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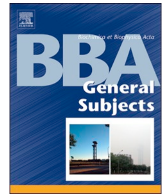

Review

The need for novel cryoprotectants and cryopreservation protocols: Insights into the importance of biophysical investigation and cell permeability

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ABSTRACT

Background: Cryopreservation is a key method of preservation of biological material for both medical treatments and conservation of endangered species. In order to avoid cellular damage, cryopreservation relies on the addition of a suitable cryoprotective agent (CPA). However, the toxicity of CPAs is a serious concern and often requires rapid removal on thawing which is time consuming and expensive.
Scope of review: The principles of Cryopreservation are reviewed and recent advances in cryopreservation methods and new CPAs are described. The importance of understanding key biophysical properties to assess the cryoprotective potential of new non-toxic compounds is discussed.
Major conclusions: Knowing the biophysical properties of a particular cell type is crucial for developing new cryopreservation protocols. Similarly, understanding how potential CPAs interact with cells is key for optimising protocols. For example, cells with a large osmotically inactive volume may require slower addition of CPAs. Similarly, a cell with low permeability may require a longer incubation time with the CPA to allow adequate penetration. Measuring these properties allows efficient optimisation of cryopreservation protocols.
General significance: Understanding the interplay between cells and biophysical properties is important not just for developing new, and better optimised, cryopreservation protocols, but also for broader research into topics such as dehydration and desiccation tolerance, chilling and heat stress, as well as membrane structure and function.

1. Introduction

Cryopreservation offers huge opportunities for both research and medical treatments. Through cryopreservation, blood banks can ensure sufficient supplies, stem cell therapies can be used to treat a range of diseases, and the field of assisted reproductive technology has undergone huge advances [1]. Furthermore, cryopreservation can be used for long-term storage of seeds [2], endangered plants [3] and animals [4]. In the area of plant cryopreservation in particular a lot of effort is being directed not only to optimising the use of CPAs to decrease cell membrane damage and toxicity, but also toward developing new cryoprotection methods and ameliorating damage arising from oxidative stress [5].

Successful cryopreservation relies on more than simply freezing cells, which exposes them to numerous stresses including dehydration and mechanical pressures. Usually, a cryoprotective agent (CPA) is added to minimise freezing damage through inhibiting ice formation, preserving cellular membranes and promoting vitrification [6,7].

The two most commonly used CPAs are glycerol and dimethyl sulfoxide (DMSO). Glycerol was first identified as a CPA in 1949 [8] while DMSO was identified in 1959 [9]. Unfortunately, both have levels of toxicity making them unsuitable for many applications, and often require extensive washing during thawing to prevent cell death or subsequent adverse reactions if used in medical treatments [10–13]. In the decades since their discovery, many other CPAs have been explored, but very few have shown the same efficacy as glycerol or DMSO [14]. Therefore, the search for new, effective, non-toxic CPAs is ongoing.

In addition to the toxicity problems of existing cryoprotectants, current methods of cryopreservation do not work for certain cell types, for example granulocytes [13] and pluripotent human stem cells [15]. Research is on-going to develop efficient cryopreservation protocols for stem cell therapies - Chen et al. [16] examined the effects of various cryoprotectants on human umbilical cord blood stem cells, and a recent review by Hunt [17] highlights the need for a holistic approach to designing stem cell cryopreservation protocols by considering not only the choice of CPA, but also the freezing container, the cooling rate and

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so on. Current methods are also not suitable for whole organs, or even whole tissue cryopreservation, due to the variety of cell types (and therefore different cryopreservation requirements) [18]. There is also the issue of achieving sufficient CPA penetration into deeper cell layers within tissue, without which deeper tissue layers suffer extensive damage [1].

Thus, there is an obvious need for new CPAs that can overcome the limitations of existing protocols. CPAs may be classified as either non-penetrating—meaning they do not enter the cells and instead act in the extracellular space—or penetrating—meaning that they do enter the intracellular space. Both classes will be discussed later, but the focus of this review is on penetrating CPAs.

In 1969, Karow published a review entitled “Cryoprotectants – A New Class of Drugs” [14]. Now, more than 50 years later, we are following up on that work to discuss the progress made in developing new methods of cryopreservation and in identifying new CPAs. Despite a half a century of research, there is still a lot that remains unknown about CPAs and the search for alternatives to DMSO and glycerol continues. In 2004, Fuller's group detailed the mechanisms of action of known CPAs and re-iterated that many potential CPAs are toxic at the concentrations required for cryopreservation [19]. The same group conducted an extensive review in 2017 of the different types of CPAs, including alcohols, sugars, and polymers [7]. That review again highlighted problems with CPA toxicity and the modes of action of the different CPA groups. The review presented here builds on this by specifically focusing on the rational design of novel CPAs based on our current understanding of how CPAs function. The last few years have seen targeted investigations that have specifically modified potential CPAs to improve their activity e.g. by making them better able to penetrate cells. Other work has focused on using naturally produced molecules such as amino acids as CPAs. The outcomes of these targeted and novel approaches will be summarised here, with an outlook toward rational design of future CPAs.

2. Mechanisms of damage during freezing

For many cells, low temperatures are not in themselves damaging, but freezing is often lethal. There are a number of different mechanisms that may cause damage during freezing, including mechanical damage due to ice crystals, and solute damage due to changes in concentration of electrolytes [6]. There is some disagreement in the literature about which mechanisms are the most important, however it is likely that the most pertinent mechanism of damage is different for different cell types, and is a function of their permeability, lipid composition and intracellular makeup.

