The Development of Cryoprotectants and Cryoprotective Solutions

By

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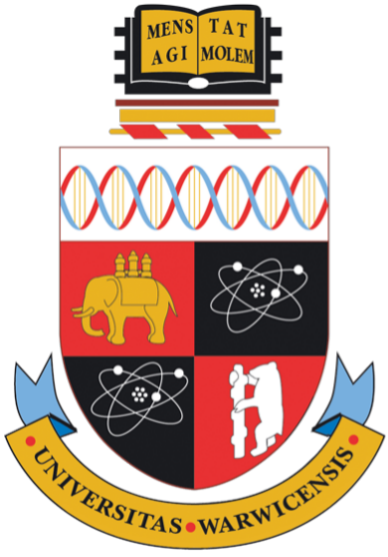

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Figure A1.1: The **(A)** post-thaw recovery and **(B)** post-thaw viability of Jurkat cells which were previously adapted to freezing by undergoing 10 freeze-thaw-grow cycles. Adapted Jurkats were cooled at – 1 °C min^{-1} to – 80 °C and warmed in a 37 °C water bath for 5 minutes. Data represents the mean ± SD of at least three independent experiments (*P < 0.05 from untreated control).

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List of Abbreviations

δ	Delta scale
µL	Microliter
% v/v	Percent volume by volume
% w/v	Percent weight by volume
°C	Degrees Celsius
AFP	Antifreeze protein
AHD	Alkaline haematin D-575
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
CAR	Chimeric antigen receptor
Da	Dalton
DCS	Differential scanning calorimetry
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
ECACC	European Collection of Authenticated Cell Cultures
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EG	Ethylene glycol
FA	Fluorescein isothiocyanate labelled Annexin V

FACS	Fluorescence activated cell sorting
FITC	Fluorescein isothiocyanate
g	Gram
HES	Hydroxyethyl starch
Hz	Hertz
IRI	Ice recrystallisation inhibition
K	Kelvin
kDa	Kilodalton
M	Molar
MGS	Mean grain size
mL	Millilitres
mM	Millimolar
Mn	Number average molecular weight
mOsm	Milliosmole
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium
MW	Molecular weight
MWCO	Molecular weight cut-off
NK	Natural killer
NMR	Nuclear magnetic resonance
NPSM	Non-penetrating small molecule
PBS	Phosphate-buffered saline
PBSC	Peripheral blood stem cell
PEG	Poly(ethylene) glycol

PI	Propidium iodide
PMGS	Poly(methyl glycidyl sulfoxide)
PVA	Polyvinyl (alcohol)
RBC	Red blood cell
ROS	Reactive oxygen species
SD	Standard deviation
TBAF	Tetra-*n*-butylammonium fluoride
T_g	Glass transition temperature
TH	Thermal hysteresis
TMS	Trimethylsilyl
TMS-trehalose	2,2',3,3',4,4'-hexa-O-trimethylsilyl-trehalose

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work presented (including data generated and data analysis) was carried out by the author except in the cases outlined below:

- Some of the polyampholyte used in **Chapter 3** was synthesised by Dr Thomas Congdon and Dr Kathryn Murray.
- The confocal microscopy of red blood cells in **Chapter 3** was carried out by Dr Ruben Tomás.
- Much of the general workflow for the blood cryopreservation and AHD assays in **Chapter 3** and **Chapter 4** was optimised by Dr Christopher Stubbs.
- The Biotage Selekt automated flash column, which was used to purify some batches of trehalose-6,6'-dibutanoate, in **Chapter 4** was operated by Collette Guy.
- The high vacuum system used to remove residual solvent from samples in **Chapter 4** was operated by Ioanna Kontopoulou and Panagiotis Georgiou.
- The cell lines used in **Chapter 4** and **Chapter 5** were usually maintained by myself, but were occasionally maintained by Ruben Tomás, Kathryn Murray, Natalia Gonzalez-Martinez, Ola Alkosti, Qiao Tang, and Yanan Gao.

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Abstract

Cryopreservation is the use of low temperatures to preserve biological materials. This has applications for the transport and storage of medical products such as red blood cells (RBCs) and cell therapies. Cold is damaging to cells because of ice formation, which necessitates the use of cryoprotectants to mitigate this damage. Current cryoprotectants have limited effectiveness, and are mostly based on organic solvents introduced more than 50 years ago, making the development of novel cryoprotectants and cryoprotectant formations a crucial endeavour to enable new therapies. **Chapter 1** is an introduction which provides an overview of the principles of cryopreservation. **Chapter 2** is a literature review comparing the effectiveness of trehalose to that of other sugars. This informed the choice of sugar to be used in subsequent chapters. It also collects many studies on trehalose in one place, providing a useful resource for cryopreservation research. **Chapter 3** details the development of a multi-component cryoprotectant solution for RBCs. This solution is an improvement over current blood preservation methods because it can be rapidly washed out, decreasing time to treatment. **Chapter 4** details the design, synthesis, and testing of a trehalose ester as a method to transport trehalose into the intracellular space. Attempts are made to identify conditions under which this molecule can be used as a cryoprotectant. **Chapter 5** explores methods for the cryopreservation of Jurkat cells, which are a model for T-cell therapies. First, a multi-component DMSO-free cryoprotectant solution is developed. Then the effect of L-proline on Jurkat cryopreservation is investigated. The **Appendix** provides preliminary data from a directed evolution experiment where Jurkat cells are exposed to repeated freeze-thaw-grow cycles to select for freeze tolerance. In summary, several approaches to cryopreservation were explored, resulting in the development of a rapid washout cryoprotectant solution for RBCs, a DMSO-free cryoprotectant solution for Jurkat cells, and the generation of data which will be useful for future research.

Chapter 1 – Introduction

1.1 Introduction

In the absence of a preservation strategy, all biological material deteriorates over time. Cell lines for research in continuous culture mutate or become senescent,[1] red blood cells for transfusion lyse,[2] organs and tissues for transplant become non-viable and must be discarded.[3–5] Cryopreservation is the science of preserving biological materials at low temperatures, allowing for storage and transport. Unfortunately, the low temperatures required for long-term storage cause the water in the material to freeze, resulting in ice formation. This ice formation is highly destructive, causing damage through mechanical forces,[6–9] and through the solute concentration effect (also known as solution effects injury).[10–12] Thus, a mechanism of cryoprotection must be used to prevent ice formation or protect the material from its effects. There are two key methods of cryopreservation: freezing and vitrification. Freezing typically uses low concentrations of cryoprotectant (typically 5 – 10 % dimethyl sulfoxide) and low cooling rates, resulting in ice formation.[13] Vitrification typically uses higher cryoprotectant concentrations (mixtures containing 30 – 65 % total cryoprotectant) and high cooling rates which prevent ice from forming. In freezing, cryoprotectants limit ice formation, allowing cells to survive in channels of unfrozen liquid, also called the unfrozen fraction.[14] As the temperature is lowered and ice grows, these unfrozen channels get smaller and the cryoprotectants they contain get more concentrated until the liquid in the channels reaches its glass transition temperature (T_g) and undergoes a phase change to a glass [15]. The more cryoprotectant present, the larger the size of the channels, so more cells are able to survive within.[11] In the vitrification method, no ice

is able to form, and the entire sample undergoes a phase change from liquid to glass when T_g is reached. With either method, once the sample has reached its T_g, chemical reactions can no longer occur, and cryopreservation is complete. The sample can now be stored in liquid nitrogen for very long periods of time without degradation.[16] When the material is warmed, ice undergoes recrystallisation, which is the conversion of small ice crystals into larger ice crystals. (Ice recrystallisation also occurs during cooling or storage at high sub-zero temperatures but for practical purposes is mostly considered a problem during warming). Likewise, upon warming, vitrified material undergoes devitrification, the transition from glass to crystal. Both ice recrystallisation[17] and devitrification[18] are damaging to the cells, which is why cryopreserved material is usually warmed as rapidly as possible during the thawing process. Although vitrification can be a highly effective cryopreservation strategy, the vitrification process is time-consuming and labour-intensive without specialist equipment due to the need for cryoprotectant concentration loading ramps. The high concentrations of cryoprotectant required for vitrification also make it unattractive for use in cell therapies due to cryoprotectant toxicity. This thesis is mostly focused on the freezing method, but vitrification is also sometimes discussed for context.

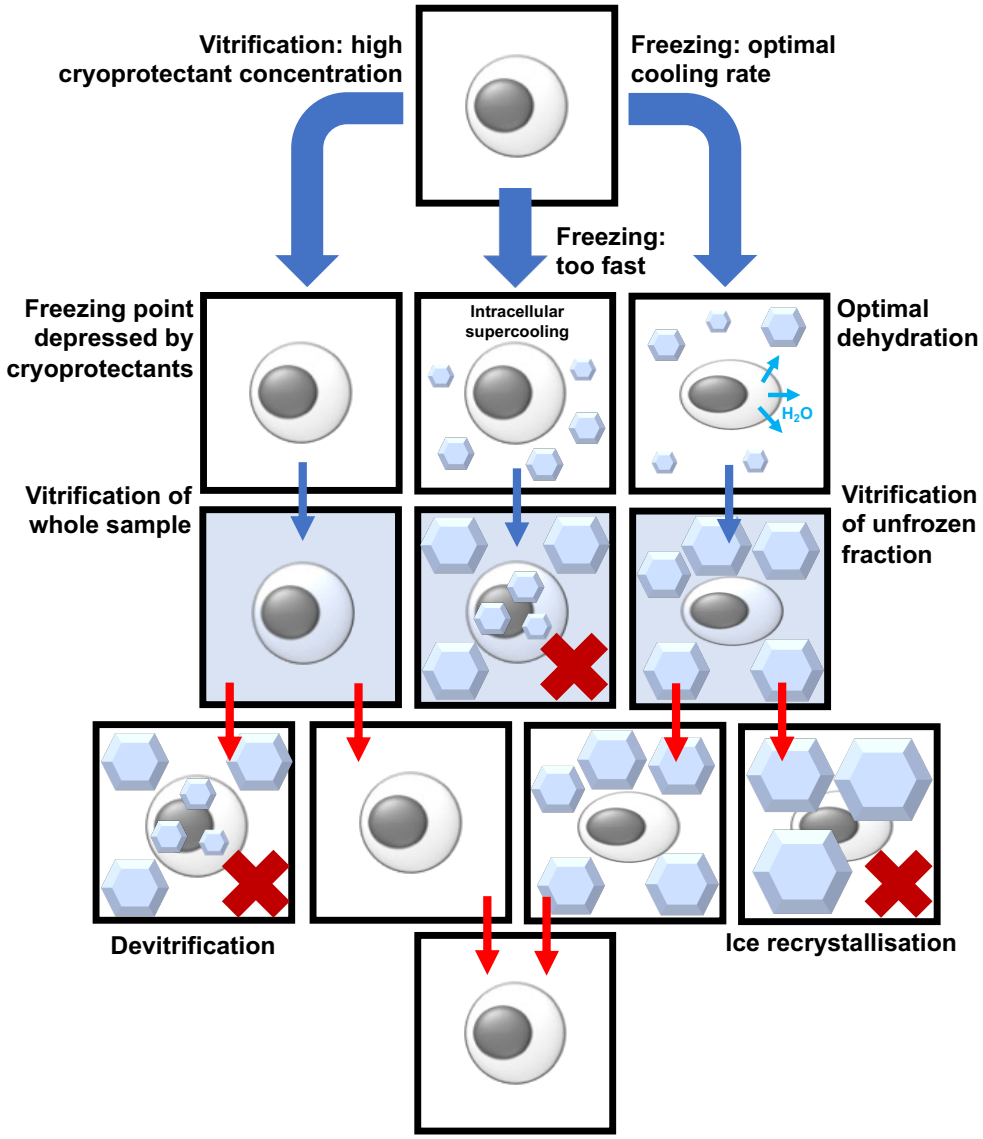

Figure 1.1. The mechanisms of cryopreservation. In the vitrification method, high cryoprotectant combinations and/or rapid cooling is used to prevent ice formation, vitrifying the whole sample. In the freezing method, an optimal cooling rate is used to allow moderate dehydration of the cell, preventing intracellular ice formation. Extracellular ice growth concentrates the cryoprotectants in the unfrozen fraction of the sample until vitrification of the unfrozen fraction occurs. If the cooling rate is too fast, there is insufficient time for water to exit the cell, resulting in intracellular supercooling and ice formation. Devitrification and/or ice recrystallisation occur if the warming rate is too high.

1.2 Mechanisms of cold-induced damage

1.2.1 Freezing damage

To preserve biological material using cold, it must be cooled to very low temperatures. Typically, – 80 ºC is used for short-term storage and liquid nitrogen temperatures are used for long-term storage. Cold, by itself, is not very harmful to biological material. However, most biological material contains water, which freezes at 0 ºC (or slightly lower when supercooled). It is the process of freezing which causes most of the damage to biological material when it is exposed to temperatures below 0 ºC. When water freezes, it first forms tiny ice crystals. The crystals rapidly grow, taking up water from their surroundings and excluding anything dissolved in the water. This has two effects on biological material. Firstly, damage is caused by mechanical forces as the ice expands.[7–9] There is some debate as to whether or not extracellular ice causes damage to cells in suspension,[19] but extracellular ice is unarguably damaging to tissues and organs as these not only rely on the viability of individual cells, but also on their macrostructure and arrangement of the cells to perform their function.[20] Intracellular ice is usually lethal to cells, damaging them by puncturing membranes or delicate intracellular components.[6] Secondly, because ice excludes solutes, the concentration of solutes in the extracellular environment increases, resulting in the dehydration of the cells and resulting in an increased intracellular solute concentration. The intracellular space of a cell is a highly regulated environment, precisely optimised for biological processes. Increasing the concentrations of intracellular solutes causes damage through multiple mechanisms: for example, increasing the concentration of ions can denature enzymes.[10,21] Cells are also damaged by hyperosmotic shock during

warming, when the osmolarity of the solution rapidly drops as the solutes are diluted by water from the melting ice.[22]

1.2.2 Ice recrystallisation

Ice recrystallisation, a form of Ostwald ripening, is a phenomenon where many small ice crystals in a frozen sample become fewer, larger, ice crystals. This process damages cells through mechanical forces as the unfrozen channels between ice crystals (where the cells reside) change shape.[23] Ice recrystallisation occurs because smaller ice crystals have greater curvature at their edges than larger ones, and so have a lower melting point. Thus, these smaller ice crystals melt sooner than larger ones, freeing water that then becomes part of a larger ice crystal.[24,25]

1.2.3 Chilling injury

The other mechanism by which cold damages biological material is known as chilling injury. This occurs before freezing, as the cell is cooled through low above-zero temperatures. The reduced molecular motion in the lipid bilayers of the cell's membrane causes a phase change from a liquid to a gel. Lamellar and nonlamellar forming lipids undergo this phase transition at different temperatures, causing the formation of regions containing high proportions of nonlamellar lipids. On rewarming, non-lamella structures are formed from these regions which compromise membrane integrity.[26,27]

1.2.4 Oxidative stress

Damage through oxidative stress is a secondary consequence of other forms of cryopreservation-induced damage. Cryopreservation has been shown cause the production of reactive oxygen species (ROS) such as superoxide.[28] This results from cryopreservation-induced damage to the mitochondria,[29] endoplasmic reticulum[30], and possibly peroxisomes.[31] ROS cause damage by reacting with proteins,[32] lipids[33] and DNA,[34] and can lead to apoptosis or necrosis.[35–37]

1.3 Cryoprotectants

Cryoprotectants, also known as cryoprotective agents (CPAs), protect cells from the damaging effects of ice formation, and can prevent ice formation entirely when used at sufficient concentrations. There are three broad classes of cryoprotectant: solvents (penetrating cryoprotectants), non-penetrating small molecules (NPSMs), and macromolecules. Solvents, in the context of cryopreservation, are small molecules that can penetrate through the cell membrane and act in the intracellular space. Non-penetrating small molecules do not penetrate the cell membrane in significant quantities unless the cell uses passive or active transport to move them. This class includes sugars and amino acids. Macromolecules are high molecular weight polymers and proteins; they cannot penetrate the cell membrane and so are only active in the extracellular space.

1.3.1 Solvents

Solvent cryoprotectants, also known as penetrating cryoprotectants, are water-soluble, usually somewhat hydrophobic, small molecules that can cross the cell membrane unaided and act in the intracellular space. The first solvent cryoprotectant discovered was glycerol, when it was found to enable the cryopreservation of spermatozoa.[38] Glycerol is also used to cryopreserve RBCs for blood banking.[39–41] Dimethyl sulfoxide (DMSO), also known as Me_2SO, is the most widely used cryoprotectant and is the current "gold standard" cryoprotectant for most cell types.[42] Further to DMSO and glycerol, many small molecules that are water-soluble and membrane-permeable can act as solvent cryoprotectants. Notable examples are ethylene glycol, propylene

glycol, 1,2-butanediol, methanol, and formamide. The main limitation of using most of these is toxicity, which will be discussed in more detail in the next section.[43]

Solvent cryoprotectants have several mechanisms of action. Firstly, they prevent ice formation in the intracellular space by dehydrating the cell, reducing the amount of freezable water. When a cell is exposed to a hypertonic concentration of solvent cryoprotectant, there is first an efflux of water out of the cell, resulting in cell shrinking. This is rapidly followed by an influx of the cryoprotectant as the concentration of cryoprotectant in the intracellular environment equilibrates with that of the extracellular environment, resulting in cell swelling.[44] Secondly, the presence of intracellular cryoprotectant dilutes intracellular solutes, preventing solute concentration damage.[11] Thirdly, they reduce ice formation in the extracellular space through colligative effects; the solvent molecules hydrogen bond to water molecules that would otherwise hydrogen bond to other water molecules, disrupting the water-water hydrogen bonding necessary for ice formation. The result of this is an increase in the size of the non-frozen glassy channels in which cells can survive.[14] Counterintuitively, more hydrophobic solvents form stronger solvent-water hydrogen bonds which translates into greater inhibition of ice formation due to greater disruption of the water-water hydrogen bond network. Unfortunately, with increasing hydrophobicity comes increasing toxicity.[45–48] The space of possible solvent cryoprotectants has largely been explored, and advances in the area of solvent cryoprotectants come in the form of complex multi-component cryoprotectant solutions, which exploit the properties of and interactions between different solvents. This is most studied for vitrification solutions, where the high concentrations of

cryoprotectant make the toxicity and viscosity of the solution very important.[49] However, solvent combinations have been explored for use in slow freezing as well.[50]

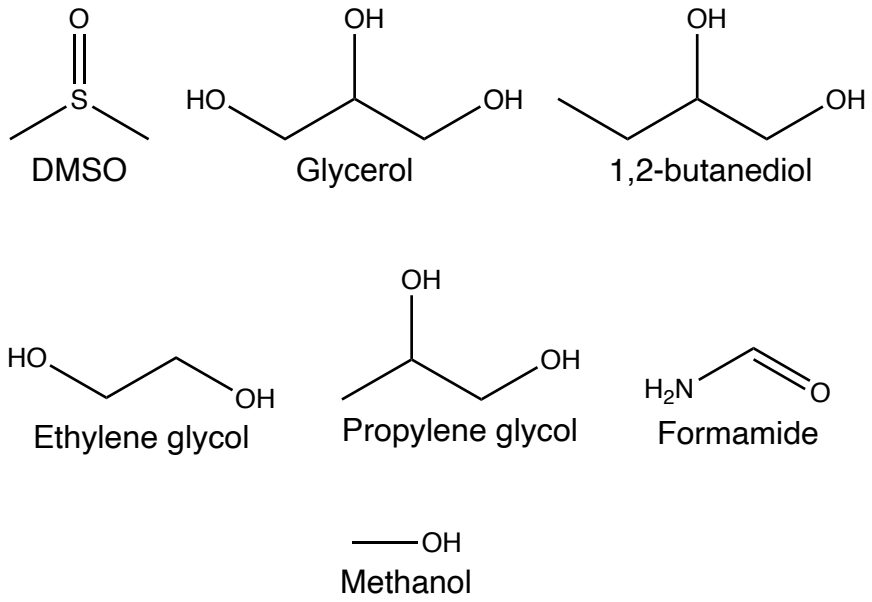

Figure 1.2. A selection of common solvent cryoprotectants.

1.3.2 Non-penetrating small molecules

Non-penetrating small molecules (NPSMs) are water-soluble small molecules that are too polar to cross the cell membrane. This category of cryoprotectants includes sugars such as sucrose, trehalose, and mannitol. It also includes amino acids such as L-proline and L-alanine. At sufficient concentrations, NPSMs share the colligative mechanism of action with solvents, but only in the extracellular space. NPSMs also share the solute dilution effect with solvents, although again this only occurs in the extracellular space.[51] If the cell possesses transporters or channels that allow the influx or efflux of NPSMs, such as L-proline,[52] the cell may use these to regulate osmolarity and cell volume. In this case the NPSM is known as an osmolyte or osmoprotectant. Non-osmolyte NPSMs can also reduce intracellular ice formation by contributing to the

pre-freeze dehydration of the cell.[53] Novel NPSMs include ice recrystallisation inhibitors (IRIs) such as aryl-glycosides[54] and L-phenylalanine derivatives,[55] which reduce ice recrystallisation, and antioxidants such as melatonin[56] which reduce damage caused by oxidative stress.[36] Ice recrystallisation inhibition is discussed below in the context of macromolecular cryoprotectants. For a detailed discussion of trehalose, see **Chapter 2**. For a detailed discussion of L-proline, see **Chapter 5**.

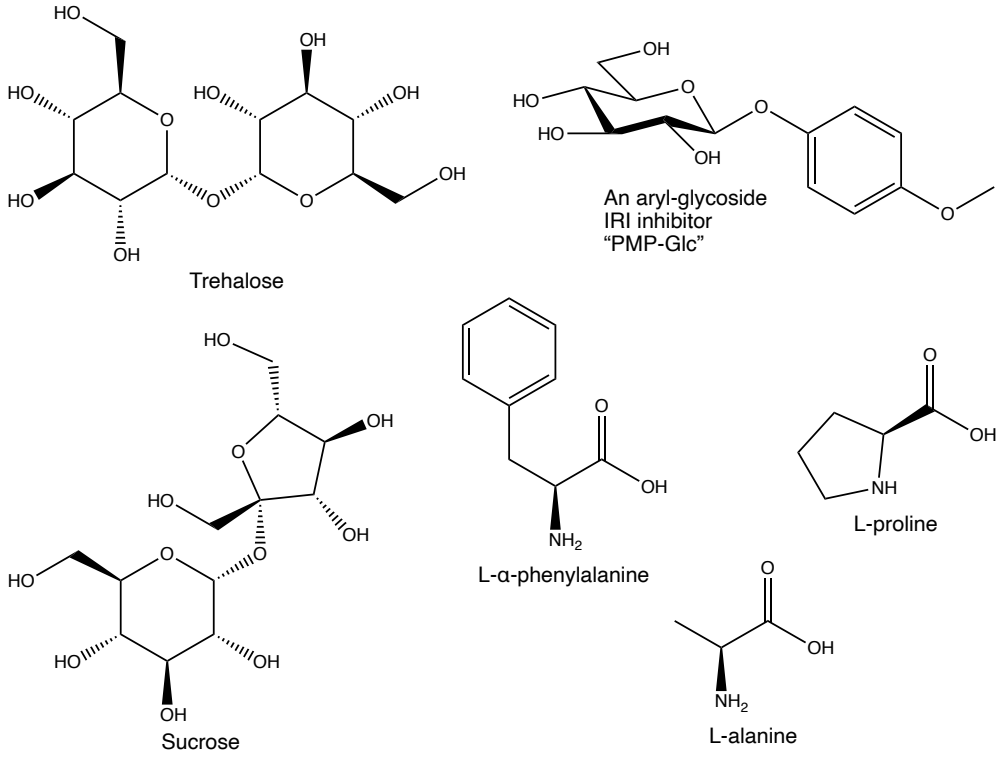

Figure 1.3. A selection of common NPSM cryoprotectants.

1.3.3 Macromolecules

Macromolecules (proteins, polysaccharides, and synthetic polymers) are much larger than NPSMs and solvents, and so cannot penetrate the cell membrane (although a small amount may be taken up by endocytosis). Macromolecules work through a wide range of mechanisms including water-binding, ice recrystallisation inhibition, ice nucleation inhibition, thermal hysteresis/freezing point depression, and ice

restructuring. “Antifreeze” (as in “antifreeze activity” or “antifreeze proteins”) is a general term used to describe these mechanisms, although papers will sometimes use the term to describe a specific mechanism, usually hysteresis/freezing point depression, preferring the term “ice binding proteins” or “ice structuring proteins” when talking about proteins which bind ice but don’t necessarily depress freezing point.[57,58] Here, “antifreeze protein” (AFP) refers to any protein that interacts with ice or the freezing/thaw process. Antifreeze proteins are expensive to produce, therefore research has focused on understanding the properties of antifreeze proteins and creating polymers that can mimic their activity.

Water-binding macromolecules provide cryoprotection by reducing the amount of freezable water. This reduces ice formation, increases the size of the unfrozen fraction of the sample and increases T_g. Examples of water-binding macromolecules include the polyremes hydroxyethyl starch (HES),[59] and Poly(methyl glycidyl sulfoxide) (PMGS).[60] These polymers also reduce intracellular ice formation by contributing to the pre-freeze dehydration of the cell.[53] Ice recrystallisation inhibitors (IRIs) prevent ice recrystallisation. Some IRIs work by binding ice and directly blocking new water molecules from being added to the surface,[58] other IRIs do not bind directly to the ice crystals and instead disrupt the semi-ordered water layer which surrounds ice crystals.[61] Examples of IRIs include the antifreeze glycoproteins from Antarctic Notothenioid fishes,[62] and the polymers poly(vinyl alcohol) (PVA),[63] and poly(l-alanine-*co*-L-lysine).[64] Some macromolecules have non-colligative thermal hysteresis (TH) activity, causing the freezing point of the sample to be depressed while the melting point remains the same. All solutes have colligative TH effects but macromolecules such as the AFPs from *Marinomonas primoryensis*[62] have thermal

TH which far exceeds that which would be predicted by their concentration. Ice nucleation inhibitors prevent heterozygous ice nucleation, an example of this is poly(glycerol) which inhibits the ice-nucleating activity of proteins from *Pseudomonas syringae.*[65] PVA also exhibits ice nucleation inhibition.[66] Supercooling occurs when a sample is cooled below 0 ºC without freezing. When the sample does freeze, this can cause additional damage by intracellular ice formation if the cell is not sufficiently dehydrated. Ice nucleators such as the ice-nucleating proteins of *P. syringae*[67] circumvent this problem by causing nucleation at a higher temperature, resulting in cellular dehydration which prevents intracellular ice from forming.[68,69] Poly(ampholyte)s are polymers with both positive and negative charges. Poly(ampholyte)s are hypothesised to provide cryoprotection by two mechanisms. Firstly, they form a viscous matrix in solution, reducing cellular dehydration during freezing to a more optimal level. Secondly, this matrix prevents the spread of extracellular ice into the cell, which would otherwise cause intracellular ice formation.[70] There is also evidence that Poly(ampholyte)s interact with the cell membrane, although the specific cryoprotective effect of this is yet to be explored.[71] Matsumura *et al*. synthesized poly(ampholyte)s based on ε-poly-L-lysine, which allowed for good cell recovery after cryopreservation without DMSO.[72] However, ε-poly-L-lysine is very expensive. At the time of writing, 2022, Merck sells poly-L-lysine for £1,380 per gram. Later, the Gibson group discovered that the cryoprotective ability of poly(ampholyte)s are maximised when they have alternating positive and negative charges in a 1:1 ratio,[73] and used this information to synthesize the highly effective poly(ampholyte), poly(vinyl ether -alt- maleic acid mono(dimethylamino ethyl) ester), from poly(methyl vinyl ether-*alt*-maleic anhydride), which is commercially available at affordable prices. Poly(vinyl ether -alt- maleic acid

mono(dimethylamino ethyl) ester), which is simply referred to as “polyampholyte” in this thesis, is used in **Chapter 3** to cryopreserve red blood cells as part of a multi-component solution, and in **Chapter 5** as part of a DMSO-free solution for Jurkat cells.

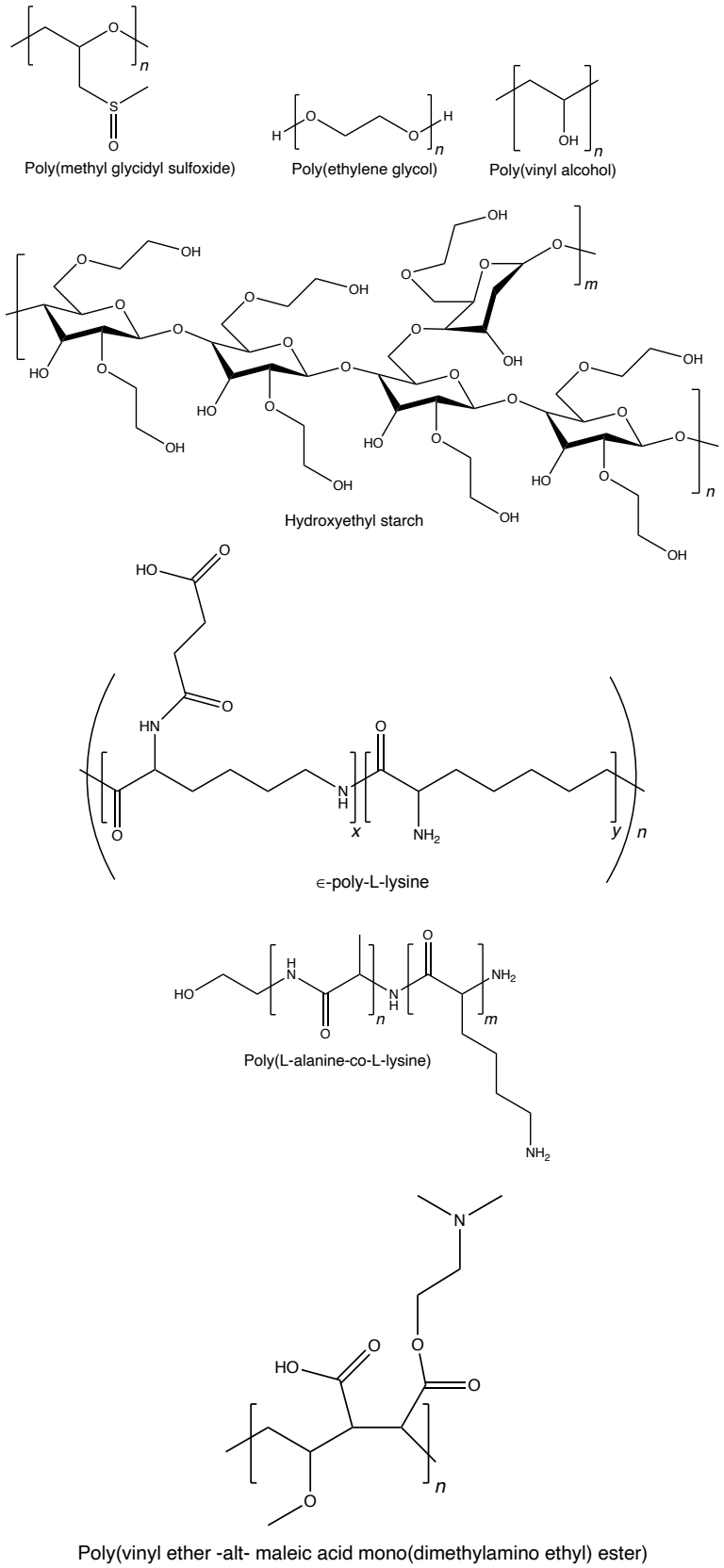

Figure 1.4. A selection of common macromolecule cryoprotectants.

1.4 Cryoprotectant toxicity

Higher concentrations of cryoprotectants result in less ice formation. In the absence of toxicity, very high concentrations of cryoprotectant could be used to prevent all ice formation, allowing for complete vitrification of any biological material. Unfortunately, the amount of cryoprotectant that can be used is limited by toxicity.[74] Each class of cryoprotectant causes general toxicity and specific toxicity. General toxicity is caused to all biological material due to the nature of the cryoprotectant class. For example, all penetrating solvents exert toxicity at sufficient concentrations by dissolving cell membranes.[75] Specific toxicity is caused by biochemical interactions between specific cryoprotectants and specific types of biological material. For example, ethylene glycol is toxic on the whole organism level because it is metabolised by the liver into several toxic compounds including oxalic acid, which causes kidney damage.[76] If two cryoprotectants can be found that don't have overlapping mechanisms of toxicity, they can each be combined at sub-toxic concentrations to make a solution with a total higher cryoprotectant concentration while remaining sub-toxic.[77] This principle is exploited in **Chapter 3** to produce a cryoprotectant solution for red blood cells.

1.4.1 Solvent toxicity

Solvents are toxic in the general sense because most of them permeabilise cell membranes at low concentrations (≈ 3 – 10 %),[78,79] and can entirely dissolve cell membranes at high concentrations (≈ 40 %).[75,80] Additionally, if high concentrations of solvent are added to a sample too quickly, the cells will be damaged by osmotic shock. In the specific sense: DMSO can denature proteins,[81] ethylene glycol,[76] and

methanol[82] have toxic downstream metabolites, and glycerol can cause membrane stiffening[83] and oxidative stress in kidney tissue.[84] Most other solvents exhibit various other specific toxicities which make them unsuitable for certain cell types.[77]

NPSMs cause toxicity in the general sense because excess concentrations cause hyperosmotic stress. Osmotic stress occurs when there is a significant osmolarity difference between the intracellular and extracellular space. Osmolality is the concentration of osmotically active particles in a solution. For example, a 100 mM NaCl solution is a 200 mOsmol kg^{-1} solution because the ions disassociate and are both osmotically active in solution. Physiological osmolality is around 280 mOsmol kg^{-1}, and cells can be damaged by dehydration if the osmolality of the extracellular solution is too far from this value.[85] As previously mentioned, one form of cryopreservation-induced damage is hypoosmotic shock on warming. This is exuberated by high concentrations of NPSMs; red blood cells can survive in a high osmolality solution before cryopreservation, but then lyse when cryopreserved in the same solution.[22] NPSMs rarely exhibit significant specific toxicities, although biological inertness may be a consideration. For example, trehalose is preferred over sucrose because it is a non-reducing sugar and therefore can't undergo a Millard-like reaction with proteins.[86]

1.4.2 Macromolecule toxicity

Macromolecules can be very effective cryoprotectants because each molecule can bind to many molecules of water or exhibit a range of other effects discussed above. Thus, compared to small molecules, macromolecules can have a large cryoprotective effect without contributing much to the total osmolality of the solution. An exception

to this is poly(ampholytes), and other charged polymers which release ions into the solution. Macromolecules therefore do not share a general mechanism of toxicity, but some have specific toxicities. For example, when prism plane binding AFPs are present at high concentrations, they exhibit dynamic ice shaping, whereby ice grows only at the basal plane causing the ice to grow into a bipyramidal needle shape. This shape change correlates with an increase in cell death.[87,88]

1.4.3 Cryoprotectant toxicity neutralisation

Some cryoprotectants reduce the toxicity of other cryoprotectants in solution. There are two known examples of this: formamide is a solvent cryoprotectant that neutralises the general toxicity of DMSO. This occurs because formamide has a higher dielectric constant than water, allowing it to decrease the hydrophobicity of a water-DMSO mixture, reducing the ability of DMSO to dissolve the cell membrane.[89,90] Trehalose and other sugars can neutralise the toxicity of solvent cryoprotectants, this may be a result of protein and/or membrane stabilisation.[91]

1.4.4 Post-transfusion toxicity

Most solvent cryoprotectants are toxic inside the human body. The most relevant example of this is DMSO toxicity which can occur during cell therapy infusions. DMSO toxicity has caused fatal cardiac arrhythmias,[92] severe respiratory depression,[93] and various other effects such as vomiting, hypotension, and cramps.[94] Theoretically, this toxicity could be prevented by washing the DMSO out of the therapy product before transfusion. However, with the exception of red blood cell (RBC) transfusions, this is often not done in practice. Possible reasons for not removing DMSO prior to

transfusion include time constraints, contamination risk, increased cost, cell loss and cell clumping during the washout process.[95]

1.5 Cryopreservation conditions and other concepts

1.5.1 Cooling and warming rates

Each cell type has an optimal cooling rate which maximizes post-thaw recovery when cryopreserved by freezing. If a sample is cooled too fast, water in the intracellular space will supercool, and then freeze, because water does not have enough time to exit the cell. If the sample is cooled too slowly, the cell loses too much water and is damaged by dehydration.[19] The optimal warming rate is usually as high as possible to prevent ice recrystallisation. However, it is possible to overheat the sample if the external temperature applied is above physiological levels. This is why a 37 ºC water bath is used for most purposes. The rate of warming achieved with even a 95 ºC water bath is not damaging in its own right,[96] but rapidly warming a large sample is difficult because ice is a poor thermal conductor.[97] (Water is an even worse thermal conductor, but samples don't usually need to be cooled as rapidly as they need to be thawed). Nano-warming has been developed as a technology to rapidly warm larger samples, whereby a sample is cryopreserved in a solution with silica-coated iron oxide nanoparticles, which can be excited by radiofrequency radiation, allowing the sample to be evenly and rapidly warmed.[98]

1.5.2 Cell type

Optimal cryopreservation conditions depend on cell type. Different cell types have different levels of permeability to water, which affects the optimal cooling rate.

Different cell types also have different levels of permeability to penetrating cryoprotectants, which affects the choice of cryoprotectant and the optimal period of incubation with the cryoprotectant. As mentioned above, some cryoprotectants are especially toxic to certain cell types.[99,100]

1.5.3 Cell density

Cell density upon freezing affects cell recovery after thawing. In cell culture, most cell types will signal to each other and can detect the density of the culture using the concentration of chemokines in the media. If the cell density upon freezing is too low, cells will proliferate slowly post-thaw, which could be misinterpreted as a poor cryopreservation result.[101] Further, freezing cell suspensions at suboptimal density reduces the number of cells that can be frozen in a given volume. If cell density upon freezing is too high, there may not be enough space in the unfrozen channels between ice crystals for all the cells to survive.[102]

1.5.4 Recovery versus viability

It is very important to make a distinction between cell recovery and viability. A "viable" cell is defined differently depending on the experiment, but it normally means that the cell is alive, has an intact membrane, and/or exhibits metabolic activity. Percentage recovery is the proportion of cells which have survived cryopreservation or other procedure, while remaining viable. Viability is the proportion of recovered cells that are viable, defined by the number of viable cells in the sample divided by the sum of the viable and non-viable cells in the sample, expressed as a percentage. Viability gives very little information about the success of the cryopreservation procedure on its own because many cells are completely destroyed by freezing damage

and so will not be detected as non-viable cells when viability is measured. A sample with low recovery may have very high viability, which would lead to misleading conclusions if only viability were reported. Therefore, we use recovery as the primary measure of cryopreservation success and only report viability where it adds useful information to the picture.[105]

1.6 Uses of cryopreservation

Cryopreservation is used for the long-term storage of biological materials. This can take the form of seed banking for agriculture,[106] the storage of tissue samples for research and diagnostics,[107] food preservation,[108] the storage of gametes for fertility treatment,[109] animal husbandry,[110] vaccine cold chains,[111] and prolonging the viability of donated organs for transplant.[112] This thesis focuses on the development of technology for blood banking, as well as technology for the transport of cell therapies. It should be noted that a great deal of expense is incurred during the process of transporting cryopreserved materials. Care must be taken at every stage of the cold chain to ensure that the temperature of the sample never rises above its maximum storage temperature, as improper thawing of even part of the sample can cause degradation. A key example of this is the cold chains used to transport ribonucleic acid (RNA) vaccines. RNA is a very unstable type of molecule, and even a single mutation in a strand of mRNA can render the whole strand non-functional.[111]

1.6.1 Blood banking

Blood is a suspension of red blood cells, white blood cells, and platelets, in a water/salt/protein mix (plasma). RBCs transport oxygen throughout the body and

remove carbon dioxide. Whole blood transfusions are used to treat trauma and surgery,[113] while RBC transfusions are used to treat anaemia.[114] In either case, it is the RBCs which are the most important component of these treatments. The medical blood supply comes from donations, and this supply fluctuates, which can result in shortages of certain blood types or blood products.[115] One contributing factor to these shortages is the wastage of expired blood products,[116,117] with the shelf life of RBCs being 42 days when refrigerated (depending on local regulations).[118] To prolong blood shelf life, RBCs can be cryopreserved, allowing them to be stored for very long periods of time (at least 37 years) at – 80 ºC.[119] This is especially useful for rare blood types which may receive infrequent donations, and in remote areas, especially for military operations, where a supply of liquid blood cannot be continuously maintained.[120,121] Frozen blood may also be used to maintain the blood supply when the liquid blood supply is exhausted due to a surge in demand or a lack of donors.[122] Current cryopreservation techniques for blood cryopreservation require the use of 20 – 40 % glycerol.[119,123,124] The large volumes, concentrations, and high viscosity of cryoprotectant used here necessitates its removal before the blood can be transfused. However, the hydrophilic nature of glycerol means that the washout process is time-consuming, taking 30 – 60 minutes for each unit of blood.[125] See **Chapter 3** for more information on RBC cryopreservation.

