00935-12.

[39] J. Suh, D. Wirtz, J. Hanes, Efficient active transport of gene nanocarriers to the cell nucleus, PNAS. 100 (2003) 3878–3882.

[40] E.M.M. Manders, Measurement of co-localization of objects in dual-colour confocal images, J. Microsc. 169 (1993) 375–382.

[41] K.W. Dunn, M.M. Kamocka, J.H. McDonald, A practical guide to evaluating colocalization in biological microscopy, AJP Cell Physiol. 300 (2011) C723–C742. doi:10.1152/ajpcell.00462.2010.

[42] K. Goyal, L.J. Walton, A. Tunnacliffe, LEA proteins prevent protein aggregation due to water stress, Biochem. J. 388 (2005) 151–157. doi:10.1042/BJ20041931.

[43] J.P. Acker, X.-M. Lu, V. Young, S. Cheley, H. Bayley, A. Fowler, et al., Measurement of trehalose loading of mammalian cells porated with a metal-actuated switchable pore, Biotechnol. Bioeng. 82 (2003) 525–532. doi:10.1002/bit.10599.

[44] S.S. Buchanan, S.A. Gross, J.P. Acker, M. Toner, J.F. Carpenter, D.W. Pyatt, Cryopreservation of Stem Cells Using Trehalose : Evaluation of the Method Using a Human, Stem Cells Dev. 305 (2004) 295–305.

[45] G. Malich, B. Markovic, C. Winder, The sensitivity and specificity of the MTS tetrazolium assay for detecting the in vitro cytotoxicity of 20 chemicals using human cell lines, Toxicology. 124 (1997) 179–192.

[46] J.K. Kaushik, R. Bhat, Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose, J. Biol. Chem. 278 (2003) 26458–26465. doi:10.1074/jbc.M300815200.

[47] V.G. Almeida, M.C.X. Pinto, F.A.G. Moura, Trypan blue exclusion assay by flow cytometry, Brazilian J. Med. Biol. Res. 47 (2014) 307–315. doi:10.1590/1414-431X20143437.

[48] A. Tunnacliffe, J. Lapinski, Anhydrobiotic engineering, Nat. Biotechnol. 18 (2000) 473. doi:10.1038/75237.

[49] S. Thirumala, J.M. Gimble, R. V Devireddy, Evaluation of methylcellulose and dimethyl sulfoxide as the cryoprotectants in a serum-free freezing media for cryopreservation of adipose-derived adult stem cells., Stem Cells Dev. 19 (2010)

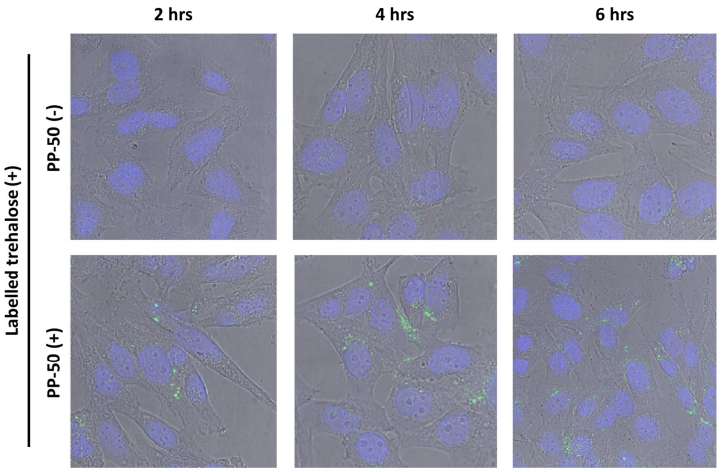
513–22. doi:10.1089/scd.2009.0173.

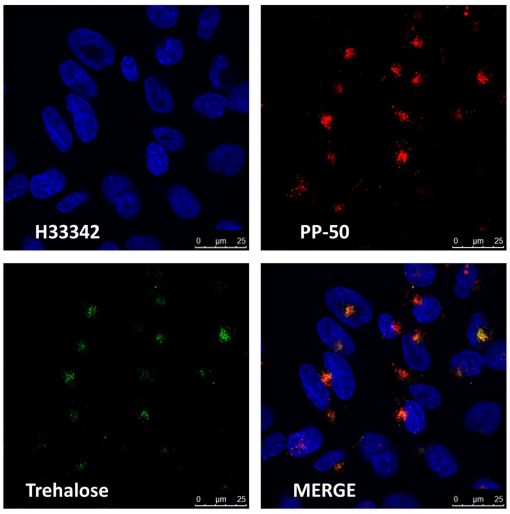
List of Figures

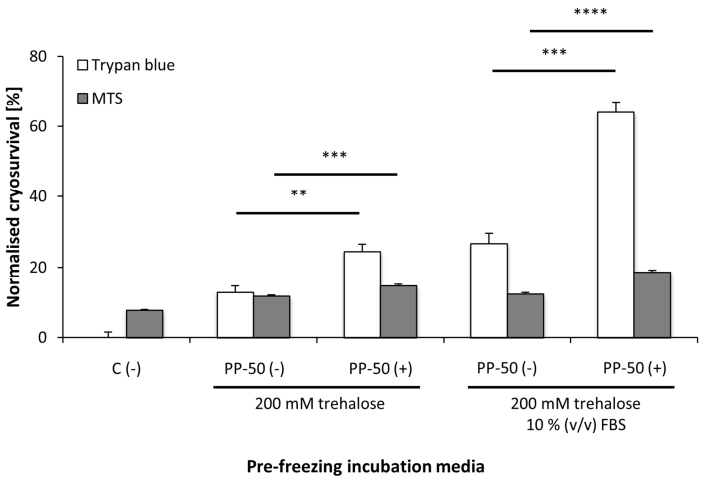
Figure 1: Confocal microscopy analysis of SAOS-2 cells incubated with labelled trehalose in the presence and absence of PP-50. Cells were incubated with for different periods of time with 50 mM of FITC-trehalose in the presence or absence of 250 µg/ml PP-50. After incubation, cells were stained with 5 µg/ml propidium iodide (red fluorescence) and 5 µg/ml Hoechst H33342 (blue fluorescence). Data are representative of a minimum of three separate experiments. Scale bar represents 30 µm.

Figure 2: Colocalisation analysis of labelled PP-50 with labelled trehalose by confocal microscopy. Cells were incubated with 250 µg/ml PP-50/AF647 (red fluorescence) and 50 mM FITC-trehalose (green fluorescence) for 4 hrs. Subsequently cells were stained with 5 µg/ml Hoechst H33342 (blue fluorescence) for 15 min. Colocalisation is shown in yellow. Data are representative of a minimum of three separate experiments. Scale bar represents 25 µm.

Figure 3: Cryosurvival of SAOS-2 cells incubated with different media measured by trypan blue and the MTS assay. Error bars represent the standard deviation of three replicates. C(-) represents the negative control of freezing, i.e. untreated cells frozen in media composed of 100 % (v/v) DMEM, whereas C(+) represents the positive control of freezing, i.e. untreated cells frozen in media composed of 90 % (v/v) DMEM and 10 % (v/v) Me_2SO. Data were normalised to the positive control of freezing C(+). **P<0.01, ***P<0.001, ****P<0.0001.






Trypan blue
MTS
Normalised cryosurvival [%]
80
60
40
20
0
****
***
***
**
C (-)
PP-50 (-)
PP-50 (+)
PP-50 (-)
PP-50 (+)
200 mM trehalose
200 mM trehalose
10 % (v/v) FBS
Pre-freezing incubation media