The formation of an ice crystal is stochastic, which means that the probability of ice formation is proportional to the volume of water available: at a given sub-zero temperature, ice is more likely to form in a large volume than a small volume [20]. As the volume of any individual cell is much smaller than the volume of the extracellular aqueous environment, ice will normally form in the extracellular space first [14].

Ice, however, is a poor solvent – water molecules need to align very specifically with each other in order to form crystalline ice, and the inclusion of any contaminant (salts or other solutes) would disrupt the ice structure. Therefore when an ice crystal forms, it forms as very pure water ice, with very low concentrations of other molecules [21]. Thus, the formation of ice outside the cells leads to freeze-concentration of the solutes into a co-existing unfrozen fraction. This fraction will have a much higher concentration of salts, sugars, proteins etc. than the isotonic solution [22,23]. High solute concentrations could damage cells in a number of ways such as influencing bilayer structure, changing protein conformations or creating osmotic stress on thawing [6,18,19,24–27]. The importance of electrolyte concentration for cell survival may vary between cell types and may be especially relevant to red blood cells due to their high water permeability and simple structure. The removal of water by ice also leads to dehydration, which can lead to membrane phase changes and cell damage [6,20,28–31]. Other mechanisms of damage may be more important to other cell types [22]. Mazur has reviewed the various contributing factors to damage due to slow cooling, including high electrolyte concentrations and dehydration effects [26]. The physical formation of ice inside the cell depends on the concentration of solutes, the presence of nucleation sites, and the rate of freeze/thaw. At rapid cooling rates, the intracellular water will be supercooled and eventually ice will nucleate [6,26,32]. Other factors such as the presence of pores, and the proximity of extracellular ice may also influence intracellular ice formation, and this is discussed elsewhere [6,20,24,33]. Most importantly, formation of intracellular ice is almost universally lethal and so must be avoided [26].

These contrasting mechanisms (solute effects vs. ice damage) led to Mazur developing the ‘Two Factor Hypothesis’ [32,34]. At slow cooling rates the cells will be exposed to high solute concentrations and undergo dehydration, both of which can be toxic. However, if the cooling rate is too fast, there will be intracellular ice formation [34]. The two-factor hypothesis suggests that there may be an ideal intermediate cooling rate that leads to maximum cell viability, represented by the classical inverted ‘U’curve as illustrated in Fig. 1 (black solid curve in the bottom figure). It must be understood that this ‘ideal’ intermediate cooling rate will vary between cell types depending on factors such as size and permeability [32,35].

However, this inverted ‘U’ curve cannot be applied for thermal stabilisation by the process known as vitrification. For conventional cryopreservation, cell injury is related to the high concentration of CPA used, leading to cell damage due to osmotic stress and interruptions in the normal metabolic pathways at super-zero temperatures prior to cooling and after warming the cells [36–38]. But in the case of vitrification with low concentrations of CPAs, cells are brought to the vitrified phase using ultra-fast cooling, without any ice formation or freeze concentration. Therefore, the traditional inverted ‘U’ curve for cell viability vs. cooling rate can be modified and extended into the ultra-fast cooling region where cell viability increases with increasing cooling rate (black dotted line in Fig. 1- Bottom). This phenomenon typically occurs when the cooling rate is very high (thousands to millions of °C/Sec) and CPA concentration is very low (< 2–4 M) [38].

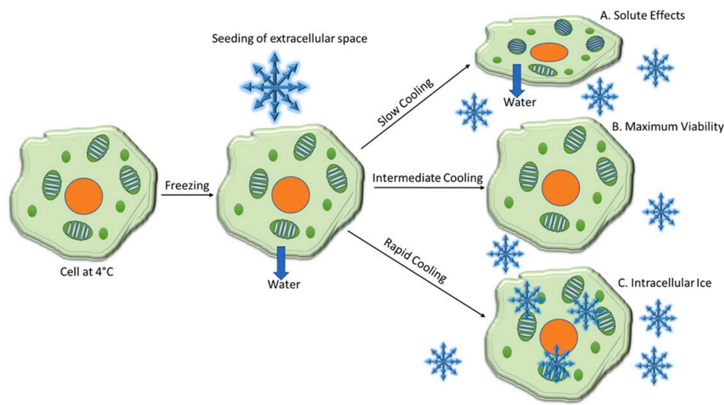
It should be noted that the stability of cells in the optimal cooling rate regime described above is due to the fact that the combination of CPA and dehydration leads to the intracellular contents becoming very viscous and eventually forming a glass – that is, the intracellular contents vitrify, thus preventing further dehydration and providing long term stability. This is different to the “vitrification” regime, where the entire contents of the sample (intracellular and extracellular) are vitrified due to ultra-fast cooling.

Thus, when seeking to minimise cell damage and death during cryopreservation there are two primary mechanisms of damage which must be considered and controlled: i) solute effects and dehydration, and ii) intracellular ice formation.

3. Mechanism of action of CPAs based on biophysical properties

Cryoprotective agents (CPAs) are used to protect biological samples from freezing damage during cryopreservation. This is achieved by different mechanisms depending on the CPA, but even for well-known CPAs there is still some doubt as to the exact mechanism of protection [19,28,40–42]. The potential mechanisms are discussed briefly below, but have been reviewed in more depth elsewhere [7,19,40,43].