1.6.2 Cell therapy

Cell therapies involve transfusing living cells into patients. Examples of cell therapies include chimeric antigen receptor (CAR) T-cell therapy,[126] natural killer (NK) cell therapy[127] and peripheral blood stem cell (PBSC) transplantation.[128] Cryopreservation can be used for the transport of cell therapies. For example, in CAR T-cell therapy,

autologous cells are frozen for transport to a centralised processing fertility after being harvested at the hospital. After processing, the modified cells are cryopreserved again for transport back to the patient.[129] The use of cryopreservation to transport autologous cell therapies (like CAR T) greatly reduces the costs involved with cell processing; it allows one centralised modification centre to be used instead of many local ones, meaning that equipment and staff costs can be reduced through economics of scale. Allogeneic transplantation of cells such as PBSCs is logistically complex; the availability of the donor, recipient and medical team must all be coordinated within a narrow window since the donated PBSCs quickly expire. The use of cryopreservation to create allogeneic cell banks would greatly reduce this logistic complexity because the required cell infusions would be immediately available off the shelf.[130] Cryopreserved cell products incur risks downstream of the manufacturer such as processing errors and contamination. An ideal cell product would be cryopreserved in a ready-to-administer state, but this requires the cells to be cryopreserved using a cryoprotectant formulation that can be tolerated by the patient.[131] Unfortunately, with conventional solvent cryoprotectants, there is a trade-off between cryoprotection and toxicity. See **Chapter 5** for more information on cell therapy cryopreservation.

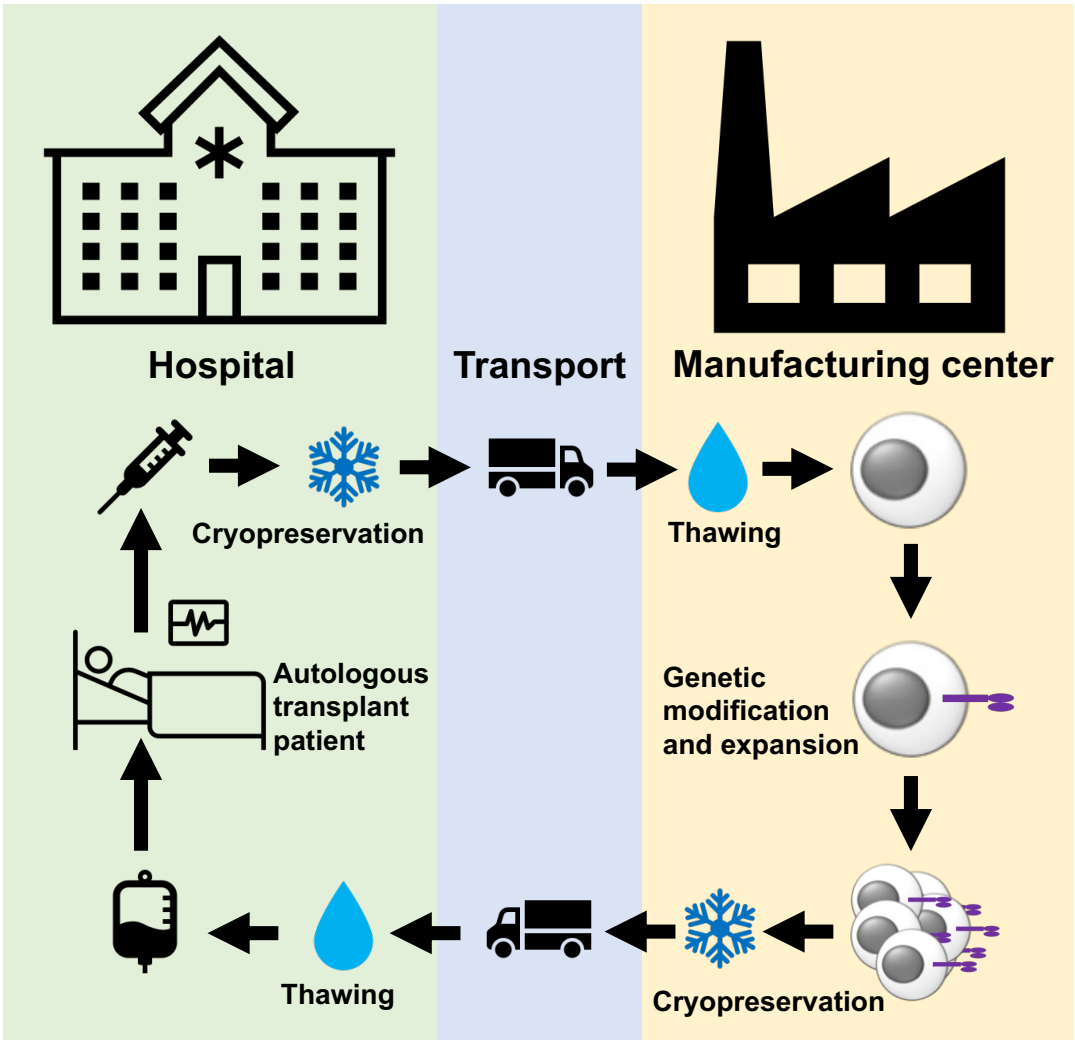

Figure 1.5. The logistics of CAR T-cell therapy. Cells are obtained from the patient at a local hospital and transported to a centralised manufacturing centre. Cryopreservation is utilised during both transport steps.

1.7 Project aims and objectives

The aim of the work presented in this thesis is to develop novel cryoprotectant solutions and cryopreservation techniques. Specifically: to develop a rapid washout cryoprotectant solution for RBCs, to explore the use of DMSO alternatives for cryopreserving Jurkat cells, and to engineer trehalose so that it can enter cells and provide intracellular cryoprotection. This research will improve the efficacy of blood transfusions and cell therapies.

1.8 Thesis summary

Chapter 1, the current chapter, is the first introduction, outlining the basic concepts in the field of cryobiology. **Chapter 2** is a second introduction, which goes into detail about the use of trehalose in cryopreservation. **Chapter 3** details the development of a rapid washout cryopreservation solution for RBCs. **Chapter 4** details the design, synthesis, and testing of trehalose-6,6'-dibutanoate, a method to deliver trehalose into the intracellular space. **Chapter 5** details the development of a DMSO-free cryopreservation solution for Jurkat cells using glycerol and various polymers. The use of L-proline for the cryopreservation of Jurkat cells is also explored, comparing it to other amino acids. The **Appendix** is a short chapter which provides a preliminary exploration into the directed evolution of Jurkat cells for cryosurvival.

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Chapter 2 – Trehalose as a Cryoprotectant for Cellular Cryopreservation

2.1 Abstract

Cryopreservation has applications in the fields of immune therapy, stem cell therapy, reproductive technology, blood banking, regenerative medicine, organ banking and more. During cryopreservation, cryoprotectants are essential to protect cells from the damage caused by exposure to sub-freezing temperatures. Unfortunately, commonly used solvent cryoprotectants such as DMSO and ethylene glycol are toxic to cells at the concentrations needed to provide optimal cryoprotection and may need to be washed out before clinical use. This has led to a search for cryoprotectants and cryoprotectant solutions with reduced toxicity. Trehalose is a non-toxic sugar which is used by many organisms in nature to aid in survival at extreme temperatures and desiccation. Here we discuss the use of trehalose as a cryoprotectant in experimental settings and compare it to other sugars. We also discuss some of the techniques for loading trehalose into the intracellular space, a feat that can allow for solvent-free cryopreservation.

2.2 Introduction

Cryopreservation is the practice of storing biological materials at low temperatures to halt metabolic processes and hence prevent degradation. Cryopreservation can be applied to proteins,[1] cells,[2] tissues,[3,4] and occasionally organs.[5,6] Cryopreservation is essential to cell culture-based research as it allows for the storage of cell lines, and has medical applications such as transporting stem cells,[7] and blood for transfusion.[8] Current cryopreservation techniques are far from ideal; a proportion of cells always die during the process and their functionality is often reduced. One of the major limiting factors in cryopreservation is cryoprotectant toxicity; cryopreservation requires the use of solvent cryoprotectants such as DMSO which can have adverse effects when the thawed cells are transplanted into patients.[9]

Cryoprotectant toxicity also limits the concentration of cryoprotectant that can be used; the mechanism of cryoprotectant action is concentration-dependent, however, there becomes a point where further increases in concentration lead to toxicity that outweighs any protective effects. This has motivated research into non-toxic cryoprotectants, which could allow for the reduction or elimination of solvents. This chapter will discuss the use of trehalose, a non-toxic sugar cryoprotectant found in the natural world.

Trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) is a water-soluble non-reducing disaccharide made up of two glucose subunits, joined by a 1,1-glycosidic bond.[10] It has 8 hydrogen bond donors and 11 hydrogen bond acceptors. Trehalose is synthesised in nature by a wide variety of non-mammalian organisms as an energy source and is used in some organisms to protect against freezing and desiccation,

allowing them to survive over winter or in other harsh environments. These organisms include the rice water weevil *Lissorhoptrus oryzophilus*,[11] the codling moth *Cydia pomonella*[12] and some tardigrade species such as *Macrobiotus richtersi*.[13] In the past, trehalose was expensive to make, but the development of an efficient manufacturing process in 1994 made this much cheaper, leading to increased research interest.[14] Trehalose has two proposed protective mechanisms, which have been termed the 'vitrification hypothesis' and the 'water replacement hypothesis'. These mechanisms are usually discussed in the context of preservation by desiccation or anhidrosis in nature but are also applicable to cryopreservation.

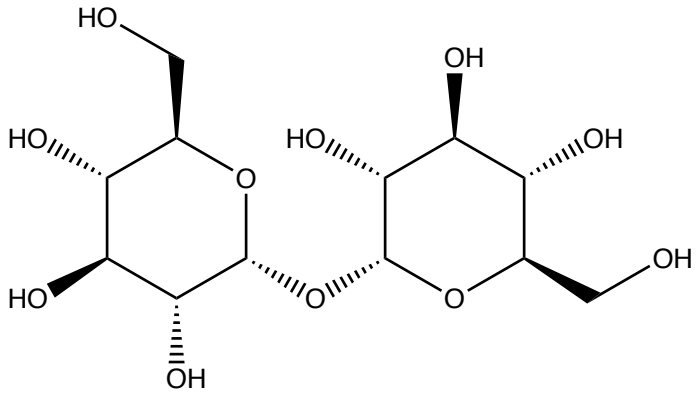

Figure 2.1. The structure of trehalose, a disaccharide made up of two glucose subunits, joined by a 1,1'-glycosidic bond.

2.2.1 The vitrification hypothesis

The vitrification hypothesis holds that trehalose protects biological materials by forming a high-viscosity glass-like state, protecting the material by inhibiting the movement of molecules and slowing metabolic activity. The transition of a liquid trehalose solution to a glassy state is achieved by concentrating the trehalose through either desiccation or freeze-induced dehydration. During cryopreservation by freezing, the cells survive within the unfrozen (vitrified) fraction of the sample.[15]

Trehalose has several properties that contribute to the formation of this fraction: adding trehalose to a solution increases its glass transition temperature (T_g),[16] resulting in less temperature-dependent freeze concentration before vitrification occurs. Trehalose is a kosmotrope,[17] it orders water molecules around itself, altering the structure of the surrounding hydrogen bond network in such a way that it can't form ice.[18] This network is over three hydration shells wide,[19] so each molecule of trehalose can prevent many molecules of water from freezing.[20] Trehalose has been found to reduce the size of ice crystals in a concentration-dependent manner.[21]

2.2.2 The water replacement hypothesis

Under normal conditions, proteins and other cell components are stabilised by bound water. The water replacement hypothesis holds that trehalose stabilises these components by acting as a replacement for this water.[22,23] During cryopreservation, trehalose protects proteins from cold denaturation,[24] and cryoprotectant toxicity.[25] Trehalose also lowers the temperature at which the gel-to-liquid crystal phase transition occurs.[26,27] During desiccation, trehalose stabilises the cell membrane by hydrogen bonding to phospholipids,[28] so trehalose may play a similar role in stabilising the cell membrane during cryopreservation. In addition to stabilisation, trehalose likely also counteracts solute concentration damage when it is used at sufficient concentrations, as would any biocompatible, water-soluble, small molecule.

2.2.3 Rationale

This chapter has several purposes: firstly, to compare and evaluate the cryoprotective effect of trehalose with that of other sugars. This chapter was drafted before experimental work, and the findings were used to inform the choice of sugar

cryoprotectant used for the blood cryopreservation solution developed in **Chapter 3**, and the choice of sugar cryoprotectant to be engineered in **Chapter 4**. We hypothesised that the inclusion of a sugar cryoprotectant would be beneficial for our cryopreservation solutions, but it was not previously clear how trehalose compares to other options. Secondly, this chapter aims to provide the reader with information about trehalose, and about previous research regarding its delivery into the intracellular space. Thirdly, this chapter aims to provide a catalogue of the studies where trehalose has been used as a cryoprotectant. The details of these studies are included where relevant information can be discussed, but other studies are referenced only to note their presence in the literature.

2.3 Trehalose as a (primarily) extracellular cryoprotectant

The need for non-toxic cryoprotectants has resulted in research into sugars as a supplement or replacement for traditional solvent cryoprotectants such as DMSO, ethylene glycol, propylene glycol and glycerol. Trehalose has gained particular attention due to its unique properties among the sugars, which will be discussed in more detail in section 2.4. Trehalose has been tested as a cryoprotectant in an extensive range of cell types and other biological materials, with largely positive results. In most studies, the addition of trehalose to the standard solvent cryoprotectant for that cell type – usually 10 % DMSO – increases cell recovery and/or improves post-thaw proliferation and other functional outcomes. A recurring trend throughout these studies is that trehalose has an optimal concentration – usually between 100 mM to 400 mM – beyond which adding further trehalose becomes detrimental to post-thaw outcome.[29–31] This is most likely general toxicity caused by cellular dehydration through osmosis rather than through specific trehalose toxicity. Importantly, trehalose can often lower the amount of solvent needed for cryopreservation while providing an equivalent or better post-thaw outcome. Occasionally, extracellular trehalose can be used without solvent cryoprotectants,[32–34] although this may be due to the presence of intracellular trehalose, which can enter the cell through endocytosis or a membrane phase transition during cooling; while this section is about the extracellular use of trehalose, small amounts of trehalose are likely to be present intracellularly in many of these studies through endocytosis during incubation, permeabilization of the cell membrane by DMSO, and freeze-induced uptake. These concepts are discussed in section 2.5.

2.3.1 Stem cell preservation

Non-human stem cells: in murine spermatogonial stem cells, adding 50 mM trehalose to the standard 10 % DMSO freezing media improved viability after storage for one week (90 % vs 76 %).[35] Adding 200 mM trehalose did not improve viability, but did improve long-term proliferation (49 % vs 28 %). For murine microencapsulated mesenchymal stem cells, 5 % DMSO, 10 %, glycerol, 10 %, 5 % trehalose, or any combination of trehalose and less than 10 % DMSO, all resulted in lower recovery than 10 % DMSO.[36] In mesenchymal stem cells from multiple animals, DMSO can be partially replaced with trehalose or poly(ethylene glycol) (PEG), although trehalose was not more effective than PEG.[37]

Human pluripotent stem cells: cryopreservation can be improved by replacing the standard 10 % DMSO with 500 mM trehalose and 10 % glycerol. Relative viability was increased by approximately 20 – 30 % depending on the type of cell. Phenotype and functionality were maintained.[38]

Human umbilical cord blood stem cells: in Chen *et al.*[29] 2.5 % DMSO and 30 mM trehalose was at least as good as 10 % EG and 2 % DMSO, or 10 % DMSO and 2.0 % dextran-40 at maintaining viability, trehalose slightly reduced the amount of post-thaw apoptosis. In Zhang *et al.*[39] freezing mediums containing 146 mM trehalose + 10 % or 5 % DMSO resulted in proliferation that was as high as the non-frozen control group. Trehalose has also been successfully used in combination with taurine and catalase for cord blood stem cell preservation.[40]

Human peripheral blood stem cells: in Martinetti *et al.*[34] the use of 1 M trehalose alone gave higher long- and short-term viability than DMSO alone, or DMSO combined with trehalose. The 1 M trehalose concentration used here is much higher than the typical optimal concentration of 100 – 400 mM in most cell types; it is possible that the slow addition of freezing media to the cells in this study allowed a higher concentration of trehalose to be reached without causing too much osmotic stress. For marrow and blood stem cells Scheinkönig *et al.*[41] found that 500 mM trehalose provides a similar level of cryoprotection to DMSO.

Human adipose-derived stem cells: cryopreservation with 250 mM trehalose alone resulted in very poor viability compared to 5 % DMSO (11 % vs 75 %).[42] In Pu *et al.*[31] 200 mM trehalose allowed the reduction of DMSO to 3.3 % while maintaining good cryoprotection.

2.3.2 Spermatozoa

Spermatozoa are different from other cell types in that they have a much larger surface area to volume ratio, have a more densely packed cytoplasm, and contain less freezable water, which makes them less vulnerable to intracellular ice formation.[43] Thus, optimal spermatozoa cryopreservation differs from that of more typical cells, with protocols often preferring fast freezing or vitrification to slow freezing.[44] Trehalose has been added to the "freezing extender" (a term referring to a freezing media used for spermatozoa) and tested in the cryopreservation of spermatozoa from many different animals.

Rabbit: spermatozoa vitrification with 4 % DMSO was improved with the addition of 100 mM trehalose as measured by increased motility (43 % vs 25 %), membrane integrity (45 % vs 24 %) and mitochondrial membrane integrity (53 % vs 28 %). Trehalose also decreased ROS and improved catalase function.[45]

Ram: post-thaw outcome was improved in multiple studies, with the optimal trehalose concertation typically being 50-100 mM[46–52] Some studies found that higher concentrations of around 200 mM are ineffective,[53,54] while others have found positive effects at higher concentrations.[55,56] These differences may be due to the total osmolality of the solution; in an extender with a high initial osmolality, less trehalose can be added before the total osmolality becomes too high and dehydrates the cells. Trehalose has also been combined with low-density lipoprotein for use as an extender.[57]

Equine: trehalose has had mixed results in equine spermatozoa, one study reported a benefit to using a small amount of trehalose.[58] Other studies reported that trehalose does not improve post-thaw outcome.[59–61]

Other animals: trehalose also improved cryopreservation outcomes for the spermatozoa of goats,[62,63] carp,[64] and oysters.[65] Trehalose did not provide benefit for the cryopreservation of European brown hare spermatozoa.[66]

2.3.3 Tissue preservation

Tissue preservation is challenging for several reasons; the large volume to surface area to ratio makes it difficult for cryoprotectant to reach every part of the tissue, and to

ensure a uniform cooling rate. Extracellular ice formation is particularly damaging to the tissue macrostructure – unlike individual cells which can move freely within the media, allowing them to fit within the unfrozen portion of the sample. Several studies have found a cryoprotective benefit to using trehalose: In one study, the viability of adipose tissue was increased by 80 % through the addition of 250 mM trehalose to an unspecified standard freezing media.[67] In another study, the addition of 500 mM trehalose to 10 % DMSO increased the post-thaw live cell count of human foetal skin (65 % vs 44 %), improved post-thaw morphology, graft necrosis, and shrinking.[68] Trehalose can also reduce post-thaw tissue pigmentation.[69] Dog tracheas have been preserved for transplant in 10 % DMSO and 100 mM trehalose, resulting in a 100 % animal and graft survival rate (n = 6) after 184 days when transplanted. This study did not report cell recovery or viability.[70] The viability of bovine calf testicular tissue, cryopreserved with 10 % DMSO, was higher with increasing trehalose concentrations of up to 438 mM.[71] In one of the most impressive examples of cryopreservation to date, Arav *et al.*[3] used 10 % DMSO and 500 mM trehalose, combined with directional freezing or vitrification to preserve rat hind limbs for at least 7 days at – 80 °C (freezing) and liquid nitrogen temperatures (vitrification). These limbs were then thawed and allotransplanted, all limbs (n = 6) survived until extracted for analysis after 1-3 days.

2.3.4 Other cell types

Human: viability of hepatocytes was improved (63 % vs 47 %) with the addition of 200 mM trehalose to 10 % DMSO.[72] Trehalose and salidroside increased the survival of red blood cells (RBCs) after cryopreservation. However, adding salidroside to glycerol is slightly more effective than replacing glycerol with trehalose 61 % vs 56

% respectively.[73] Trehalose and catalase protect the proteins on the surface of human hematopoietic cells.[74]

Non-human: in murine embryos, 1.5 M glycerol and 100 mM trehalose was the optimal cryoprotectant combination for freezing. Interestingly at 2.0 M glycerol, increasing trehalose concentration increases viability in a dose-dependent manner, even beyond the optimal trehalose concentration at optimal and suboptimal glycerol concentrations. This suggests that glycerol prevents damage by preventing dehydration damage, and/or trehalose protects cells from glycerol toxicity.[75] For *Trypanosoma brucei*, studies have found that 400 mM trehalose in host blood, or 200 mM trehalose *in vitro*, without DMSO or glycerol, is optimal for cryopreservation. In these studies, the replacement of 7.5 % DMSO with trehalose alone resulted in drastic increases in survival rate, depending on the form of the trypanosome.[76,77] In porcine spermatogonia stem cells, 200 mM trehalose alone, compared to 10 % DMSO, did not significantly increase the recovery of testis cells, but did increase proliferation capacity and the recovery of germ cells from thawed testis cells and tissue.[78] In ecto-mycorrhizal basidiomycetes, trehalose addition to DMSO improves post-thaw viability of most sub-types.[79] Finally, trehalose has been used to cryopreserve multiple cell types by pre-freeze dehydration.[80]

2.4 Comparison with other sugars

To what extent is trehalose unique among the sugars? Has trehalose proven beneficial as a cryoprotectant only to the extent that any sugar can act as a cryoprotectant? One advantage that trehalose has over many other sugars is that it's non-reducing, meaning that it does not undergo a Maillard-like reaction whereby it would react with the amino groups of proteins. Further, the glycosidic bond of trehalose is more stable than that of sucrose (another non-reducing disaccharide) and is less susceptible to hydrolysis.[81] Trehalose is a disaccharide, which appear to be better cryoprotectants than monosaccharides.[82,83] Although this isn't always the case, for example: in some studies, the monosaccharides galactose[84] and inositol[85] have been more effective than disaccharides. One explanation for the relative success of disaccharides could be that, at any given molarity, disaccharides have more polar groups while exerting the same osmotic pressure. Thus one important consideration when comparing monosaccharides with disaccharides is that the experimental concentrations of sugars in cryoprotectant solutions are usually measured in terms of molarity, rather than % weight; comparing 100 mM monosaccharide with 100 mM disaccharide can be misleading because the former tend to be approximately half the molecular weight of the latter, and critically – contain just over half as many polar groups per mol, resulting in fewer hydrogen bonds with water. Future studies would benefit from making weight-to-weight comparisons. Trehalose is most often compared to sucrose – another non-reducing disaccharide. The two have a similar structure; trehalose being made up of two glucose subunits and sucrose of one glucose and one fructose subunit. They have equal molecular weights and an equal number of hydrogen bond donors and acceptors. However, there are a number of ways in which trehalose appears to be a

better cryoprotectant than sucrose: in solution, trehalose forms stronger hydrogen bonds with water than sucrose,[86] and becomes associated with more molecules of unfrozen water, in this way each molecule of trehalose is able to disrupt the hydrogen bonding network of a greater number of water molecules, preventing them from forming ice.[87,88] Trehalose has superior ice growth inhibiting properties compared with sucrose.[89] Unlike sucrose, trehalose is able to displace water bound to the carbonyl groups of phospholipid membranes, displacing more water.[90] Trehalose has a larger hydrated volume than sucrose, which is correlated with protein protection, and more sucrose is needed to provide the same level of protein protection as trehalose.[20,91] Trehalose and sucrose cause a dynamic slowing effect on the surrounding water network, this effect is stronger in trehalose.[92] Some of these effects may occur because sucrose has more intramolecular hydrogen bonding, resulting in fewer and/or weaker intermolecular hydrogen bonds to interact with water.[87] Trehalose solutions have a higher glass transition temperature than sucrose,[93,94] however, a lower concentration of sucrose is needed to vitrify in aqueous solution at any given cooling rate.[95] During freeze-drying where water content is very low and samples are stored at room temperature, trehalose would be the superior stabiliser as it would be more likely to remain above its glass transition temperature, even if it absorbed a small amount of water. Conversely, during cryopreservation, sucrose may be more useful (at least with regard to its vitrifying properties) in both vitrification, and in slow freezing where vitrification occurs due to freeze concentration. Sucrose also has greater solubility in water,[96] making it less likely to precipitate out of solution at high concentrations and low temperatures. In some organisms that use trehalose as a cryoprotectant, anti-freeze proteins may be used to mitigate this solubility problem.[97] A summary of the comparative advantages of trehalose and sucrose can be seen in Table 1. While

trehalose is – in theory – a better cryoprotectant than other sugars, the results of experimental cryopreservation studies where trehalose has been directly compared to other sugars are mixed. Discussed below are cryopreservation studies where trehalose has been tested and found to be a better, worse, or equivalent cryoprotectant compared to other sugars.

Table 2.1. The advantages of trehalose versus sucrose as cryoprotectants.

Trehalose	Sucrose
Higher Tg at any given concentration (106 ºC vs 60 ºC for the pure sugars).[94]	Lower C_v at any given volume (74 % vs 58 % at 20 µl).[95]
Higher hydration number (4.598 vs 4.127 at 66 % w/v).[87]	Greater solubility (68.1 % vs 52.3 % at 30 ºC).[96]
Larger hydrated volume (62.5% vs 87 % water for a 1.5 M solution).[20]	-
Superior ice growth inhibiter ("Approximately twice as effective").[89]	-
Displace water bound to the carbonyl groups of phospholipid membranes.[90]	-
Causes more dynamic slowing of the surrounding water network.[92]	-
More effective for protein stabilisation.[91]	-

2.4.1 Studies where trehalose outperforms other sugars

In human embryonic stem cells, cryopreservation with 10 % DMSO + 200 mM trehalose, and adding trehalose to the recovery media, resulted in a greater number of undifferentiated cells surviving compared to DMSO alone. No similar improvement was seen with sucrose.[98] In human amniotic fluid stem cells, trehalose was slightly better than sucrose in the absence of solvent, although both were inferior to solvents.[99] In bovine spermatogonial stem cells, 20 % DMSO + 200 mM trehalose improved stem cell recovery relative to DMSO alone, DMSO + sucrose, or to DMSO + PEG (19 % vs 7 % vs 5 % respectively). The cells preserved with trehalose also showed the lowest

post-thaw apoptosis.[100] Trehalose was also better than sucrose for mouse oocyte vitrification,[101] and improved post-thaw outcome relative to sucrose in human spermatozoa.[102] In ram spermatozoa, trehalose was better than sucrose and slightly better than raffinose at 100 mM.[103] The presence of trehalose or sucrose in a cryoprotectant solution reduces solvent cryoprotectant toxicity to RBCs, but this effect is higher for trehalose.[25] In mouse neuroblastoma cells, trehalose gave higher post-thaw survival than sucrose (35 % vs 20 %). Cells frozen in trehalose benefit from trehalose preincubation, whereas cells frozen in sucrose do not benefit from preincubation with sucrose. The most effective cryoprotectant combination was trehalose + L-proline in a 1:1 ratio, at a total concentration 112.5 mM. This gave a post-thaw survival of 51 %.[104] Inositol is a non-reducing monosaccharide that can confer freeze tolerance to cricket cells, but not to crickets, while trehalose can confer freeze tolerance to both cricket cells and to whole crickets.[105] Inositol is inferior to trehalose for protection during lyophilisation.[106]

2.4.2 Studies where other sugars outperform trehalose

Multiple sugars (trehalose, sucrose, sorbitol, glucose and mannitol) were tested for the cryopreservation of *Catharanthus roseus* cells*,* it was found that trehalose resulted in the lowest survival rate and sorbitol gave the highest.[107] Sucrose was marginally better than trehalose for post-thaw survival in saltwater crocodile spermatozoa preservation.[108] Mentioned above, Chaytor *et al.*[84] measured the IRI activity of six different sugars (galactose, glucose, melibiose, lactose, trehalose, sucrose). They found that 220 mM lactose has the best IRI activity. Viability in a 200 mM solution of these sugars was then measured in human hepatocytes, both after incubation at 37 °C, and after freeze-thaw. Galactose and lactose gave the best viability after incubation

at 37 ºC. Galactose gave the best post-thaw viability, while glucose gave the worst. Trehalose was not better than sucrose. While this study does show that the selection of sugar can affect post-thaw outcome, the differences in post-thaw survival were not large (with the exception of glucose, which was by far the worst cryoprotectant here). Another study assessed the effect of multiple sugars (lactose, galactose, glucose, fructose, lactulose, melibiose, trehalose, sucrose) on post-thaw boar sperm quality. It was found that disaccharides were better than monosaccharides, and those disaccharides that contain glucose were better than does which did not. Trehalose increased membrane permeability in this study, resulting in worse post-thaw outcome than other sugars.[83]

2.4.3 Studies where trehalose and other sugars were equivalent

Rat hepatocyte viability after cryopreservation was slightly higher (80 % vs 75 %) with 200 mM trehalose in the absence of DMSO, than with 5 % DMSO. The addition of trehalose or sucrose to 5 % DMSO did not improve viability, and the addition of glucose decreased viability.[32] In mouse spermatogonial stem cells, trehalose did not significantly increase recovery, but almost doubled post-thaw proliferation. Trehalose was more effective than sucrose and maltose in this respect. Lactose was as effective as trehalose. Fructose, galactose, xylose, and raffinose did not increase proliferation compared to the control. Glucose and mannose did increase proliferation, but not as much as the disaccharides.[82] In Rodrigues *et al.*[30] post-thaw recovery with 5 % DMSO + 146 mM trehalose was 85 %, compared to 79 % recovery with 10 % DMSO for the preservation of human cord blood hematopoietic stem cells. Although there was no difference between using trehalose and sucrose in this study. Nynca *et al.*[109] reports that sperm cryopreservation can be improved with glucose, sucrose, or trehalose, but

trehalose was not more effective than other sugars for most Salmonidae species. The addition of 250 mM trehalose or 321 mM lactose to boar sperm freezing extender had no beneficial effects.[110] In Woelders *et al.*[111] bull Sperm, isotonic trehalose and sucrose were equally beneficial at the optimal fast cooling rate, and in Chen *et al.*[112] trehalose and sucrose provided equal benefit to bull spermatozoa motility. For the vitrification of human spermatozoa, trehalose and sucrose were equally effective at preserving motility, and were both slightly better than 3-O-methyl-D-glucopyranose, raffinose and stachyose.[113] In dog spermatozoa vitrification, trehalose was not more effective than sucrose.[114]

2.5 Use as an intracellular cryoprotectant

Trehalose is most effective when it is in both the intracellular and extracellular space.[115–117] In the intracellular space, it is able to inhibit intracellular ice, prevent cellular dehydration caused by both extracellular ice and extracellular cryoprotectants, and stabilise intracellular proteins. However, trehalose is a polar molecule, and does not cross the cell membrane by diffusion, nor are there any trehalose-specific transporters in most mammalian cells. Various methods have been used to load trehalose into the intracellular space of cells which do not naturally produce it namely; electroporation, membrane permeabilising polymers, genetic engineering, microinjection, nanoparticles, liposomes, freeze-induced uptake, molecular engineering, and natural endocytosis through preincubation. For an in-depth review of the topic, see Stewart and He.[118] The ideal method for trehalose permeation would have low cytotoxicity, not be time-consuming or labour-intensive, and would allow for a high enough concertation of trehalose to be loaded that it could entirely replace solvents.

2.5.1 Preincubation, fluid-phase endocytosis, and osmotic manipulation

Fluid-phase endocytosis, or "cellular drinking" is where cells internalise the surrounding fluid using vesicles. This allows for the non-specific uptake of molecules from the surrounding environment. In this way, cells can be slowly loaded with trehalose by incubating them in a trehalose solution for a number of hours. Trehalose pre-incubation was first used in carrot and tobacco cells where it allowed for trehalose to be used as the sole cryoprotectant.[119] While trehalose does not cross cell membranes by diffusion, it can be taken up by Clathrin mediated endocytosis.[120] This process is

slow – cells have to be incubated for up to 24 hours before freezing – and the maximum concentration of trehalose that can be loaded is limited. Preincubation with trehalose has been used to improve the cryosurvival of mesenchymal stem cells,[121] and is more effective than simply adding trehalose to the cryopreservation media without preincubation.[122] In Stokich *et al.*[123] human hepatocyte cell monolayer recovery was improved by a 24-hour preincubation with 100 mM trehalose in addition to the standard 10 % DMSO, resulting in 39 % and 10 % viability respectively. In Campbell *et al.*[124] preincubation with 200 mM trehalose before preservation with 400 mM trehalose greatly increased metabolic activity measured 7 days after thawing in bovine endothelial cells. In Hara *et al.*[125] 1.3 M trehalose in the absence of solvent increased proliferation compared to glycerol for human embryonic kidney cells (36 % vs 10 %). For cattle ovarian granulosa, a 30-minute preincubation with 200 – 400 mM trehalose, before flash freezing with trehalose + 5 % DMSO and 5 % EG, greatly improved viability (70 % vs 15 %).[126] It is possible to force trehalose to cross the cell membrane by creating large osmotic pressure differences between the intracellular and extracellular spaces. However, it only allows for small amounts of trehalose to be loaded and it can damage cells. This has been attempted in red blood cells, allowing for up to 43 mM trehalose to be loaded.[127,128] This has also been attempted in T-lymphocytes, but only allowed 2.2 mM to be loaded.[129]

2.5.2 Freeze-induced uptake

When trehalose is simply added to the extracellular space immediately prior to freezing, it can be mistakenly considered to only be an extracellular cryoprotectant. However, the phase change in the cell membrane during freezing, combined with freezing-induced osmotic effects and membrane damage, allows trehalose and other

small molecules to enter the cell. Counterintuitively, the amount of trehalose loaded by freeze-induced uptake increases with freezing rate, resulting in a faster optimal cooling rate for cryoprotection from freeze-induced uptake than with DMSO.[130] This is unfortunate because most cell types require low freezing rates for optimal cryopreservation. Beattie *et al.*[131] was the earliest study using intracellular trehalose to preserve mammalian cells. Here, human pancreatic islets were cryopreserved with 2 M DMSO + 300 mM trehalose, which resulted in improved cell recovery compared to 2 M DMSO + 11.1 mM glucose (94 % vs 58 %). Insulin production was also greatly improved. Intracellular trehalose was measured and was found to increase as temperature was lowered. The threshold temperature for freeze-induced uptake appears to be 15 °C, as only very small amounts of intracellular trehalose were detected before this temperature was reached. In mouse neuroblastoma cells, both preincubation and freezing with trehalose contribute to increased cell recovery. Trehalose positively combines with L-proline at equal total concentration, even when L-proline was added to the freezing media, meaning that L-proline protects cells through a mechanism other than inducing freeze tolerant metabolism.[104] In Zhang *et al.*[132] a 500 mM extracellular trehalose concentration was optimal for mouse embryonic fibroblasts, resulting in 50 % survival. 160 mM trehalose could be detected inside cells after preservation. Preincubation with 25 mM trehalose had no positive effect. Freeze-induced uptake may also occur in platelets[133] and spermatozoa.[134]

2.5.3 Electroporation

Electroporation is the application of an electric current to cells which causes the formation of pores, resulting in a temporary increase in membrane permeability. In Shirakashi *et al.*[135] mouse myeloma cells were loaded with 100 mM trehalose from

an extracellular trehalose concertation of 290 mM. Higher intracellular concentrations were achievable but required damaging voltages to be used. In Dovgan *et al.*[136] human umbilical stem cells were electroporated and incubated with 200 mM trehalose for 25 minutes, which resulted in improved cell recovery relative to 10 % DMSO (86 % vs 60 %). For human adipose-derived stem cells, electroporation with trehalose increased recovery compared to trehalose incubation, as measured by trypan blue excision but not when measured by MTT assay.[137]

2.5.4 Polymers

A polymer known as 'PP-50' has been used to increase membrane permeability in a reversible pH-responsive manner.[138] It can be used to permeabilise human red blood cells to trehalose resulting in 251 mM trehalose uptake from 700 mM solution after a 9h incubation period. In ovine red blood cells, PP-50 allowed 123 mM trehalose loading from a 360 mM extracellular solution, with a 9-hour incubation. This resulted in 83 % survival between the end of incubation and thawing. However, PP-50 causes some haemolysis, and this was not accounted for in the post-thaw haemolysis lysis assay, as RBCs were washed after incubation, which removed free haemoglobin. Elsewhere in the paper, it was found that 22 % haemolysis occurs during loading, resulting in a probable final survival of around 61 %. So, while this shows that the level of trehalose loading archivable with PP-50 greatly improves survival, it does not present PP-50 as a good way of achieving it. Nonetheless, PP-50 can be used to increase cryosurvival with trehalose uptake.[115] The PP-50 trehalose combination was also used to improve the cryosurvival of osteosarcoma cells, although does not result in better cryosurvival than that with DMSO.[139,140]

2.5.5 Carrier peptides

A cargo peptide was developed by Wei *et al.* that non-covalently binds to trehalose and carries it across the cell membrane. The peptide was non-toxic, but only very small amounts of trehalose could be loaded into the cell.[141]

2.5.6 Gene expression for trehalose synthesis

Trehalose can be synthesised from glucose using trehalose 6-phosphate synthase and trehalose 6-phosphate phosphatase. Cells can be transfected with genes that encode these enzymes, but the intracellular trehalose concentration that can be achieved is low.[142,143]

2.5.7 Transporter proteins and channels

Most cell types have an endogenous receptor called P2Z (or P2X7) which forms pores when ATP is present extracellularly. In human hematopoietic stem cells, it allowed for 200 mM trehalose to be loaded from 200 mM extracellular trehalose after a 60-minute incubation period, resulting in 91 % post-thaw viability as measured by proliferation. This is compared to 11 % viability after freezing with 200 mM extracellular trehalose alone.[116] Other pores and channels can be added artificially. For example, cells can be genetically engineered to express the trehalose transporter TRET1. In Chinese hamster ovary cells, this allowed 23 mM trehalose uptake after incubation in 400 mM trehalose for 4 hours. This did not affect the cell's proliferation.[144] This technique was later used to protect Chinese hamster ovary cells during cryopreservation.[145] H5 is a pore-forming agent which has been engineered from α-haemolysin. H5 forms pores in the cell membrane which can be opened and closed by altering zinc ion concentration. This allowed for 80 % cryosurvival in

murine fibroblasts, 70 % cryosurvival for human keratinocytes,[146] and 92 % post-thaw viability for human hemopoietic stem cells.[147] This approach allows for high concentrations of trehalose to be loaded quickly, but the potential of H5 to cause an immune response may limit its use in cells that are to be used clinically.

2.5.8 Microinjection

Microinjection allows for small volumes of liquid to be directly introduced into the intracellular space. It allows very high concentrations of trehalose to be loaded but is extremely labour-intensive and poses challenges when used on cells which are smaller than oocytes. In Eroglu *et al.*[148] human oocytes were microinjected with 150 mM trehalose and frozen in 500 mM trehalose, resulting in 66 % survival. In a later study, mouse oocytes were injected with 500 mM trehalose and frozen in 500 mM trehalose solution, giving 80 % survival. Using 500 mM extracellular trehalose only gave just 15 % survival. Reducing intracellular trehalose concentration to 80 mM and adding 500 mM DMSO (4 % w/v) resulted in 97 % survival. These oocytes were able to develop into embryos at the highest rate. One explanation for the increased cryosurvival in the presence of DMSO, is that DMSO is able to cross intracellular membranes and enter the organelles.[149]

2.5.9 Nanoparticles

Nanoparticles can encapsulate trehalose and deliver it into the cell. Thermally responsive nanoparticles can be used to load up to 300 mM trehalose into murine fibroblasts, from a 40 minute incubation followed by cold shock.[150] In human adipose-derived stem cells, 24-hour incubation with pH-responsive nanoparticles and 200 mM extracellular trehalose allowed for a post-thaw viability equivalent to that given by 10

% DMSO.[151] Nanoparticles that increase membrane permeability have been used to load 51 mM trehalose into red blood cells from 350 mM extracellular trehalose, after a 7 hour incubation period, resulting in 91 % post-thaw survival.[152]

2.5.10 Liposomes

Holovati and Acker[153] developed liposomes that can be used to deliver trehalose into RBCs. These liposomes were used to carry a small volume of 300 mM trehalose and were incubated with red blood cells for 4 hours (for an estimated total of 15 mM intracellular trehalose), then frozen with 300 mM trehalose. This resulted in 67 % post-thaw recovery, compared to 27 % recovery when incubated in extracellular trehalose alone. The concentration of intracellular trehalose alone provided minimal cryoprotection, but surprisingly the liposomes themselves provided a very large degree of cryoprotection; cells incubated with liposomes containing no trehalose and frozen in 300 mM extracellular trehalose gave 60 % recovery.[154] The ability of liposomes to improve cryopreservation could cause misleading results when using them to deliver cryoprotectants. For example, Motta *et al.*[155] used liposomes to assess the effect of intracellular and extracellular trehalose on umbilical cord blood stem cell preservation but did not control for the effects of the liposomes themselves. However, this cryoprotective ability is useful in its own right, and could be used as part of a multi-component cryoprotectant solution to deliver low concentrations of non-penetrating cryoprotectants such as antifreeze proteins. In Stoll *et al.*[156] liposomes allowed for up to 80 mM trehalose to be loaded into human RBCs, dependent on liposome concentration. This slightly increased post-thaw recovery, but post-thaw recovery did not increase with increasing liposome concentration above 1 mM, which was the lowest liposome concentration used.

2.5.11 Ultrasound

In Zhang *et al.*[157] ultrasound was used to induce non-specific permeability in human platelets. This technique allowed only 30 mM of trehalose to enter the cell after 30 minutes of treatment, which is insufficient for cryoprotection. This method also altered platelet morphology.