CPAs are separated into two broad categories: penetrating and non-penetrating [7,14,19,40,42,44,45]. Fig. 2 shows a schematic representation of cellular responses to these two types of CPAs. Penetrating CPAs are generally small, non-ionic molecules that can easily diffuse through cell membranes. Non-penetrating CPAs include small molecules which cannot penetrate membranes (such as sugars) as well



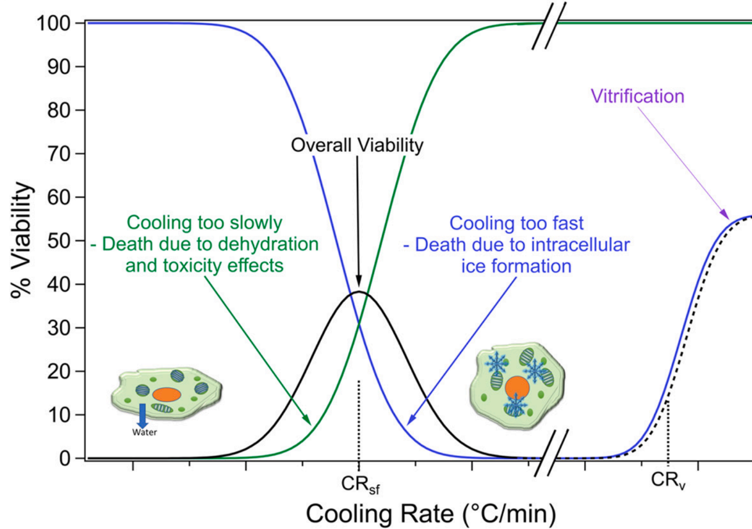


Fig. 1. Top: Illustration of the effects of different cooling rates on cell survival during freezing; with slow cooling (A), there is shrinkage due to water loss and dehydration; solute concentration effects may cause cell death. With intermediate cooling (B) there is a balance between solute effects and intracellular ice formation, leading to maximum viability. With rapid cooling (C) there is supercooling of the intracellular space which leads to intracellular ice formation and cell death. Bottom: Graphical representation of Mazur's Two-Factor Hypothesis, highlighting damage due to cooling too slowly or too quickly, with an optimal cooling rate (CR_{sf}) which maximises survival. The optimal cooling rate is dependent on cell type and cryoprotectant concentration. When the cooling rate is higher than a critical cooling rate (CRv), cells are vitrified without freezing (or ice formation) and high cell survival ensues. A*dapted from Mazur (1972,1977)* [35,39], *He (2011)* [38] and *Zhao and Fu (2017)* [25].

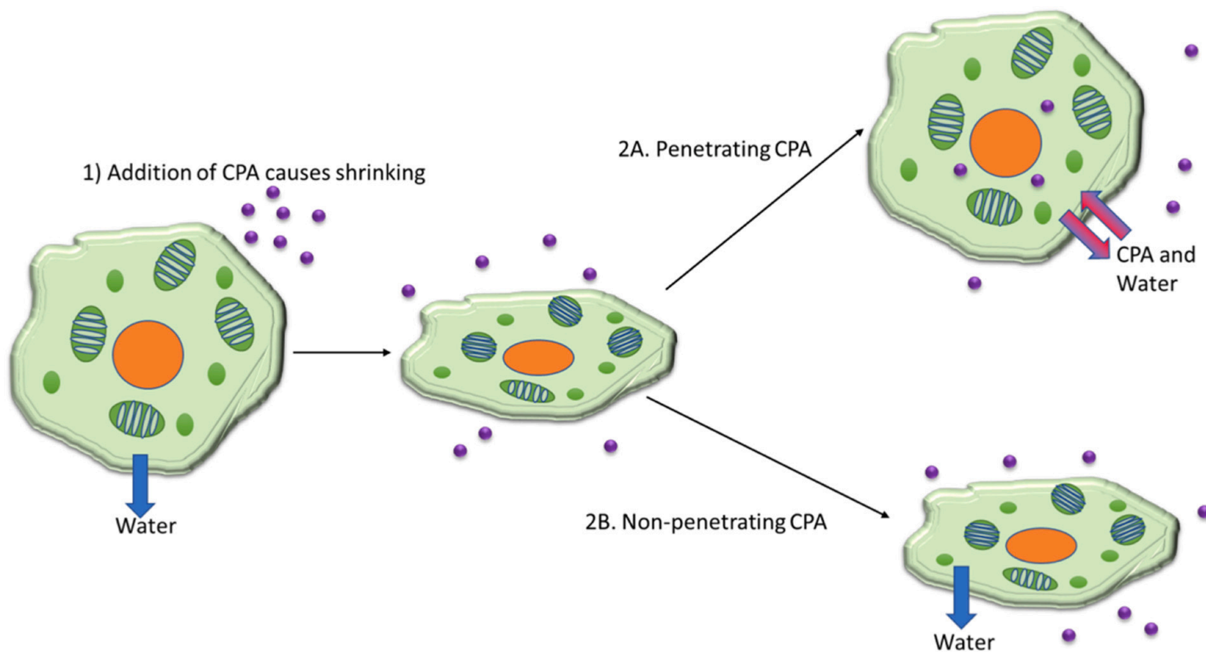


Fig. 2. Schematic representation of the effects of cryoprotective agents. Initially on addition of a CPA (1), osmotic pressure forces water out of the cell which results in shrinkage. If the CPA is penetrating (2A) then it will enter the cell, thus reversing the osmotic balance and causing the cell to swell back to near its original size. If the CPA is non-penetrating (2B) then the cell will continue to shrink until it reaches osmotic equilibrium with the extracellular fluid. The amount of shrinkage will depend on the increase in osmolality, so is generally minimal when adding large biopolymers.

as large, long chain polymers, added to aqueous cryoprotective solutions (commonly called vitrification solutions). These molecules inhibit ice growth, but do not enter cells. Non-penetrating cryoprotectants are usually less toxic than penetrating cryoprotectants at the same concentration. They reduce the amount of penetrating cryoprotectants needed by mimicking outside the cell the cryoprotective effects of proteins inside the cell. Note that in the case of large non-penetrating CPAs such as biopolymers, dehydration effects are usually minimal.

The focus of this review is on penetrating CPAs, which are the most widely used type, but a brief description of non-penetrating CPAs is provided here for completeness. Non-penetrating CPAs such as sugars or long-chain polymers remain on the outside of cells as they cannot permeate the membrane [7,13,14,43]. Primarily, non-penetrating CPAs act by increasing the osmolarity of the extracellular environment which results in cell dehydration, thus requiring slow cooling rates. Thus, there is a higher solute concentration inside of the cell which inhibits intracellular ice formation [40]. For the same reason, non-penetrating CPAs can be included in the thawing media to prevent osmotic shock and lysis by slowing the return of water to cells and thus stopping traumatic expansion [7,19]. It has been postulated that non-penetrating CPAs can absorb to the cell membrane surface, thus inhibiting ice formation in the immediate vicinity of the cell by preventing the necessary formation of crystal lattices and promoting vitrification [30,46,47]. However, at low hydrations, large molecules such as non-penetrating polymers are excluded from the bilayer region and have no effect on membrane phase transitions [30,46–48].

As discussed in the introduction, glycerol and DMSO are the two most commonly used CPAs. Both are penetrating CPAs, and both have some toxicity which limits their use [10–13]. In order to pass through the cell membrane and enter a cell, penetrating CPAs are often small, non-ionic molecules that are highly water soluble at room temperature [9,40,43,49]. Common groups of penetrating CPAs include molecules consisting of sulfoxides, alcohols, amides, and imides [43].

Penetrating CPAs have multiple mechanisms of action, although as discussed above, the full contribution of these to the preservation of different cell types is not yet fully understood. Because of their penetrating nature, and thus their ability to interact with cellular components including enzymes, penetrating CPAs tend to be more toxic than non-penetrating CPAs [19].

3.1. Preventing ice formation

By increasing the glass transition temperature and/or reducing the freezing point of the solvent, a penetrating CPA can prevent ice formation inside of the cell [20]. As discussed above, intracellular ice formation can lead to both mechanical damage and solute concentration effects and is almost universally lethal [26].

Some CPAs increase membrane permeability, which allows further leakage of water from the cell and decreases the chance of intracellular ice formation [42,44,50]. Penetrating CPAs can also prevent or inhibit ice recrystallization during thawing [51]. Ice recrystallization, a form of Ostwald ripening, can be defined as the growth of large ice crystals at the expense of smaller ones, thereby reducing the overall surface energy of the system [52]. This growth and coarsening of ice crystals can lead to mechanical damage of biological membranes at or around zero degrees and can occur during the freeze-thaw cycles of cryopreservation [53]. Many studies that investigate new CPAs use inhibition of ice recrystallization as one of the primary screens to identify potential candidates [54–56]. However, inhibition of ice recrystallization alone does not mean that a molecule will be an effective CPA. For example, many naturally derived antifreeze molecules might inhibit ice formation at moderately low temperatures, but as the temperature is further decreased below the depressed freezing point, these molecules cause uncontrolled ice crystal growth [33].

3.2. Protein stabilisation

Solutes and CPAs that are excluded from the hydration shell of proteins can enhance stability during freezing. This is because unfolding of the protein would expose more surface area, requiring more exclusion of the solute which is energetically unfavourable. Thus protein stability is favoured [11]. This is in contrast to solutes such as urea which directly bind to and destabilise proteins [11].

Note that this mechanism for protein stabilisation is different to the ‘water replacement hypothesis’, which suggests that CPAs interact directly with the protein, thus taking over the role of water [44,57]. Whilst this does appear to be a potential mechanism for protection during desiccation, it has been argued that such bound water is not removed during freezing, and therefore CPA binding to the protein is irrelevant [28]. In fact, a detailed study by Crowe et al. demonstrated that the mechanisms of damage and protection during dehydration were different to that during freezing. Therefore, some molecules may stabilise proteins during dehydration (e.g. trehalose) while others cannot (e.g. glycerol). Similarly, some molecules may stabilise proteins during freezing but have no protective effect during dehydration such as proline [29].

3.3. Membrane interactions

One of the key requirements for cell survival is that the membrane retains its integrity. Without a selectively permeable barrier, the cell's contents will be lost, and the cell will die. The lipids that provide the structure to cellular membranes undergo phase transitions with changing temperature and with changing hydration. During freezing, the membrane is exposed to both [24]. These transitions can not only lead to ‘leakiness’ of the membrane, but may also cause intramembrane protein aggregation or loss [30,46,58].

Several studies have been carried out on the effects of non-penetrating CPAs such as sugars, especially disaccharides, and their mechanisms of action are becoming clearer [30,46,59–65]. During dehydration, these solutes are known to protect cellular membranes by lowering deleterious phase transition temperatures (Fig. 3) [47,66,67]. Studies of the effects of sugars on membrane phase transitions at different hydrations suggest that the phase transition modification arises due to non-specific osmotic and volumetric effects [46,47]. In order to explain how sugar molecules help to protect from lethal phase transitions, two molecular scale theories have been proposed: the water replacement hypothesis and the hydration forces explanation [46], which are not mutually exclusive, and are discussed in detail elsewhere [59–63,68].

The effects of penetrating cryoprotectants on membranes have been less widely studied, with most attention being given to DMSO and glycerol [58,69–75]. CPAs may bind to the cell membrane, either the polar or non-polar region depending on the chemical composition of the CPA [69]. Some CPAs can protect membranes from phase transition damage by inhibiting fusion e.g. of adjacent membranes [28], or by modifying the phase transition behaviour of the lipids (Fig. 3) [30,46,58].