2.5.12 Modified trehalose

Trehalose itself can be chemically modified so that it can penetrate the cell membrane. In Abazari *et al.*[158] trehalose was acetylated at six of its hydroxyl groups, greatly increasing its hydrophobicity. Rat hepatocytes were incubated with 30 mM acetylated trehalose, resulting in an intracellular acetylated trehalose concentration of 80 mM within 1 hour, and of up to 300 mM after 8 hours. Trehalose concentration lagged behind this with a concentration of around 100 mM after 12 hours. Despite acetic acid production from the de-acetylation process, this method had minimal toxicity.

2.5.13 Trehalose removal

While trehalose has almost no direct toxicity at concentrations required for cryopreservation, trapped intracellular trehalose can cause osmotic damage when trehalose is removed from the extracellular solution.[130] However, after thawing, trehalose will be eliminated from cells due to cell division, and possibly by exocytosis and/or conversion to glucose.[159] Therefore, to maximise cell survival, the osmolarity of the recovery media can be progressively lowered to maintain osmotic balance, although this strategy is labour-intensive. Alternatively, using a lower intracellular concentration of trehalose in conjunction with a penetrating cryoprotectant can mitigate osmotic damage.[149]

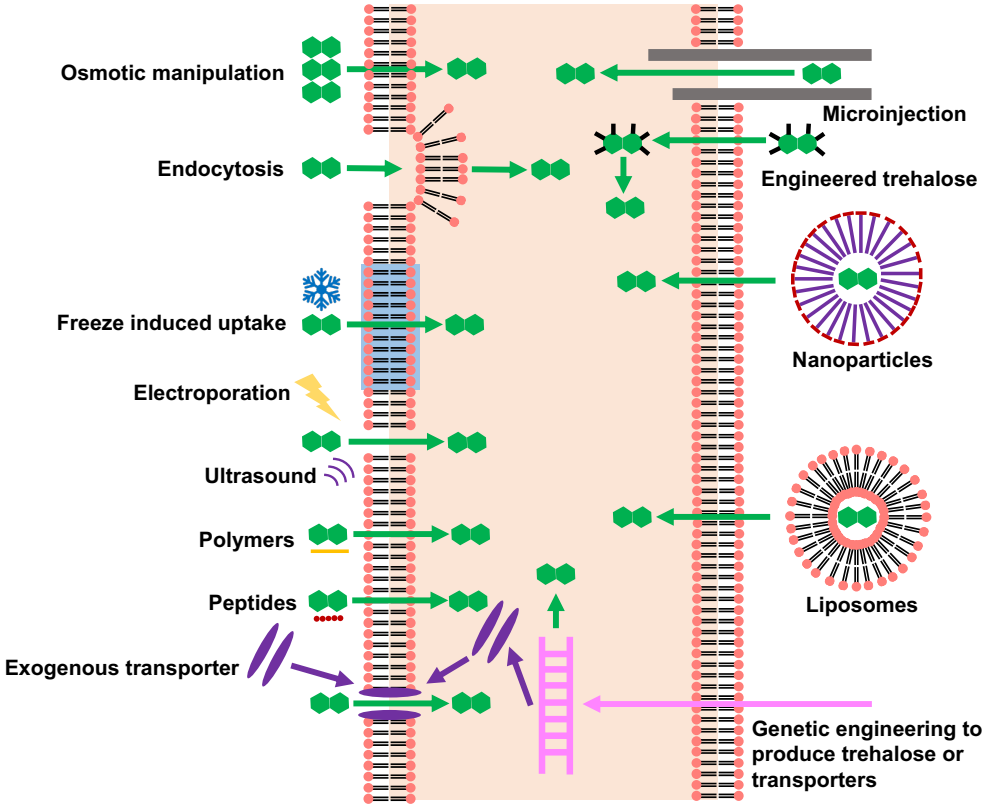

Figure 2.2. Methods for loading trehalose into the intracellular space.

2.6 Conclusions

When used as an extracellular cryoprotectant in conjunction with a penetrating solvent cryoprotectant such as DMSO, trehalose provides additional cryoprotection, improves post-thaw outcome and/or allows for the reduction of solvent. Trehalose has unique properties (Table 2.1) which – at least theoretically – indicate that it is the best choice of cryoprotectants among the mono- and di-saccharides. However, studies which compare different sugars for cryopreservation have found mixed results. Some studies found that trehalose provides an improvement over other sugars.[25,98–106] While other studies have found that other sugars are equally,[30,32,82,109,112–114] or more effective.[83,84,107,108] Nonetheless, trehalose represents a good (although perhaps not always optimal) choice of sugar, and most cryopreservation protocols would benefit from the addition of 100 – 400 mM trehalose to the freezing media. Many studies discussed in this review demonstrate the importance of penetrating cryoprotectants, these cryoprotectants reduce intracellular ice formation at super-optimal freezing rates, and are essential for preventing damage by solute concentration.[160] The concentration of intracellular solutes is also one of the mechanisms of damage caused by cellular dehydration,[161] this limits the total osmolality of non-penetrating extracellular cryoprotectants that can be used as these will dehydrate the cell. In this way, the use of intracellular cryoprotects allows for a higher concentration of non-penetrating extracellular cryoprotectants to be used without causing this type of damage. For an example of this, see the glycerol/trehalose concentration matrix in Table 2 of Honadel *et al*.[75] The downside is that most intracellular cryoprotectants are solvents, which cause damage to cells through direct toxicity and must be removed if the cells are to be used clinically. One solution to this problem is to deliver trehalose

to the intracellular space. Out of all the trehalose delivery mechanisms discussed here, it seems that thermally responsive nanoparticles are one of the most effective solutions, delivering a large amount of intracellular trehalose after only a short incubation period, and a cold shock.[150] An alternative and currently unexplored solution is the chemical modification of trehalose to make it membrane permeable.[158] This technique may be just as effective and offers a simpler loading protocol.

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Chapter 3 – Red Blood Cell Cryopreservation with Minimal Post-Thaw Lysis Enabled by a Synergistic Combination of a Cryoprotecting Polyampholyte with DMSO/Trehalose

3.1 Abstract

Blood transfusions, primarily consisting of red blood cells (RBCs), are essential in medicine for treating trauma patients and replacing blood lost during surgical procedures. However, fresh blood accessibility is not guaranteed, and shortages can happen. Cryopreservation of RBCs typically requires cryopreserving them with 20 – 40 % glycerol, which involves time-consuming washing steps post-thaw. Here we explore mixtures of multiple cryoprotectants, which each function by a different mechanism, to enable their advantages, whilst minimising toxicity of each component: specifically, DMSO, polyampholyte, and trehalose. Using a high-throughput screen, and optimisation, we show that the optimal composition allows 97 % recovery of red blood cells after cryopreservation, even using non-optimal freezing conditions. We demonstrate that the solution is non-cytotoxic and that the RBCs survive cryoprotectant washout with 80 % total recovery after a suboptimal washout protocol. Upon testing with an osmotic fragility assay and flow cytometry, no differences were found between fresh and post freeze-wash-thaw RBCs. Finally, to assess its potential for clinical usage, we used our solution to cryopreserve 400 mL of blood in a blood bag resulting in 84 % post freeze-thaw-wash recovery. Our solution may be used for the storage of RBCs where rapid washout is essential, or where suboptimal freezing/thawing conditions are used. These results also demonstrate how

cryoprotectants with different mechanisms of action can be combined to enable improvements in cryoprotection whilst mitigating toxicity effects.

3.2 Disclaimer

- This work was published in *Biomacromolecules* **2022**, *23* (2), 467–477.[1] The figures, figure legends, and some of the text in this chapter are similar or identical to that of the article.
- Polyampholyte was produced in various batches by Dr Thomas Congdon, Dr Kathryn Murray, and myself (3.4).
- Confocal imaging was conducted by Dr Ruben Tomás (3.4).
- Much of the general workflow for the blood cryopreservation and AHD assays was optimised by Dr Christopher Stubbs.

3.3 Introduction

A consistent supply of human blood, primarily consisting of RBCs, is a medical necessity for treating blood loss incurred through injury or surgery. Fresh (non-frozen) human blood units can be stored for a maximum of 42 days before they must be discarded.[2] The transfusion of cryopreserved blood has been shown to be just as safe and effective as fresh blood,[3] and so the cryopreservation of blood is used to extend its storage time. The main uses of frozen blood are maintaining the availability of rare blood types and the supply of large amounts of blood to cope with surges in demand during natural disasters and other large-scale emergencies.[4] Frozen blood is also needed for military use, to treat battlefield injuries in remote locations, and on ships and submarines that are away from land for months at a time.[5] While cryopreservation of non-rare blood types for general civilian use is uncommon, blood supply, particularly type O, fluctuates by time of year.[6] Frozen blood supplies may also be useful in remote rural areas.[7,8] There is some evidence that fresh RBCs decline in quality over time, leading to unfavourable patient outcomes; this is controversial, but highlights another advantage of frozen blood units, that they can be stored indefinitely[9] (although current regulations limit this time to 10 years), without sustaining any loss in quality beyond that caused by the freeze-thaw-wash process. Regardless of quality, non-frozen blood must be discarded sooner or later, leading to wastage, whereas a switch to using frozen blood would avoid this. In the veterinary setting, advances in blood banking are needed to ensure a constant supply of blood to varied and potentially uncommon animals, for which maintaining a constant supply of fresh blood is unpractical due to infrequent or highly localised need.[10–13] Finally, long-term RBC

cryopreservation has use in research and diagnostic settings for long-term storage of samples, where testing would otherwise be limited to freshly drawn blood.[14]

3.3.1 Current methods for blood cryopreservation

There are two widely used clinical protocols for freezing RBCs. The first uses 40 % glycerol and slow freezing, which is the most common method in the USA.[15,16] The second uses 20 % glycerol and fast freezing, which is more common in Europe.[17] DMSO can also be used for freezing RBCs, although this is less common.[18] All of these methods work by freezing, rather than vitrification, a separate physical process in which no ice crystals are allowed to form, resulting in the transition of the sample to a glassy state. After freezing, the cryoprotective solution must be removed from the blood units before transfusion. In the clinical setting, cryoprotectant washout is performed using an automated system, such as the ACP 215 cell processor. This machine slowly reduces the concentration of glycerol in the solution, allowing glycerol to diffuse out of the RBCs at a rate which does not cause osmotic stress. The key disadvantage of this system is the time it takes to remove the glycerol, with washout taking around 30 – 60 minutes per unit of blood.[19] This may result in the patient dying from blood loss before thawed blood can be provided.[20] This is especially relevant to battlefield injuries, where blood loss from bullet injuries is a common cause of death.[21] This problem is compounded when multiple units of blood are needed simultaneously because most facilities are not equipped to wash multiple units of blood in parallel.[22] This problem can be somewhat mitigated by periodically thawing cryopreserved blood, even when it's not needed in order to maintain a ready-

to-use supply. However, this inevitably leads to wastage, and is not suitable for emergencies when a large amount of blood is needed at once.

3.3.2 Alternatives to glycerol

Glycerol is a hydrophilic molecule relative to other solvent cryoprotectants, having a logP of -1.8 while DMSO has a logP of -0.9. It, therefore, crosses the cell membrane relatively slowly, and must be used at high concentrations to be effective. Both of these factors increase washout time. Alternative cryoprotectants that are not currently used for blood cryopreservation include DMSO, trehalose, polyampholyte and poly(ethylene glycol). These cryoprotectants are discussed below (Figure 3.1). DMSO is more hydrophobic than glycerol and has a much lower effective concentration (10 % vs 20 – 40 % (v/v)). Consequently, DMSO crosses the cell membrane more quickly than glycerol.[23,24] It is therefore likely that a DMSO-based cryopreservation solution could be removed from a unit of thawed blood much faster than glycerol. DMSO acts via a similar cryoprotective mechanism to glycerol; it acts in the intracellular space to inhibit intracellular ice-formation and to counter the solute concentration effect (whereby ice formation causes cellular dehydration, causing toxicity by concentrating intracellular solutes). DMSO is not currently used to cryopreserve RBCs in a clinical setting. This may be due to fears over toxicity if transfused in large volumes,[25] or perhaps lack of effectiveness compared to glycerol. Although this latter point is controversial with studies reporting differing levels of cryoprotection with DMSO.[10,26] Both of these concerns are addressed in this study, as the solutions that are developed here are both less toxic to RBCs and more effective for their cryopreservation. Polyampholytes are polymers with mixed positive and negative charges. They have

little IRI activity[27,28] but are thought to reduce cellular dehydration, prevent the spread of intracellular ice into the cell, and to protect the cell membrane.[29,30] Matsumura *et al*. found that poly(L-lysine) based polyampholytes provided high levels of cryopreservation.[31] However poly(L-lysine) based cryoprotectants are expensive to make at the scale needed for blood storage. As a cheaper alternative, the Gibson group synthesised their own polyampholyte: poly(vinyl ether -alt- maleic acid mono(dimethylamino ethyl)ester), henceforth referred to as "polyampholyte", using commercially available starting materials, namely poly(methyl vinyl ether -*alt*- maleic anhydride. The Gibson group used 100 mg mL^{-1} of this polyampholyte under fast freeze/fast thaw conditions, without manual nucleation, or any solvent, to obtain ≈ 85 % immediate post-thaw recovery of RBCs.[32] Trehalose has also been used to cryopreserve blood, although most work has focused on delivering trehalose into the intracellular space as proof of concept, as trehalose cannot permeate the cell membrane in quantities sufficient for cryopreservation.[33–37] Trehalose exerts most of its cryoprotective properties in the extracellular space, reducing ice formation by disrupting the network of hydrogen bonds between water molecules.[38-39] Trehalose also acts by stabilising proteins,[40-41] stabilising the cell surface membrane,[42-43] and reducing the toxicity of other cryoprotectants – which has been demonstrated using red blood cells.[44] Poly(ethylene glycol) (PEG) is a water-soluble polymer which can replace water, reducing ice formation in much the same way as DMSO and trehalose, but being a polymer with a high MW, it causes less osmotic stress because high MW polymer solutions have low osmolarity relative to their concentration by weight. These four cryoprotectants have different (although often overlapping) mechanisms of action, but critically, they also have different mechanisms of toxicity. For example,

the optimal concentration of trehalose is limited by its osmotic dehydration effects, but this problem is not caused by PEG, which has its own means of causing toxicity at higher-than-optimal concentrations. Therefore, rather than focussing on a single alternative cryoprotectant for blood, we hypothesise that using a combination of cryoprotectants with different mechanisms of action could have synergistic cryoprotective effects. Specifically, we hypothesise that combining DMSO, trehalose and a macromolecular compound would give superior cryoprotective properties than using each one individually. We aim to develop a multicomponent cryopreservation solution for red blood cells and investigate the effectiveness of combining cryoprotectants with different mechanisms of action.

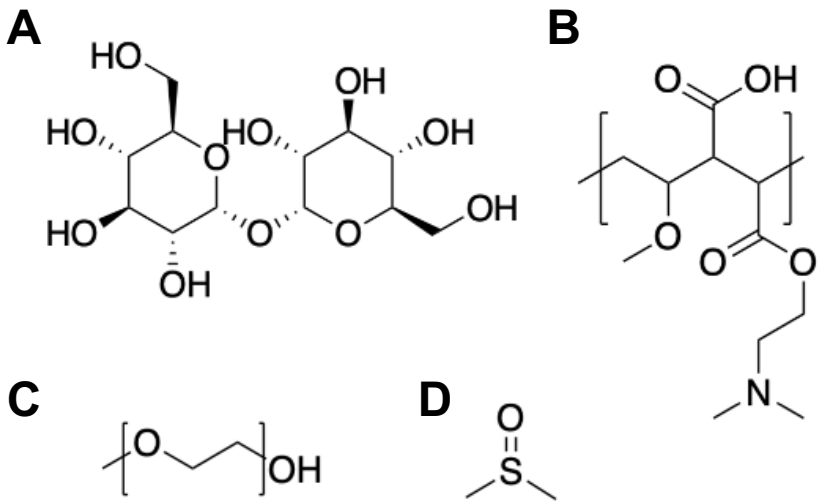

Figure 3.1. The cryoprotectants used in this study; **(A)** trehalose; **(B)** polyampholyte (poly(vinyl ether-*alt*-maleic acid mono(dimethylamino ethyl)ester)); **(C)** poly(ethylene glycol) Mn = 4,000 g mol^{-1}; **(D)** dimethyl sulfoxide (DMSO).

3.4 Results and Discussion

3.4.1 Synergy between trehalose and DMSO

Our first aim was to evaluate how mixing different ratios and concentrations of two common cryoprotectants, DMSO and trehalose, could benefit RBC cryopreservation. Due to the large number of variables, and to maximise the number of combinations screened, liquid handling robots were employed. The liquid handling robot was programmed to distribute 50 μL of 2× concentration cryoprotectant solutions into a 96 well plate, in a matrix format, before mixing 100 μL of ovine blood into each well (Figure 3.2). The plate was then incubated at room temperature for 10 minutes, before rapid cooling in liquid nitrogen vapour. After 20 minutes, RBCs were thawed for 5 minutes in a water bath at 37 °C. An alkaline haematin D-575 (AHD) assay was used to determine the percentage lysis of the RBCs. The AHD assay measures the amount of haemoglobin in the solution by converting haemoglobin to alkaline haematin D-575, a stable compound with an absorption peak at 575 nm.[46] Advantages of the AHD assay over the classical cyanhaemiglobin method include increased stability of the resulting haemoglobin complex, and improved safety of the reagents.[47] The equation: $\text{Recovery} = 100 - \%(\text{lysis})$ gives an estimation of the percentage of RBCs surviving with intact membranes (Figure 3.3). Unless otherwise specified, all cryoprotectant solutions used Dulbecco's phosphate-buffered saline (DPBS) (8 g L^{-1} sodium chloride, 0.2 g L^{-1} potassium chloride, 0.2 g L^{-1} monobasic potassium phosphate, and 1.15 g L^{-1} dibasic sodium phosphate) as the carrier.

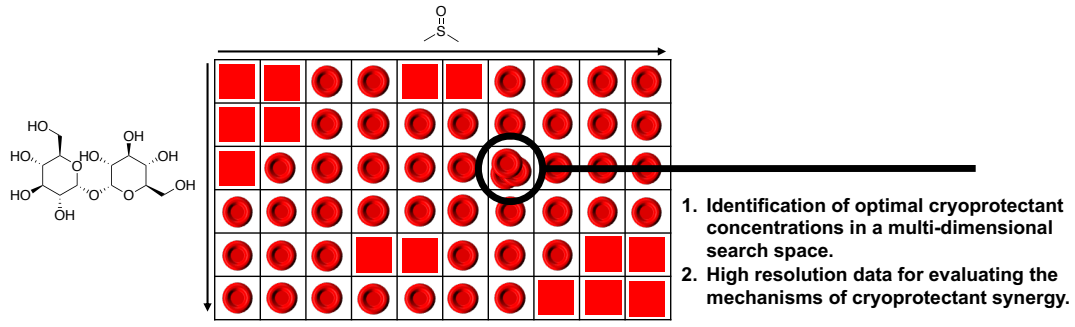

Figure 3.2. A schematic showing the high throughput assay used to test the cryoprotection afforded by different concentrations of DMSO and trehalose. The assay is in a matrix format, with increasing DMSO concentrations along the X-axis and increasing trehalose concentrations down the Y-axis.

A liquid handling robot was used to mix 100 µL cryoprotectant solution in each well, before mixing the solution with 100 µL of ovine blood. Blood was then frozen in liquid nitrogen vapour and thawed in a 37 ºC water bath. Haemolysis was assessed using an AHD assay and the results are shown in Figure 3.3.

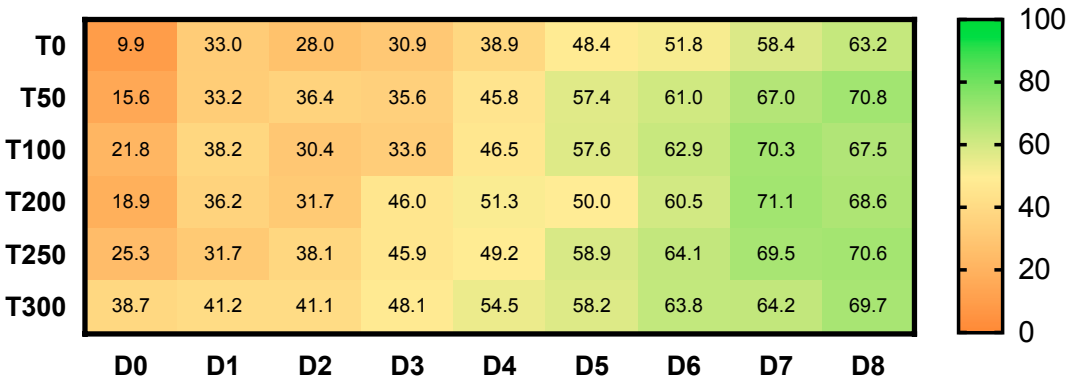

Figure 3.3. The percentage recovery of RBCs after being rapidly frozen in the liquid nitrogen vapour phase and thawed for 5 minutes at 37 °C. X-axis labels indicate final DMSO concentration (% v/v, e.g., D1 = 1 % DMSO). Y-axis lab labels indicate final trehalose concentration (mM, e.g., T250 = 250 mM trehalose). Data represents the mean of three independent experiments.

We used the above method to assess the effectiveness of mixtures with DMSO concentrations between 0 % and 8 %, and trehalose concentrations between 0 mM and 300 mM. The cryoprotectant combination that resulted in the highest recovery was 7 % DMSO and 200 mM trehalose. This peak recovery (71 %) was similar to that obtained by using 40 % glycerol (75 %)[16] in a laboratory setting and higher than using DMSO (40 %, canine RBCs with 10-minute incubation)[10] or trehalose alone (55 %).[48] As DMSO concentration increased along the X-axis, recovery tended to increase. When trehalose was not present, recovery increased to 63 % and would likely have continued increasing up to some optimal point before decreasing again. Likewise, as the concentration of trehalose was increased in the absence of DMSO, recovery tended to increase up to 39 % at 300 mM and would likely have continued increasing until an optimal point was reached. The mixing of trehalose and DMSO brings the optimal >

8 % DMSO concentration down to 7 % and the optimal trehalose concentration down to 200 mM.

While the range of concentrations tested here was limited, enough of the two-dimensional concentration space was explored to identify an optimal pair of cryoprotectant concentrations and show that cryoprotectant concentrations need to be optimised along multiple axes as the concentration of each cryoprotectant affects the optimal concentration of the others.

3.4.2 Development of a multi-component cryoprotectant solution

Guided by the above results, where a maximum recovery of 70 % was achieved using 7 % DMSO and 200 mM trehalose, we set out to further enhance it by adding in a macromolecular cryoprotectant, polyampholyte, which is shown in Figure 3.1. Polyampholyte has been shown to increase both RBC and nucleated cell recovery post-thaw[49] and so is an ideal cryoprotectant to explore as a third component. We attempted to use the same high throughput assay to test 0 – 300 mM trehalose with 0 – 140 mg mL^{-1} polyampholyte. However, at high concentrations of polyampholyte (above around 50 mg mL^{-1}), we found that the polyampholyte sequestered haemoglobin into a separate layer during the centrifugation step, this made the AHD assay unusable inside 96 well plates. This phenomenon was not apparent when cryovials were used for freezing, therefore, we reverted to mixing the cryoprotectant solutions manually in cryovials rather than using the liquid handling robot. This method resulted in lower throughput. The cryoprotectants investigated here were DMSO (D), trehalose (T), PEG (P), and polyampholyte (Pa), and combinations

thereof. Due to the lower throughput assay, we selected optimal concentrations of the individual cryoprotectants to assess how the addition of the polyampholyte affected RBC recovery post-thaw. DMSO was used at 10 % v/v, PEG and Pa at 100 mg mL^{-1}, and trehalose at 100 mM. Working concentrations of trehalose were determined by a series of optimisation experiments (see appendix Figures 3.18 – 3.20). The combinations are noted by combining abbreviations, for example: PaD is 100 mg mL^{-1} polyampholyte and 10 % DMSO.

In each of these experiments, 1000 µL of cryoprotectant solution was used to resuspend a pellet made from 500 µl of blood. 1 mL of the resulting suspension was transferred to cryovials and incubated for 10 minutes at room temperature. In the first series of experiments (fast freeze/fast thaw) cryovials were plunged into liquid nitrogen for at least 20 minutes. Cryovials were then transferred to a 37 °C water bath for 5 minutes. Post-thaw recovery was assessed using an AHD assay. PaD and PaDT, gave 91 % and 96 % post-thaw recovery respectively (Figure 3.4). The effectiveness of blood cryopreservation with PaD (100 mg ml^{-1} polyampholyte, 10 % DMSO) and PaDT (100 mg ml^{-1} polyampholyte, 10 % DMSO, 100 mM trehalose) has not been previously studied. However, the addition of polyampholyte to DMSO has demonstrated benefits in nucleated cells. The cryoprotective effect of Pa alone was surprisingly variable. This result was not seen in previous work,[50] and may be due to differences between batches.

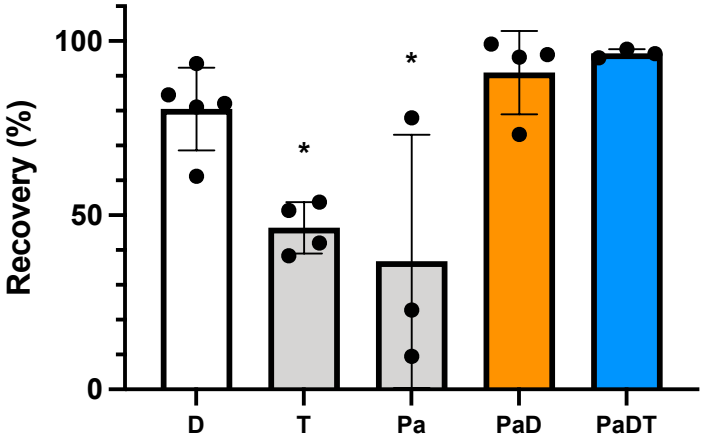

Figure 3.4. The percentage recovery of RBCs after being rapidly frozen in liquid nitrogen and thawed for 5 minutes at 37 °C. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant. Pa = 100 mg mL^{-1} polyampholyte, D = 10 % DMSO, T = 100 mM trehalose. Data represents the mean ± SD of at least three independent experiments (*P < 0.05 from 10 % DMSO).

We next explored the effect of adding a fourth component – poly(ethylene glycol). It was necessary here to change the protocol to use suboptimal conditions as the high recovery obtained when using PaD and PaDT made it almost impossible to discern further improvements. Therefore, for the second series of experiments, cryovials were warmed at room temperature until thawed, instead of warming in a 37 ºC water bath (fast freeze/slow thaw). The optimal warming rate for cells is generally much higher than that achieved at room temperature, as evidenced by a decreased recovery using 10 % DMSO from 81 % using fast warming to 68 % using slow warming, and PaD from 90 % to 78 %. To our surprise, PaDT still remained highly effective during the slow warming protocol, achieving 95 % recovery. Adding 100 mg mL^{-1} PEG to make PaDTP (100 mg ml^{-1} polyampholyte, 10 % DMSO, 100 mM trehalose, 100 mg ml^{-1}

PEG) resulted in a recovery of 98 % (Figure 3.5). One thing to highlight here is that PaDT affords robust protection regardless of warming rate. PaDTP may also afford indifference to warming rate, but this was not tested under fast warming conditions. The difference in recovery between PaD and PaDT under fast and slow warming conditions highlights the protective role of trehalose during the warming process. Trehalose does have some ice recrystallisation activity,[51] but may protect the cells from the effects of ice recrystallisation via its other mechanisms of protection.

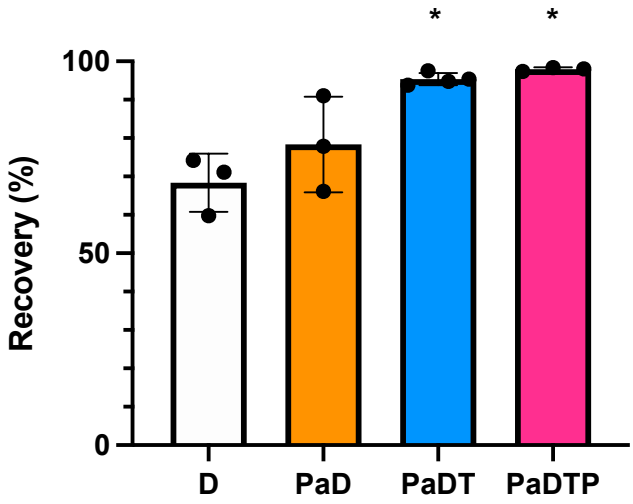

Figure 3.5. The percentage recovery of RBCs after being rapidly frozen in liquid nitrogen and slowly warmed at room temperature. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant. Pa = 100 mg mL^{-1} polyampholyte, D = 10 % DMSO, T = 100 mM trehalose, P = 100 mg mL^{-1} poly(ethylene glycol). Data represents the mean ± SD of at least three independent experiments (*$P < 0.05$ from 10 % DMSO).

One mechanism by which ice causes damage to cells is by increasing the concentration of ions in solution to damaging levels. We hypothesised that removing these ions from solution would mitigate this form of damage. An experiment was performed to verify

this, using the protocol above, under fast thaw/fast freeze conditions. We found that RBCs frozen with 300 mM trehalose had increased recovery when the carrier solution was water rather than DPBS (65 % vs 46 %) (Figure 3.6). The absence of ions in the water does not cause osmotic bursting of the cells as the extracellular trehalose in the cryopreservation solution maintains osmotic balance.

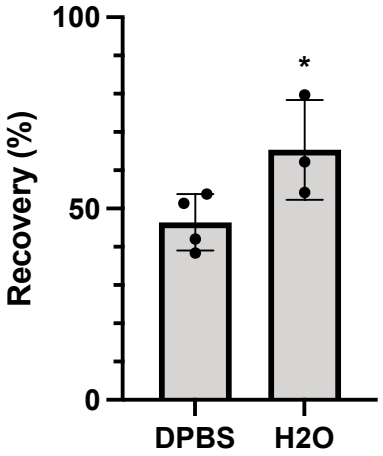

Figure 3.6. The percentage recovery of RBCs after being rapidly frozen in liquid nitrogen and thawed for 5 minutes at 37 °C. Prior to freezing, RBCs were incubated for 10 minutes with 300 mM trehalose in DPBS or water. Data represents the mean ± SD of at least three independent experiments (*$P < 0.05$ from DPBS).

The results above (Figure 3.5) demonstrated that is it possible to achieve high recovery regardless of freezing rate. We, therefore, decided to use the most challenging set of conditions for blood (slow freeze/slow thaw), to test all combinations of cryoprotectants. In this final series of cryopreservation experiments, cryovials were cooled at – 1 °C min^{-1} to – 80 °C, and warmed at room temperature until thawed. Under these conditions, the effectiveness of 10 % DMSO was reduced from 81 % (fast freeze/fast thaw), to 54 % (slow freeze/slow thaw) (Figure 3.4 vs Figure 3.6). Here,

we tested every possible combination of the four cryoprotectants. We also tested PaDT and PaDTP with DPBS substituted for water. When cryopreserving RBCs using the fast freeze/fast thaw method, the most effective solution using DPBS as a carrier was PaDP (92 %). The most effective solution using water as a carrier, and the most effective solution overall, was PaDT-H_2O (100 mg ml^{-1} polyampholyte, 10 % DMSO, 100 mM trehalose, in water) (97 %) (Figure 3.7). Of the individual cryoprotectants, DMSO has the greatest cryoprotective effect, and was an essential component in these solutions. Adding trehalose and polyampholyte enhanced the cryoprotective effect of DMSO, almost doubling recovery, but we were unable to achieve this cryoprotective effect without it, likely because DMSO is needed to prevent ice formation in the intracellular space and/or because it is needed to protect the cell from ice and cryoprotectant induced dehydration (the solute effect).

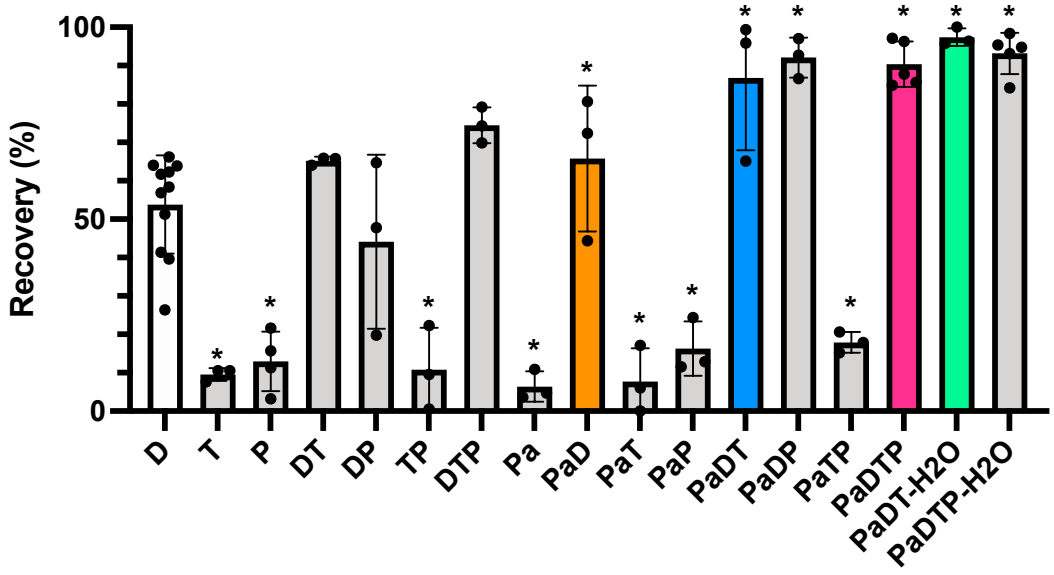

Figure 3.7. The percentage recovery of RBCs after being slowly cooled at – 1 °C min^{-1} to – 80 °C and slowly warmed at room temperature. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant. Pa = 100 mg mL^{-1} polyampholyte, D = 10 % DMSO, T = 100 mM trehalose, P = 100 mg mL^{-1} poly(ethylene glycol). Data represents the mean ± SD of at least three independent experiments (*P < 0.05 from 10 % DMSO).

3.4.3 Cytotoxicity

Having developed an effective cryoprotectant solution, we evaluated its cytotoxicity and the toxicity of each combination of its components. To test this, 1000 µL cryoprotectant was used to resuspend a pellet made from 500 µL of blood. The blood was then incubated with cryoprotectant for up to 1 hour. At the one-hour time point, only PaD caused significant toxicity (92 % recovery). The addition of trehalose or poly(ethylene glycol) to the PaD mixture (PaDT or PaDP) resulted in a mixture that

was not significantly toxic at the 1-hour time point (Figure 3.8). DMSO is believed to cause toxicity at low concentrations.[53] This was not detectable here when DMSO was used alone, but was apparently exuberated by the presence of polyampholyte. The addition of trehalose or poly(ethylene glycol) seems to neutralise this effect; poly(ethylene glycol) is known to stabilise proteins[54] and so like trehalose,[55] may also have this effect on the cell surface membrane. This demonstrates effective cryoprotectant toxicity neutralisation[56] and shows that the most effective solution, PaDT-H_2O, is not toxic after 1-hour exposure at room temperature. We also found no progressive toxicity when measured at the 15, 30, 45, and 60 minute time points (see Figure 3.9). This also demonstrates that the haemolysis which occurred during freezing experiments was due to freezing damage rather than cryoprotectant toxicity.

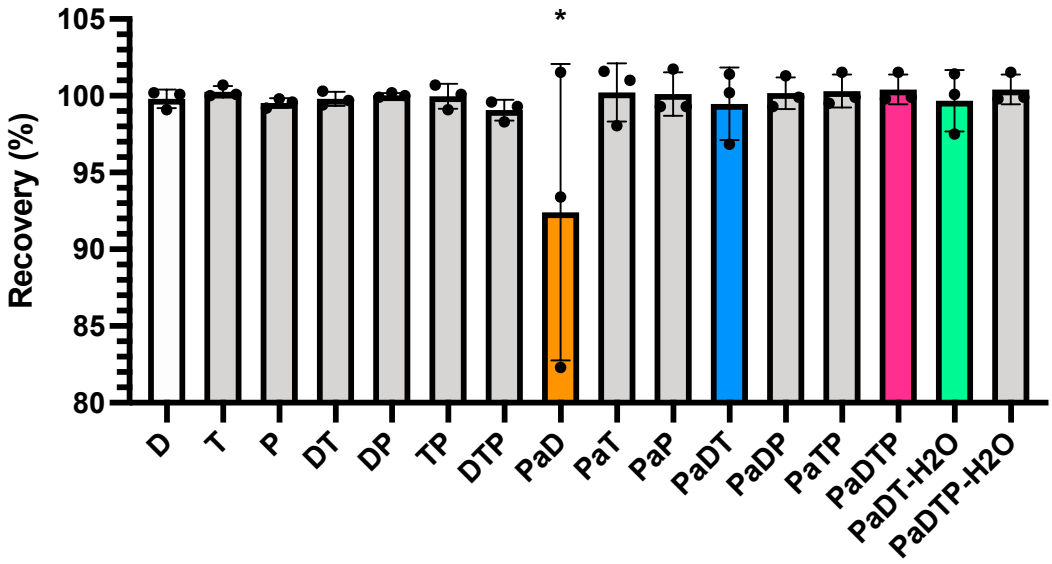

Figure 3.8. The percentage recovery of RBCs after being incubated with cryoprotectant for 1 hour. Pa = 100 mg mL^{-1} polyampholyte, D = 10 % DMSO, T = 100 mM trehalose, P = 100 mg mL^{-1} poly(ethylene glycol). Data represents the mean ± SD of three independent experiments. (*P < 0.05 from 10 % DMSO).

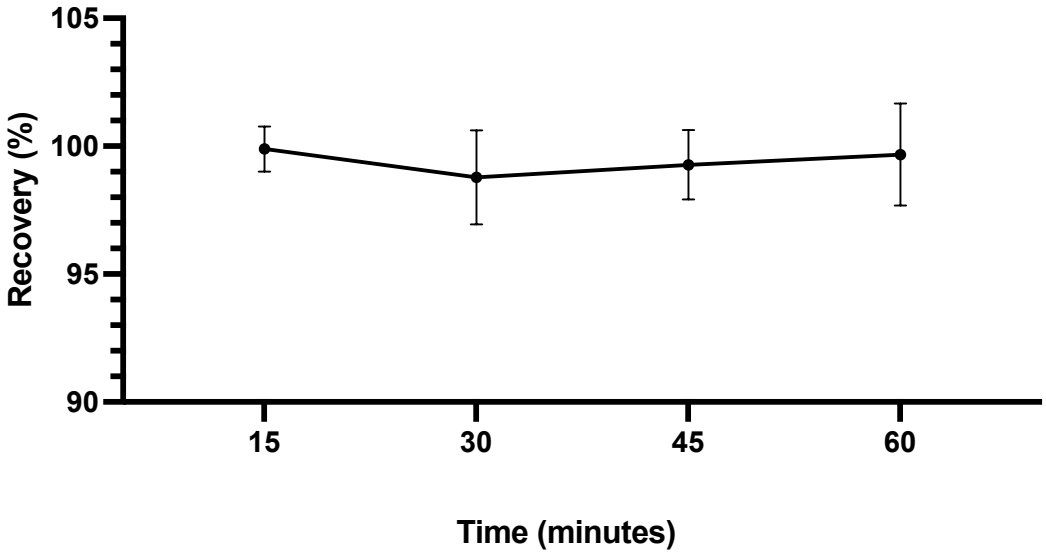

Figure 3.9. The percentage recovery of RBCs at various time points while being incubated with PaDT-H_2O (100 mg ml^{-1} polyampholyte, 10 % DMSO, 100 mM trehalose, in water). Data represents the mean ± SD of three independent experiments. There was no statistically significant decrease in cell recovery over the one-hour time period.

3.4.4 Post-washout recovery

For blood to be used clinically, cryoprotects must be removed from the blood post-thaw. Thus, we wanted to assess the post-washout viability of RBCs after cryopreservation with PaDT-H_2O. After thawing RBCs at room temperature, the cryoprotectant was removed using four 1-minute centrifugation steps. After each centrifugation, the supernatant was collected and replaced with a lower concentration of the cryoprotectant solution, without polyampholyte, using Alsever's solution as the carrier. Cryoprotectant concentrations at each step were 80 %, 40 %, 20 %, and 0 % respectively (with 100 % being 10 % DMSO + 100 mM trehalose). Washout solutions

were initially chilled to 4 ºC before usage to minimise cryoprotectant toxicity, but that resulted in a large amount of haemolysis. Therefore, the washout process was kept as close to 37 ºC as possible by pre-warming the solutions and the thawed RBCs. We hypothesise that this enabled DMSO to quickly exit the RBCs, thus reducing the equilibrium time needed for DMSO washout. Polyampholyte was not present in any of the washout solutions as its presence results in higher haemolysis during washout, and polyampholyte does not enter the cell, so a gradual reduction of its concentration during washout is not needed. This also demonstrates that the red blood cells remain intact without further stabilisation by polyampholyte. The final post-washout recovery was 80 %. This value was obtained by combining post-thaw haemolysis with the haemolysis caused by each of the four wash steps. For comparison, pre-washout recovery values (immediate post-thaw recovery from previous experiments) are shown for DMSO and PaDT-H_2O, which are 54 % and 97 % respectively (Figure 3.10).