Interactions between membrane lipids and DMSO have been studied by numerous methods and summarised elsewhere [76]. DMSO destabilizes the lamellar liquid crystal phase in favour of the gel phase [58], but can also inhibit membrane compression by partitioning between the intermembrane space and forming a physical barrier [77]. At low concentrations, DMSO causes membrane thinning and increased fluidity, while at high concentrations it causes pore formation [70,72,77,78]. DMSO can dehydrate the lipid headgroup area [79–81] and a recent study demonstrated that while DMSO decreases the hydrated headgroup volume by competing for interactions with water, glycerol strengthens the H-bond network of water [82]. These interpretations are further supported by recent Langmuir monolayer experiments, which also highlighted that CPAs have differential

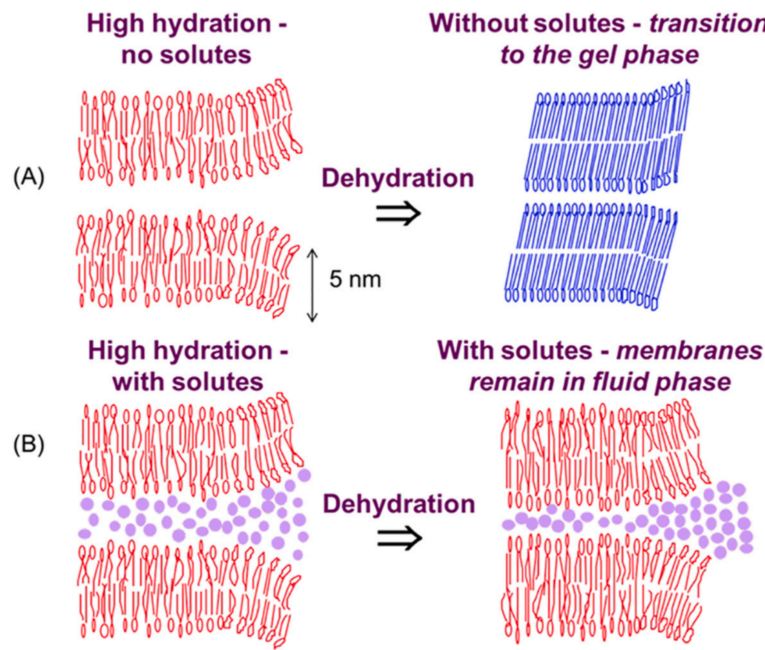


Fig. 3. Schematic representation of the effect of dehydration on membrane lipid organisation. A) Without solutes, dehydration causes a transition from the liquid to the gel phase. B) With solutes, the cell membrane remains in the fluid phase. *Image adapted from Bryant* et al. [46].

interactions with different lipid species [83].

So, while both DMSO and glycerol are effective CPAs, it appears that their mechanisms of interaction with membranes are different. Over the last decade, Molecular Dynamics techniques have shed considerable light on the mode of action of a range of CPAs, and also highlighted the fact that membrane composition is critical– for example, addition of sterols significantly reduces the damaging effects of some CPAs [84,85].

3.4. Optimising CPAs

Because different cryoprotectants have different mechanisms of protection, often mixtures are used to maximise cell viability [19,44,86]. Examples of this include plant vitrification solutions (PVS) which combine a number of CPAs. These mixtures form glasses and avoid ice crystallization during both cooling and thawing [87,88]. Different types of PVS are used for different species of plants including citrus callus [89], wasabi [90], and asparagus [91]. Variations on PVS are made by modifying the concentration of the constituent CPAs [92]. Similarly, mixtures of CPAs have demonstrated greater protective capacity than individual CPAs for the cryopreservation of mammalian and bacterial cells [19,93]. Furthermore, some studies have shown that mixtures of CPAs can result in reduced toxicity [86]. Such effects have been linked to the synergistic action of combined CPAs – for example it has been shown that replacing some DMSO with glycerol or ethylene glycol still achieves vitrification but with significantly reduced membrane damage [94].

Toxicity of CPAs may also be reduced by adding them at a lower temperature. Equilibration times and temperatures can also be modified to minimise toxicity [95]. Alternatively, stepwise addition or gradually increasing CPA concentration can reduce toxicity [96]. Similarly, stepwise removal during thawing can minimise osmotic stress [95].

4. Rational design of Novel CPAs

As discussed by Elliott *et al.*, despite more than half a century of studies into cryopreservation, the same few CPAs continue to be used [19]. Therefore, there is a need for targeted, rational design of new cryoprotectants.

While non-penetrating CPAs are important to cryopreservation and can greatly improve outcomes when used as an additive, penetrating CPAs offer the greatest potential for freezing new cell types, or even tissues and organs, because they inhibit intracellular ice formation [19,49,97].

Based on the above discussion, the ideal penetrating CPA will promote vitrification, inhibit ice recrystallization and reduce membrane/protein damage without being in itself too toxic. Therefore, many investigations into new CPAs begin with a few simple experiments to test: a) Does the solute penetrate the cell? b) Does the solute/water mixture have a glass transition in a reasonable temperature range? c) Does the solute inhibit intracellular ice formation and/or recrystallisation?

In order to better understand the cryopreservation protocols best suited to different cell types, an understanding of a cell's biophysical properties is vital. These properties include the osmotically inactive volume (V_b), which is the portion of the cell volume that does not respond to osmotic pressure and is calculated using the Boyle van't Hoff relationship (Eq. (1)) [98].

$$\frac{V}{V_o} = \frac{V_w^o}{V_o}\frac{\pi_o}{\pi} + \frac{V_b}{V_o} = (1-b)\frac{\pi_o}{\pi} + b \tag{1}$$

where V is the cell volume, V_o is the total cell isotonic volume, $V_w{}^o$ is the isotonic volume of water in the cell, and π and π_o are the osmolality and isotonic osmolality respectively. By exposing the cell to different anisotonic (hypertonic and hypotonic) solutions containing an impermeable solute and allowing for equilibration, the relative volumes can be determined. Plotting the normalised volume $\frac{V}{V_o}$ vs. the normalised inverse osmolality $\frac{\pi_o}{\pi}$ then yields a straight line whose intercept is equal to the osmotically inactive volume fraction $b = \frac{V_b}{V_o}$. A schematic of this is shown in Fig. 4.

The osmotically inactive volume can be used to give an indication of the 'safe' volume changes a cell can experience without loss of viability: some protocols use a two-step or gradual addition of CPA, rather than a one-step addition which could lead to the cell volume change exceeding 'safe' levels [99,100]. By knowing the V_b value, a protocol for adding CPA can be designed to avoid these extreme volume changes. However, it is more complicated than this simple picture – in particular it has been reported that the osmotically inactive volume is temperature dependent: the variation of inactive volume with temperature and number

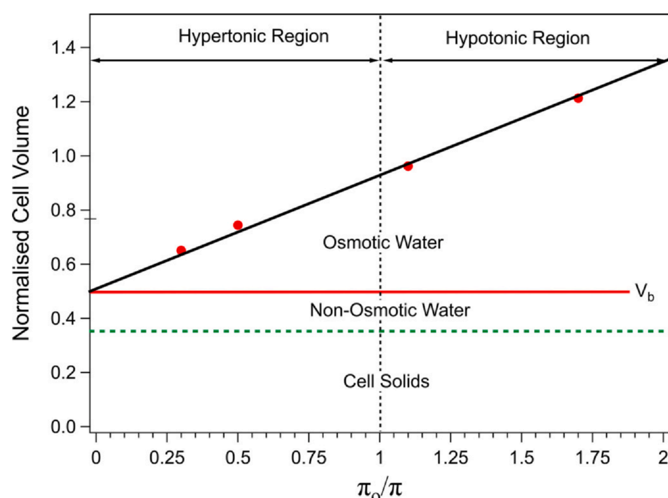


Fig. 4. A Boyle-Van't Hoff plot used to calculate the osmotically inactive volume of a cell. Normalised cell volume is plotted against normalised inverse osmolality and the fitted straight line is extrapolated to find the normalised volume at infinite osmolality which is b. The difference between the osmotically inactive volume (indicated by the red line) and the non-water volume (cell solids, indicated by the green dashed line) is sometimes called "unfreezable" water, though this terminology has been questioned [31]. Hypertonic and hypotonic regimes are highlighted.

of osmotic cycles, and how this is related to designing CPA protocols, is described in detail by Casula *et al.* [101]. An early study by Gilmore *et al.* [102] analysed the effect of various cryoprotectant solutes on water permeability of human spermatozoa in order to better understand the parameters relevant for optimising preservation protocols [102].

In addition to V_b, the water permeability (hydraulic conductivity) and the CPA/solute permeability of a cell are important for optimising cryopreservation protocols [103,104]. This information can be used to predict a cell's response to hypo- or hyper-osmotic solutions, the concentration of CPA inside a cell at a given time point, and the concentration/volume of water inside a cell under certain conditions [105,106]. For example, if a given cell type has a low permeability to the CPA, then it will probably require extended incubation in the CPA before freezing to allow adequate penetration, as has been demonstrated previously for glycerol [45] and other CPAs [12,107].

4.1. Testing potential CPAs

In order to test if a molecule has the potential to act as a CPA, there are several key questions that can be asked.

- Can the CPA inhibit ice formation during cooling and promote vitrification?
- Is the toxicity of the CPA low enough that exposure during cryopreservation protocols does not lead to irreparable damage?
- Can the CPA penetrate cells, and if so, how quickly?

The thermal properties of solutions containing the potential CPA can be measured routinely using methods such as differential scanning calorimetry (DSC) [20,97,108,109]. As these are widely known, they are not discussed further here.

Inhibition of ice recrystallization is measured by splat cooling assays where the mean size of ice crystals during thawing is measured [12,33,97,110]. An effective recrystallization inhibitor will result in observably smaller crystals.

The most critical of these tests is the determination of the ability of CPAs to penetrate cells, as without this none of the other properties matter. Permeability can be measured in a variety of ways which were reviewed by McGrath [111], including stopped-flow light scattering [112–114], electronic particle counters [102], differential scanning calorimetry [115], and micropipette perfusion [116]. Each of these methods have advantages and disadvantages depending on cell type, availability, and the conditions under examination [111].

One of the simplest methods of measuring CPA penetration is a 'shrink-swell' light microscopy experiment, whereby changes in cell size are observed as the suspending solution is changed from an isotonic solution to one containing the CPA. If the CPA is permeable, the cell will initially shrink due to water leaving the cell, but will swell again as the CPA enters the cell and restores osmotic equilibrium [117]. If the cell does not swell and return to near its starting size, then the CPA cannot penetrate [118]. Both of these cases are shown in Fig. 5.