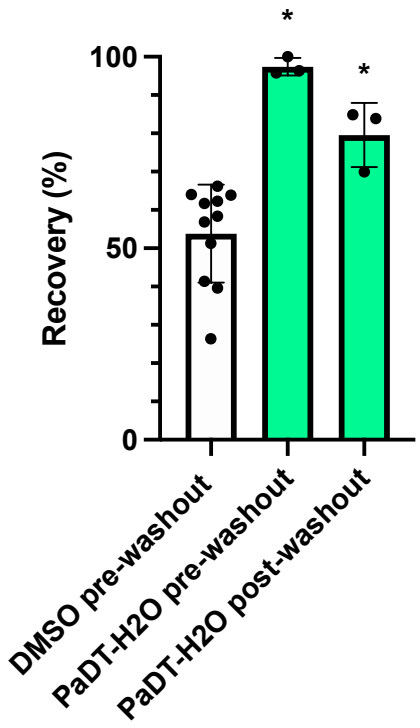

Figure 3.10. The pre- and post-washout percentage recovery of RBCs after being slowly cooled at – 1 °C min^{-1} to – 80 °C and slowly warmed at room temperature. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant (10 % DMSO, 100 mM trehalose and 100 mg mL^{-1} polyampholyte in water). "Pre-washout" indicates immediate post-thaw recovery, "post-washout" indicates recovery after cryoprotectant removal and suspension in Alsever's solution. Data represents the mean ± SD of at least three independent experiments. (*$P < 0.05$ from pre-washout).

3.4.5 Osmotic fragility

As a further quality control measure, we used an osmotic fragility assay to determine the resistance of the blood to osmotic shock after thawing. Blood was resuspended in different concentrations of NaCl solution: (1, 0.5, 0.25, 0.063, 0.031, 0.016, and 0 M (water)). Results show that the lysis threshold for haemolysis is the same in the post-

washout and control samples, suggesting no loss of integrity. Note that control lysis is higher because RBCs had already been lost from the post-washout group during washout (Figure 3.11).

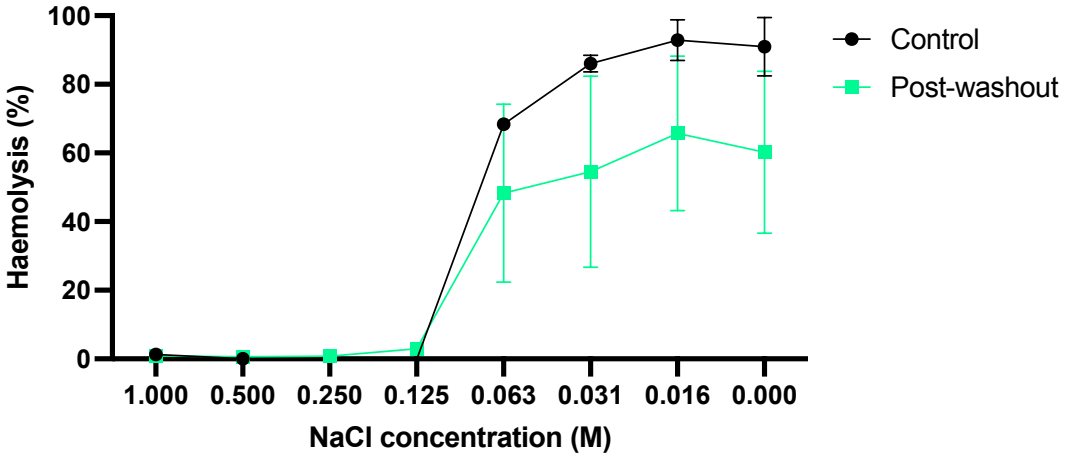

Figure 3.11. Percent haemolysis of RBCs after being suspended in NaCl solutions of different concentrations. Data represents the mean ± SD of at least three independent experiments.

3.4.6 Flow cytometry and phase contrast microscopy

Flow cytometry (Figure 3.12a-d) was used to assess RBC morphology as an assessment of post freeze-thaw-wash health. Optical complexity (side scatter) is increased in crenated cells, or may indicate cellular damage which manifests in an altered cytoskeleton. Blebbing is another cause of increased side scatter, which indicates cytoskeletal damage or apoptosis.[57,58] Fresh and post freeze-wash-thaw RBCs suspended in Alsever's solution were compared against each other, and to fresh RBCs suspended in hypotonic and hypertonic NaCl solutions (0.125 M and 0.5 M respectively). Results show that fresh and post freeze-thaw-wash RBCs have very similar light scattering properties, indicating that post

freeze-thaw-wash RBCs have unchanged morphology and good post-thaw health. Likewise, phase contrast microscopy (Figure 2.12e-g) showed normal morphology. Although post-freeze-wash-thaw RBCs are slightly turgid, this is probably a result of residual DMSO, which is normal for post-wash cells.

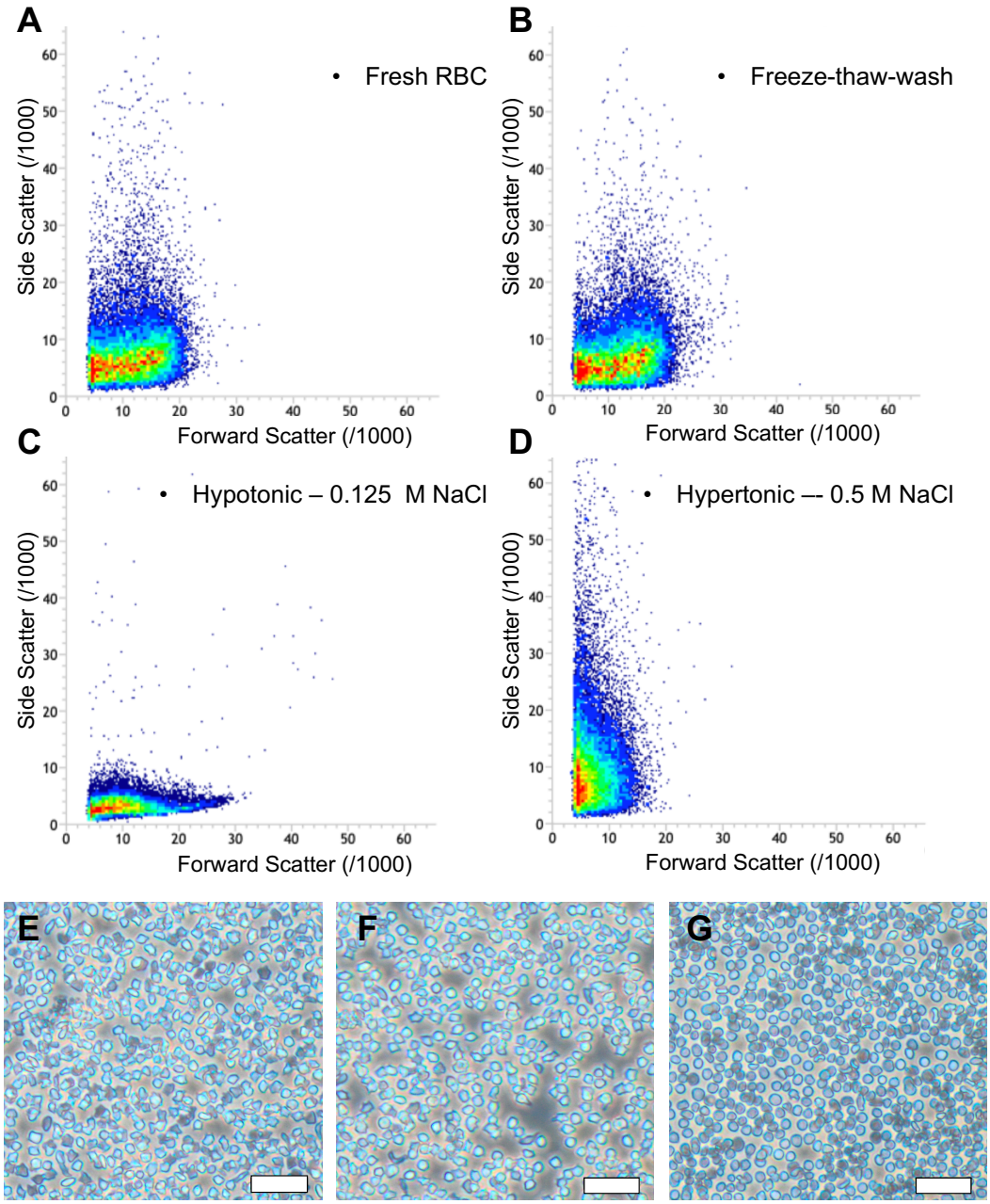

Figure 3.12. **Top.** Flow cytometry plots showing forward scatter and side scatter of **(A)** fresh and **(B)** post freeze-thaw-wash RBCs in Alsever's solution. For comparison are fresh RBCs in **(C)** hypotonic and **(D)** hypertonic conditions. **Bottom.** Phase contrast microscopy images of RBCs. **(E)** Fresh in Alsever's solution. **(F)** Fresh in DPBS. **(G)** Post freeze-thaw-wash in Alsever's solution. Scale bar represents 20 mm.

3.4.7 Confocal microscopy

Live confocal microscopy (Figure 3.13) was used to further investigate the post-thaw morphology of the RBCs. Cells were cryopreserved in PaDT-H_2O, thawed, washed, and compared to fresh non-frozen control RBCs. A higher proportion of the frozen cells were found to be crenated compared to the control. This may correspond to the minor differences in forward scatter and side scatter observed by flow cytometry. It is unknown how this would affect cell function, but this may be resolved by optimising the washing protocol.

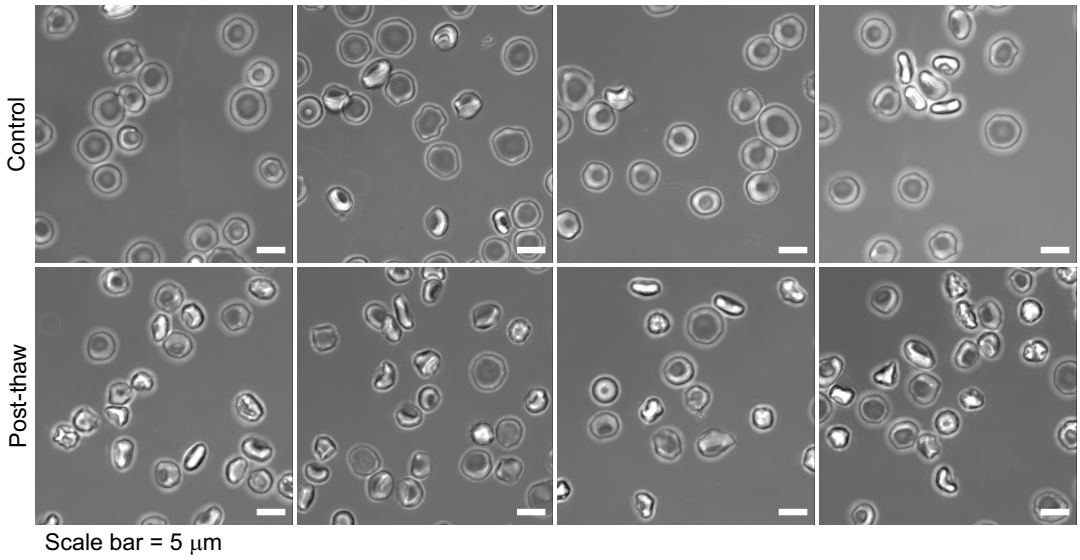

Figure 3.13. Confocal microscopy of post freeze-thaw-wash RBCs cryopreserved with PaDT-H_2O, compared to fresh RBCs. 4 representative images are presented for each group.

3.4.8 ATP assay

Post-thaw adenosine triphosphate (ATP) concentrations were compared to investigate metabolic function. ATP is necessary for RBCs to carry out homeostatic processes such as the maintenance of the cytoskeleton, membrane,

and ion transport.[59,60] ATP concentration was measured using a CellTiter-Glo® 3D Cell Viability Assay (Promega), which is a fluorescence-based luciferase assay. RBCs were cryopreserved with 10 % DMSO or PaDT-H_2O, thawed and washed. The RBCs were stored for 24 hours at 4 ºC prior to the assay to allow for regeneration and/or leakage of ATP; it is normal for ATP to be depleted directly after cryopreservation, but a failure to regenerate ATP or its leakage into the cytoplasm indicates a longer-term metabolic failure. Cryopreservation is energetically taxing as the cell expends energy to mitigate stress, thus post-thaw regeneration of ATP indicates a functional metabolic system.[61] It was found that the ATP concentration of post freeze-thaw-wash blood cryopreserved using PaDT-H_2O was similar to that of the fresh control (3.1mM vs 3.7 mM), and that these values were significantly higher than that of cells cryopreserved with DMSO-alone Figure (3.14). ATP loss in cells cryopreserved with PaDT-H_2O does not exceed haemolysis, (20 % haemolysis vs 16 % loss of ATP) therefore the loss of ATP is likely due to cell loss during cryopreservation and washout, rather than being due to a loss of metabolic function.

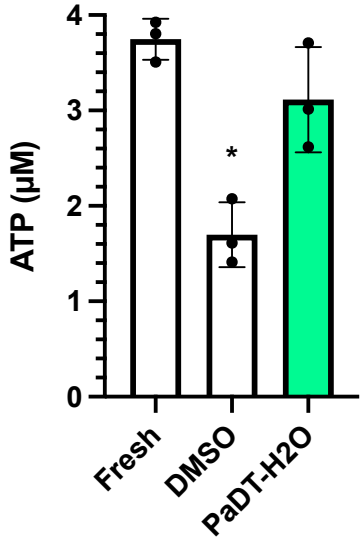

Figure 3.14. ATP quantification comparing fresh (non-frozen) blood with that cryopreserved by DMSO or optimal cryoprotectant mixture post-thaw. RBCs were cooled at – 1 °C min^{-1} to – 80 °C and slowly thawed in air at room temperature before cryoprotectant washout and storage at 4 ºC for 24 hours. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant (10 % DMSO or 10 % DMSO, 100 mM trehalose and 100 mg mL^{-1} polyampholyte in water). Data is for total ATP in the samples, not normalised to cell recovery. Data represents the mean ± SD of at least three independent experiments. (*P < 0.05 from the fresh control).

3.4.9 Repeated freezing

The robustness of our solution was then tested using a second freeze/thaw cycle. RBCs were frozen in liquid nitrogen and thawed in a 37 ºC water bath, then cryoprotectant was removed and replaced with fresh cryoprotectant, and the RBCs were frozen in liquid nitrogen a second time. After the second freezing, this yielded 90 % post-thaw recovery and 75 % recovery after washout. This is only 5 % less than the post freeze-

thaw-wash recovery for RBCs frozen only once. This is further evidence that the integrity of the RBCs is maintained using our solution. Differences between groups here were not statistically significant.

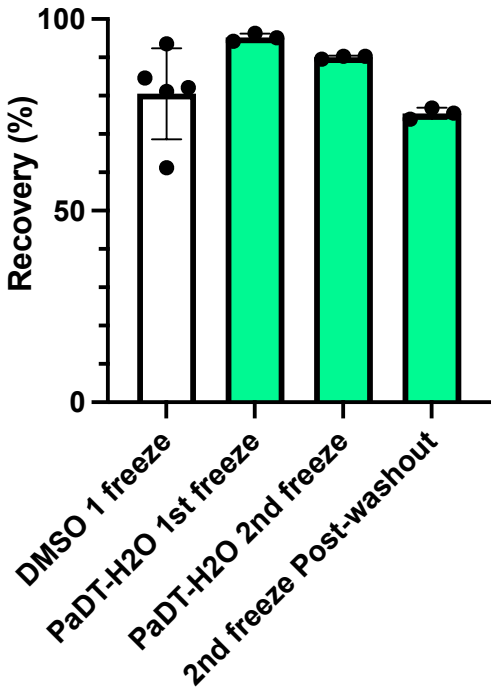

Figure 3.15. The percentage recovery of RBCs after being rapidly frozen in liquid nitrogen and thawed for 5 minutes at 37 °C, frozen and thawed a second time, and after cryoprotectant was washed out. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant. Pa = 100 mg mL^{-1} polyampholyte, D = 10 % DMSO, T = 100 mM trehalose. Data represents the mean ± SD of at least three independent experiments.

3.4.10 High volume recovery

To establish the viability of our cryoprotectant solution for clinical use, 400 mL blood was frozen in PVC blood bags (Praxisdienst). The bags were cooled at an uncontrolled rate in a – 80ºC freezer to – 80ºC, then heated in a 37 ºC water bath

until thawed. This yielded 85 % post-thaw recovery, and a post freeze-wash-thaw recovery of 83 % (Figure 3.16). Surprisingly, there was minimal haemolysis from washout. This recovery is higher than the 80 % minimum standard required by the American Association of Blood Banks,[63] and demonstrates the potential for clinical use.

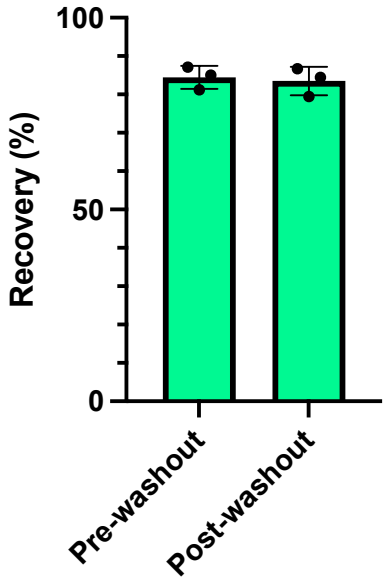

Figure 3.16. The percentage recovery of RBCs after 400 mL of blood was placed into blood bags and cooled at an uncontrolled rate to – 80 ºC, and slowly warmed to 37 ºC in a water bath. A sample was taken from each bag, the cryoprotectant removed, and the sample resuspended in Alsever's solution. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant (100 mg mL^{-1} polyampholyte, 10 % DMSO, 100 mM trehalose, 100 mg mL^{-1} poly(ethylene glycol). Data represents the mean ± SD of three independent experiments.

3.4.11 Differential scanning calorimetry

The mechanism of action of polyampholyte is unclear,[45] but it has only a small impact on ice formation and ice growth. To determine whether vitrification of the

cryoprotectant solutions was occurring, solutions were analyzed using differential scanning calorimetry (DSC). It was found that ice forms in each cryoprotectant solution when cooled at 10 ºC min^{-1}, ruling out vitrification at the experimental 1 ºC min^{-1} cooling rate (Figure 3.17). The frozen faction was smallest in solutions with multiple components. These solutions were also the top-performing formulations for cryopreservation, suggesting that the reduction of ice formation is important for cryopreservation. Polyampholyte and trehalose have only weak ice recrystallisation inhibition activity[45,64] but may still reduce ice formation through colligative interference and water-binding. One proposed mechanism of action of DMSO is the reduction of ice crystal size.[65]

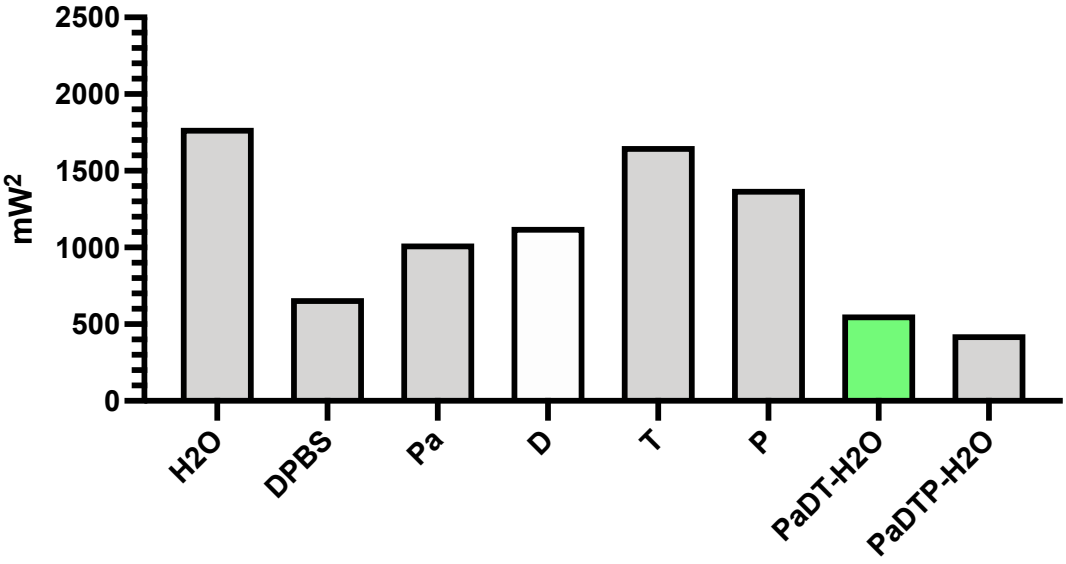

Figure 3.17. Differential scanning calorimetry analysis of cryoprotectant solutions. 40 µL samples were analyzed and the data presented is the integration of the peak corresponding to ice formation during cooling at 10 ºC min^{-1}. The formulation used for large-volume freezing, PaDT-H_2O, is indicated in green, 10 % DMSO is indicated in white. Pa = 100 mg mL^{-1} polyampholyte, D = 10 % DMSO, T = 100 mM trehalose, P = 100 mg mL^{-1} poly(ethylene glycol).

3.5 Conclusion

In this study, we explored the synergy between cryoprotectants with different mechanisms of action. A high throughput assay was developed to generate high-resolution data on the concentration-dependent cryoprotective effects of DMSO and trehalose. We found that there was an optimal "hotspot" at 200 mM trehalose, 7 % DMSO, whereby adjusting the concentrations only decreased the effectiveness of the solution. We now have a high throughput assay for determining the recovery of RBCs that can be adapted and applied to nucleated cells. This is used in **Chapter 5** for the cryopreservation of Jurkat cells.

Through the process of exploring cryoprotectant synergy, a highly effective multi-component cryoprotectant solution was developed. This solution allowed for 84 – 97 % recovery of RBCs (depending on volume) even under freezing and thawing rates that we found to be suboptimal. This is comparable to current glycerol-based solutions, which result in recoveries of around 85 %.[66] Post-washout recovery was lower (80 %), but still meets the requirements for clinical use.[63] Our washout protocol is limited by equipment, and it is expected that post-washout recovery would be much higher using a medical cell processing device,[67] which would enable the washout of cryoprotectant via linear reduction of its concentration in solution, drastically reducing osmotic stress from washout. The removal of glycerol from blood using this method takes 40 minutes,[66] in which time a patient may die if no other blood is available. Conversely, our DMSO-based solution can be removed with minimum equilibration time; we were able to resuspend RBCs in Alsever's solution, with no deliberate incubation time at

all. Our solution did not exhibit significant toxicity, as no haemolysis was detected after incubation for 1 hour.

3.6 Experimental

3.6.1 Materials

Dimethyl sulfoxide (DMSO) (analytical reagent grade), poly(methyl vinyl ether-alt-maleic anhydride) average Mn ≈ 80 000 Da (impurities: < 2 % benzene) and Dulbecco's phosphate-buffered saline (1.15 g L^{-1} dibasic sodium phosphate, 0.2 g L^{-1} potassium chloride, 8 g L^{-1} sodium chloride, and 0.2 g L^{-1} monobasic potassium phosphate) (DPBS) was from Sigma-Aldrich. Trehalose dihydrate (purity > 98 %) was from Carbosynth. Poly(ethylene glycol) (PEG) average Mn = 4,000 g mol^{-1} was from Aldrich. 2-dimethylaminoethanol (purity 99 %) was from Acros Organics. Tetrahydrofuran (THF) (laboratory reagent grade) was from Merk. BD FACSFlow sheath fluid was from BD biosciences. Sheep's blood in Alsever's solution (not defibrinated) was from TCS Biosciences. Polyampholyte was synthesised in-house in several batches as described below. Milli-Q ultra-pure water (> 18.2 MΩ cm^{-1} 2 ppb) was used throughout.

3.6.2 Nuclear magnetic resonance spectroscopy

^{1}H NMR were recorded on a Bruker DPX-400 at 298 K. Coupling constants (J) are reported in hertz (Hz). Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet. Chemical shifts are quoted in parts per million (ppm), with the residual solvent as the internal standard.

3.6.3 Differential scanning calorimetry

DSC was carried out using a DCS 1 STAR System (Mettler Toledo). In these experiments, 40 μL of sample was added to the pan, and cooled at 10 ºC min^{-1} from 20 ºC to – 100 ºC, before warming at 10 ºC min^{-1} back to 20 ºC. These cooling and warming steps were repeated, and measurements were taken during the second cooling/warming cycle.

3.6.4 Flow cytometry

Flow cytometry was performed using a BD Influx cell sorter (BD Biosciences). 20,000 events were recorded per sample. The nozzle used was 100 μm in diameter. The sheath pressure was 20 psi, and the sample pressure was approximately 21 psi. The voltage settings were configured such that normal RBCs appear at low values for forward and side scatter. To avoid clogging the machine, 100 μL blood was diluted with 10 mL Alsever's solution or the desired NaCl solution. Data was analysed using BD FACS Sortware software.

3.6.5 Polyampholyte synthesis

Polyampholyte – poly(vinyl ether -alt- maleic acid mono(dimethylamino ethyl)ester) – was produced in several batches. Typically, poly(methyl vinyl ether -*alt*- maleic anhydride) (10 g) (average $M_n \approx 80\,000$ Da), was dissolved in THF (100 mL) at 50 °C in a 500 mL conical flask with a magnetic stirrer. 2-dimethylaminoethanol (11 g, excess) was then added, resulting in the formation of a waxy pink solid in a purple solution. Water (100 mL) was added, and the reaction was left overnight at 50 °C while stirring, resulting in a yellow solution. The product was purified by dialysis

(Spectra/Por, 12 – 14 kDa MWCO), and dried by sublimation resulting in the formation of a light-yellow solid.

^{1}H NMR (D_2O): δ 1.6 (CH_2) 2H s; 2.8 (NCH_3) 6H s; 3.2 (NCH_2) 2H t; 3.3 (C*H*) 3H m, 3.9 (OCH_2) 2H t; 4.4 (OC*H*) 1H s.

3.6.6 Blood preparation

Ovine blood (10 mL) in Alsever's solution (TCS Biosciences, UK) was transferred into a centrifuge tube and centrifuged at 367 × g for 5 minutes (Spectrafuge 6C, Labnet). The supernatant was discarded and replaced with 7 mL DPBS, and mixed by inversion, for a final haematocrit of 30 %.

3.6.7 High throughput freezing and AHD assays

After blood preparation, a liquid handling robot (Pipetmax, Gilson) was used to mix 50 µL of two different 2× concentration cryoprotectants for a final volume of 100 µL, in each well of a 96 well plate, leaving the edge wells empty to avoid edge effects during freezing. The liquid handling robot then mixed 100 µL ovine blood with the cryoprotectants for a final volume of 200 µL per well. The plate was incubated for 10 minutes before being rapidly cooled in liquid nitrogen vapour. After at least 20 minutes, the plate was removed from the vapour and rapidly warmed in a 37 °C water bath for 5 minutes. The positive control (DPBS) and negative control (lysate) (100 µl blood, 100 µl lysis buffer) were added at this time. The plate was then centrifuged at 2,250 × g for 5 minutes (5804R, Eppendorf), and the AHD assay was conducted as follows: 8 µl of the resulting supernatant from each well was transferred to 100 µl AHD solution (100 mL water, 2.5 g X-100, 0.5 g NaOH) in a 96 well plate. Each well

was then stirred with the end of a spatula to ensure mixing. Absorbance of the AHD plate was read at 580 nm. Recovery was calculated as: $(1 - (\text{Absorbance} - \text{DPBS}/(\text{lysate} - \text{DPBS}))\times 100$. Lysis buffer was 0.32 M sucrose, 5 mM $MgCl_2$, 10 % w/v triton X-100, 10 mM tris HCl pH 7.8.

3.6.8 Vial freezing assay

After blood preparation, 500 µl blood was transferred to centrifuge tubes and centrifuged for 5 minutes at 2000 × g (Prism Mini, Lab net). The pellet was mixed with 1000 µl cryoprotectant solution, transferred to cryovials, and incubated for 10 minutes. After incubation, cryovials were plunged into liquid nitrogen or inserted into a cryovial cooler (Coolcell LX, Corning), and cooled at – 1 °C min^{-1} for at least 2 hours. Vials were warmed in a 37 °C water bath for 5 minutes or at room temperature until thawed. The blood was then centrifuged for 5 minutes at 2000 × g (Prism Mini, Lab net). The AHD assay was carried out as above, except that 40 µl of supernatant from each sample was transferred to 750 µl AHD solution.

3.6.9 Haemolysis assay calibration

To verify that the AHD haemolysis assay was working correctly, known quantities of lysed blood were added to DPBS or to DPBS containing 100 mg mL^{-1} PEG, and an AHD assay was conducted. We found that our method of estimating haemoglobin present in solution is accurate. The addition of 100 mg mL^{-1} PEG (which can precipitate haemoglobin at higher concentrations) does not interfere with results (Figure 3.18).

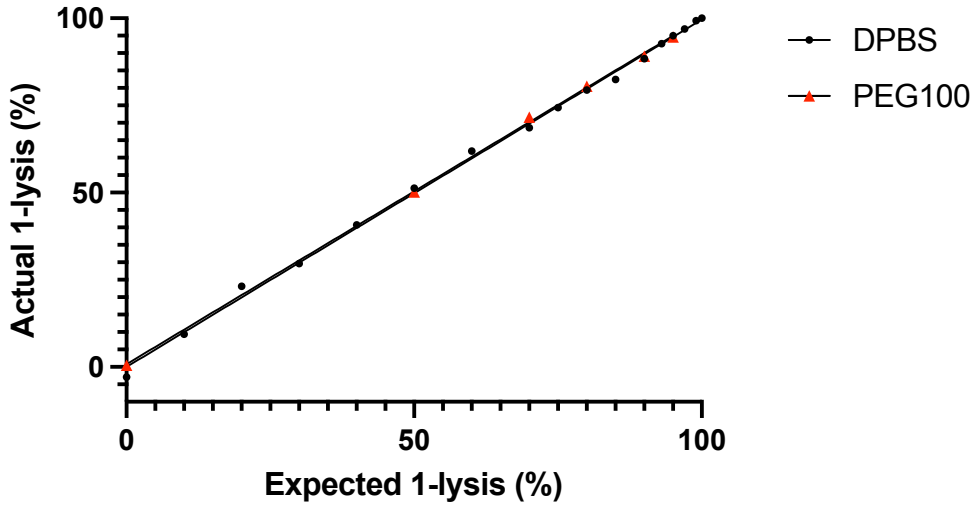

Figure 3.18. Experimental haemolysis values obtained after adding a known volume of lysed blood to DPBS or 100 mg mL^{-1} poly(ethylene glycol). The X-axis shows the blood lysate concentration, which is directly proportional to the amount of blood lysate that would be in the solution for a given percentage of haemolysis. The Y-axis shows the experimental haemolysis equivalent value obtained.

3.6.10 Toxicity assay

After blood preparation, 500 µl blood was transferred to centrifuge tubes and centrifuged for 5 minutes at 2000 × g (Prism Mini, Lab net). The pellet was mixed with 1000 µl cryoprotectant solution, transferred to cryovials, and incubated for 15, 30, 45, or 60 minutes. The blood was then centrifuged for 5 minutes at 2000 × g (Prism Mini, Lab net). The AHD assay was carried out with 40 µl of supernatant from each sample transferred to 750 µl AHD solution.

3.6.11 Washout

Thawed blood was transferred into centrifuge tubes and centrifuged for 1 minute at 2000 × g. The pellet was then resuspended in pre-warmed (37 ºC) 80 % DT cryoprotectant solution (10 % DMSO, 100 mM trehalose, diluted using Alsever's solution, and without polyampholyte). The blood was then centrifuged again and resuspended in 40 % DT cryoprotectant solution. This was repeated for 20 % cryoprotectant solution. Finally, the blood was resuspended in Alsever's solution. Blood was then centrifuged a final time to collect any remaining free haemoglobin. The haemolysis resulting from each step was added together to give a final percentage recovery.

3.6.12 Osmotic fragility assay

After the cryoprotectant washout, blood was centrifuged for 1 minute at 2000 × g (Prism Mini, Lab net). The pellets were resuspended in 1000 µl NaCl solution, at concentrations of: 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, and 0 M. Then an AHD assay was performed. NaCl solutions were prepared by dilution in serial dilution in deionised water.

3.6.13 High-volume freezing assay

PVC blood bags (Praxisdienst) were washed to remove the included anti-coagulant solution. After blood preparation, 200 mL 2× concentrated cryoprotectant solution was added to 200 mL blood and transferred to the bags, which were frozen in a – 80 ºC freezer to – 80 ºC. Bags were then removed and thawed in a 37 ºC water bath. 1 mL samples were taken, washed, and the AHD assay was carried out as above.

3.6.14 Luciferase assay

5 μL of recovered RBCs were added to 95 μL Alsever's solution in a 96 well plate. 100 μL CellTiter-Glo® 3D Reagent (Promega) was added, and the solution was thoroughly mixed. The solution was then left at room temperature for 30 minutes. A plate reader (Synergy HT, BioTek) was then used to measure luminescence (visible light ~740 to ~380 nm). Background noise was controlled for by subtracting the luminescence value of samples that were incubated with Alsever's solution. The standard curve was produced by adding 0.011 g adenosine 5'-triphosphate disodium salt hydrate (Thermo Scientific) to 1 L of distilled water which was diluted by serial dilatation in a 96 well plate. The luciferase assay was conducted as above. The initial 20× dilution of the original samples was accounted for when calculating the final ATP concentration, with the presented value representing the ATP concentration of the undiluted sample.

3.6.15 Confocal microscopy

Live RBC confocal imaging was conducted with a Zeiss LSM 710 inverted microscope, with a multichannel spectral imaging detector and three photomultiplier detectors (GaAsP, multialkali and BiG.2). RBCs were diluted 500-fold and placed on glass bottom dishes (MatTek) (No. 1.5, 35 mm). Brightfield images were obtained with a C-Apochromat 63x/1.20 W Korr M27 objective lens and x3.0 zoom. Z-stacks were spaced z = 0.3 μm apart with the whole cell being imaged. Image collection and processing was conducted using Zeiss ZEN (black edition) 2.3 lite and utilised maximum intensity projection processing.

3.6.16 Statistics

Statistical analysis was performed using Prism 8 software (GraphPad). Statistical significance was determined by ordinary one-way ANOVA using Dunnett's test for multiple comparisons, except for experiments where only two conditions were compared, in which case an unpaired t-test was used. P values below 0.05 were considered to be statistically significant.

3.7 References

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3.8 Appendix

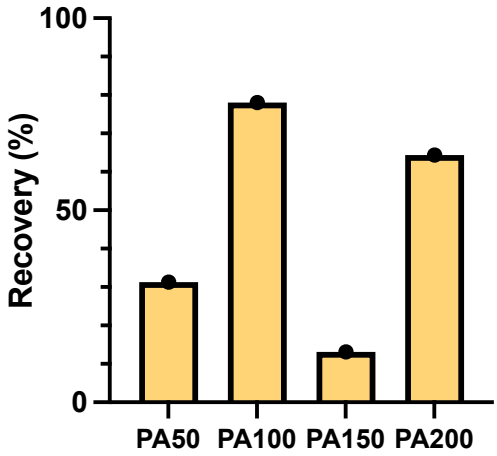

Figure 3.18. Optimisation of polyampholyte concentration. The percentage recovery of RBCs after being rapidly frozen in liquid nitrogen and thawed for 5 minutes at 37 °C. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant. The optimal concentration of polyampholyte in the absence of other cryoprotectants was 100 mg mL^{-1}, yielding 78 % recovery. Later experiments would reveal that the effectiveness of polyampholyte is highly variable and did not usually give recovery rates this high. However, PaDT gave such good results that no further optimisation was needed.

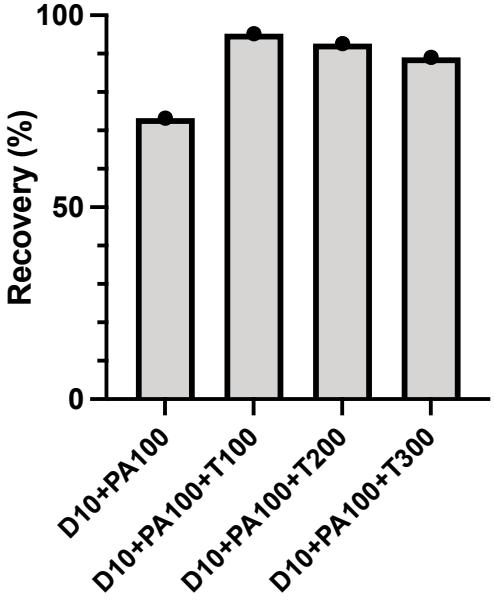

Figure 3.19. Optimisation of trehalose concentration. The percentage recovery of RBCs after being rapidly frozen in liquid nitrogen and thawed for 5 minutes at 37 °C. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant. The optimal concentration of trehalose combined with 10 % DMSO and 100 mg mL^{-1} polyampholyte was 100 mg mL^{-1} trehalose, yielding 95 % recovery.

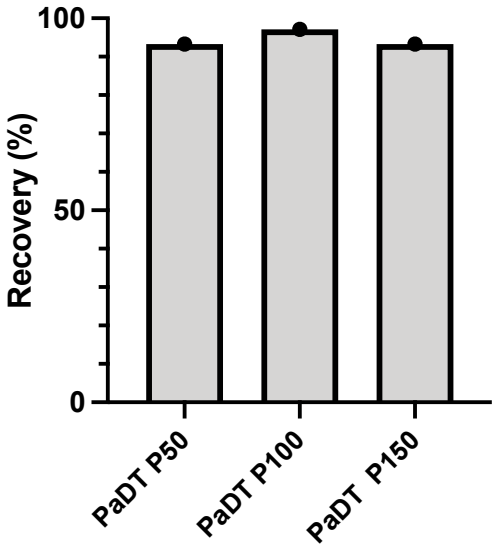

Figure 3.20. Optimisation of PEG concentration. The percentage recovery of RBCs after being rapidly frozen in liquid nitrogen and thawed for 5 minutes at 37 °C. Prior to freezing, RBCs were incubated for 10 minutes with cryoprotectant. The optimal concentration of PEG combined with 10 % DMSO, 100 mg mL^{-1} polyampholyte, and 100 mg mL^{-1} trehalose was 100 mg mL^{-1} PEG, yielding 97 % recovery.

	D0	D1	D2	D3	D4	D5	D6	D7	D8
	18.8	28.5	27.3	31.6	39.1	47.3	48.0	57.0	57.0
T0	-	6.7	24.4	27.8	38.3	47.6	52.7	59.8	67.0
	10.8	63.8	32.2	33.3	39.3	50.3	54.8	58.5	65.7
	21.1	19.1	24.6	32.4	42.6	51.2	60.9	66.4	67.2
T50	13.5	22.8	29.5	30.8	43.0	55.6	56.9	68.3	70.4
	12.3	57.8	55.1	43.5	51.8	65.3	65.3	66.4	74.7
	21.5	21.9	27.0	24.6	43.0	51.2	57.8	64.5	55.9
T100	31.2	41.7	36.2	40.9	50.1	61.9	60.7	71.2	70.4
	12.7	51.0	28.1	35.2	46.5	59.6	70.2	75.1	76.2
	15.6	23.4	15.2	31.6	39.1	31.3	55.9	67.6	71.9
T200	11.4	30.3	34.1	50.1	53.9	62.4	65.7	72.9	66.6
	29.6	54.8	45.7	56.3	60.8	56.3	60.0	72.8	67.2
	19.9	21.1	22.3	28.1	25.0	50.0	52.0	66.4	72.7
T250	22.8	25.3	40.0	52.7	58.1	60.7	66.6	70.4	66.6
	33.3	48.7	52.1	57.0	64.5	66.0	73.6	71.7	72.4
	25.4	22.3	19.5	26.2	34.8	50.0	51.2	56.6	70.3
T300	46.8	51.0	51.0	53.9	59.8	59.4	61.9	57.3	70.8
	43.9	50.3	52.9	64.2	69.0	65.3	78.4	78.8	67.9

	D0	D1	D2	D3	D4	D5	D6	D7	D8
T0	9.8	33.0	28.0	30.9	38.9	48.4	51.8	58.5	63.2
T50	15.6	33.2	36.4	35.6	45.8	57.4	61.0	67.0	70.7
T100	21.8	38.2	30.4	33.6	46.5	57.6	62.9	70.2	67.5
T200	18.9	36.2	31.7	46.0	51.3	50.0	60.5	71.1	68.5
T250	25.3	31.7	38.1	45.9	49.2	58.9	64.0	69.5	70.6
T300	38.7	41.2	41.1	48.1	54.5	58.2	63.9	64.3	69.7

Figure 3.21. High throughput cryoprotectant screening showing individual datapoints from Figure 3.3 **(top)** and mean RBC cell recovery **(bottom)**. X-axis labels indicate final DMSO concentration (% v/v, e.g., D1 = 1 % DMSO). Y-axis lab labels indicate final trehalose concentration (mM, e.g., T250 = 250 mM trehalose). Recovery is reported as 100 % – haemolysis.

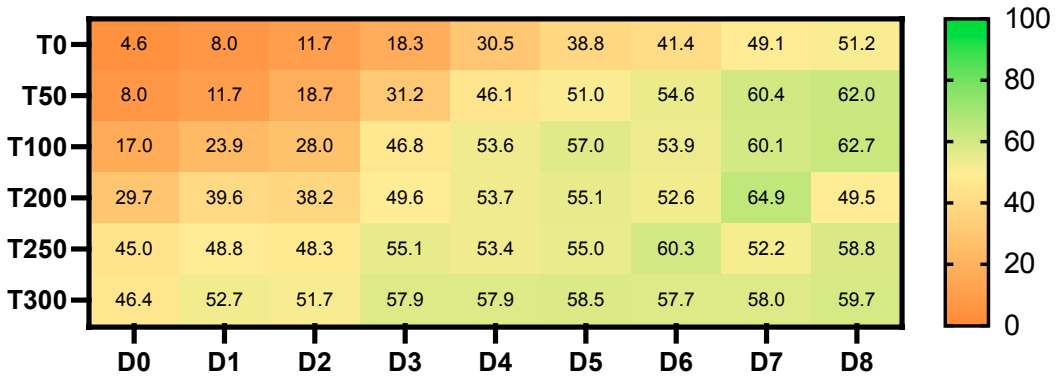

Figure 3.22. The percentage recovery of RBCs after being rapidly frozen in the liquid nitrogen vapour phase and thawed for 5 minutes at 37 °C. RBCs were cooled at a haematocrit of 21 % rather than the 30 % used in other experiments. X-axis labels indicate final DMSO concentration (% v/v, e.g., D1 = 1 % DMSO). Y-axis lab labels indicate final trehalose concentration (mM, e.g., T250 = 250 mM trehalose). Data represents the mean of two independent experiments.

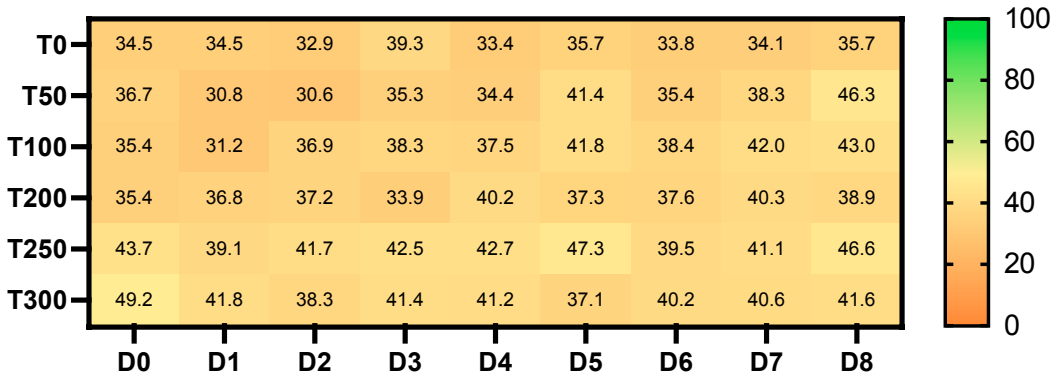

Figure 3.23. The percentage recovery of RBCs after being rapidly frozen in the liquid nitrogen vapour phase and thawed for 5 minutes at 37 °C. Prior to freezing, cells were centrifuged at 2,250 × g for 5 minutes to form a pellet. X-axis labels indicate final DMSO concentration (% v/v, e.g., D1 = 1 % DMSO). Y-axis lab labels indicate final trehalose concentration (mM, e.g., T250 = 250 mM trehalose). Data represents the mean of two independent experiments.

Figure 3.24. The percentage recovery of RBCs after being rapidly frozen in the liquid nitrogen vapour phase and thawed for 5 minutes at 37 °C. X-axis labels indicate final betaine concentration (mM, e.g., B1 = 1 mM betaine). Y-axis lab labels indicate final trehalose concentration (mM, e.g., T200 = 200 mM trehalose). (n = 1). From this we concluded that trehalose and betaine do not work synergistically, rather they exert their strongest cryoprotective effects when they are used alone. This is likely because they have a similar mechanism of action, and both increase the osmolality of the solution.

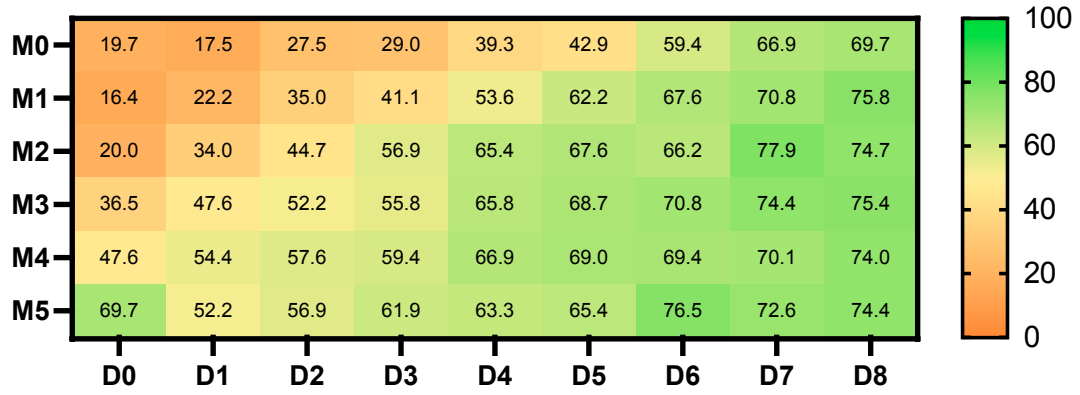

Figure 3.25. The percentage recovery of RBCs after being rapidly frozen in the liquid nitrogen vapour phase and thawed for 5 minutes at 37 °C. X-axis labels indicate final DMSO concentration (% v/v, e.g., D1 = 1 % DMSO). Y-axis labels indicate final methanol concentration (% v/v, e.g., M1 = 1 % methanol). (n = 1).

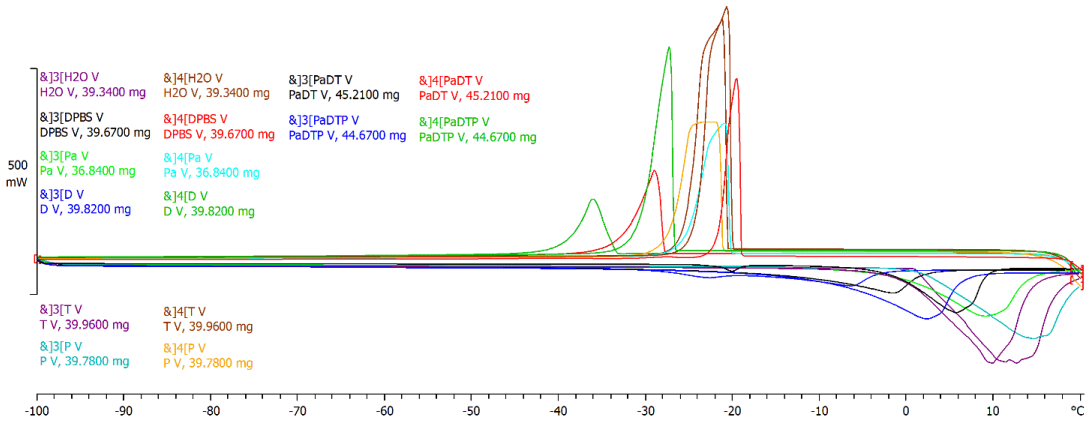

Figure 3.26. Differential scanning calorimetry traces showing heat transfer during sample cooling from 20 ºC to – 100 ºC (plotted above the X-axis) and heating from – 100 ºC to 20 ºC (plotted below the X-axis) each at 10 ºC min^{-1}. Y-axis shows heat transfer in mW^2, X-axis shows reference temperature. Measurements were taken during the second cooling/warming cycle.

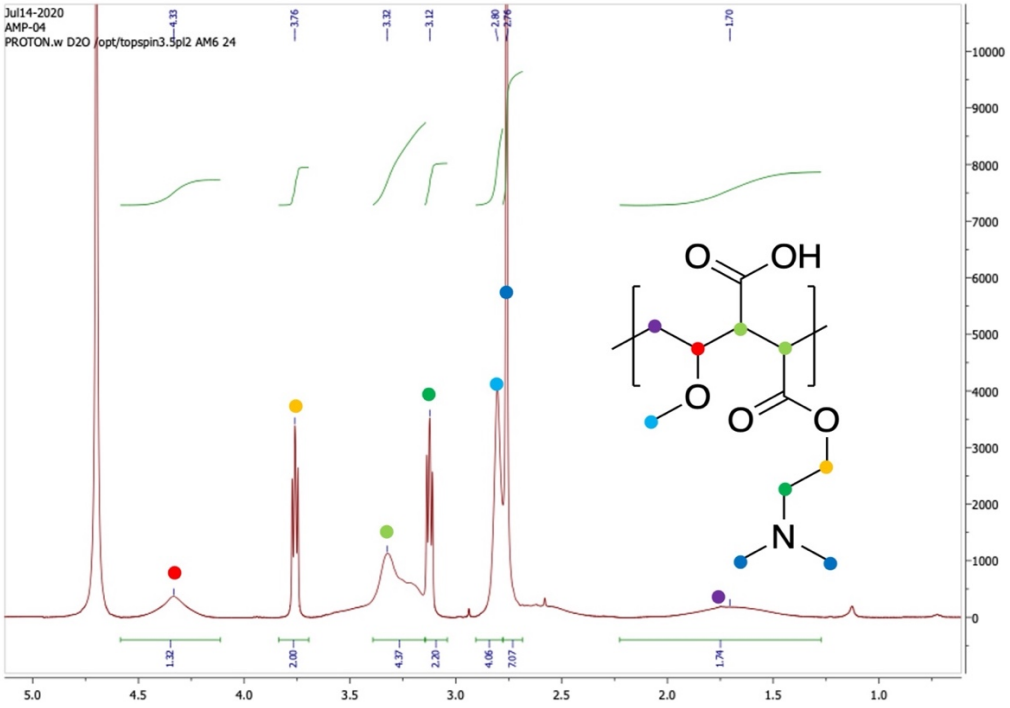

Figure 3.27. 1H NMR spectrum of polyampholyte (Bruker DPX-400 at 298 K). Chemical shifts are quoted in parts per million (ppm). The residual solvent was used as the internal standard.

Chapter 4 – Engineered Trehalose Derivatives to Promote Membrane Permeability

4.1 Abstract

Trehalose is a non-penetrating small molecule cryoprotectant. Under normal conditions, it exerts its cryoprotective effect only in the extracellular space as it is unable to cross the cell membrane. However, it is more effective when it is also present in the intracellular space. Several methods have been employed to deliver trehalose into the intracellular space, but all of these have drawbacks. One as yet unexplored method is to chemically engineer trehalose so that it is capable of crossing the cell membrane. Here we select a trehalose modification to test by assessing predicted membrane permeation and toxicity characteristics of esterified trehalose derivatives, finding trehalose-6,6'-dibutanoate to be a good candidate. We then synthesise trehalose-6,6'-dibutanoate using both synthetic and enzymatic techniques, and test trehalose-6,6'-dibutanoate for the cryopreservation of red blood cells, Jurkat cells and A549 cells. We demonstrate the ability of trehalose-6,6'-dibutanoate to cross the cell membrane using a cell swelling assay. However, we were not able to identify any conditions in which trehalose-6,6'-dibutanoate exerts a cryoprotective effect.

4.2 Disclaimer

- The Biotage Selekt automated flash column, which was used to purify some batches of trehalose-6,6'-dibutanoate, was operated by Dr Collette Guy.
- The high vacuum system used to remove residual solvent from samples was operated by Ioanna Kontopoulou and Panagiotis Georgiou.
- Much of the general workflow for the blood cryopreservation and AHD assays was optimised by Dr Christopher Stubbs.
- The cell lines were usually maintained by myself, but were occasionally maintained by Dr Ruben Tomás, Dr Kathryn Murray, Natalia Martinez, Ola Alkosti, Qiao Tang, and Ya Nan Gao.

4.3 Introduction

Trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) is a small molecule cryoprotectant which cannot penetrate the cell membrane unaided. Trehalose is hypothesised to work as a cryoprotectant by two major mechanisms: in the vitrification hypothesis, trehalose hydrogen bonds to water molecules, reordering the structure of hydrogen bonds in the solution[1] which lowers the amount of ice formed and increases the size of the unfrozen pocket where cells can survive during slow freezing.[2] In the water replacement hypothesis, trehalose binds to and stabilises proteins[3] and membranes,[4] allowing these components to survive the harsh conditions of dehydration and freezing where less water is available. Here, trehalose either binds to proteins directly as a substitute for water, traps a layer of water against the surface of the protein, or holds the proteins in a stable conformation.[5] Although cell surface proteins can be protected by trehalose in the extracellular space, the water replacement hypothesis is especially relevant where trehalose is present in the intracellular space; most proteins critical for cell function are in the intracellular space and the membrane is best protected when trehalose is present on both sides.[6] With regards to the vitrification hypothesis, intracellular trehalose helps protect against the effects of intracellular ice formation and may help reduce solute concentration damage by diluting the freeze-concentrated intracellular ions.

Several methods to transport trehalose inside the cell have been demonstrated in academic research, although all have drawbacks:[7] trehalose can be forced to cross the membrane by rapid freezing combined with a high extracellular trehalose concentration, resulting in a membrane phase change. This is known as freeze-induced

uptake.[8] Unfortunately, this requires a high freezing rate which is suboptimal for most cell types and only small amounts of trehalose can be loaded this way. Electroporation,[9] ultrasound,[10] polymers,[11] and pore-forming proteins[12] can be used to increase membrane permeability. However, increasing membrane permeability is a trade-off that allows more trehalose to be loaded, but also causes more cell death. Carrier peptides[13] bind to trehalose and carry it across the membrane, subverting the toxic effects caused by indiscriminate membrane permeabilization. Peptides are difficult to make at scale, but polymers could be used to similar effect. Trehalose can be loaded into liposomes, although this method only allows small amounts of trehalose to enter the cell.[14] Trehalose loaded into nanoparticles has been used to load high amounts of trehalose into the cell, but a long incubation time is needed.[15] Genetic engineering can be used to make cell lines which can synthesize trehalose,[16] or to produce transporter proteins or channels,[17] which allow extracellular trehalose to enter the cell. The drawback with all genetic engineering approaches is that they are labour-intensive, require specially engineered cell lines, and genetic engineering may limit the translational use of the cells due to safety or regulatory concerns. Microinjection allows for high concentrations of trehalose to be loaded into the cell, but is highly labour-intensive, making it suitable only for situations in which very low numbers of cells are needed, such as oocytes.[18] See **Chapter 2** for a full discussion of these methods.

A promising but largely unexplored approach is to chemically modify trehalose to be membrane permeable. Abazari *et al.*[19] modified trehalose with 2-8 acetyl groups attached via ester bonds. The trehalose ester then entered the cells and was metabolised

by intracellular esterases to form trehalose and acetic acid. This method was not tested for cryoprotective effectiveness, and only acetyl modifications were tested for membrane permeability. Trehalose-2,2',3,3',4,4'-hexacetate was the most successful compound tested, it entered rat hepatocytes resulting in an intracellular concentration of 300 mM after 8 hours. However, it took much longer for Trehalose-2,2',3,3',4,4'-hexacetate to be converted to trehalose once inside, reaching only around 100 mM after 12 hours.

Here we aim to design and test trehalose esters as cryoprotectants. The optimal trehalose derivative should be membrane permeable, non-toxic, and rapidly metabolised into trehalose. It should also be water-soluble without first needing to be dissolved in another solvent. One way to predict whether a molecule will cross the cell membrane is to use the octanol-water partition coefficient, which measures the ratio of a molecule's concentration between immiscible solvents. This can be determined experimentally, but can also be predicted from the molecule's physical properties such as polar surface area, hydrogen bond donor and acceptor counts, molecular volume, and charge. Much of the information relevant to the membrane permeability of molecules comes from research into drugs which cross the blood-brain barrier (BBB). In the absence of specific transporters or channels, drugs which cross the BBB must cross through the cell membrane of the endothelial cells which form the capillaries in the brain. This is necessary because the cells which make up the BBB form tight junctions, lack fenestration and engage in only low levels of pinocytosis. For the purposes of crossing the BBB, Pajouhesh and Lenz,[20] recommends that the logP of a molecule be between 0 and 3, and that MW should be below 400-600.

4.4 Results and discussion

4.4.1 Design

The first thing to consider when designing a trehalose derivative is post-metabolic toxicity, as the trehalose ester will be converted to trehalose and a carboxylic acid inside the cell. We assembled a list of carboxylic acids which would be produced by various trehalose esters. Each carboxylic acid considered is found naturally in food, and none are carcinogenic. Post-hydrolysis acetic acid had minimal toxic effects in Abazari *et al.*[19] pKa is similar between acids and slightly increases with chain length, so carbocyclic acids with higher chain lengths are unlikely to cause excessive acidity. To give a rough estimation of toxicity, we took LD50 values from the PubChem hazardous substances databank,[21] preferring mouse intravenous (IV) data where available (Table 4.1). To predict the toxicity of hypothetical trehalose derivatives, we then divided by molecular weight and multiplied by number of esterified groups (more esterified groups will result in more acid being released per trehalose molecule) to give relative predicted toxicity per mol of trehalose loaded into the cell (Table 4.2). This is summarised by the equation: $\text{Toxicity} = \left(\frac{1}{\text{LD50}} \times \text{N}\right) \times 100$ where *N* is the number of esterified groups. The LD50 of IV administration is normally much lower (i.e., more toxic) than the LD50 of oral administration, making direct comparison difficult.[22] We, therefore, didn't attempt to predict toxicity where mouse IV data was unavailable.

Table 4.1. Toxicity of carboxylic acids.

Metabolite	pKa	LD50 (mg kg^{-1})	LD50 (mmol kg^{-1})
Acetic acid	4.76	525 (Mouse IV)	8.7
Propanoic acid	4.88	625 (Mouse IV)	8.4
Butanoic acid	4.82	800 (Mouse IV)	9.1
Pentanoic acid	4.84	1290 (Mouse IV)	12.6
Hexanoic acid	4.88	1725 (Mouse IV)	13.3
Heptanoic acid	4.89	7000 (Rat oral)	-
Octanoic acid	4.89	10080 (Rat oral)	-
Benzoic acid	4.19	1940 (Mouse oral)	-

The 1-octanol water partition coefficient (logP) is the ratio of molecules in the 1-octanol and water phases of a water-octanal system. It is a measure of lipophobicity and correlates with membrane permeability. Here we use two freely available machine learning tools to predict logP of trehalose derivatives: Molinspiration[23] and ALOGPS.[24] Molinspiration's interactive logP calculator (Molinspiration property engine v2018.10) publicly available at https://www.molinspiration.com was trained using 12,000 experimental logP values for drug-like molecules. From this the hydrophobicity values for around 200 fragments were also identified, allowing the prediction of a molecule's logP from its composite fragments. The algorithm takes into account intramolecular hydrogen bonding and charge interactions. ALOGPS 2.1,[24–27] publicly available at http://www.vcclab.org/web/alogps was trained using the experimental logP values of 12,908 organic molecules from the Physical/Chemical Property Database (PHYSPORP) database.[28] The ALOGPS program takes into account electrotopological state when making predictions. We used Molinspiration and ALOGPS to predict the logP values for 37 trehalose derivatives (Table 4.2). According to Abazari *et al.*[19] trehalose-2,2',3,3',4,4'-hexacetate, with its averaged logP of -0.41 across both tools, MW of 594.52 and relative predicted toxicity of 69.0 mol^{-1} successfully penetrates the cell membrane and is converted to trehalose without damaging the cells. Pajouhesh and Lenz[29] recommend a logP between 0 and 3, and

that MW should be below 400 – 600. These values give some known positive parameters to aim for.

Table 4.2. Properties of modified trehalose compounds

Molecule	Molinspiration (LogP)	ALOGPS (LogP)	Average (LogP)	MW (g mol^{-1})	Relative predicted toxicity per mol of trehalose
Hex	3.66	4.02	3.84	86.18	-
Glycerol	-1.60	-1.93	-1.765	92.09	-
DMSO	-0.69	-1.09	-0.89	79.13	-
Trehalose	-4.30	-2.98	-3.64	342.30	-
Trehalose-6,6'-diacetate	-2.94	-1.75	-2.35	426.37	23.0
Trehalose-2,2',3,3'-tetraacetate	-1.97	-0.85	-1.41	510.44	46.0
Trehalose-2,2',3,3',4,4'-hexacetate	-1.00	0.18	-0.41	594.52	69.0
Trehaloseoctoacetate	0.41	0.82	0.615	664.57	92.0
Trehalose-6,6'-dipropanoate	-2.22	-1.31	-1.77	454.43	23.8
Trehalose-2,2',4,4'-tetrapropanoate	-0.53	0.39	-0.07	566.55	47.6
Trehalose-2,2',4,4',5,5'-hexapropanoate	1.16	1.80	1.48	678.68	71.4
Trehaloseoctopropanoate	3.29	2.85	3.07	790.81	95.2
Trehalose-6,6'-dibutanoate	-1.10	-0.81	-0.96	482.48	22.0
Trehalose-2,2',3,3'-tetrabutanoate	1.71	1.04	1.375	622.66	44.0
Trehalose-2,2',4,4',5,5'-hexabutanoate	4.52	2.95	3.735	762.84	65.9
Trehaloseoctobutanoate	7.77	4.45	6.11	903.02	87.9
Trehalose-6,6'-dipentanoate	-0.09	-0.11	-0.10	510.53	15.9
Trehalose-2,2',3,3'-tetrapentanoate	3.73	2.16	2.945	678.77	31.7
Trehalose-2,2',4,4',5,5'-hexapentanoate	7.55	4.40	5.975	847.00	47.6
Trehaloseoctopentanoate	9.57	5.26	7.415	1015.24	63.5
Trehalose-6,6'-dihexanoate	0.92	0.52	0.72	538.59	15.0
Trehalose-2,2',3,3'-tetrahexanoate	5.75	5.23	5.49	734.88	30.1
Trehalose-2,2',4,4',5,5'-hexahexanoate	9.28	5.09	7.185	931.17	45.1
Trehaloseoctohexanoate	10.18	6.00	8.09	1127.46	60.2
Trehalose-6,6'-diheptanoate	1.93	1.13	1.53	566.64	-
Trehalose-2,2',3,3'-tetraheptanoate	7.77	4.19	5.98	790.99	-
Trehalose-2,2',4,4',5,5'-hexahepttanoate	9.89	5.90	7.90	1015.33	-
Trehaloseoctoheptanoate	10.56	6.63	8.60	1239.67	-
Trehalose-6,6'-dioctanoate	2.94	1.77	2.36	594.70	-
Trehalose-2,2',3,3'-tetraoctanoate	9.03	5.12	7.08	847.09	-
Trehalose-2,2',4,4',5,5'-hexaictanoate	10.27	6.55	8.41	1099.49	-
Trehaloseoctooctanoate	10.83	7.12	8.98	1351.89	-
Trehalose-6,6'-dibenzoate	0.51	0.31	0.41	550.51	-
Trehalose-2,2',3,3'-tetrabenzoate	4.92	3.00	3.96	758.73	-
Trehalose-2,2',4,4',5,5'-hexabenzoate	8.85	4.68	6.77	966.95	-
Trehaloceoctooctobenzoate	9.97	6.16	8.07	1175.16	-
Trehalose-1-C8	-0.70	-0.58	-0.64	468.50	-
Trehalose-1-C9	-0.20	-0.22	-0.21	482.52	-
Trehalose-1-C10	0.30	0.21	0.26	496.55	-
Trehalose-1-C11	0.81	0.53	0.61	510.58	-
Trehalose-1-C12	1.32	0.90	1.11	524.60	-

The limiting factor in Abazari *et al.* for trehalose loading was not the ability of acetylated trehalose to cross the membrane, but the conversion of acetylated trehalose to trehalose by intracellular esterases. To overcome this bottleneck, trehalose derivatives with fewer modified groups, and thus fewer ester bonds for the enzymes to break, are preferable. Although, this would leave more OH groups exposed, reducing membrane permeability. Additionally, a lower number of ester groups added per trehalose molecule would result in lower toxicity, allowing a higher amount of trehalose to be loaded before toxicity becomes a limiting factor. Therefore, a diester is likely to be the most effective option. The average logP value of trehalose-6,6'-dibutanoate, is -0.96, which is close to the logP trehalose-2,2',3,3',4,4'-hexacetate (-0.41), which has been established to be cell permeable,[19] and close to the recommend logP of between 0 and 3.[29] The relative predicted toxicity of trehalose-6,6'-dibutanoate is 22.0 mol^{-1}, this is lower than trehalose-2,2',3,3',4,4'-hexacetate at 65.9 mol^{-1}, which is established to have low toxicity.[19] Based on these results, we chose to synthesize and investigate trehalose-6,6'-dibutanoate. Trehalose-6,6'-dipentanoate and trehalose-6,6'-dihexanoate were also considered as options, but they have a higher molecular weight, and their long hydrocarbon chains may alter the membrane permeation characteristics in unpredictable ways, for example, long chain carboxylic acids insert themselves into the cell membrane rather than cross it,[30] and so we wanted to avoid the possibility of this happening with our trehalose derivative.

4.4.2 Synthesis

We initially synthesised trehalose-6,6'-dibutanoate using a protocol based on Khan *et al.*[31] This was a three step process: 1. Trehalose was reacted with N,O-bis(trimethylsilyl)acetamide in the presence of tetra-*n*-butylammonium fluoride (TBAF) to protect all eight alcohol groups with trimethylsilyl (TMS) groups. The product was then reacted with K_2CO_3 to deprotect the primary alcohol groups at the C6 positions, forming 2,2',3,3',4,4'-hexa-O-trimethylsilyl-trehalose (TMS-trehalose). 2. TMS-Trehalose was reacted with butanoic acid in the presence of 4-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) to esterize the unprotected C6 primary alcohol groups, forming the ester TMS-trehalose-6,6'-dibutanoate. 3. Finally, the TMS protecting groups were removed by exposing TMS-trehalose-6,6'-dibutanoate to Dowex-H^+ ion exchange resin to obtain trehalose-6,6'-dibutanoate. This method was time-consuming, requiring a purification step with silica gel column chromatography between each of the three reaction steps, and resulted in a poor overall yield (14 %) (Figure 4.1).

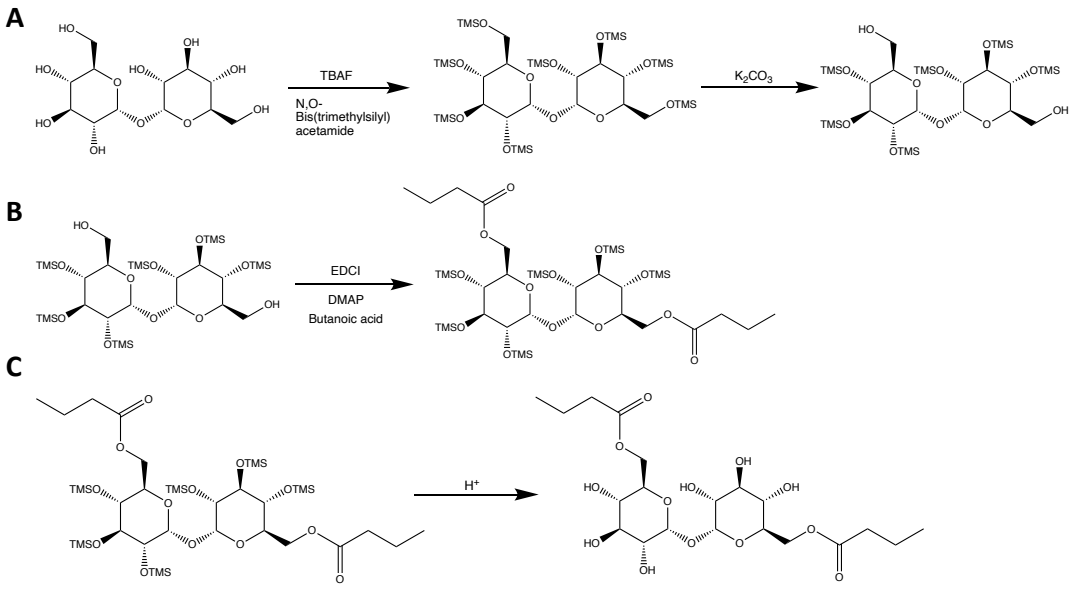

Figure 4.1. Chemical synthesis. **(A)** Trehalose-6,6'-dibutanoate was reacted with N,O-bis(trimethylsilyl)acetamide to protect all alcohol groups. The product was then reacted with K_2CO_3 to deprotect the primary alcohol groups. **(B)** The now protected trehalose is esterified with butanoic acid. **(C)** The secondary alcohol groups are deprotected using Dowex H^+ ion exchange resin.

To overcome these problems, we searched the literature for a more concise synthetic method. We found that according to John *et al.*[32] the primary alcohol groups at the C6 positions on trehalose can be selectively esterified with vinyl butyrate in a condensation reaction using Novozyme 435 (*Candida antarctica* lipase B immobilised on hydrophobic acrylic resin) as a catalyst, in an anhydrous environment (acetone) forming trehalose-6,6'-dibutanoate. The secondly alcohol groups do not react due to selectivity of the enzyme.[32,33,34] We, therefore, synthesised trehalose-6,6'-dibutanoate using this method. When the reaction was heated at 45 ºC for 48 hours, the yield was only 3 – 5 %, but when heated at 60 ºC for 96 hours the yield increased to 18 %, a

slight improvement over the synthetic method. More importantly, the enzymatic method allowed for several reactions to be conducted in parallel before purification in a single column chromatography step, greatly speeding up the process (Figure 4.2).

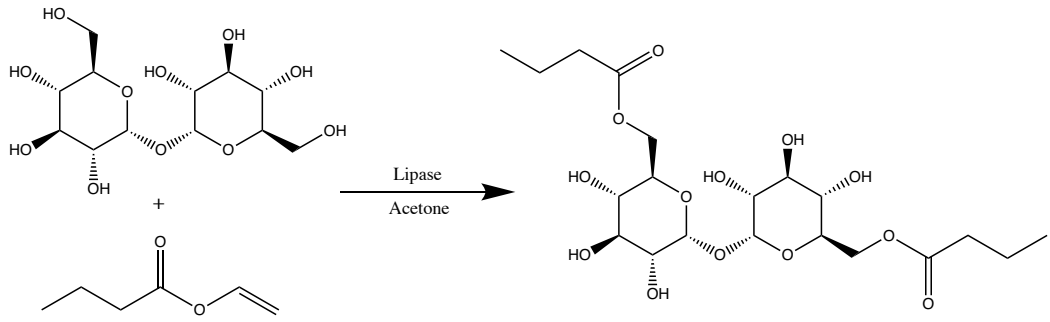

Figure 4.2. Enzymatic synthesis. Trehalose-6,6'-dibutanoate was synthesised from trehalose and vinal butyrate using Novozyme 435 as an enzymatic catalyst in the presence of acetone.

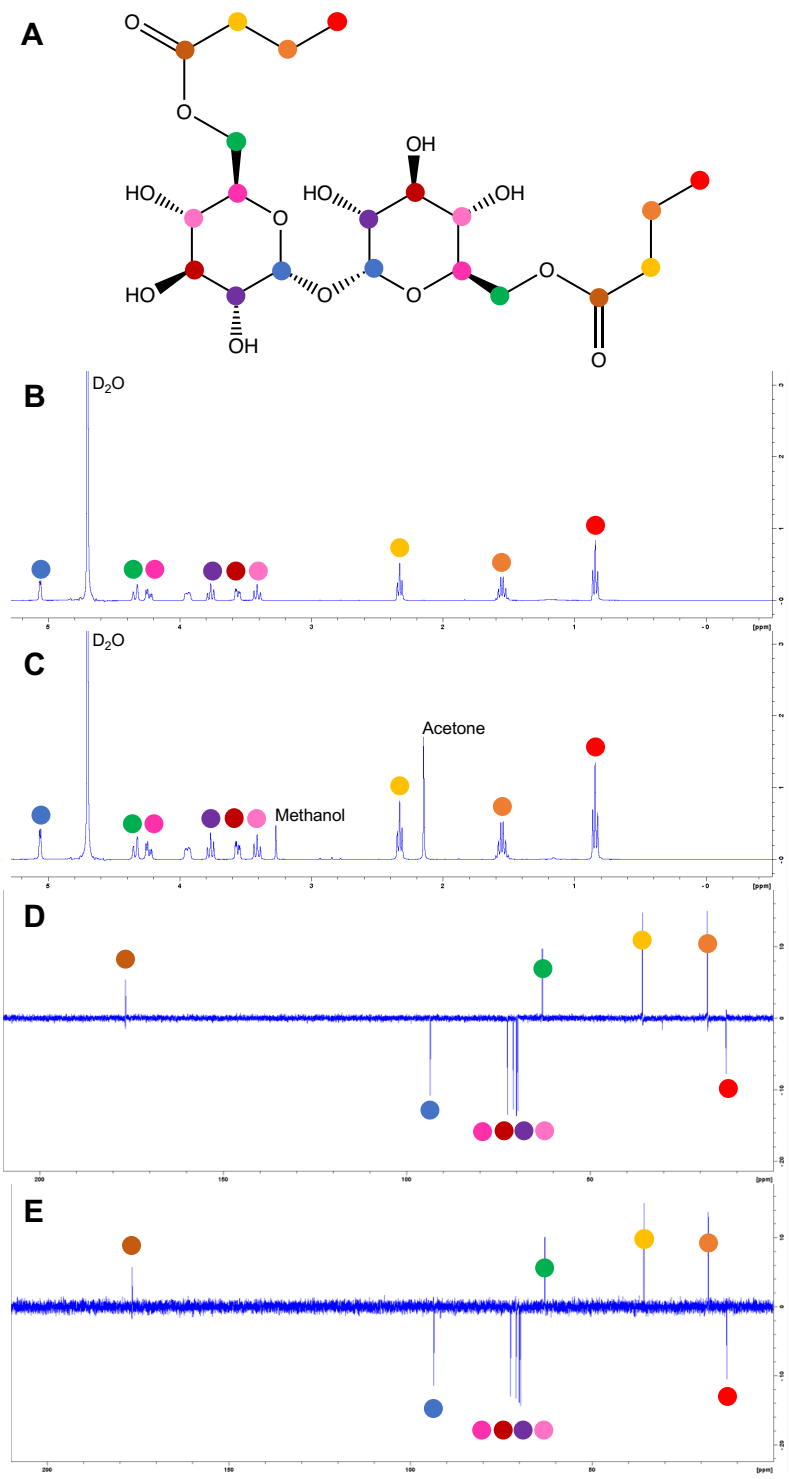

Figure 4.3. **(A)** Structure trehalose-6,6'-dibutanoate with proton/carbon environments designated by colour. **(B)** ^{1}H NMR and **(D)** ^{13}C NMR of trehalose-6,6'-dibutanoate made by the synthetic method. **(C)** ^{1}H NMR and **(E)** ^{13}C NMR of trehalose-6,6'-dibutanoate made by the synthetic method.

4.4.3 Testing on RBCs

To test trehalose-6,6'-dibutanoate for cryoprotective effect, we first attempted to cryopreserve red blood cells (RBCs) without solvent cryoprotectants. Note that limited trehalose-6,6'-dibutanoate was available for testing, therefore some screening experiments used were not repeated (n = 1). Without repeats, little information can be obtained by comparing successful conditions. However, in this context, false positives are much less likely than false negatives, so screening many conditions without repeats allows for the detection of conditions under which the cryoprotectant may potentially work. In each experiment, a pellet of RBCs was prepared from 500 μl of blood. The pellet was then resuspended in 1000 μL of cryoprotectant solution and incubated at room temperature for 1 hour (unless otherwise specified). After incubation, the RBCs were plunged into liquid nitrogen for at least 20 minutes, then thawed in a 37 ºC water bath. Haemolysis was measured using an alkaline haematin D-575 (AHD) assay.[35,36] In the first screen, 200 mM trehalose (shown in Figure 4.4 as T200) was combined with 0, 10, 50 and 100 mM of the ester trehalose-6,6'-dibutanoate (shown in Figure 4.4 as E0 – E100). It was found that cryopreservation with 200 mM trehalose alone resulted in a recovery of 43 %. However, when 200 mM trehalose was combined with 10 mM or more trehalose-6,6'-dibutanoate the recovery dropped drastically (Figure 4.4a). No haemolysis was observed when the trehalose/trehalose-6,6'-dibutanoate solution was added to the RBCs, suggesting that most of the damage occurred during cryopreservation. We suspected that the low recovery was caused by the intracellular trehalose concentration being too high relative to the extracellular osmolarity, causing osmotic shock upon freezing or thawing. So, for the second screen, we combined 10 mM trehalose-6,6'-dibutanoate with 300 – 1500 mM trehalose. It was found that

increasing the trehalose concentration to 1500 mM restored post-thaw recovery to 36 %, but the recovery previously achieved with 200 mM trehalose alone was not exceeded (Figure 4.4b). In order for trehalose-6,6'-dibutanoate to deliver trehalose into the intracellular space, it must first cross the cell membrane and then be hydrolysed to trehalose. This process takes time, according to Abazari *et al*. intracellular trehalose was still increasing after 8 – 12 hours incubation with Trehalose-2,2',3,3',4,4'-hexacetate,[19] and Zhong *et al.* used an incubation period of 48 hours when testing sugar alcohol propanoates.[37] To see if a longer incubation time would increase the cryoprotective effect, we incubated the RBCs with 10 mM trehalose-6,6'-dibutanoate and 300 mM or 400 mM trehalose for 48 hours prior to cryopreservation (Figure 4.4c). This resulted in post-thaw recoveries of 30 % in both cases. However, this was less than the recovery achieved by 48 hours incubation with 300 mM trehalose. Overall, none of the conditions screened using RBCs resulted in a recovery higher than that which can be obtained from cryopreservation with extracellular trehalose alone.

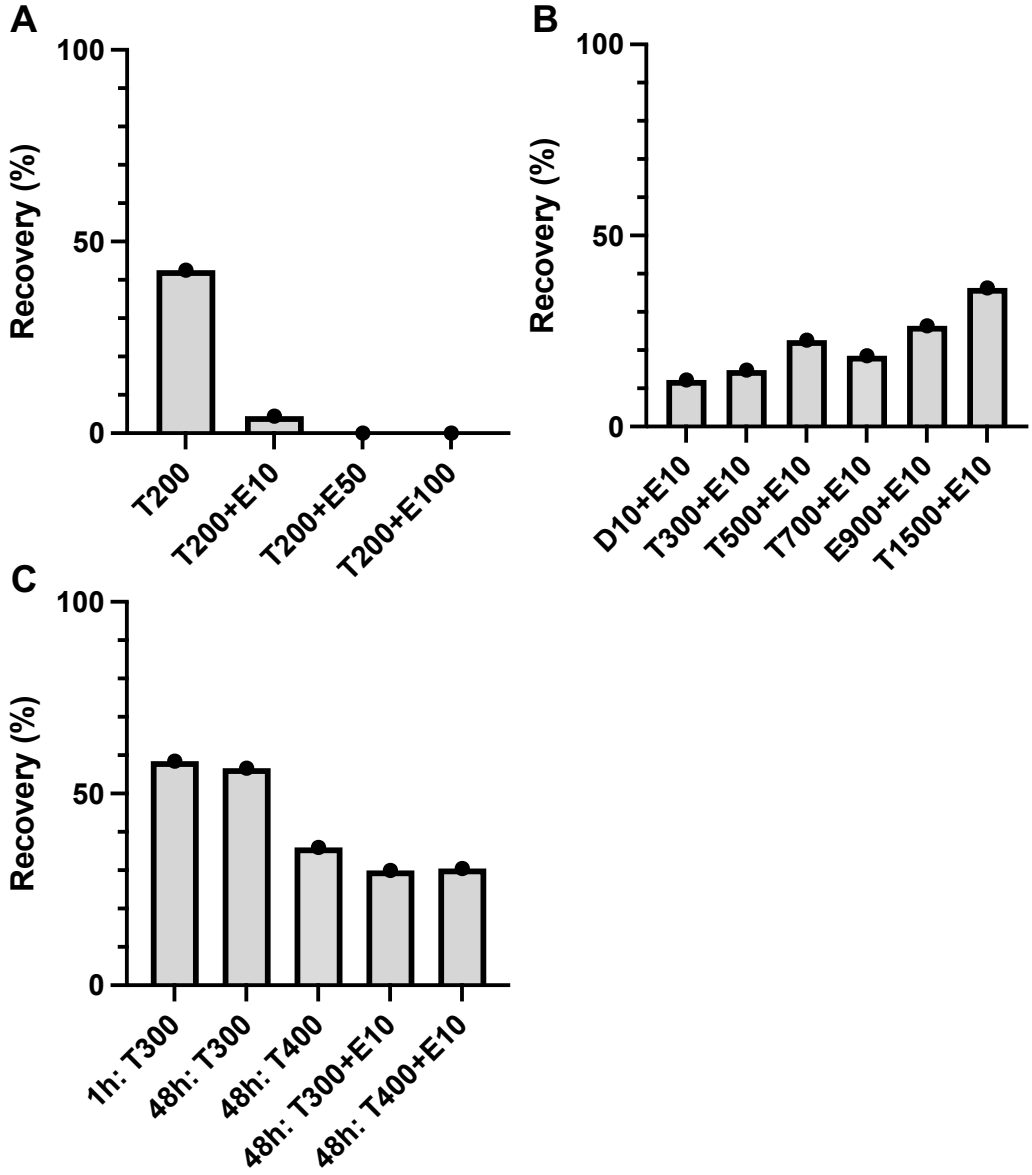

Figure 4.4. The percentage recovery of RBCs after being plunged into liquid nitrogen and warmed in a 37 °C water bath. Prior to freezing, RBCs were incubated for 10 minutes with 0 –100 mM trehalose-6,6'-dibutanoate and/or 200 – 1500 mM. T = trehalose, E = trehalose-6,6'-dibutanoate (ester).