The above microscopic method can be further improved by using microfluidic technologies. In recent years, several microfluidic devices with perfusion chambers [119] or single cell traps [117,120,121] have been developed, some of them specifically for the measurement of cell permeability [25,119,122–124]. Microfluidic devices are now considered essential technology in cell biology, as they enable the user to: (i) monitor the changes of cellular conditions following the perfusion of

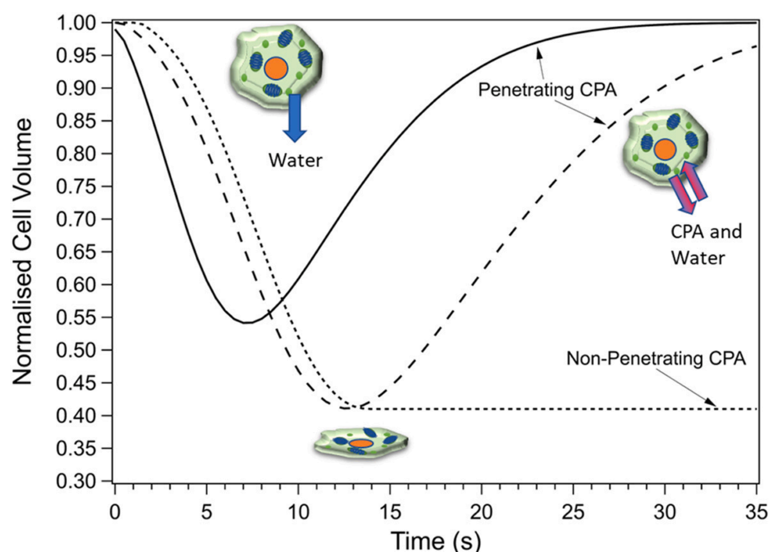


Fig. 5. Change in cell volume during shrink-swell experiments as a result of osmotic changes with penetrating and small non-penetrating CPAs. The solid and long-dash lines show example behaviour of penetrating CPAs that have different permeabilities and osmolalities where the rate of shrink/swell are different.

various media; (ii) confine cells within a chamber/trap to prevent imaging ambiguity due to overlapping of cells or their movement out of the focal plane; (iii) study the single cell response to osmotic pressure and calculate the cell membrane parameters; (iv) use low cell numbers and reagent volumes; and (v) perform kinetic analysis of non-adherent suspension cells by maintaining their position in place over long times without any dislodgement during image acquisition [119,120]. Technological advances in high-resolution three dimensional (3D) printing with direct laser writing (DLW) using multi-photon polymerization has enabled the fabrication of microfluidic devices in a single step [125–128].

The shrink-swell behaviour can be modelled to extrapolate permeability parameters and related activation energies. Mazur and colleagues [129,130] introduced the first one-parameter model for calculating solute permeability. This was refined to a two-parameter model called 2P [131,132] for finding water and solute permeability. Kedem and Katchalsky [132] developed the three-parameter model (also called the Kedem–Katchalsky (KK) formalism) which is most commonly used today. The KK formalism adds a 'σ' term to the 2P, which is the term representing the interaction between solute and solvent [133]. An Arrhenius plot is used to find the activation energies of these permeability parameters [133].

4.2. Recent developments in CPAs

Many synthetic CPAs are based on naturally occurring compounds found in cold-resistant plants and animals. One of the most common protectants found in nature are antifreeze proteins and glycoproteins. These were first identified in arctic fish and their protective capacity attributed to binding to ice crystals [134]. Since then, many attempts have been made to isolate or design analogous antifreeze proteins and mimics [7,43].

Many anti-freeze proteins successfully inhibit ice formation at moderately low temperatures, but further cooling leads to uncontrolled oriented crystal growth which can be damaging [33]. Thus, anti-freeze proteins must be designed by taking into account the effect of molecular modifications such as side groups, and chain lengths, etc. in order to maximise cryoprotective properties [33]. C-linked anti-freeze proteins offered some protection during cryopreservation, but not as much as DMSO [135]. Extensive simulations have been carried out to better understand how anti-freeze proteins provide protection, including the mechanisms of binding to ice [136,137].

Similarly, glycolipids have been identified as offering protection against freeze damage in some species [138]. Similar to the (glyco) proteins, the activity of glycolipids could come from binding to nucleation sites and preventing crystal formation, or alternatively, glycolipids could stabilise the cell membrane. Further investigations have shown that glycolipids can inhibit vesicle fusion during freeze drying, especially when used in combination with glucose [139].

In addition to (glyco)proteins and glycolipids, other carbohydrate derivatives have undergone extensive testing as potential CPAs. Many cold-resistant species use sugars as natural CPAs, for example, trehalose and sucrose are well-known naturally occurring CPAs. However, these naturally occurring sugars are produced (and act) intracellularly [43]. They present a problem as artificial CPAs because they are non-penetrating, and therefore cannot be easily introduced to the interior of cells where they would have an effect [11,140]. This has led to attempts to modify naturally-occurring carbohydrate CPAs to make them more permeable while still retaining their cryoprotective activity [141]. Alternative methods such as electroporation or genetic modification have been used to get such CPAs inside cells [11,142].

Carbohydrates offer interesting insights into the mechanisms of cryoprotectant activity. One study found that disaccharides were twice as effective at preventing ice recrystallization as monosaccharides *but* at high concentrations the disaccharides were more toxic to the cell lines tested [51]. Ultimately, this work showed that some sugars could be used in combination with low concentrations of DMSO to achieve successful cryopreservation, with less toxicity. In fact, at 200 mM, many of the carbohydrates tested (including D -galactose and D -lactose) had the same cryoprotective activity and resulted in the same cell viability following freeze-thaw as 5% DMSO [51]. Another important finding of this study was that many of the sugars were effective CPAs but at physiological temperatures were highly cytotoxic. Ideally, a CPA would be non-toxic at physiological temperatures to avoid cell death during thawing or incubation. This study found that D -galactose may be ideal because at 200 mM D-galactose results in 78% viability at physiological temperatures and 75% viability post-cryopreservation.

Similarly, glyceryl glucoside (GG, α-D-glucosylglycerol) which is a natural glycerol derivative found in alcoholic drinks, is found to be a better alternative cryoprotectant as this compound has lower genotoxicity than glycerol and lower cytotoxicity than DMSO [143].