4.4.4 Testing on A549s

Having failed to find a cryoprotective effect on RBCs, we next tested trehalose-6,6'-dibutanoate for cryoprotective effect on A549 cells. A549 is an immortalised human lung epithelial cell line. Unlike Jurkats, these cells are adherent, meaning that they adhere to the tissue culture plastic rather than being in suspension. This property allows the cells to be more readily imaged, which will be important for determining cryoprotectant uptake. For each experiment, unless otherwise specified, 300,000 cells per well were plated in 24 well plates with 500 μL complete media and incubated for 24 hours. After incubation, media was aspirated, leaving the cells in the plate. 500 μL serum-free media containing trehalose-6,6'-dibutanoate was added to the plate and the cells were incubated for the desired time. After incubation, cells were counted, then either transferred to cryovials or left on the plate, removing the trehalose-6,6'-dibutanoate. 500 μL complete media containing trehalose was then added. Cells were then frozen to – 80 ºC for at least 12 hours, either at – 1 ºC min^{-1} or at an uncontrolled rate on a 48 well plate. After freezing, cells were thawed using a 37 ºC water bath, resuspended in fresh media, incubated for 24 hours, then counted. All incubations longer than 10 minutes took place in an incubator at 37 ºC with 5 % CO_2. The 10-minute incubations with the final freezing media were at room temperature.

For initial testing with A549s, cells were incubated with 5 mM or 10 mM trehalose-6,6'-dibutanoate for 1 hour, then resuspended in 400 mM trehalose. DMSO + 400 mM trehalose, and 400 mM trehalose were used as controls. For two of the conditions, the trehalose-6,6'-dibutanoate was made up the day before in media that contained fetal bovine serum (FBS) and stored in the fridge, this is labelled "(old)" in Figure 4.5a.

The cells, now in 400 mM trehalose solution, were cooled to – 80 ºC at a freezing rate of – 1 ºC min^{-1}, stored at – 80 ºC for at least 12 hours, then thawed in a 37 ºC water bath. It was found that cells incubated with 10 mM fresh trehalose-6,6'-dibutanoate in serum-free media, then cryopreserved with 400 mM trehalose, showed survival comparable with those cryopreserved with 5 % DMSO + 400 mM trehalose (≈ 30 %). Cells incubated with trehalose-6,6'-dibutanoate that had been made up the day before in media containing FBS had a worse recovery (18 % vs 31 % for 10 mM trehalose-6,6'-dibutanoate). This indicates that the trehalose-6,6'-dibutanoate had decomposed during storage, either due to the serum esterases present in the FBS or through unassisted hydrolysis, though the former seems more likely. Consequently, going forward we decided to make the trehalose-6,6'-dibutanoate solutions fresh on the day they were needed, in FBS-free media, storing in the – 20 ºC freezer if necessary. To test trehalose-6,6'-dibutanoate for toxicity, A549s were incubated with trehalose-6,6'-dibutanoate for 24 hours before counting. It was found that 10 mM fresh trehalose-6,6'-dibutanoate causes a large reduction in recovery compared to the untreated control (115 % vs 61 %), meaning that trehalose-6,6'-dibutanoate exhibits a large amount of toxicity within 24 hours incubation. Incubation with fresh 10 mM trehalose-6,6'-dibutanoate made up in media without FBS resulted in a lower cell recovery than 5 mM or 10 mM trehalose-6,6'-dibutanoate that had been made up in media with FBS (61 % vs 133 % and 187 % respectively) (Figure 4.5b). This may also be due to the decomposition trehalose-6,6'-dibutanoate by serum esterizes.

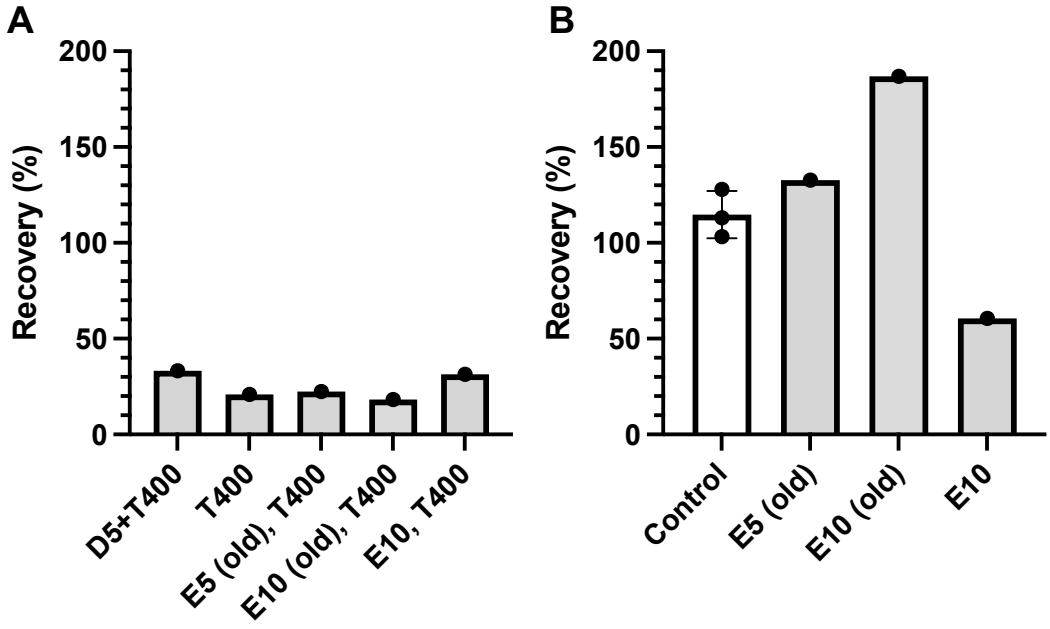

Figure 4.5. **(A)** The percentage recovery of A549s after being cooled at – 1 °C min^{-1} to – 80 °C and warmed in a 37 °C water bath. Prior to freezing, RBCs were incubated for 1 hour with 0 – 10 mM trehalose-6,6'-dibutanoate, which was replaced with 400 mM trehalose, and/or 5 % DMSO, and incubated for a further 10 minutes. **(B)** The percentage recovery of A549s recovered after being incubated with trehalose-6,6'-dibutanoate for 24 hours. T = trehalose, E = trehalose-6,6'-dibutanoate (ester), D = DMSO, old = trehalose-6,6'-dibutanoate that had been made up in media containing FBS and stored overnight.

With a potentially useful cryoprotectant combination found, we wanted to determine the optimal incubation time. Cells were incubated with 10 mM trehalose-6,6'-dibutanoate for 1, 2 or 4 hours, then cryopreserved as above with 400 mM trehalose. It was found that incubation for 1 or 2 hours resulted in a higher recovery than incubation for 4 hours (19 % vs 18 % vs 9 % for 1, 2 and 4 hours respectively) (Figure 4.6). 19 % is notably lower than the recovery that would be expected when

cryopreserved with 5 % DMSO (30 – 40 %), but a 5 % DMSO control was not included in this optimisation experiment so no conclusion can be drawn. Although these results were not statistically significant, this experiment indicates that the optimal incubation time is an hour (or less), probably due to the toxicity trehalose-6,6'-dibutanoate, as previously demonstrated.

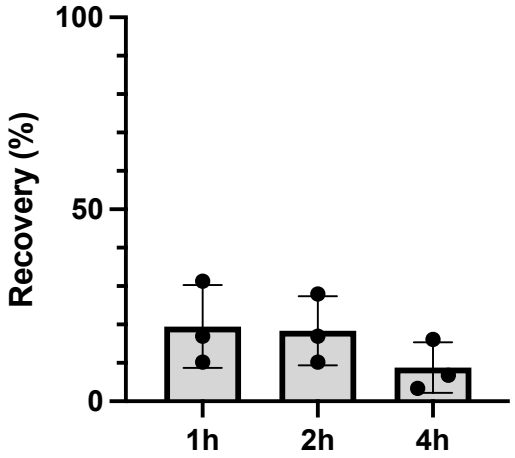

Figure 4.6. The percentage recovery of A549s after being cooled at – 1 °C min^{-1} to – 80 °C and warmed in a 37 °C water bath. Prior to freezing, A549s were incubated for 1, 2, or 4 hours with 10 mM trehalose-6,6'-dibutanoate, which was replaced with 400 mM trehalose and incubated for a further 10 minutes. T = trehalose, E = trehalose-6,6'-dibutanoate (ester). Data represents the mean ± SD of at least three independent experiments.

To further identify optimal conditions and cryoprotectant combinations for the use of trehalose-6,6'-dibutanoate, three screening assays were conducted. In the first screen (Figure 4.7a), cells were incubated with 0, 5, or 10 mM trehalose-6,6'-dibutanoate for 1 hour, and cryopreserved in complete media containing 5 % or 10 % DMSO or trehalose. Here, the cells were not transferred to cryovials. Instead, the cells remained

in the 48 well plate. The 48 well plate was placed on a corning “coolsink”, a metal plate that insured that all wells of the 48 well plate were in contact with the bottom of the – 80 ºC freezer to achieve a rapid and consistent freezing rate. In the second screen (Figure 4.7b), after incubation with 0, 5, 10, or 20 mM trehalose-6,6’-dibutanoate for 1 hour, cells were incubated with complete media containing DMSO or trehalose for 10 minutes. The DMSO/trehalose media was then removed prior to cryopreservation using the plate method described above. In the third screen (Figure 4.7c), cells were incubated with 10 or 20 mM trehalose-6,6’-dibutanoate for 1 hour, and cryopreserved with DMSO or trehalose in cryovials. The first screen revealed that 20 mM trehalose-6,6’-dibutanoate incubation, followed by cryopreservation with 300 mM trehalose, is a potentially effective cryoprotectant combination. The second screen did not show any useful cryoprotectant combinations, although even DMSO failed to cryopreserve more than 14 % of the cells, indicating that the cryopreservation conditions hindered cryopreservation rather than the cryoprotectants. Likewise, the third screen did not show any useful cryoprotectant combinations. The previously successful (incubation with 10 mM trehalose-6,6’-dibutanoate, cryopreserve with 400 mM trehalose) combination did not result in a recovery above 6 % in any of the screens.

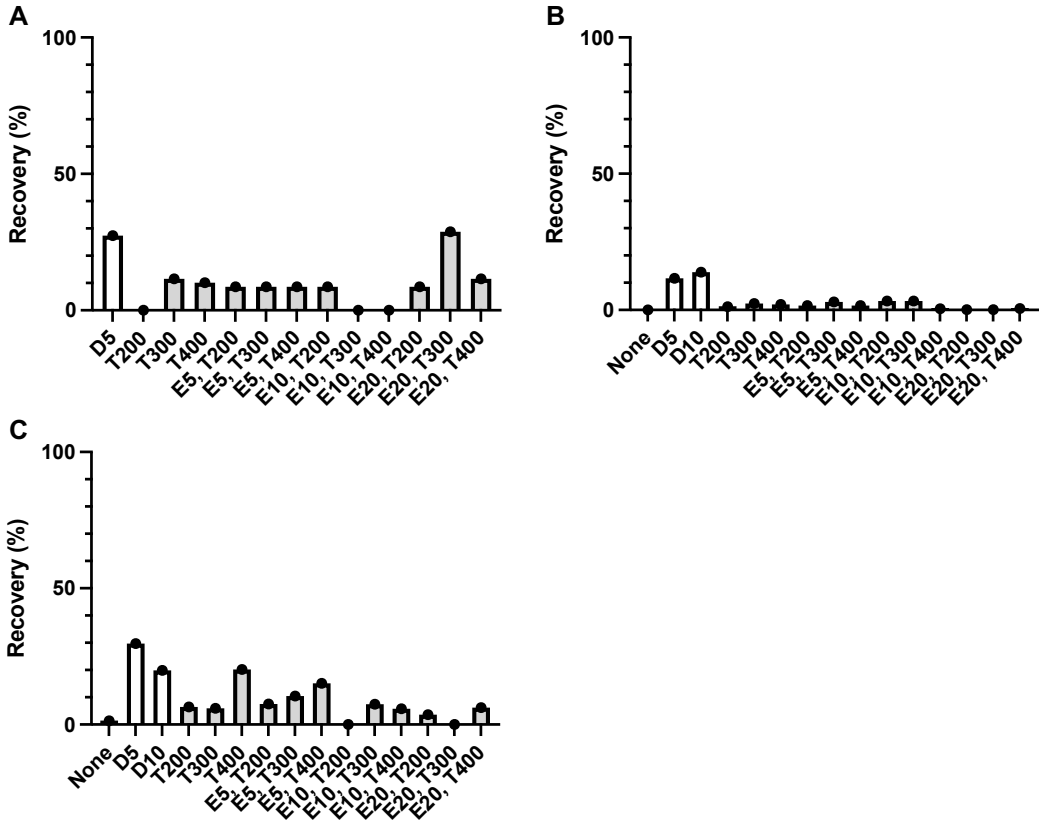

Figure 4.7. The percentage recovery of A549s after being cooled to – 80 °C and warmed in a 37 °C water bath. Prior to freezing, A549s were incubated for 1 hour with 0 – 400 mM trehalose-6,6'-dibutanoate, which was replaced with 200 – 400 mM trehalose, or 5 – 10 % DMSO, and incubated for a further 10 minutes. T = trehalose, E = trehalose-6,6'-dibutanoate (ester), D = DMSO. **(A)** Cells were cooled at an uncontrolled rate in a 48 well plate. **(B)** Cells were cooled at an uncontrolled rate in a 48 well plate, all media having been aspirated. **(C)** Cells were cooled at – 1 °C min^{-1} in cryovials.

Having identified two potentially useful cryoprotectant combinations from the above screens, we conducted an experiment to compare them against 5 % DMSO, 300 mM, and 400 mM trehalose controls. Cells were incubated with 10 mM or 20 mM trehalose-

6,6'-dibutanoate for 1 hour, resuspended in 400 mM or 300 mM trehalose respectively, transferred to cryovials, and incubated for 10 minutes. The cells were then cooled to – 80 ºC at a freezing rate of – 1 ºC min^{-1}, stored at – 80 ºC for at least 12 hours, and thawed in a 37 ºC water bath. This protocol was identical to those above, except that cells were seeded at a density of 200,000 per well. This was done to avoid cell clumping, which can lead to misleadingly low cell counts. It was found that both combinations failed to cryoprotect the cells, resulting in 0 % recovery compared to the 5 % DMSO control 35 % (Figure 4.8). These results are statistically significant. This data is in contrast with earlier experiments where 10 mM trehalose-6,6'-dibutanoate and 400 mM trehalose resulted in 31 % (Figure 4.5, n = 1) and 19 % recovery (Figure 4.6, n = 3).

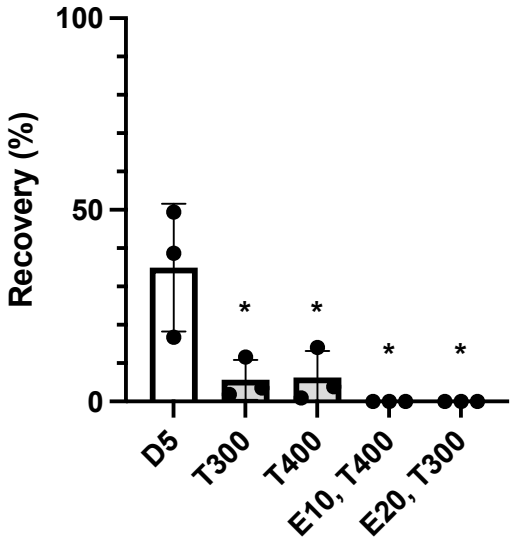

Figure 4.8. The percentage recovery of A549s after being cooled at – 1 °C min^{-1} to – 80 °C and warmed in a 37 °C water bath. Prior to freezing, A549s were incubated for 1 hour with 0, 10, or 20 mM trehalose-6,6'-dibutanoate, which was replaced with 300 or 400 mM trehalose and incubated for a further 10 minutes. T = trehalose, E = trehalose-6,6'-dibutanoate (ester). Data represents the mean ± SD of at least three independent experiments. (*$P < 0.05$ from 5 % DMSO).

We suspected that the poor cryopreservation results were due to toxicity, so a toxicity assay was conducted. Theoretically, trehalose-6,6'-dibutanoate would be metabolised by the cells into butanoic acid and trehalose, with butanoic acid being potentially toxic. We, therefore, compared the toxicity of trehalose-6,6'-dibutanoate with butanoic acid. A549 cells at a density of 300,000 per well were seeded and incubated for 24 hours. Cells were then incubated with 0 – 20 mM butanoic acid or 0 – 20 mM trehalose-6,6'-dibutanoate for 1 hour and counted. It was found that incubation with butanoic acid caused no statistically significant toxicity, while incubation with trehalose-6,6'-dibutanoate caused a dramatic and statistically significant drop in cell recovery, with

2.5 mM resulting in a recovery of 65 % and higher concentrations resulting in a negligible recovery (Figure 4.9).

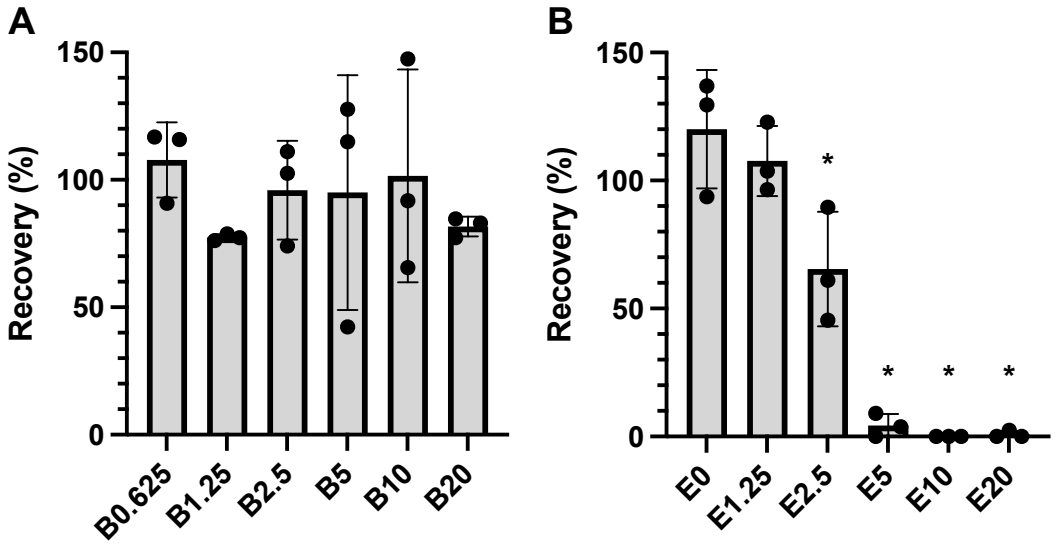

Figure 4.9. **(A)** The percentage recovery of A549s after being incubated with 0 – 20 mM butanoic acid for 1 hour. **(B)** The percentage recovery of A549s recovered after being incubated with 0 – 20 mM trehalose-6,6'-dibutanoate for 1 hour. Data represents the mean ± SD of at least three independent experiments. (*P < 0.05 from 0 mM).

To investigate one possible cause of the toxicity, we formed the hypothesis that trehalose-6,6'-dibutanoate had a surfactant effect on the cells. To test this, 5 mM and 10 mM trehalose-6,6'-dibutanoate was made up with serum-free media and vigorously shaken. It was observed that the 5 mM and 10 mM solutions formed foam, indicating a surfactant effect (Figure 4.10). This effect may be responsible for disrupting the cell membranes and/or causing the A549s to detach from the plate.

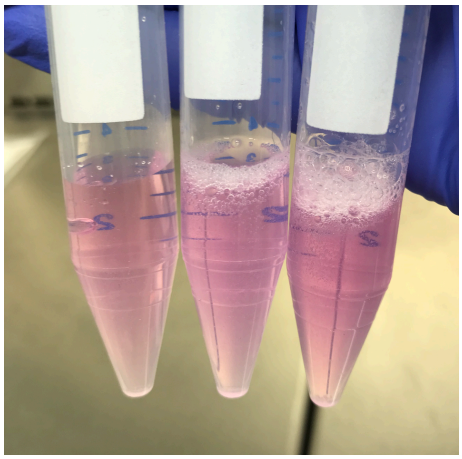

Figure 4.10. Left to right: solutions of 0 mM, 5 mM, and 10 mM trehalose-6,6'-dibutanoate in FBS-free media, immediately after being vigorously shaken.

The uptake of trehalose-6,6'-dibutanoate into the cells was examined using a cell swelling assay; if trehalose-6,6'-dibutanoate is penetrating the cell membrane and accumulating inside the cells, the volume of the cells should increase, as this was the case in Zhong *et al.* when they measured cell NIH-3T3 cell diameters after incubation with 10 mM sugar alcohol propanoates.[37] 100,000 A549 cells per well were seeded in a 24 well plate, and were incubated for 24 hours in 500 μL complete media. After incubation, the media was removed and replaced with 0 – 5 mM trehalose-6,6'-dibutanoate in 500 μL serum-free media. The cells were then incubated, and cell area was measured over a 24-hour period. A large amount of cell-to-cell variability was observed, dependent on factors such as position in the well, and the presence of nearby cells. To overcome this, 30 cells were measured per well across three different locations within the well, for a total of 90 cells measured for each timepoint and each concentration across three independent repeats (Figure 4.11). Although the data is noisy, a pattern began to emerge at the 4-hour time point: cells incubated with trehalose-6,6'-dibutanoate concentrations between 0.306 and 2.5 increased in area

relative to the 0 mM control. The pattern becomes especially prominent at the 24-hour time point where cells incubated with 1.25 mM trehalose-6,6'-dibutanoate show the largest increase in area relative to the control, with this increase becoming smaller the further away the concentration is from this point. Much of the "increase" here is driven by a reduction in the area of the 0 mM control cells, this reduction is likely due to changes in cell morphology as the cells adapt to their new environment (the 48 well plate) and proliferate. These factors should also apply to the cells incubated with trehalose-6,6'-dibutanoate. Only some of these values are statistically significant, but this is likely due to the small sample size rather than lack of effect. Overall, these results provide evidence that trehalose-6,6'-dibutanoate does cross the cell membrane and enter the intracellular space.

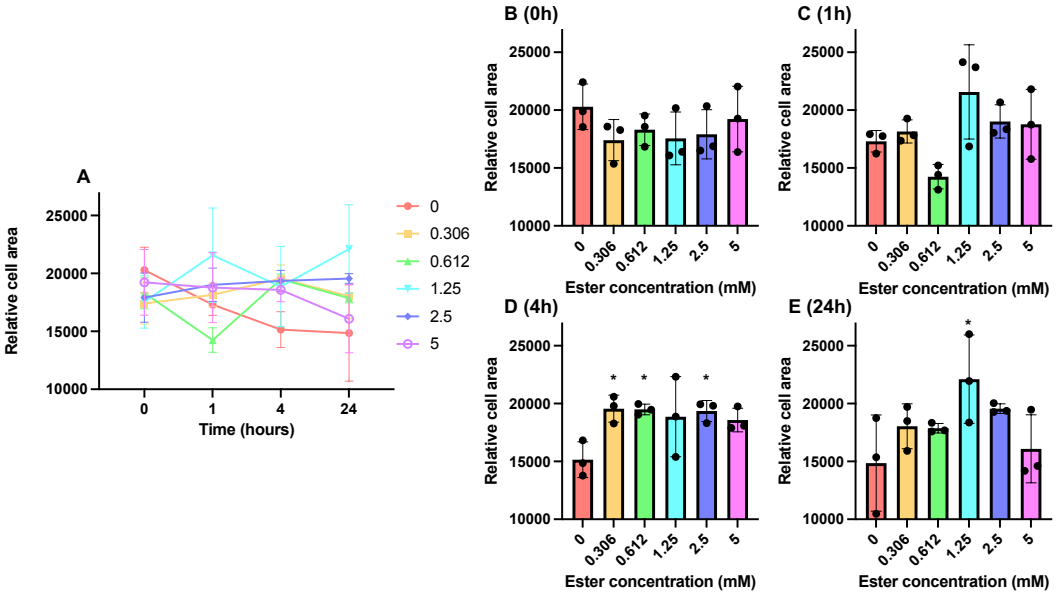

Figure 4.11. A549 cells were incubated with 0 – 5 mM trehalose-6,6'-dibutanoate. Cell area was measured at the 0, 1, 4, and 24-hour timepoints. **(A)** All time points. **(B)** Immediately at the start of incubation. **(C)** 1 hour. **(D)** 4 hours. **(E)** 24 hours. Data represents the mean ± SD of at least three independent experiments. (*P < 0.05 from 0 mM trehalose-6,6'-dibutanoate).

4.4.5 Testing on Jurkats

It is possible that the toxicity trehalose-6,6'-dibutanoate is specific to A549 cells, so we tested trehalose-6,6'-dibutanoate with Jurkat cells. In an attempt to mitigate toxicity, we used lower concentrations of trehalose-6,6'-dibutanoate, and incubated cells with trehalose and trehalose-6,6'-dibutanoate at the same time to mitigate any osmotic stress caused by trehalose-6,6'-dibutanoate uptake. Jurkats were incubated with 0 – 5 mM trehalose-6,6'-dibutanoate and 300 mM trehalose for 24 hours, in 24 well plates at a cell density of $1x10^6$ mL^{-1} at a volume of 1 mL. It was found that 400 mM trehalose was toxic to the cells, reducing recovery to 4 %. However, the addition

of 2.5 mM and 5 mM trehalose-6,6'-dibutanoate significantly improved recovery (58 % recovery in both cases) (Figure 4.12a). The surviving cells that had been incubated with 400 mM trehalose and 2.5 mM and 5 mM trehalose-6,6'-dibutanoate for 24 hours were then cooled at – 1 °C min^{-1} to – 80 °C and stored at – 80 °C for at least 12 hours. Cells were then thawed in a 37 ºC water bath for 5 minutes and counted. Unfortunately, the cryoprotectants failed to cryoprotect the cells, with recovery not exceeding 5 % (Figure 4.12b).

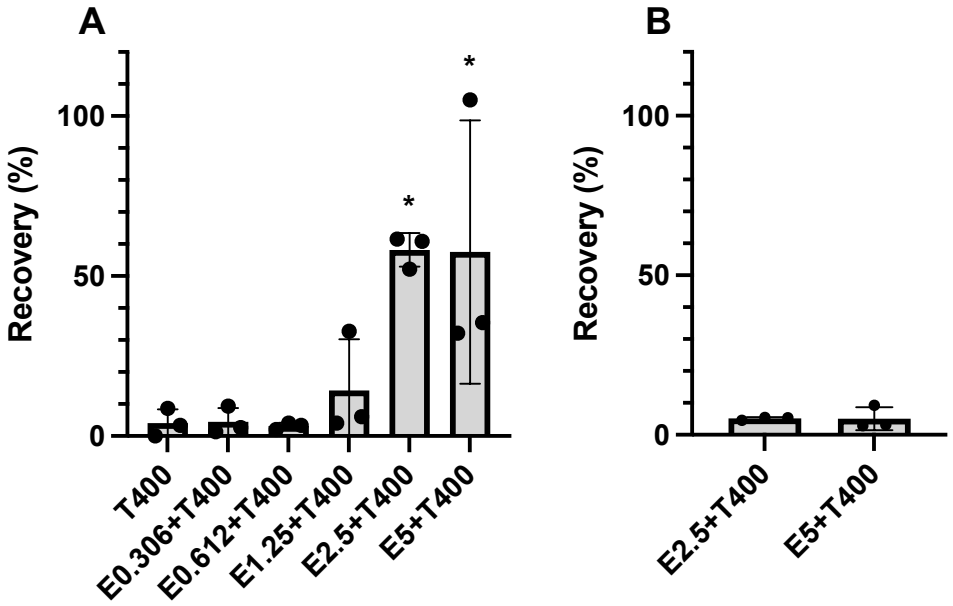

Figure 4.12. The percentage recovery of Jurkat cells after **(A)** being incubated with 0 – 5 mM trehalose-6,6'-dibutanoate and 400 mM trehalose for 24 hours. **(B)** the cells were then cooled at – 1 °C min^{-1} to – 80 °C and warmed in a 37 °C water bath. Data represents the mean ± SD of at least three independent experiments. *P < 0.05 from 0 mM trehalose-6,6'-dibutanoate.

Robert Ben's group[38] has developed small molecule ice recrystallisation inhibitors by adding hydrophobic modifications to sugars. They found that these molecules were able to enter cells and reduce intracellular ice recrystallisation.[38,39] Due to the similarity between these modifications and ours, we decided to use a splat assay[40] to assess the ice recrystallisation inhibition (IRI) activity of trehalose-6,6'-dibutanoate: it was found that a 10 mM trehalose-6,6'-dibutanoate solution formed ice crystals with a mean grain size (MGS) of 86 % of the PBS control (Figure 4.13). This was not statistically significant. This result shows that trehalose-6,6'-dibutanoate has minimal IRI activity, for comparison, poly(vinyl alcohol), a polymer with moderate IRI activity, results in a MGS (% PBS) of 20 % at the same concentration (0.5 mg ml^{-1}).[41,42]

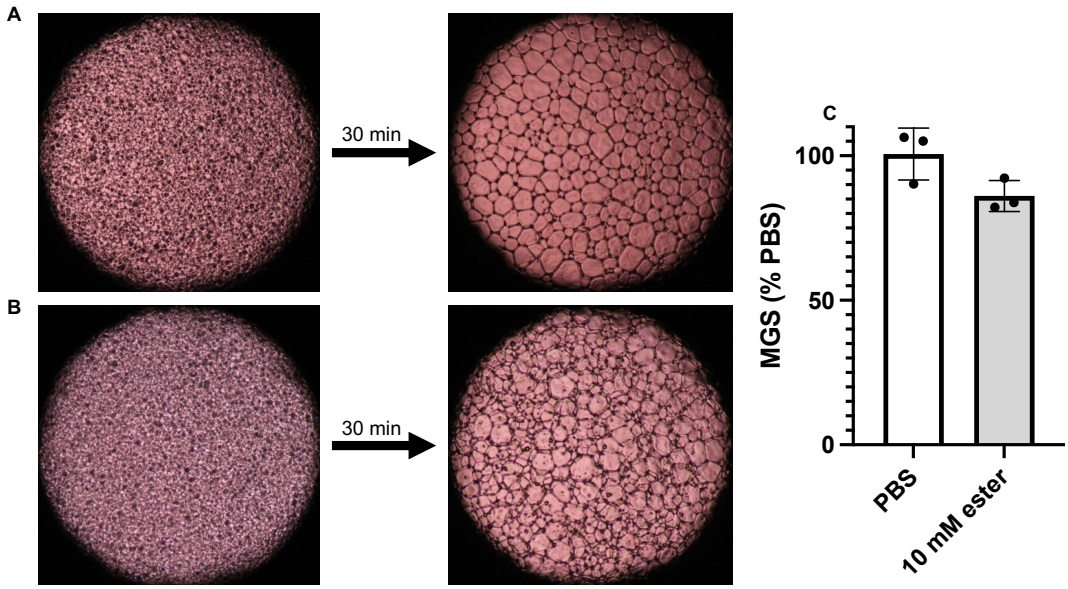

Figure 4.13. Drops of **(A)** PBS or **(B)** 10 mM trehalose-6,6'-dibutanoate in PBS were dropped onto – 80 °C coverslips to form ice wafers. The wafers were imaged immediately, and after 30 minutes at – 8 °C. 40× magnification. **(C)** Relative mean grain size (MGS) of the ice crystals. Data represents the mean ± SD of at least three independent experiments.

4.5 Conclusions

In this study, we have used the predicted membrane permeation and toxicity parameters of trehalose derivatives to identify trehalose-6,6'-dibutanoate as a potential novel cryoprotectant. We then synthesised this molecule using both a synthetic and an enzymatic method, and tested it for cryoprotective effect on RBCs, A549s, and Jurkats. Many conditions were tested, with variations in extracellular trehalose concentration, incubation time, and freezing method. Initial screening indicated that in A549s, incubation with 10 or 20 mM trehalose-6,6'-dibutanoate for 1 hour before cryopreservation with 400 mM or 300 mM trehalose respectively, resulted in a low-

moderate cryoprotective effect, potentially rivalling DMSO. However, further testing failed to replicate this result. We then found that trehalose-6,6'-dibutanoate was toxic to A549 cells at the concentrations we used for cryopreservation, which was surprising because butanoic acid was well tolerated. One explanation for this is that butanoic acid is non-toxic in the extracellular space at low concentrations, but becomes toxic when it accumulates in the intracellular space. We also found that trehalose-6,6'-dibutanoate exhibits a surfactant effect which may contribute to the toxicity. We then tested trehalose-6,6'-dibutanoate on Jurkat cells, finding that some combinations of trehalose-6,6'-dibutanoate and trehalose are only partially toxic to Jurkat cells after 24 hours incubation (≈ 60 % recovery). However, these trehalose-6,6'-dibutanoate/trehalose combinations failed to cryoprotect the surviving Jurkat cells. Zhong *et al.*[37] used sorbitol modified with propyl groups to successfully deliver sorbitol into fibroblasts. This study found that butyl modifications were more toxic than propyl modifications, so is possible that propyl modifications to trehalose would be successful in delivering trehalose into the cell without toxicity.

4.6 Experimental

4.6.1 Materials

Anhydrous trehalose, used for enzymatic and chemical synthesis, was from Carbosynth. Trehalose dihydrate used for biological experiments, and $MgSO_4$ was from Alfa Aesar. Methanol, K_2CO_3, petroleum ether, and NaCl was from Fisher. DMAP was from Acros Organics. Novozym 435 was from Strem Chemicals. Anhydrous acetone was from VWR chemicals. Vinal butyrate, diethyl ether, butanoic acid, anhydrous dimethylformamide, isopropanol, TBAF, N,O-

bis(trimethylsilyl)acetamide, ethyl acetate, EDCI, Silica, CH_2CL_2, acetone, Dowex-H^+ ion exchange resin, and Dulbecco's phosphate-buffered saline (1.15 g L^{-1} dibasic sodium phosphate, 0.2 g L^{-1} potassium chloride, 8 g L^{-1} sodium chloride, and 0.2 g L^{-1} monobasic potassium phosphate) (DPBS) was from Sigma-Aldrich. Sheep's blood in Alsever's solution (not defibrinated) was from TCS Biosciences. Solutions were made by dissolving cryoprotectants in culture media (details of culture media below), and were sterile filtered using 0.2 μm syringe filters obtained from Fisher.

4.6.2 Nuclear Magnetic Resonance Spectroscopy

^1H and ^{13}C nuclear magnetic resonance spectroscopy (NMR) was recorded on a Bruker DPX-400 at 298 K. Coupling constants (J) are reported in hertz (Hz). Abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; p, pentet; m, multiplet. Chemical shifts are quoted in parts per million (ppm), with the residual solvent as the internal standard.

4.6.3 Chemical Synthesis of trehalose-6,6'-dibutanoate

Anhydrous trehalose (1 g) was dissolved in anhydrous Dimethylformamide (DMF) (4 mL). TBAF (0.16 mL), and N,O-bis(trimethylsilyl)acetamide (5.6 mL) were then added and the mixture was stirred for 1.5 hours under nitrogen gas at room temperature. The reaction was then quenched with 1 mL isopropanol, diluted with 60 mL methanol, and cooled to approximately 0 °C by partially submerging the reaction flask in ice cold water. A solution of 0.364 g K_2CO_3 in 80 mL methanol was added and the solution was stirred at approximately 0 °C for 2 hours. The solution was neutralised with 0.4 mL acetic acid and the solvent was removed *in vacuo* yielding a yellow oil. The oil was dissolved in diethyl ether and impurities were removed by

partitioning with brine. The organic layer was collected, dried using $MgSO_4$, and solvent was removed *in vacuo* yielding a yellow oil. This was purified using silica gel flash column chromatography with the solvent system being petroleum ether:ethyl acetate 4:1, yielding 2,2',3,3',4,4'-hexa-O-trimethylsilyl-trehalose (TMS-trehalose) as a yellow oil.

^{1}H NMR (toluene D8): δ 0.1 (Si(CH_3)$_3$) 9H t; 3.6 (C*H*) 2H t; 3.7 (C*H*) 2H t; 3.8 (C*H*) 2H t; 4.2 (C*H*) 2H d; 4.3 (CH_2OH) 4H t; 5.1 (C*H*O) 2H s.

TMS-Trehalose (950 mg) was dissolved in anhydrous Ch_2Cl_2 (48 mL). Added to this were butanoic acid (1.4 mL), EDCI (982 mg), and DMAP (140.8 mg). This mixture was stirred at room temperature under nitrogen gas for 48 hours. A precipitate was formed, removed by filtration, and washed with ethyl acetate to extract any remaining product. The organic portions were combined, washed with water and brine, and dried using $MgSO_4$. Solvent was removed *in vacuo* yielding a yellow oil. This was purified using silica gel flash column chromatography with the solvent system being petroleum ether:ethyl acetate 5:1, yielding TMS-trehalose-6,6'-dibutanoate as a yellow oil.

^{1}H NMR ($CDCl_3$): δ 0.1 (Si(CH_3)$_3$) 9H t; 0.8 (CH_3) 6H t; 1.3 (CH_2) 4H s; 1.7 (CH_2) 4H t; 3.9 (C*H*) 2H t; 4.0 (C*H*) 2H t; 4.06 (C*H*) 2H t; 4.1 (OC*H*) 2H d, 4.3 (CH_2OCO) 4H d, 5.0 (C*H*O) 2H s.

TMS-trehalose-6,6'-dibutanoate (266 mg) was dissolved in CH_2Cl_2 (50 mL) and methanol (50 mL). Dowex-H^+ ion exchange resin (28.6 mg) was added, and the solution stirred at room temperature for 30 minutes. The mixture was then filtered, and solvent removed *in vacuo* yielding a white powder. This was purified using silica

gel flash column chromatography with the solvent system being acetone:methanol 5:1, yielding trehalose-6,6'-dibutanoate as a white powder.

^{1}H NMR (D_2O): δ 0.8 (C*H*$_3$) 6H t; 1.6 (C*H*$_2$) 4H p; 2.4 (C*H*$_2$) 4H t; 3.4 (C*H*) 2H t; 3.6 (C*H*) 2H t; 3.8 (C*H*) 2H t; 4.3 (C*H*$_2$OCO) 4H d; 5.1 (C*H*O) 2H s.

4.6.4 Enzymatic Synthesis of trehalose-6,6'-dibutanoate

Trehalose-6,6'-dibutanoate was produced via the enzymatic method in several batches. Typically, anhydrous trehalose (3.7 g) was dissolved in anhydrous acetone (100 mL) and vinyl butyrate (3.4 g). Novozym 435 (1.5 g) and a spoonful of 4 Å molecular sieves (Sigma) were added. The mixture was then heated at 45 °C and stirred under nitrogen gas for 48 hours. Alternatively, the mixture was heated at 60 °C and stirred under nitrogen gas for 96 hours, using a water condenser to prevent evaporation of the acetone. This resulted in the formation of a yellow liquid and a white-brown solid. The solid was removed by filtration, and an additional 100 mL of acetone was used to extract any remaining product from the solid. This extraction was performed three times. The yellow liquid was then concentrated *in vacuo* resulting in a yellow-white powder. The powder was purified using silica gel flash column chromatography with the solvent system being ethyl acetone:methanol:water 17:4:1, yielding trehalose-6,6'-dibutanoate as a white powder.

^{1}H NMR (D_2O): δ 0.8 (C*H*$_3$) 6H t; 1.6 (C*H*$_2$) 4H p; 2.4 (C*H*$_2$) 4H t; 3.4 (C*H*) 2H t; 3.6 (C*H*) 2H t; 3.8 (C*H*) 2H t; 4.3 (C*H*$_2$OCO) 4H d; 5.1 (C*H*O) 2H s.

4.6.5 Blood preparation and cryopreservation

Ovine blood (10 mL) in Alsever's solution (TCS Biosciences, UK) was transferred into a centrifuge tube and centrifuged at 367 × g for 5 minutes (Spectrafuge 6C, Labnet). The supernatant was discarded and replaced with 7 mL Dulbecco's buffered phosphate solution (DPBS) (8 g L^{-1} sodium chloride, 0.2 g L^{-1} potassium chloride, 0.2 g L^{-1} monobasic potassium phosphate, and 1.15 g L^{-1} dibasic sodium phosphate) (Sigma-Aldrich), and mixed by inversion, for a final haematocrit of 30 %. After blood preparation, 500 µl blood was transferred to centrifuge tubes and centrifuged for 5 minutes at 2000 × g (Prism Mini, Lab net). The pellet was mixed with 1000 µl cryoprotectant solution (containing trehalose-6,6'-dibutanoate and/or trehalose), transferred to cryovials, and incubated for the desired time. After incubation, cryovials were plunged into liquid nitrogen for at least 20 minutes. Vials were warmed in a 37 °C water bath for 5 minutes or at room temperature until thawed.

4.6.6 AHD assay

Thawed blood was then centrifuged for 5 minutes at 2000 × g (Prism Mini, Lab net). 40 µl of supernatant from each sample was transferred to 750 µl AHD solution. 200 µl of the resulting solution was added to a 96 well plate in triplicate. The positive control was DPBS, and the negative control (lysate) was 100 µl blood mixed with 100 µl lysis buffer. The absorbance of the plate was read at 580 nm. Recovery was calculated as: $(1 - (\text{Absorbance} - \text{DPBS}/(\text{lysate} - \text{DPBS})) \times 100$. Lysis buffer was 0.32 M sucrose, 5 mM $MgCl_2$, 10 % w/v triton X-100, 10 mM tris HCl pH 7.8.