Other carbohydrate molecules have been tested for inhibition of ice recrystallization, but not yet for cryopreservation activity, including carbohydrate-based surfactants [55,56].

Sugar surfactants show some promise for stabilising proteins during freeze-thaw and freeze-drying [144]. The authors attributed the stabilising activity to direct interaction between the surfactant and the proteins, with shorter tailed surfactants offering better protection because they were less likely to self-aggregate than longer tailed surfactants [144]. They are also less likely to dissolve lipid membranes.

In a recent study, n-octyl (thio)glycoside derivatives showed promising glass transition behaviour, and some could penetrate human monocytes (THP-1 cells [145]) without causing lysis [97]. These results provide useful insight for further modification and fine-tuning of analogue molecules to maximise permeability and glass transition behaviour.

Similarly, *O*-aryl glycosides have been investigated by the Ben group for cryopreservation activity. Some derivatives at low concentrations in conjunction with 15% glycerol could achieve the same cryopreservation activity as 40% glycerol on its own, which is promising for the purposes of reducing toxicity post-thaw [146].

Betaine and its analogues are found in cold-resistant plants and fish [147,148]. Some betaine analogues can preserve enzymes during freezing to the same level, or even better, than traditional CPAs such as DMSO. In fact, some enzymes retained activity even after 100 freeze-thaw cycles. The authors propose that protection comes from the analogues promoting ice nucleation which prevents aggregation [148]. Betaine has also demonstrated protection against fusion of vesicle membranes during freeze-thaw cycles, probably through interaction with the non-polar regions of the lipids [69]. Even more importantly, betaine has demonstrated even better CPA activity than DMSO for a number of cell types (HeLa, MCF-10 and GLC-82), including when used with rapid-freeze protocols [107]. Furthermore, betaine can be used for cryopreservation of whole blood with a one-step removal process; a method that would result in haemolysis if applied to blood preserved with glycerol [149].

Proline is a known natural CPA and has shown some promise as a synthetic additive for cryopreservation [43]. Proline has also been shown to inhibit fusion of vesicles and stabilise membranes during freezing, but did not interact with phospholipid headgroups, suggesting an alternate mode of protection [69]. Proline is also known to increase the allowed change in surface area of a cell membrane (as occurs during shrinking and swelling) before cell death occurs, and acts as a protein stabiliser [24]. As discussed above, betaine has CPA activity, but cell viability was improved even further by combining betaine and proline, even at very low concentrations [149]. This demonstrates that even a simple amino acid such as proline could have huge potential as a non-toxic CPA.

Foetal bovine serum (FBS) is often used in cell culture and sometimes in cryopreservation. However, there is inherent variation between batches as well as immunological, infectious, ethical, and religious concerns around its use. Sericin—a protein derived from

silkworm cocoons—offers a possible alternative [150]. Sericin has been used instead of FBS in the freezing media for a number of cell types, including Chinese-hamster ovaries [151], adipose tissue-derived stem cells [142], human mesenchymal stromal cells [150], and mouse hybridoma cells [152]. However, in all of these studies, the main CPA was DMSO at 10%, and this could not be replaced by sericin. Therefore, while sericin might offer a serum-free option for cryopreservation it does not in itself offer sufficient CPA activity.

Deep eutectic solvents (DESs) are composed of a hydrogen bond donor and a hydrogen bond acceptor. They are characterised by having freezing points at much lower temperatures than either of the components [153]. Many DESs have glass transitions which makes them of interest for cryopreservation [154–156]. In addition, DESs are highly tuneable and can be made from non-toxic components including amino acids and sugars, or even betaine and proline, which as discussed above are already promising CPAs. However, so far there has been only one investigation into using DESs for cryopreservation. That study used a DES composed of trehalose and glycerol (at a 1:30 mol ratio) and found equitable cell viability as those preserved using DMSO [108].

Examining the work that has been done so far can help to streamline the search for new CPAs. In addition, predictive modelling can help to identify promising CPAs and reduce the number of required tests [157]. For example, a recent study used a differential evolution algorithm to successfully design a CPA solution that functioned even better than DMSO for the target cell type, reducing the number of required experiments from over a thousand to just eight [158].

5. Conclusion

The most commonly used CPAs (DMSO and glycerol) are not only toxic, which makes them unsuitable or inefficient for many clinical applications, but they are also ineffective for hundreds of cell types. Therefore, new, non-toxic CPAs must be developed.

In order to efficiently design CPAs and cryopreservation protocols, a number of factors must be considered including the biophysical properties of the cells to be preserved, the penetration ability of the CPA, the rate of cooling, and the ice inhibition capacity of the CPA. These parameters will provide vital information that will help fine-tune cryopreservation parameters such as cooling rate, or pre-incubation time.

As well as optimising the cryopreservation procedure, there is huge scope for developing new CPAs. The research outlined above highlights that glycerol and DMSO are just two options, but that there are many more which are worthy of further exploration, including: sugar derivatives, amino acids, other additives (such as betaine), and deep eutectic solvents.

Many of the studies detailed above demonstrated that combinations of CPAs are more effective and less toxic than individual CPAs on their own. Whilst this can make it harder to quantify, and creates exponentially more combinations to test, it appears that this is the most promising avenue for future development of CPAs.

Despite more than fifty years of research, the development of novel CPAs is still very much in its infancy. However, with improved experimental methods such as microfluidics, predictive modelling, and molecular simulations, the library of promising CPAs is likely to increase exponentially which offers significant promise for the many applications that rely on cryopreservation. This includes medical applications, such as blood and organ banks, and storage of seeds and endangered species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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