4.6.7 Cell culture

A549 and E6.1 Jurkat cells were from the European Collection of Authenticated Cell Cultures (ECACC). The cell lines were maintained in T175 flasks (Greiner Bio-One Ltd) and incubated at 37 ºC in a humid atmosphere with 5 % CO_2. Complete culture media was Ham's F-12K (Kaighn's) (F-12K) (Thermo Fisher) for A549 cells, and Advanced RPMI 1640 (Thermo Fisher) for Jurkat cells, each supplemented with 1 % Antibiotic-Antimycotic (Thermo Fisher) and 10 % non-USA origin fetal bovine serum (Merck). A549 cells were passaged to remain below 100 % confluence. Jurkat cells were passaged every 4 days to maintain a cell density not exceeding $1.5x10^6$ mL^{-1}.

4.6.8 Cell counting

Cells were counted using a Countess automated cell counter with Countess cell counting chamber slides (both Thermo Fisher). Percentage recovery was defined as $\frac{\text{Live cells recovered}}{\text{Live cells frozen}} * 100$ and viability was defined as $\frac{\text{Live cells recovered}}{\text{Total cells thawed}} * 100$. Live and dead cells were distinguished using the trypan blue exclusion method,[43] whereby live cells exclude the dye while dead cells were stained. The stock trypan blue was obtained from Merck and diluted two-fold with DPBS (Sigma-Aldrich).

4.6.9 A549 Cryopreservation and toxicity assays

A549 cells were taken from the main cell line when at least 80 % confluent. Cells were detached by washing with Dulbecco's phosphate-buffered saline (DPBS) then adding 10 mL 0.25 % trypsin-EDTA solution and incubating at 37 ºC in a humid atmosphere with 5 % CO_2 for 5 minutes. Cells were resuspended in fresh F-12K media at a concentration of 400,000 mL^{-1}, and 500 µL media containing 200,000 cells was added

to each well in a 24 well tissue culture plate (Corning). The plate was incubated as above for 24 hours to allow attachment. After incubation, the media was removed and replaced with 500 µL FBS-free F-12K media containing the trehalose-6,6'-dibutanoate, and the plate was incubated as above for the desired time. For the toxicity assay, cells were then detached by adding 500 µL 0.25 % trypsin-EDTA solution, incubated as above for 5 minutes and counted. For cryopreservation, cells were either: detached with 500 µL trypsin-EDTA, resuspended in 500 µL complete F-12K containing trehalose and/or DMSO and transferred to 1 mL cryovials, or left on the plate with the media being replaced with 500 µL complete F-12K containing trehalose and/or DMSO. In each case, cells were incubated at room temperature for 10 minutes before cryopreservation. Cryovials were cooled at – 1 °C min^{-1} to – 80 °C using a cryovial cooler (Coolcell LX, Corning). Plates were cooled at an uncontrolled rate to – 80 °C using a coolsink® (Corning). Cells were kept at – 80 °C for at least 24 hours. After freezing, cryovials and plates were warmed in a 37 °C water bath for 5 minutes. The cells were resuspended in fresh media, and if in cryovials, were plated onto a new 24 well tissue culture plates. Finally, cells were incubated at 37 ºC in a humid atmosphere with 5 % CO_2 for 24 hours until counting. Centrifugation for the resuspension steps was conducted at 367 × g in a (Spectrafuge 6C, Labnet).

4.6.10 Jurkat cryopreservation and toxicity assays

Jurkat cells were taken from the main cell line when the cell density reached approximately $1x10^{-6}$ mL^{-1}. Cells were then resuspended at a density of $1x10^{-6}$ mL^{-1}, in FBS-free Advanced RPMI 1640 media containing trehalose-6,6'-dibutanoate and trehalose for 24 hours in a 48 well plate (Corning) at 37 ºC in a humid atmosphere

with 5 % CO_2. Cells were then counted using the trypan blue exclusion method to assess toxicity. For cryopreservation, cells were transferred to 1 mL cryovials and cooled at – 1 °C min^{-1} to – 80 °C using a cryovial cooler (Coolcell LX, Corning). Cells were kept at – 80 °C for at least 24 hours. Cryovials were warmed in a 37 °C water bath for 5 minutes, resuspended in fresh media and plated onto a new 48 well tissue culture plate (Corning). Cells were incubated at 37 ºC in a humid atmosphere with 5 % CO_2 for 24 hours until counting. Centrifugation for the resuspension steps was conducted at 825 × g in a (Spectrafuge 6C, Labnet).

4.6.11 Cell swelling assay

A549 cells were taken from the main cell line when at least 80 % confluent. Cells were detached by washing with Dulbecco's phosphate-buffered saline (DPBS) then adding 10 mL 0.25 % trypsin-EDTA solution and incubating at 37 ºC in a humid atmosphere with 5 % CO_2 for 5 minutes. Cells were resuspended in fresh F-12K media at a concentration of 200,000 mL^{-1} and 500 µL media containing 100,000 cells was added to each well in a 24 well tissue culture plate (Corning). The plate was incubated as above for 24 hours to allow attachment. After incubation, the media was aspirated and replaced with 0 – 5 mM trehalose-6,6'-dibutanoate in 500 µL serum-free media. The cells were then incubated at 37 ºC in a humid atmosphere with 5 % CO_2. 3 photos per well were taken at the 0, 1, 4, and 24 hour timepoints. Cell areas were measured using ImageJ software version 2.1.0.

4.6.12 Ice recrystallisation inhibition (splat) assay

A solution of 10 mM trehalose-6,6'-dibutanoate was made up in phosphate-buffered saline (PBS). A drop of this solution was then dropped from a height of 1.4 meters onto a – 78 °C glass coverslip, atop an aluminium plate which had been cooled using dry ice. The resulting ice wafer was quickly transferred to a – 8 °C microscope stage. After 30 minutes, an image was taken, and the number of ice crystals was manually counted using image J software. This number was compared to the number of ice crystals formed by a PBS control to determine the relative mean grain size (MGS) of the ice crystals formed by the trehalose-6,6'-dibutanoate solution.[40]

4.6.13 Statistics

Statistical analysis was performed using Prism 9 software (GraphPad). Statistical significance for all experiments was determined by ordinary one-way ANOVA using Dunnett's test for multiple comparisons, unless only two conditions were compared, in which case an unpaired t-test was used. P values below 0.05 were considered statistically significant.

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Chapter 5 – Cryopreservation of Immortalised T-cells. Discovery of a DMSO-free Cryoprotectant and pre-conditioning with L-proline

5.1 Abstract

Cell therapies such as allogeneic CAR T-cell, natural killer cell, and stem cell therapies must be cryopreserved for transport and storage. However, cryopreservation can reduce the effectiveness of these therapies, and necessitates the use of solvent cryoprotectants, namely dimethyl sulfoxide (DMSO), which can cause side effects in patients. L-proline is an amino acid osmolyte, used as a cryoprotectant by several organisms such as the codling moth *Cydia pomonella* and the larvae of the fly *Chymomyza costata*. Here we develop a DMSO-free multicomponent cryoprotectant solution for Jurkat cells. We then investigate the effectiveness of L-proline compared to D-proline and L-alanine for the cryopreservation of Jurkat cells, a model for CAR T-cell and other lymphocyte-based cell therapies. We also investigate the mechanisms by which L-proline exerts its cryoprotective effects, hypothesising that L-proline works by reducing the growth rate of the cells to prepare them for cryopreservation. It was found that a solution containing glycerol, polyampholyte, poly(ethylene glycol), hydroxyethyl starch and L-proline was as effective as 5 % DMSO for Jurkat cell cryopreservation. It was also found that 24-hour incubation of Jurkat cells with 200 mM L-proline resulted in a modest increase in cell recovery post-thaw at high cell density, and a larger increase in recovery at lower cell densities. L-alanine was as effective as L-proline at lower cell densities. Unlike L-proline and L-alanine, D-proline was found to be cytotoxic at 200 mM. We found that L-proline works through

a biological rather than physical mechanism of action, possibly by limiting cell proliferation, which is linked to its density-dependent effects. Preliminary analysis of apoptosis/necrosis profiles by flow cytometry indicate that the effect is not mediated by inhibition of apoptosis.

5.2 Disclaimer

- The cell lines were usually maintained by myself, but were occasionally maintained by Dr Ruben Tomás, Dr Kathryn Murray, Natalia Martinez, Ola Alkosti, Qiao Tang, and Ya Nan Gao.

5.3 Introduction

Cell therapies often need to be cryopreserved during the manufacturing and/or transport process. As an example: CAR T-cells are a cancer treatment consisting of autologous T-cells which have been harvested from a patient, and then genetically modified to express a chimeric antigen receptor (CAR) against a target antigen known to be expressed by the tumour cells, but not by healthy host cells. These T-cells are then expanded *in vitro* before being transfused back into the patient.[1] During this process the CAR T-cells are often cryopreserved twice, once for transport from the patient at a local hospital to a centralised lab for modification and expansion, and a second time when transporting cells from the lab back to the patient.[2] Under current cryopreservation techniques, CAR T-cell viability is reduced.[3] Although the freezing process has not been shown to directly reduce the effectiveness of CAR T-cell therapy,[3,4] other cell therapies have not fared so well. Frozen natural killer (NK) cell therapies were less effective in mice than fresh NK cells, with frozen NK cells having lower viability, and homing to different areas of the body.[5] In human melanoma patients, cryopreserved NK cells exhibited very poor viability and activity.[6] Human mesenchymal stromal cells show decreased *in vitro* function in response to cryopreservation.[7] After peripheral blood stem cell transplantation, neutrophil engraftment is limited by cryopreservation: removing DMSO post-thaw increases engraftment time, but the DMSO concentration necessary to achieve satisfactory viability can cause life-threatening toxicity in children.[8] It is likely that future types of cell therapy will face similar problems, and while advances in technology and logistics may allow for local production of cell therapies, cryopreservation would be essential for cell therapy banking. It is therefore important to develop improved

cryopreservation methods which either reduce the loss of viability during cryopreservation or reduce the DMSO concentration needed to achieve good viability. The Jurkat cell line, often referred to as “Jurkats” is an immortalised T-cell line. The ability to cryopreserve Jurkats is useful as a standalone goal because these cells are commonly used as a model to study T-cells,[10] and T-cell pathology such as leukaemia.[11] But further to this, the development of cryopreservation techniques for cryopreserving Jurkats is applicable to the cryopreservation of lymphocyte-based cell therapies such as CAR T-cells. Here we explore the reduction of DMSO in cryoprotectant solutions and formulate a DMSO-free cryoprotectant solution using glycerol, polymers, and L-proline. We also develop and explore the use of L-proline to improve the cryopreservation of Jurkat cells.

Proline is one of 22 amino acids that form the structure of proteins in biological organisms. Only the L-isomer is found in the proteins of mammals. Proline is formally an “imino” acid rather than an “amino” acid, as both the amine and carboxyl group are attached to the alpha carbon. However, it is normally referred to as an amino acid for nomenclatural simplicity. Proline is predominantly a zwitterion at physiological pH, meaning that due to its charged nature, its membrane permeability is limited without transporter proteins or channels.

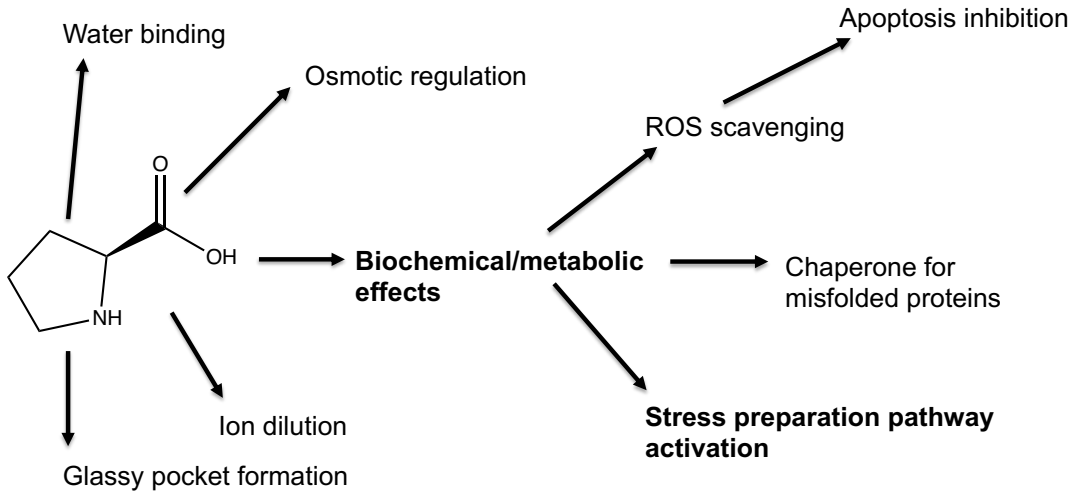

Figure 5.1. The cryoprotective mechanisms of L-proline.

In nature, L-proline is used by various organisms as an osmoprotectant.[12] For example, in halophytes such as *Armeria maritima* and *Aster tripolium*, L-proline concentration in the individual plants positively correlates to the salinity of the environment.[13] Some species of fish, such as *Fundulus diaphanus* also use L-proline, along with other amino acids to regulate their osmolarity, with more L-proline being present in the tissues when the external osmolarity is higher.[14] Some organisms use L-proline as a cryoprotectant. Examples are the wood frog *Rana sylvatica* which accumulates L-proline along with several other small molecular cryoprotectants before freezing over winter,[15] the overwintering larvae of the codling moth *Cydia pomonella*, which accumulates L-proline along with trehalose,[16] and the larvae of the drosophilid fly *Chymomyza costata* which can survive exposure to liquid nitrogen temperatures (–196 ºC or lower) in a hydrated state.[17] This last feat is particularly impressive; even tardigrades, known for their ability to survive extreme cold, can only do so by entering a state of anhidrosis.[18]

Unlike some osmolytes, such as trehalose, L-proline is membrane-permeable as it can enter the cell through Na^+ dependent transporters.[19] Inside the cell there are two categories of cryoprotective mechanism: 1. biophysical mechanisms, in which L-proline acts in much the same way as DMSO, by inhibiting intercellular ice formation and preventing solute concentration toxicity and 2. biochemical/metabolic mechanisms, where L-proline may prepare the cell to withstand freezing or protect the cell from the effects of freezing in some way.

Studies support the existence of biophysical mechanisms. In mouse oocytes, vitrification was enhanced by the addition of L-proline, with short incubation times and reduction of the solvent cryoprotectant concentrations necessary to achieve successful cryopreservation by vitrification, suggesting that recovery was improved by enhancement of vitrification.[20] L-proline was also used to cryopreserve ovine red blood cells (RBCs), human epithelial cells, mouse fibroblasts, human smooth muscle cells,[21] and human spermatozoa.[22] These experiments likewise did not pre-incubate with L-proline and cryopreservation was initiated before any metabolic effects of L-proline likely could have taken place. There are several biophysical mechanisms: colligative effects occur when a water-soluble molecule hydrogen bonds with a water molecule that might otherwise have formed a hydrogen bond with another water molecule, thus disrupting hydrogen bond formation between them. Water binding is a similar principle but is usually used to describe one solute molecule that forms hydrogen bonds with many molecules of water, reducing the amount of freezable water in the sample.[23] Like many small water-soluble molecules, L-proline is capable of both effects, and a solution of only L-proline in water vitrifies at sufficient

concentrations and cooling rates. As discussed above, L-proline is an osmoprotectant, and may prevent damage through freeze-induced dehydration/freeze-induced concentration of solutes. L-proline accumulation may allow the cells to retain water in response to extracellular dehydration without risking the creation of an ionic imbalance by relying on inorganic osmolytes.[24]

But it is clear that L-proline also has a biochemical/metabolic mechanism, with cryoprotective effects becoming apparent after exposure to L-proline over a period of time. Baily *et al*. (2015) found that pre-incubation with L-proline improved neuroblastoma cell recovery. A proposed mechanism for this was the effect of L-proline on ice crystal size. However, it was found that although a combination of trehalose and L-proline was able to affect ice crystal size, L-proline alone was not. Baily *et al.* (2015) also found that pre-incubation of cells with L-proline was able to temporally reduce cell growth rate prior to cryopreservation.[25] Baily *et al.* (2021) found that pre-incubation with L-proline improves human epithelial cell monolayer cryopreservation and improves the effectiveness of polymer ice recrystallisation inhibitors (IRIs). The osmoprotectants betaine and L-alanine where unable to improve cell recovery to the extent that L-proline did. Baily *et al.* (2021) also found that this was a biochemical, not merely biophysical, mechanism of action; cell growth was slowed prior to cryopreservation and proteomic analysis revealed that the cells were upregulating many proteins associated with the general cell stress response.[26] There are several potential biochemical/metabolic mechanisms: L-proline acts as a reactive oxygen species (ROS) scavenger,[27] which is useful for cryopreservation as the freezing process causes oxidative stress,[28] and may prevent apoptosis due to ROS-

mediated damage.[29] L-proline can also act as a chaperone, preventing the aggregation of damaged and misfolded proteins.[30] L-proline is involved in cell signalling,[24] and exposure to heightened L-proline concentrations may activate signalling pathways that prepare the cell for stress.[26]

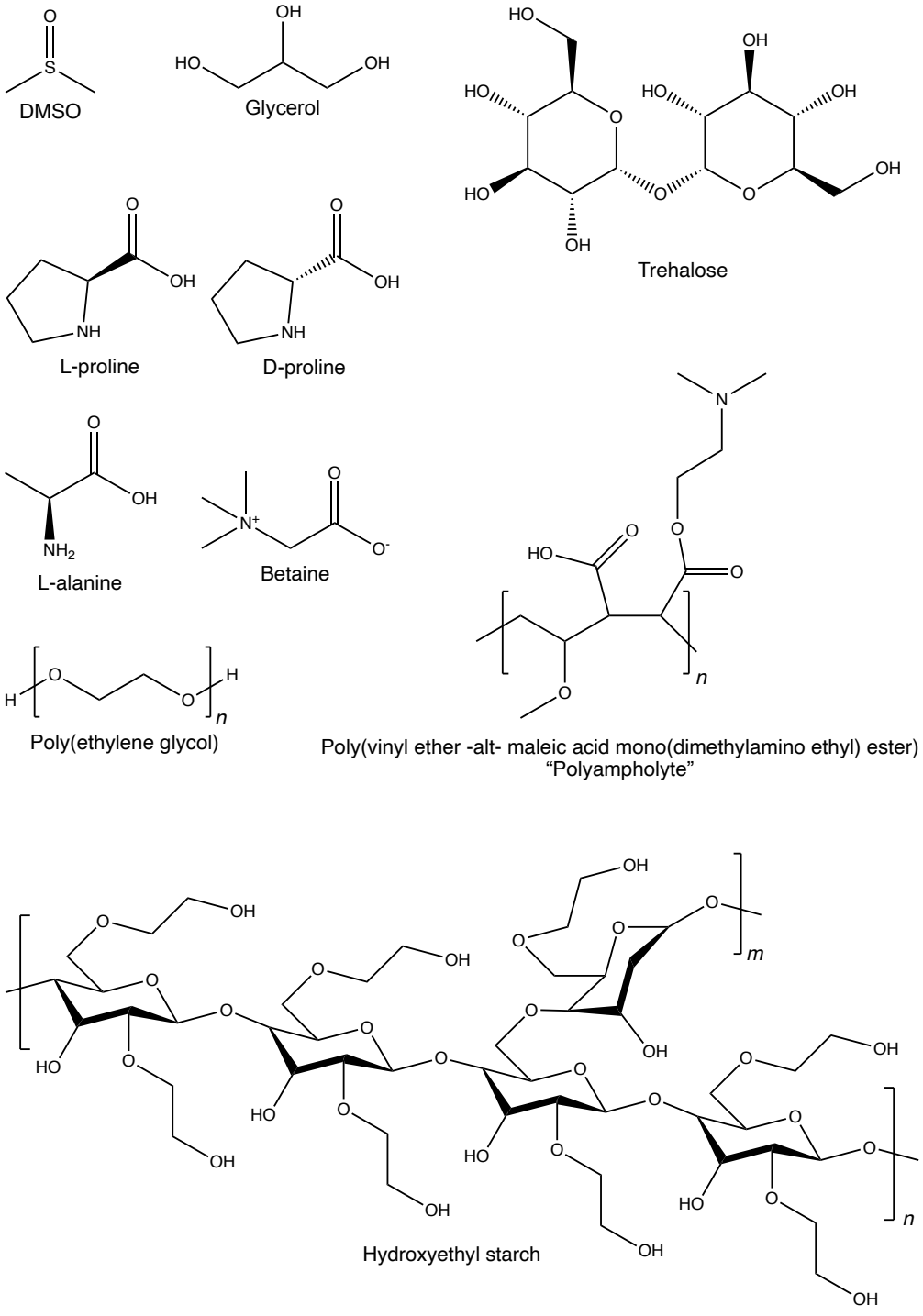

Figure 5.2. The cryoprotectants used in this chapter.

5.4 Results and discussion

5.4.1 Discovery of a DMSO-free cryoprotectant solution

Our first aim was to determine if glycerol + DMSO is a synergistic cryoprotectant combination for Jurkat cells. If so, added glycerol would increase post-thaw recovery at the same DMSO concentration or allow the DMSO concentration to be reduced for the same post-thaw recovery. To do this, we used a liquid handling robot to produce cryoprotectant matrix plates with 100 µL 2× concentration cryoprotectant solutions. We then added 100 µL Jurkat cells (cultured as described in **Chapter 4** and **Chapter 5**) to the plates at $2x10^6$ cells mL^{-1} with mutual dilution resulting in 1× concentration cryoprotectant and $1x10^6$ cells mL^{-1} and a final volume of 200 µL. Plates were cooled at – 1 °C min^{-1} to – 80 °C and stored at – 80 °C for at least 24 hours. Plates were then thawed for 5 minutes in a 37 °C water bath, resuspended in fresh media, and incubated for 24 hours at 37 ºC in a humid atmosphere with 5 % CO_2 for 24 hours. After incubation, cell recovery was assessed using a resazurin assay with a 4-hour incubation period. Here, resazurin, a non-fluorescent chemical, was metabolised by mitochondrial reductase to produce resorufin, which was strongly fluorescent.[31] It was found that DMSO and glycerol are not a synergistic cryoprotectant pair; the peak recovery achieved (51 %) was 8 % DMSO, 0 % glycerol, and no combination of DMSO and glycerol resulted in a recovery higher than that obtained with 8 % DMSO (Figure 5.3). However, 8 % glycerol with 0 % DMSO resulted in an acceptable recovery (39 %). This was not statistically different from that obtained with 8 % DMSO 0 % glycerol, but this may be due to small sample size. Overall, this data indicates that glycerol can be used as a slightly less effective alternative to DMSO.

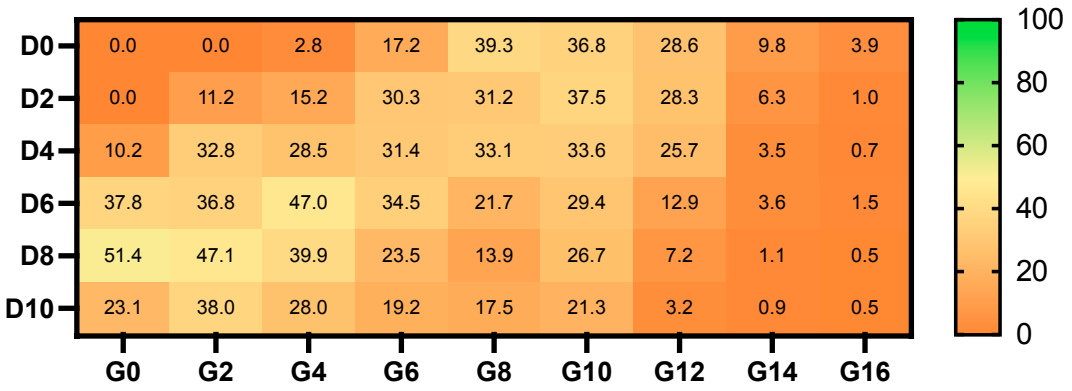

Figure 5.3. The percentage recovery of Jurkat cells after being cooled at – 1 °C min^{-1} to – 80 °C and thawed for 5 minutes at 37 °C. X-axis labels indicate final glycerol concentration (% v/v, e.g., G2 = 2 % glycerol). Y-axis labels indicate final DMSO concentration (% v/v, e.g., D2 = 2 % DMSO). Data represents the mean of three independent experiments.

Our next aim was to determine if DMSO and trehalose are a synergistic cryoprotectant pair for Jurkat cells. An assay was conducted as above with trehalose instead of glycerol. It was found that DMSO and trehalose are not a synergistic cryoprotectant pair, as the peak recovery was achieved at 8 % DMSO, 0 mM trehalose, and no combination of DMSO and trehalose resulted in a recovery higher than that obtained with 8 % DMSO (Figure 5.4).

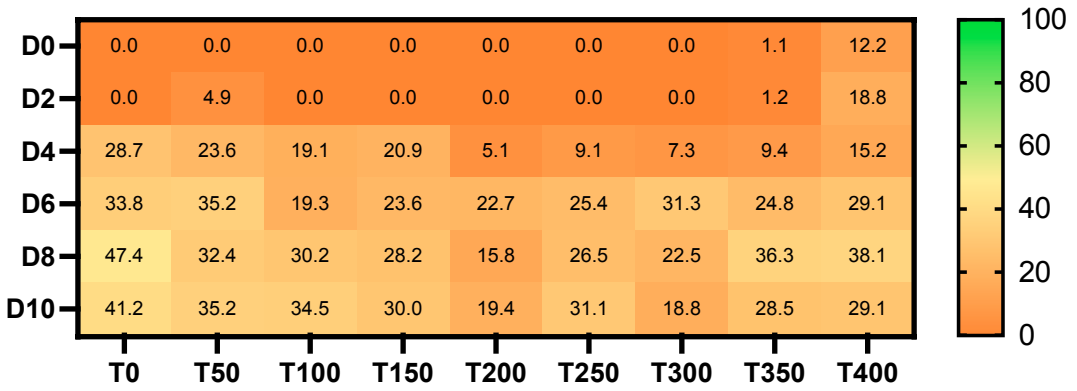

Figure 5.4. The percentage recovery of Jurkat cells after being cooled at – 1 °C min^{-1} to – 80 °C and thawed for 5 minutes at 37 °C. X-axis labels indicate final trehalose concentration (mM, e.g., T50 = 50 mM trehalose). Y-axis labels indicate final DMSO concentration (% v/v, e.g., D2 = 2 % DMSO). Data represents the mean of three independent experiments.

We then wanted to find out if polyampholyte and DMSO are a synergistic cryoprotectant pair for Jurkat cells. An assay was conducted as above with polyampholyte instead of glycerol. It was found that DMSO and polyampholyte are not a synergistic pair, as the peak recovery was achieved at 4 % DMSO, 0 % polyampholyte, and no combination of DMSO and polyampholyte resulted in a recovery higher than that obtained with 4 % DMSO (Figure 5.5).

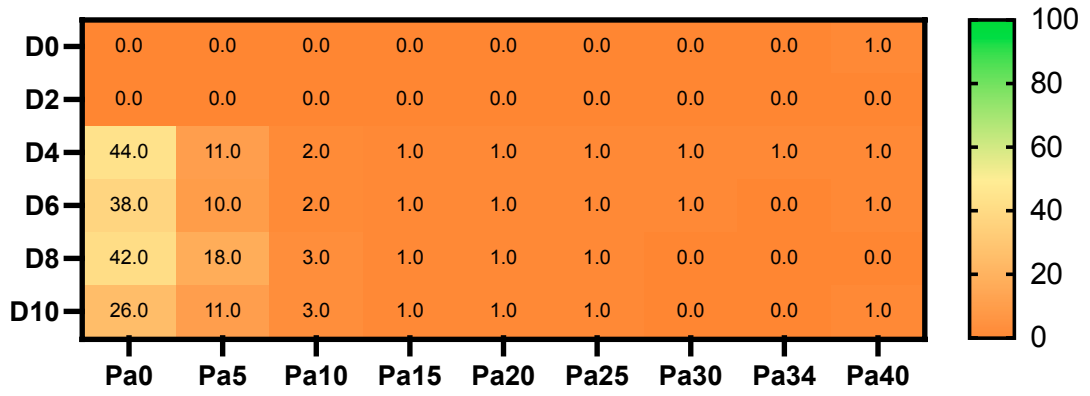

Figure 5.5. The percentage recovery of Jurkat cells after being cooled at – 1 °C min^{-1} to – 80 °C and thawed for 5 minutes at 37 °C. X-axis labels indicate final polyampholyte concentration (mg mL^{-1}, e.g., Pa5 = 5 mg mL^{-1} polyampholyte). Y-axis labels indicate final DMSO concentration (% v/v, e.g., D2 = 2 % DMSO). Data represents the mean of three independent experiments.

Having failed to find a cryoprotectant that would allow for the reduction of DMSO by synergistically combining with it, we turned our attention to DMSO-free solutions. Several cryoprotectant solutions were formulated using mixtures of 8 % glycerol (G) and 20 mg mL^{-1} of each polyampholyte (Pa), hydroxyethyl starch (HES), poly(ethylene glycol) (P) and L-proline (Pro). The cryoprotectant combinations are shown in Figure 5.6. 1 mL Jurkat cells at a concentration of $1x10^6$ cells mL^{-1} were mixed with these cryoprotectants and plunged into liquid nitrogen. Cells were left in liquid nitrogen for at least 20 minutes before warming in a 37 °C water bath for 5 minutes and resuspending in fresh media. Cells were counted immediately post-thaw (Figure 5.6a). Cell recovery is defined as $\frac{\text{Live cells recovered}}{\text{Live cells frozen}}$ expressed as a percentage, and viability is defined as $\frac{\text{Live cells recovered}}{\text{Total cells thawed}}$ expressed as a percentage. Viability of

cells was determined by the trypan blue exclusion method.[32] The experiment was conducted a second time with the most successful cryoprotectant combinations, this time counting after 24 hours incubation at 37 ºC in a humid atmosphere with 5 % CO_2 (Figure 5.6b). It was found that immediately post-thaw, all cryoprotectant combinations resulted in significantly higher post-thaw recovery than the 5 % DMSO control, with 20 mg mL^{-1} of each polyampholyte, poly(ethylene glycol), hydroxyethyl starch, and L-proline, in the absence of glycerol yielding the best recovery (89 %). However, after 24 hours incubation, the recovery of all solutions tested fell drastically, with no solution giving higher than 18 % recovery, which was not statistically higher than the 5 % DMSO control.

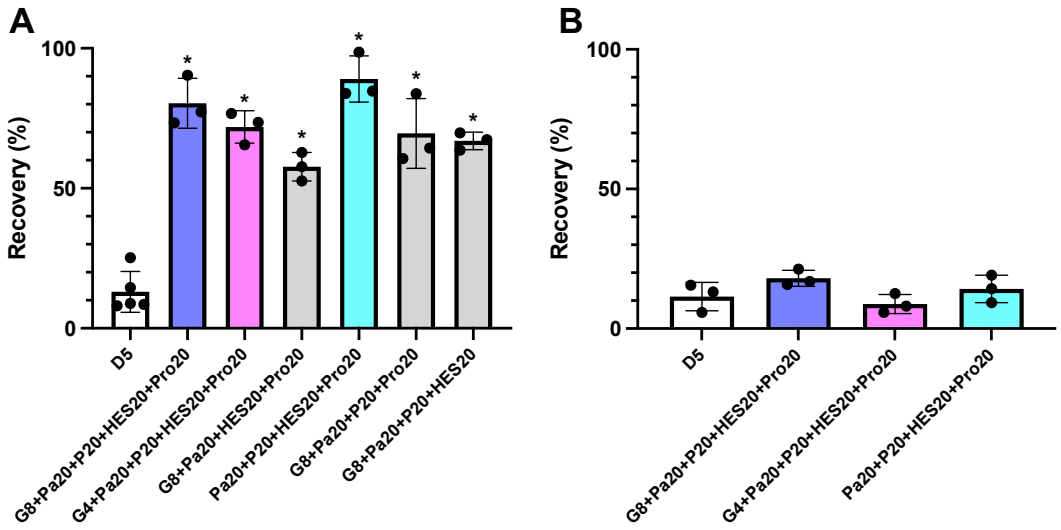

Figure 5.6. The percentage recovery of Jurkat cells after being cooled at – 1 °C min^{-1} to – 80 °C and thawed for 5 minutes at 37 °C. X-axis labels indicate final polyampholyte concentration (mg mL^{-1}, e.g., Pa5 = 5 mg mL^{-1} polyampholyte). Y-axis labels indicate final DMSO concentration (% v/v, e.g., D2 = 2 % DMSO). Data represents the mean of three independent experiments.

Plunging cryovials into liquid nitrogen results in a much higher freezing rate than the optimal freezing rate for Jurkats.[33] We, therefore, tested several formulations by freezing the cells at – 1 °C min^{-1} to – 80 °C before thawing as above and counting 24 hours post-thaw. This series of formulations focused on combining the non-penetrating components with different concentrations of DMSO. However, on the first run of the experiment, no combinations resulted in sufficient recovery relative to the control and so the experiment was terminated without repeats (Figure 5.7).

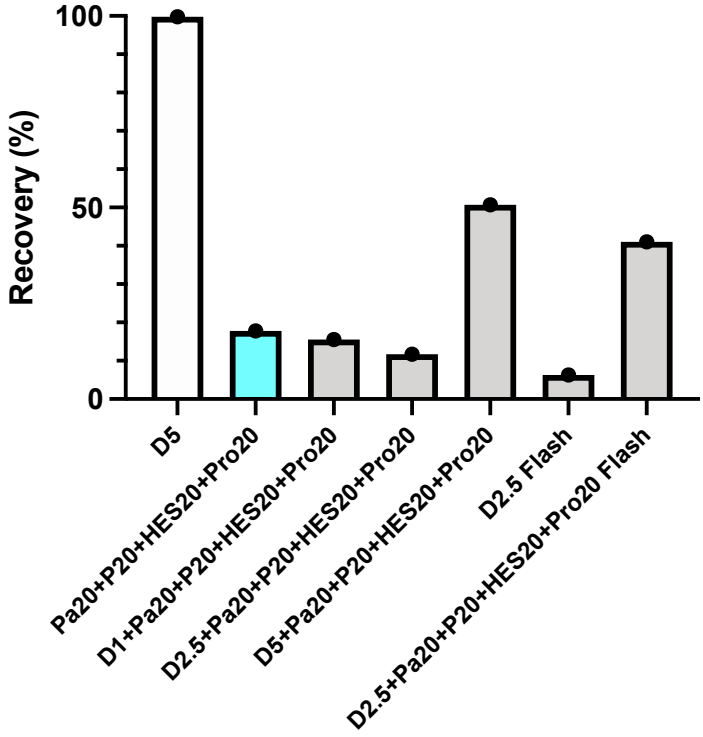

Figure 5.7. Recovery of Jurkat cells after either being frozen in liquid nitrogen or cooled at – 1 °C min^{-1} to – 80 °C, and thawed for 5 minutes in a 37 °C water bath. D = DMSO, G = glycerol, Pa = polyampholyte, P = poly(ethylene glycol), HES = hydroxyethyl starch, Pro = L-proline. Flash = frozen in liquid nitrogen.

Based on these results, we suspected that a combination without glycerol would not be successful. Where therefore tested 8 % glycerol + 20 mg mL^{-1} of each polyampholyte hydroxyethyl starch, poly(ethylene glycol) and L-proline, as this formulation gave the highest 24-hour post-thaw recovery when plunging Jurkats into liquid nitrogen (Figure 5.6b). For this test, Jurkats were cooled at – 1 °C min^{-1} to – 80 °C, and thawed for 5 minutes in a 37 °C water bath as normal. We found that this formulation gave very good recovery (79 %), almost as high as the DMSO control (86 %). This difference was not statistically significant, although this may be due to low sample size in the experimental group. The formulation gave a much higher recovery than the glycerol control (50 %). This difference was statistically significant. In an attempt to improve this formulation, we made a range of small variants to it; the 8 % glycerol was kept constant in most formulations while the concentration of one component in each formation was either doubled or halved. In one formulation, the carrier solution was water instead of Dulbecco's phosphate-buffered saline (DPBS) (signified by "-H2O" in Figure 5.8) as this substitution was beneficial for RBCs in **Chapter 3**. We also made formulations with trehalose (T), and betaine (B), with the concentration of these cryoprotectants given in mM. We found that no solution tested was more effective than 8 % glycerol and 20 mg mL^{-1} of each component with DPBS as the carrier solution.

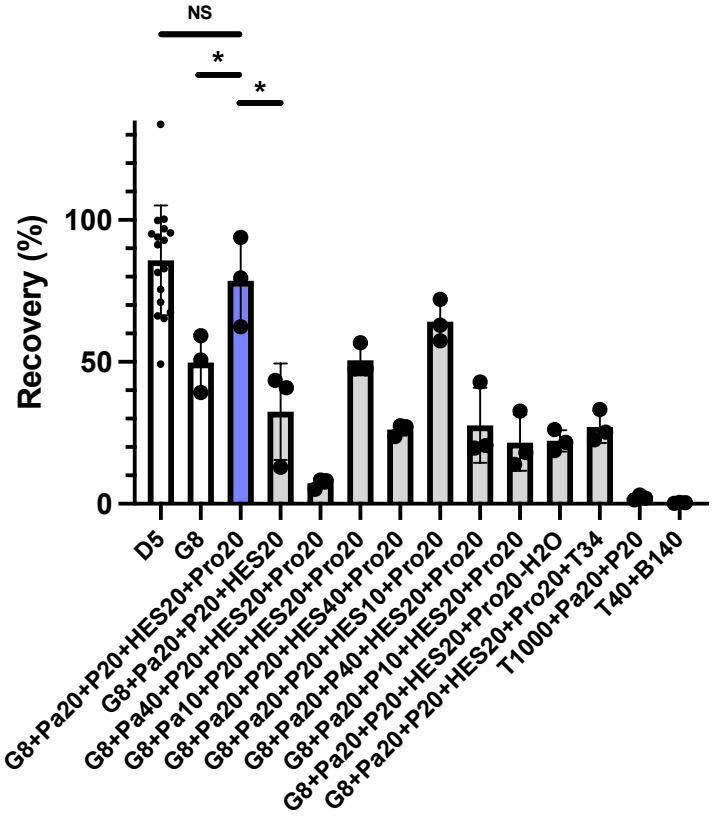

Figure 5.8. Recovery of Jurkat cells after being cooled at – 1 °C min^{-1} to – 80 °C, and thawed for 5 minutes in a 37 °C water bath. D = DMSO, G = glycerol, Pa = polyampholyte, P = poly(ethylene glycol), HES = hydroxyethyl starch, Pro = L-proline, T = trehalose, B = betaine. Data represents the mean ± SD of at least three independent experiments (*P < 0.05).

5.4.2 Pre-incubation with L-proline

L-proline was an essential component of this cryoprotectant solution, with the addition of L-proline increasing recovery from 32 % to 79 % (Figure 5.8). We wanted to explore this effect further, and hypothesised that pre-incubation with L-proline would improve the cryopreservation of Jurkat cells as it has in other cell types.[25,26] To find out if L-proline pre-incubation has a cryoprotective effect on Jurkats, cells were

incubated in a 12 well plate, at a volume 1 mL per well, with standard growth media containing 0.17 mM L-proline and 0.10 mM L-alanine, supplemented with 0 mM, 100 mM, or 200 mM L-proline for 24 hours. A cell density of $1x10^{-6}$ mL^{-1} was chosen for this experiment as it is the standard cell density used to test cryoprotectants in our group. After incubation, cell density was readjusted to $1x10^{-6}$ mL^{-1} and cells were resuspended in fresh media containing no added L-proline. 5 % DMSO was added, and the cells were incubated for 10 minutes. Cells were then transferred to 1 mL cryovials and cooled at – 1 ºC min^{-1} to – 80 ºC in a cell cooler. After spending at least 24 hours at – 80 ºC, cells were warmed in a 37 ºC water bath for 5 minutes, resuspended in fresh media and transferred to a 12 well plate. Cells were counted immediately post-thaw and then every 4 hours for the following 24 hours. It was found that cells incubated with 100 mM or 200 mM L-proline had higher immediate recovery (39 % vs 50 % vs 50 % for 0 mM, 100 mM and 200 mM respectively), and viability (43 % vs 60 % vs 63 %), and higher recovery after 24 hours (57 % vs 77 % vs 75 %) than cells with 0 mM additional L-proline (Figure 5.9). Although none of these differences were statistically significant at $P < 0.05$, this could be due to small sample size rather than lack of effect. The recovery between cells incubated with 100 mM and 200 mM was essentially the same at the 24-hour time point.

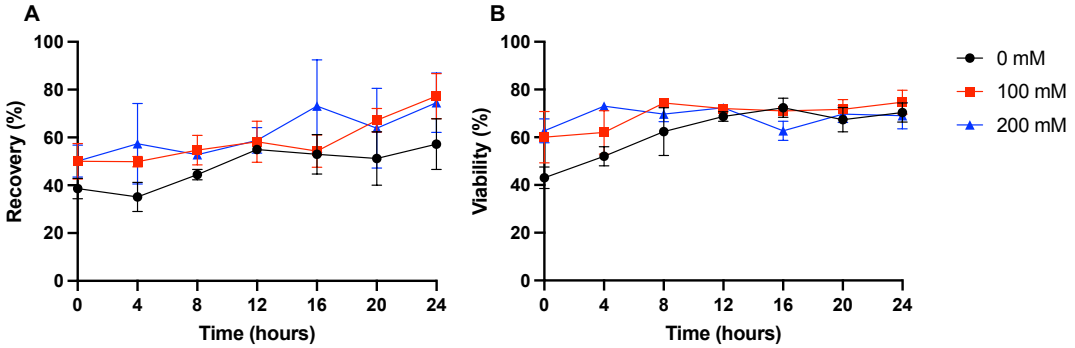

Figure 5.9. **(A)** Recovery and **(B)** viability of Jurkat cells after being frozen with 5 % DMSO at – 1 °C min^{-1} to – 80 °C and thawed for 5 minutes in a 37 °C water bath. Prior to freezing, cells were incubated for 24 hours with media containing 0, 100 or 200 mM added L-proline. This media was removed and replaced with fresh media pre-freeze. Cell count was taken every 4 hours. Data represents the mean ± SD of three independent experiments.

As L-proline is known to reduce the growth rate of lung epithelial cells,[26] we studied the effect of L-proline on cell growth over the incubation period. Cells at a density of $1x10^{-6}$ mL^{-1} were incubated in a 12 well plate, at a volume 1 mL per well, with standard growth media containing 0.17 mM L-proline and 0.10 mM L-alanine, supplemented with 0 mM, 100 mM, or 200 mM L-proline for 24 hours. Cells were counted every 4 hours for 24 hours. It was found that L-proline slows growth from the 8-hour time point, and completely inhibits growth from the 24-hour time point (Figure 5.10). This appears to be dose-dependent with 200 mM L-proline inhibiting growth more than 100 mM L-proline (34 % vs 46 % reduction in growth after 24 hours compared to control respectively), although the difference between the two L-proline concentrations tested is not statistically significant. Viability was not reduced with L-

proline incubation up to 200 mM. As 200 mM L-proline may cause a greater reduction in growth rate than 100 mM L-proline, and taking into account preliminary data (Figure 5.16), we took 200 mM forward as the experimental concentration for additives in future experiments.

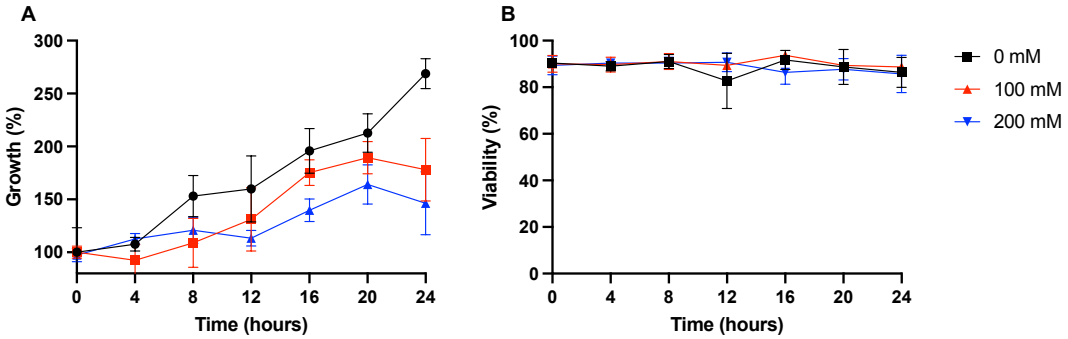

Figure 5.10. **(A)** Recovery and **(B)** viability of Jurkat cells during 24 hours incubation with media containing 0, 100, or 200 mM added L-proline. Cell count taken every 4 hours. Data represents the mean ± SD of three independent experiments.

To begin to elucidate the cryoprotective mechanism of L-proline, we compared it to two other amino acids: D-proline and L-alanine. In nature, D-proline is only found in a very small minority of proteins, with L-proline being favoured throughout the natural world.[34] In mammals, most D-amino acids are of bacterial origin.[35] Proteins display a high degree of stereoselectivity,[36,37] meaning that proteins which interact with L-proline are likely unable to interact with D-proline. An example of this is the need for separate D- and L- amino acid oxidase enzymes.[38] If D-proline had the same cryoprotective effect as L-proline, it would provide evidence that proline's cryoprotective effect is physical rather than metabolic, since cells cannot interact with D-proline on a metabolic level to the extent that they can with L-proline. L-alanine shares some of the physical mechanisms of cryoprotection with L-proline by virtue of

being a water-soluble small molecule, but may have different biochemical/metabolic properties inside the cell, so knowing its effectiveness compared to L-proline would help determine if L-proline's biochemical mechanisms of action are unique among amino acids. Cells at a density of $1x10^{-6}$ mL^{-1} were incubated for 24 hours, in a 12 well plate, at a volume of 1 mL per well, with fresh media containing 200 mM of L-proline, D-proline, L-alanine, or no additive. After incubation, cells were counted, concentration readjusted to $10x10^{6}$ mL^{-1}, and resuspended in fresh media. 5 % DMSO was added, and the cells were incubated for 10 minutes. Cells were then transferred to 1 mL cryovials and cooled at – 1 ºC min^{-1} to – 80 ºC in a cell cooler. After spending at least 24 hours at – 80 ºC, cells were warmed in a 37 ºC water bath for 5 minutes, resuspended in fresh media and transferred to a 12 well plate. Cells were counted 24 hours post-thaw. It was found that L-proline, D-proline, and L-alanine, all reduced the growth rate of the cells (by 79 %, 107 % and 72 % respectively), as determined by cell count (Figure 5.11a). However, D-proline alone caused a statistically significant reduction (25 %) in the proportion of viable cells (Figure 5.11b). Combined with negative cell growth, this implies that the low post-incubation cell counts after incubation with D-proline are due to toxicity rather than merely growth inhibition. Cells cryopreserved with L-proline exhibited slightly higher recovery than the untreated control (63 % vs 70 %), whereas cells cryopreserved with D-proline and L-alanine exhibited lower recovery (52 % and 49 % respectively) (Figure 5.11c). Although these recovery values were not statistically significant, they provided some evidence to spur further exploration. D-proline also exhibited lower post-thaw viability (Figure 5.11d). Some of the data here was carried forward from previous experiments, shown in Figure 5.9 and Figure 5.10 (0 mM and 200 mM L-proline at

the 24-hour timepoint), so that the accumulated datapoints generated under the same experimental conditions could be analysed.

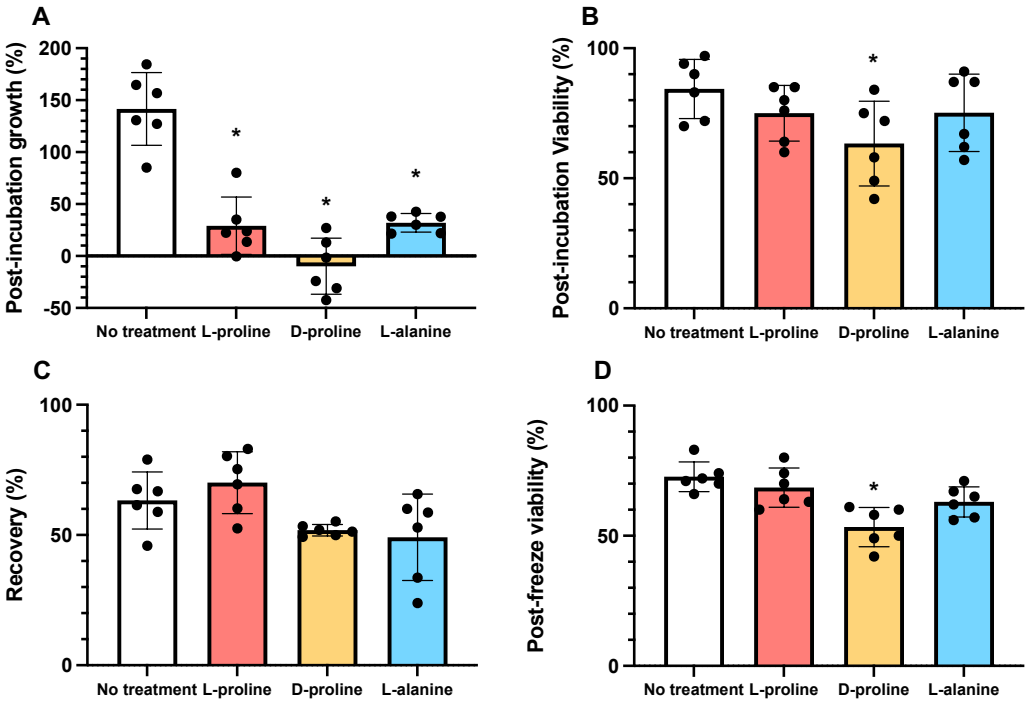

Figure 5.11. **(A)** Growth and **(B)** viability of Jurkat cells after 24 hours incubation with 200 mM additives (listed on X-axis). **(C)** Recovery and **(D)** Viability of Jurkat cells after being frozen with 5 % DMSO at – 1 °C min^{-1} to – 80 °C and thawed for 5 minutes in a 37 °C water bath, after 24 hours incubation with 200 mM additives and resuspension in fresh non-additive media. Data represents the mean ± SD of at least six independent experiments (*P < 0.05 from untreated control). Some data here was carried forward here from previous experiments, shown in Figure 5.9 and Figure 5.10, to show the accumulated datapoints generated under the same experimental conditions.

Many cell types are sensitive to cell density, exhibiting density-dependent growth rates[39,40] and recoveries[41,42] after cryopreservation. To explore the effectiveness of the

additives at different cell densities, we incubated the cells at densities of $0.5x10^6$ mL^{-1} and $0.8x10^6$ mL^{-1}. (Data from experiments at $1x10^6$ mL^{-1} is included in Figure 5.11 for comparison). Cells were incubated for 24 hours, in a 12 well plate, at a volume of 1 mL per well, with fresh media containing 200 mM of L-proline, L-alanine, or no additive, for 24 hours. After incubation, cells were counted, readjusted to $10x10^6$ mL^{-1}, and resuspended in fresh media. 5 % DMSO was added, and the cells were incubated for 10 minutes. Cells were then transferred to 1 mL cryovials and cooled at – 1 ºC min^{-1} to – 80 ºC in a cell cooler. After spending at least 24 hours at – 80 ºC, cells were warmed in a 37 ºC water bath for 5 minutes, resuspended in fresh media and transferred to a 12 well plate. Cells were counted 24 hours post-thaw. D-proline had previously been found to be toxic to the cells, masking any cryoprotective effect it may have, and was therefore not included in the cell density experiments. It was found that cells incubated with L-proline or L-alanine at a density of $0.5x10^6$ mL^{-1} exhibited the highest recoveries (78 % and 84 % respectively, compared to 54 % for the control). It seems that while L-proline has a modest, if any, cryoprotective effect at a cell density of $1x10^6$ mL^{-1}, and L-alanine has none, they both have a cryoprotective effect at $0.5x10^6$ mL^{-1} and $0.8x10^6$ mL^{-1} (Figure 5.12).

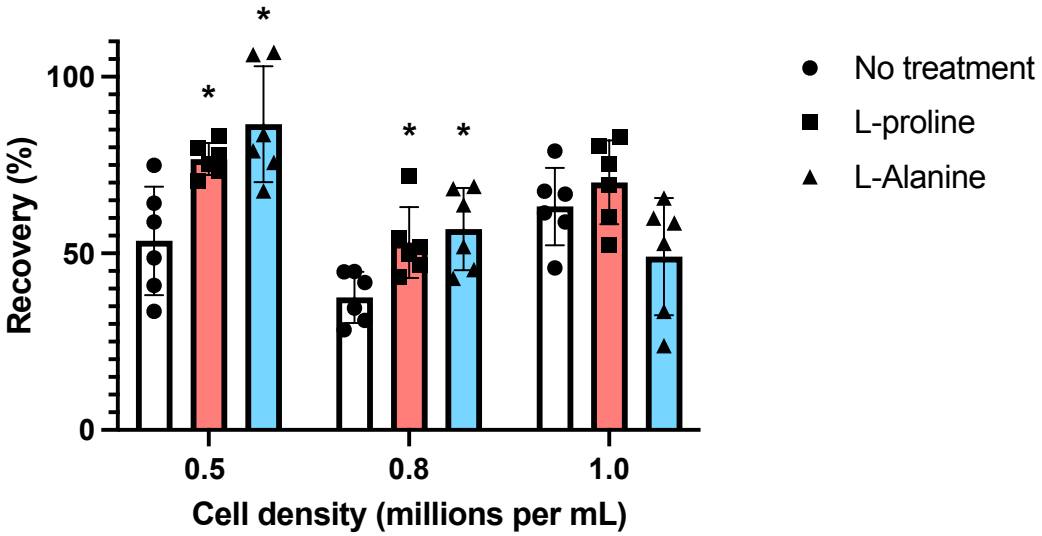

Figure 5.12. Recovery of Jurkat cells at different cell densities after being frozen with 5 % DMSO at – 1 °C min^{-1} to – 80 °C and thawed for 5 minutes in a 37 °C water bath. Prior to freezing, cells were incubated for 24 hours with 200 mM L-proline or 200 mM L-alanine. These additives were removed and replaced with fresh media pre-freeze. Data from experiments at $1x10^6$ mL^{-1} is included for comparison. (*P < 0.05 from untreated control at the same density). Data represents the mean ± SD of at least six independent experiments (*P < 0.05 from untreated control).

Extracellular L-proline was an essential component of our most effective glycerol-based solution (Figure 5.8). To find out if the presence of extracellular L-proline can enhance the cryopreservation of Jurkats with DMSO, cells at a density of $1x10^{-6}$ mL^{-1} were incubated for 24 hours, in a 12 well plate, at a volume of 1 mL per well, with fresh media containing 200 mM of L-proline, or no additive, for 24 hours. After incubation, cells were counted, readjusted to $10x10^6$ mL^{-1}, and resuspended in fresh media containing 200 mM of L-proline, or no additive respectively. 5 % DMSO was added, and the cells were incubated for 10 minutes. Cells were then transferred to 1

mL cryovials and cooled at – 1 ºC min^{-1} to – 80 ºC in a cell cooler. After spending at least 24 hours at – 80 ºC, cells were warmed in a 37 ºC water bath for 5 minutes, resuspended in fresh media and transferred to a 12 well plate. Cells were counted 24 hours post-thaw. It was found that when L-proline incubated Jurkat cells are cryopreserved with 200 mM L-proline in the freezing media, this confers no cryoprotective effect (Figure 5.13).

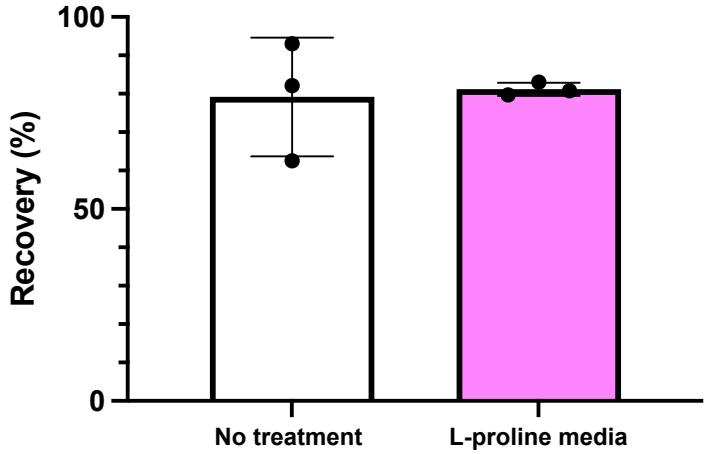

Figure 5.13. Recovery of Jurkat 5 % DMSO at – 1 °C min^{-1} to – 80 °C and thawed for 5 minutes in a 37 °C water bath. Prior to freezing, cells were incubated for 24 hours with 200 mM L-proline. L-proline was not removed prior to freezing.

We next investigated the effect of the additives on cellular metabolism. Cells at a density of $1x10^{-6}$ mL^{-1} were incubated for 24 hours, in a 12 well plate, at a volume of 1 mL per well, with fresh media containing 200 mM of L-proline, D-proline, L-alanine, or no additive. After incubation, cells were counted, readjusted to $10x10^{6}$ mL^{-1}, and resuspended in fresh media. Metabolic activity was assessed using a resazurin assay with a 3-hour incubation period.[31] This assay is usually used to count cells, but here cells were adjusted to equal numbers so that the assay became a measure of

relative metabolic activity per cell. It was found that cells incubated with D-proline showed greatly reduced metabolic activity which is consistent with the toxicity observed in earlier experiments. Cells incubated with L-proline and L-alanine also showed reduced metabolic activity, but this was not statistically significant (Figure 5.14).

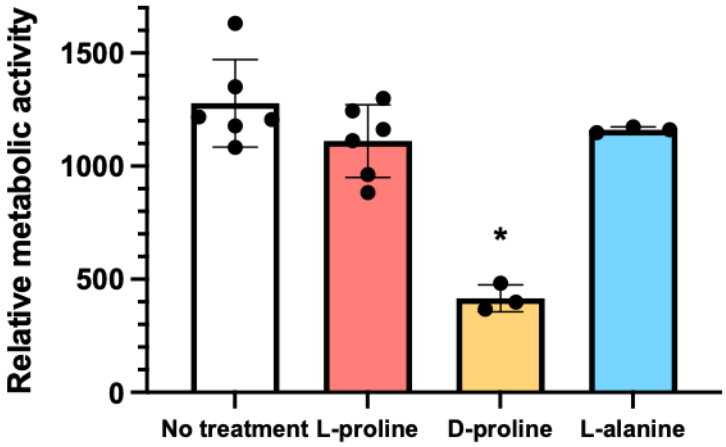

Figure 5.14. Relative metabolic activity, measured by resazurin assay, of Jurkat cells after 24 hours incubation with media containing 200 mM additives. Data represents the mean ± SD of at least three independent experiments. (*P < 0.05 from 10 % DMSO).

One mechanism of L-proline's cryoprotective effects is the inhibition of apoptosis by ROS scavenging.[27] To investigate the effect of L-proline on post-thaw apoptosis, we used a FITC Annexin V apoptosis assay, in which cells were incubated with Fluorescein isothiocyanate (FITC) labelled Annexin V (the conjugate is abbreviated as FA), and Propidium iodide (PI). The resulting fluorescence was measured by flow cytometry.[43] Annexin V binds to phosphatidylserine, which is only present on the cell surface during apoptosis. PI is a fluorescent DNA binding dye, which is only able to

enter the cells during late apoptosis or necrosis when the cell surface membrane is disrupted. Therefore, cells stained with neither FA nor PI are alive, cells stained with only FA are in early apoptosis, and cells stained with both FA and PI are in late apoptosis or necrosis. These changes must be monitored over a 24-hour period because there is a window of time in which early apoptosis can be detected: if measured too soon, apoptosis will not have started in all the cells destined for it. If measured too late, then the cells will have transitioned to late apoptosis which is indistinguishable from necrosis because the cell membrane becomes permeable to PI in both causes. Cells at a density of $1x10^{-6}$ mL^{-1} were incubated for 24 hours, in a 12 well plate, at a volume of 1 mL per well, with fresh media containing 200 mM of L-proline, or no additive. After incubation, cells were counted, concentration readjusted to $10x10^{6}$ mL^{-1}, and resuspended in fresh media. 5 % DMSO was added, and the cells were incubated for 10 minutes. Cells were then transferred to 1 mL cryovials and cooled at – 1 ºC min^{-1} to – 80 ºC in a cell cooler. After spending at least 24 hours at – 80 ºC, cells were warmed in a 37 ºC water bath for 5 minutes, resuspended in fresh media and transferred to a 12 well plate. Cells were stained, and fluorescence was analysed by flow cytometry at 1, 4, 8, and 24 hours post-thaw. There was no indication of a difference in the apoptosis/necrosis profile between the untreated control and L-proline incubated cells (Table 5.1 and Figure 5.15). However, this experiment was not repeated (n = 1) so no conclusion can be drawn.

Table 5.1. Results of flow cytometry showing the apoptosis/necrosis profile of Jurkat cells previously incubated with and without 200 mM L-proline for 24 hours. Early = early apoptosis, late = late apoptosis.

	1 hour	**4 hours**	**8 hours**	**24 hours**
Control	Live: 58 %	Live: 37 %	Live: 41 %	Live: 53 %
	Early: 19 %	Early: 46 %	Early: 49 %	Early: 31 %
	Late or necrosis: 23 %	Late or necrosis: 17 %	Late or necrosis: 10 %	Late or necrosis: 16 %
L-proline	Live: 63 %	Live: 30 %	Live: 46 %	Live: 53 %
	Early: 19 %	Early: 52 %	Early: 45 %	Early: 31 %
	Late or necrosis: 19 %	Late or necrosis: 18 %	Late or necrosis: 9 %	Late or necrosis: 16 %

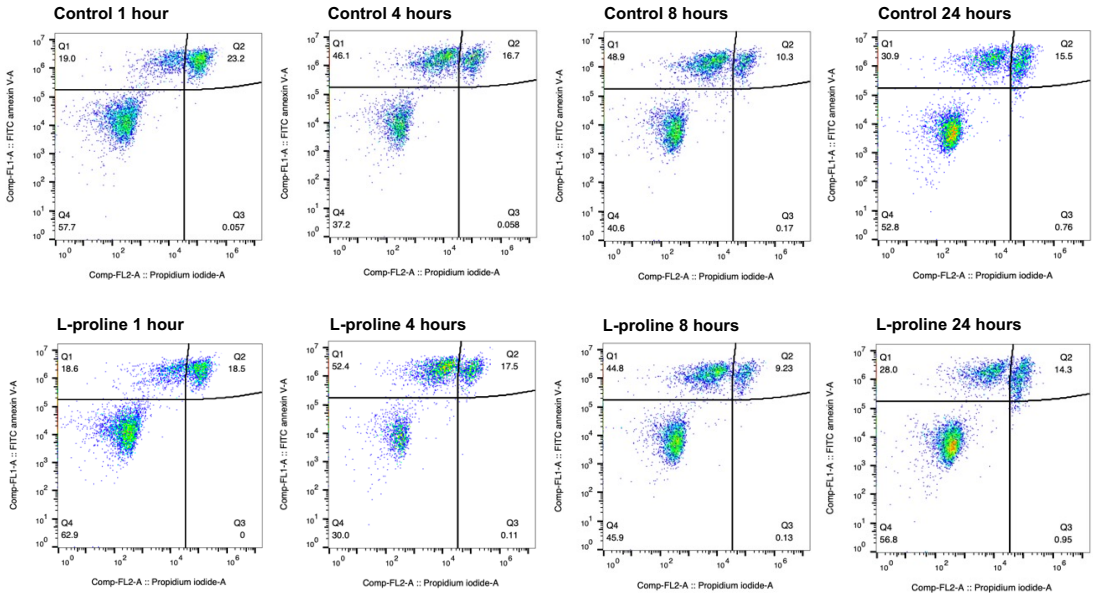

Figure 5.15. Flow cytometry plot showing the apoptosis/necrosis profile of Jurkat cells previously incubated with and without 200 mM L-proline for 24 hours. $FITC^+$ = early apoptosis. $FITC^+$ PI^+ = late apoptosis or necrosis. n = 1.

5.5 Conclusions

The first outcome of the work in this chapter was the development of a DMSO-free solution for the cryopreservation of Jurkats by using glycerol, a combination of polymers and L-proline. Glycerol and DMSO have similar toxicity values (rat oral),[44] so this may not be much of an improvement if glycerol also has similar toxicity in humans. However, unlike glycerol, DMSO is incompatible with poly(vinyl chloride)[45] which is commonly used for cell storage bags and transfer tubing,[46] so this formulation may prove advantageous where poly(vinyl chloride) systems are used. Although not all possible combinations were tested, we found that halving or doubling the concentration of any one component resulted in a reduction in post-thaw recovery. This suggests that all components are necessary for full cryoprotection. Replacing DPBS with water as the carrier solution did not result in improved recovery as it did in RBCs; it is likely the case that Jurkats are much more sensitive to changes in pH and osmolarity than RBCs. We also found that DMSO and either glycerol, trehalose or polyampholyte do not combine synergistically to form a cryoprotectant solution for Jurkat cells. This is in contrast to similar work on other cell types, where DMSO + trehalose[47] and DMSO + polyampholyte[48] have been successful combinations. Ours is not the first DMSO-free cryoprotectant solution,[49–51] but many promising DMSO-free cryoprotectant formulations are commercial secrets and their makeup is not publicly known, so there is still benefit to exploring DMSO-free cryopreservation and making the results public.[52,53] Further research should compare the effectiveness of these solutions with our solution in Jurkats and primary human T-cells. The second outcome was an exploration of the effectiveness of L-proline for the cryopreservation of Jurkat cells, which are a useful model for CAR T-cell therapies. We initially found

a small, statistically insignificant, cryoprotective effect of L-proline at a cell density of $1x10^{-6}$ mL^{-1}, then when exploring the relationship between cell density and cryoprotective effect, we found that L-proline is more effective at lower cell densities. We found that L-alanine also has a cryoprotective effect at lower cell densities, indicating that L-proline is not unique among amino acids as a cryoprotectant. D-proline was toxic to the cells, indicating that chirality is an important property, perhaps because the cell can only interact with the L-isomer on a biochemical level. For example, it could be that D-proline cannot be taken up by L-proline uptake transports, resulting in failure of equilibration and toxicity from hyperosmotic stress. An alternative explanation is that D-proline is integrated into proteins, resulting in protein misfolding. Since D-proline is not toxic to larger organisms,[54] at least not directly,[55] the former explanation is more likely. We found that L-proline and L-alanine reduce cell growth without causing significant toxicity, supporting the hypothesis that these amino acids prepare the cells for cryopreservation by slowing growth. Further supporting this hypothesis is that the cryoprotective effect is cell density-dependent;[40] Jurkats undergo more cell division at lower densities, which is where L-proline is at its most effective. At higher cell densities, the rate of Jurkat proliferation decreases due to senescence and the lower availability of nutrients in the media, so if L-proline works primarily through growth inhibition, its effectiveness would be reduced where there is less growth to inhibit. This replicates the conclusion made by Baily *et al.* (2021) regarding the slowing of cell growth, but contradicts the hypothesis that this mechanism of action is unique to L-proline.[26] Analysis by flow cytometry found no evidence to support the hypothesis that L-proline prevents apoptosis, although the flow cytometry dataset of n = 1 is too limited to refute it.

5.6 Experimental

5.6.1 Materials

L-proline, D-proline and L-alanine were obtained from Sigma-Aldrich. Glycerol and betaine were from Fisher Scientific. Poly(ethylene glycol) (PEG) average Mn = 4,000 g mol^{-1} was from Aldrich. Hydroxyethyl starch was from Carbosynth. Solutions were made by dissolving additives in culture media (details of culture media below). Polyampholyte was synthesised in-house as described in **Chapter 3**. Solutions were sterile filtered using 0.2 μm syringe filters obtained from Fisher.

5.6.2 Flow cytometry

Immediately after thawing, cells were labelled with FITC-Annexin V, and PI, and the resulting fluorescence was detected by flow cytometry. Fluorescence was measured at the one-hour timepoint. The process was repeated for measurements at the 4, 8, and 24 hour time points. Flow cytometry was performed using a BD Accuri C6 flow cytometer. 10,000 events were recorded per sample using a flow rate of 14 μL per min^{-1}. Data was analysed using FlowJo software. FITC was excited using a 488 nm laser and detected via a 533/30 nm bandpass filter. PI was excited using a 488 nm laser and detected using a 585/40 nm bandpass filter. FITC fluorescence spillover into the PI channel was colour compensated by subtracting 14.47 % of FITC from PI.

5.6.3 Cell culture

E6.1 Jurkat cells were from the European Collection of Authenticated Cell Cultures (ECACC). The cell line was maintained in T175 flasks (Greiner Bio-One Ltd), and incubated at 37 ºC in a humid atmosphere with 5 % CO_2. Complete culture media was

Advanced RPMI 1640 supplemented with 1 % Antibiotic-Antimycotic (both Thermo Fisher) and 10 % non-USA origin fetal bovine serum (Merck). Base Advanced RPMI 1640 media contained 0.17 mM L-proline and 0.10 mM L-alanine. Cells were passaged every 4 days to maintain a cell density not exceeding $1.5x10^6$.

5.6.4 Cryopreservation, recovery, and viability assays

Cells were taken from the main cell line when its cell density reached approximately $1x10^{-6}$ mL^{-1}. After any pre-incubation steps, cells were incubated at room temperature with fresh media and cryoprotectant for 10 minutes. After incubation, cells were cooled at – 1 °C min^{-1} to – 80 °C using a cryovial cooler (Coolcell LX, Corning). Cells were kept at – 80 °C for at least 24 hours. Vials were warmed in a 37 °C water bath for 5 minutes, resuspended in fresh media and plated onto a sterile 12 well tissue culture plate (Corning). Cells were incubated at 37 ºC in a humid atmosphere with 5 % CO_2 until counting. Centrifugation for the resuspension steps was conducted at 825 × g in a Spectrafuge 6C for volumes above 1 mL and at 3000 × g in a VWR Micro Star 17 for volumes 1 mL or lower. Cells were counted using a Countess automated cell counter with Countess cell counting chamber slides (both Thermo Fisher). Percentage recovery was defined as $\frac{Live\ cells\ recovered}{Live\ cells\ frozen} * 100$ and viability was defined as $\frac{Live\ cells\ recovered}{Total\ cells\ thawed} * 100$. Live and dead cells were distinguished using the trypan blue exclusion method, whereby live cells exclude the dye while dead cells were stained.[32] The stock trypan blue was obtained from Merck and diluted two-fold with DPBS (Sigma-Aldrich).

5.6.5 Resazurin assay

To conduct the Resazurin assay.[31] Resazurin solution was prepared by dissolving one resazurin tablet (Scientific Laboratory Supplies) in 30 mL fresh complete media containing no additives. Cells were resuspended in resazurin solution and incubated at 37 ºC for either 3 or 4 hours. After incubation, the plate was excited with light using a 530/25 mm bandpass filter and fluorescence was detected with a 590/35 nm bandpass filter using a synergy HT microplate reader.

5.6.6 Statistics

Statistical analysis was performed using Prism 9 software (GraphPad). Statistical significance was determined by ordinary one-way ANOVA using Dunnett's test for multiple comparisons, unless only two conditions were compared, in which case a t-test was used. P values below 0.05 were considered statistically significant.

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5.8 Appendix

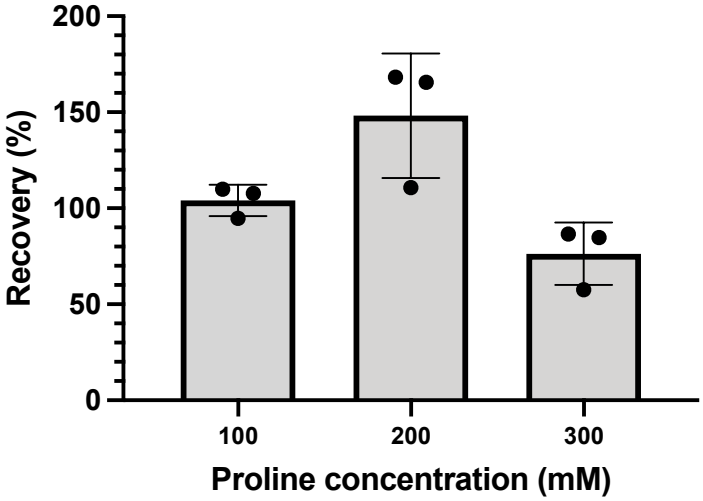

Figure 5.16. Data from a preliminary L-proline incubation experiment. Recovery of Jurkat cells at different cell densities after being frozen with 5 % DMSO at – 1 °C min^{-1} to – 80 °C and thawed for 5 minutes in a 37 °C water bath. Prior to freezing, cells were incubated for 24 hours with 100 – 300 mM L-proline. The L-proline was removed and replaced with fresh media pre-freeze.

Chapter 6 – Conclusion

The purpose of this work was to develop new cryoprotectants, cryoprotectant formulations and cryopreservation techniques for the storage and transport of cells. We hypothesised that to develop an effective cryoprotectant solution, several cryoprotectants of different classes must be used together, including a sugar cryoprotectant. Trehalose is widely known for its cryoprotective properties, so we conducted a literature review comparing the cryoprotective effects of trehalose to that of other sugars. We found mixed results, with some studies indicating that trehalose is a more effective cryoprotectant than other sugars, some indicating that it is equally effective, and a minority of studies indicating that other sugars are more effective. Overall, trehalose appeared to be at least as effective as other sugars, and possibly more so. Consequently, we decided to use trehalose as the sugar cryoprotectant in our solution for red blood cell (RBC) cryopreservation. To develop this solution, we used a high throughput assay and an iterative approach where RBCs were cryopreserved under less optimal conditions at each stage. The result was a multi-component cryoprotectant solution, consisting of DMSO, polyampholyte, and trehalose, which allowed RBCs to be cryopreserved with good recovery under suboptimal conditions. We used haemolysis, ATP, and osmotic fragility assays, flow cytometry and confocal microscopy to assess RBC viability, finding that the RBCs had overall good health post-thaw, although some damage to the RBCs was observed under confocal microscopy. This solution is advantageous compared to current RBC cryoprotectants because it can be rapidly washed-out post-thaw, decreasing time-to-treatment where RBCs are needed urgently in a medical setting. Next, we wanted to produce a DMSO-free cryopreservation solution, so we designed a trehalose ester that can be used to

deliver trehalose into the intracellular space. This would ideally fulfil the role of DMSO, replacing it as an intracellular cryoprotectant. We assessed the theoretical properties of various trehalose esters, and decided to synthesise trehalose-6,6'-dibutanoate. We found that trehalose-6,6'-dibutanoate was able to enter A549s, as measured by cell swelling assay, but when we attempted to cryopreserve RBCs, A549s, and Jurkat cells, we were unable to find cryopreservation conditions where trehalose-6,6'-dibutanoate worked as a cryoprotectant. This was possibly due to toxicity. We next decided to take the techniques that we used to develop the cryopreservation solution for RBCs and apply them to Jurkat cells. We attempted to use a high throughput assay to identify DMSO-cryoprotectant pairs that would increase cell recovery or allow the concentration of DMSO needed for adequate recovery to be reduced, but we were unable to identify any cryoprotectant that could be combined with DMSO for these effects. However, we did find that 8 % glycerol can be used as a cryoprotectant for Jurkats, although it was less effective than 8 % DMSO. We then took 8 % glycerol as our basis for a new cryoprotectant solution, and improved it by adding polyampholyte, poly(ethylene glycol), hydroxyethyl starch and L-proline. The result was a DMSO-free cryoprotectant solution that was as effective as 5 % DMSO for Jurkat cells. We noticed that L-proline was an essential component of this solution, so we decided to investigate further, comparing the effect of L-alanine and D-proline. We found that pre-incubation with L-proline and L-alanine had a cryoprotective effect on Jurkat cells which was more prominent when cells were cryopreserved at a lower cell density. Conversely, incubation with D-proline decreased cell viability during the incubation period and did not increase post-thaw

recovery, suggesting that the cryoprotective effects of L-proline are dependent on its ability to interact with biological systems, rather than being a purely physical effect.

The research presented in this thesis can be readily translated into applications for the cryopreservation of RBCs in a medical context; future research should optimise the RBC cryoprotectant solution for use on human RBCs at medically relevant volumes, and conduct *in vivo* testing in animal models to ensure safety and effectiveness. The research in this thesis will also inform the development of cryoprotectant solutions for other cell types.

Appendix – Preliminary Exploration into the Experimental Evolution of Jurkat Cells for Cryosurvival

Introduction

Many creatures have evolved to survive in cold temperatures. Examples include the wood frog *Rana sylvatica,* which has adapted to accumulate L-proline and glucose before winter,[1,2] and the tardigrade *Macrobiotus richtersi,* which has adapted to accumulate trehalose and use intrinsically disordered proteins to survive extreme temperatures through anhydrobiosis.[3,4] Experimental evolution can be used to adapt a population to artificial conditions by mimicking natural selection. For example, it is possible to produce antibiotic resistance in bacteria by exposing the population to antibiotics.[5] A similar concept has been applied to *Saccharomyces cerevisiae* (yeast), where populations were frozen to – 80 ºC in 5 freeze-thaw-grow cycles, allowing the population to regrow between each cycle. It was found that recombinant, but not isogenic strains, were able to adapt, with post-thaw viability in the recombinant yeast being higher after the fifth cycle compared to the first (≈ 72 % vs ≈ 92 % with 15 % glycerol).[6] A similar experiment has been conducted with *Escherichia coli*, finding that repeated freeze-thaw-grow cycles result in a bacterial population that grows approximately 2.3 times more rapidly than the ancestor control post-thaw, when frozen to – 80 ºC in the absence of cryoprotectant.[7] To our knowledge, an experimental evolution experiment with the goal of studying freeze tolerance has not been conducted in mammalian cells. Although primary mammalian cell populations consist of genetically identical, or near genetically identical cells, secondary cell lines that have been derived from cancers can contain much genetic diversity. For example, Jurkat cells (immortalised leukaemia-derived T-cells) have mutations in genes that are

required for genetic stability, leading to rapid mutation, and resulting in clonal heterogenicity.[8–10] Here we conduct the experimental evolution of Jurkat cells through repeated freeze-thaw-grow cycles, providing preliminary evidence that Jurkat cell lines can be cold adapted.

Methods and results

Experimental evolution

Jurkat cells were cultured and counted as described in **Chapter 4** and **Chapter 5**. The first freezing cycle was initiated when the Jurkats had been passaged 50 times after the cells were initially received. Although tests for genetic diversity were not conducted, Jurkat cells that have been through more divisions are more likely to have accumulated additional mutations, yielding higher clonal heterogenicity. The line was split into four sub-lines, one of which remained cooled at – 80 °C as a control for the duration of the experiment. The remaining three sub-lines were exposed to 10 freeze-thaw-grow cycles. In each freezing experiment, cells were transferred to 1 mL cryovials, 5 % DMSO was added, and the cells were cooled at – 1 °C min^{-1} to – 80 °C using a cryovial cooler. Cells were kept at – 80 °C for at least 2 hours. Cryovials were warmed in a 37 °C water bath for 5 minutes, resuspended in fresh media, plated onto a 12 well tissue culture plate, and incubated at 37 ºC in a humid atmosphere with 5 % CO_2 for 24 hours before counting. Cells were then either immediately subjected to the next cycle or incubated for up to an additional 144 hours (6 days) to allow the population to recover. The duration of each growth stage (24 – 168 hours) and cell density upon cryopreservation ($0.26x10^6$ – $1.51x10^6$ mL^{-1}) were non-uniform. This was to maximise the number of cycles that could be completed, but has the additional

benefit of reducing unintended selection effects that could occur by exposing the cells to stressors that are regular and predictable. For example, if the cells were frozen regularly every 24 hours at a concentration of $1x10^6$ mL^{-1}, the cells may evolve to become freeze tolerant for a short period every 24 hours by syncing circadian rhythm to growth cycle,[11] or altering growth patterns in response to reaching a certain cell density.[12] On the tenth cycle, the control line was thawed along with the three experimental lines, and all lines were cultured as normal for 2 weeks to ensure full recovery and resumption of normal cell grow patterns before testing.

Results

Cells were adjusted to a density of $1x10^6$ mL^{-1}, and 1 mL from each line was transferred into cryovials. Cells were then cryopreserved as above for at least 24 hours, thawed as above, and counted. Cells were then transferred to 12 well plates, incubated for 24 hours as above, and counted using the trypan blue exclusion method.[13] It was found that line 2 and line 3 had higher post-thaw recovery than the control (28 % vs 38 % and 36 % respectively). These differences were not statistically significant. It was found that post-thaw viability was higher than the control in all three lines (44 % vs 57 %, 66 % and 57 % respectively), with the difference between the viability of the control and line 2 being statistically significant. Statistical significance was determined by ordinary one-way ANOVA using Dunnett's test for multiple comparisons $P < 0.05$.

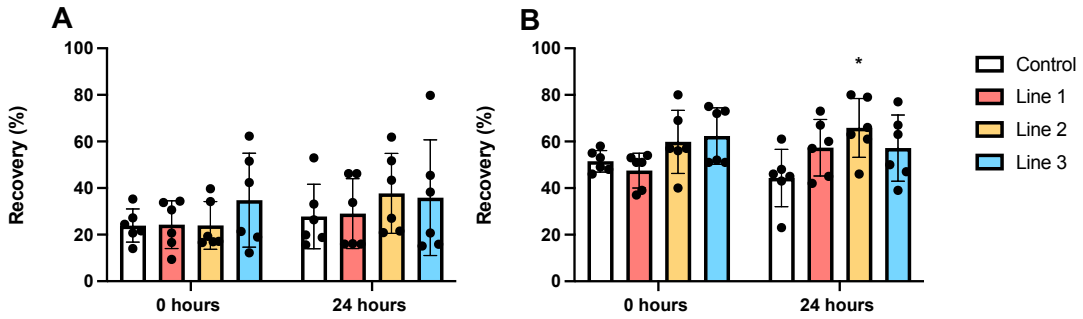

Figure A1.1. The **(A)** post-thaw recovery and **(B)** post-thaw viability of Jurkat cells which were previously adapted to freezing by undergoing 10 freeze-thaw-grow cycles. Adapted Jurkats were cooled at – 1 °C min^{-1} to – 80 °C and warmed in a 37 °C water bath for 5 minutes. Data represents the mean ± SD of at least three independent experiments (*P < 0.05 from untreated control).

Conclusions

After 10 freeze-thaw-grow cycles, all three experimental Jurkat lines showed signs of cold adaption. Line 1 showed increased viability 24 hours post-thaw, line 2 showed increased post-thaw recovery and significantly higher post-thaw viability after 24 hours, and line 3 showed increased recovery and viability immediately post-thaw. Although all but one of these results were not statistically significant, taken together the data provide preliminary evidence that cold adaption is occurring. However, the post-thaw recovery of the 5 % DMSO control in this study was lower than expected (the recovery of Jurkat cells cryopreserved under these conditions with 5 % DMSO is usually above 50 %), meaning that the relatively higher recovery of the experimental lines may not represent an absolute improvement. On the other hand, the low recovery of the control line may be due to its high passage number, a condition shared by the experimental lines. Further study should aim to expose Jurkat cell lines to 50 freeze-

thaw-grow cycles, and conduct genomic analysis to find any genetic changes responsible for cold adaption.

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