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Vitrification tendency and stability of DP6-based vitrification solutions for complex tissue cryopreservation

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Abstract

Vitrification tendency and stability of the amorphous state were analyzed by means of differential scanning calorimetry (DSC) for the vitrification solution DP6, with and without additional solutes to enhance ice suppression. This study is a part of an ongoing research effort to characterize the thermophysical and mechanical properties of DP6 and its derivatives, and their qualities as cryoprotective solutions. DP6 was determined to have a critical cooling rate necessary to ensure vitrification of 2.7°C/min. The following additional solutions were tested: DP6 + 6% (2R,3R) 2,3-butanediol, DP6 + 6% 1,3-cyclohexanediol, DP6 + 6% (0.175M) sucrose, DP6 + 12% PEG 400, and DP6 + 17.1% (0.5 M) sucrose. The additives decreased the critical cooling rate of the DP6 solution to rates below 1°C/min that were not quantifiable by the DSC techniques used. The following critical warming rates necessary to avoid devitrification were identified for DP6 and the modified solutions, respectively: 189°C/min, 5°C/min, $\approx$ 1°C/min, 15°C/min, <1°C/min, and <1°C/min. Glass transition temperatures and melting temperatures were also measured. Sucrose was the least effective additive on a per mass basis, with 1,3-cyclohexanediol appearing to be the most effective additive for suppressing ice formation in DP6.

Keywords

Cryopreservation; vitrification; DP6; synthetic ice modulator; DSC

1. Introduction

Vitrification is a promising approach for cryopreservation of complex tissues and organs that do not tolerate ice formation [17, 19, 21, 34]. Vitrification is a kinetic process, requiring cooling and rewarming rates faster than specific threshold values which depend upon the composition and total concentration of the vitrification solution used. While those threshold rates are commonly achievable and easily controlled at the outer surface of a sample, the core of larger samples will tend to cool and rewarm more slowly within critical temperature ranges due to the underlying principles of convective heat transfer. Consequently, during cooling of larger organs, there is a danger that cooling rates may fall below the critical

values to ensure vitrification [12]. This problem can be mitigated by using higher concentrations of cryoprotective agents or cryoprotectants that have stronger glass-forming tendencies, but both strategies often come at the cost of higher risk to the viability and functionality of the cryopreserved material (toxicity and/or osmotic damage) [20, 24, 25, 27].

The design of cryoprotectant solutions concentrated enough to permit vitrification of multi-milliliter samples while still having tolerable toxicity remains challenging. Progress has been made by using mixtures of ingredients to stay below toxicity thresholds of individual agents [20], combining cryoprotectants that exhibit mutual toxicity reduction [22], adding non-penetrating cryoprotectants [10, 17], and recently by adding agents that directly inhibit ice nucleation, growth, or recrystallization directly [34, 36].

One conceptual approach to the development of new vitrification solutions suitable for large samples is to start with a base solution that has proven utility for smaller samples, and then form new compositions by adding specific new solutes that enhance the solution's stability against ice crystallization and growth. This has been done for the vitrification solution DP6, which is defined as a mixture of 3 M dimethylsulfoxide and 3 M propylene glycol in a suitable vehicle solution [34]. In particular, the solutions DP6 + 6% 2,3-butanediol, DP6 + 6% 1,3-cyclohexanediol, and DP6 + 12% PEG 400 have been studied [11, 13–15, 30]. Meso-isomer-depleted 2,3-butanediol is a penetrating cryoprotectant and strong glass former [7]. 1,3-cyclohexanediol is a synthetic ice blocker with a molecular structure suggestive of activity similar to an antifreeze protein [16, 18, 34]. Polyethylene glycol (PEG) is a non-penetrating water soluble polymer [32]. Conceptualized as additives to the base solution DP6, these agents may be considered synthetic ice modulators (SIMs) of the base solution [14].

In the present study, we have used differential scanning calorimetry (DSC) to measure the vitrification tendency and stability of the amorphous state of vitrification solutions made from DP6 and the aforementioned SIMs. Additionally, DP6 mixed with either 0.175M sucrose or 0.5 M sucrose were studied due to emerging utility of these two new solutions (unpublished data).

2. Materials and Methods

2.1. Solutions

Solutions were prepared using ingredients from Sigma-Aldrich. The 2,3-butanediol used was the 2R,3R stereoisomer (Sigma-Aldrich #237639). 1,3-cyclohexanediol was a mixture of cis and trans isomers (#C101109). PEG 400 was polyethylene glycol of number average molecular mass (M_n) 400 (#202398). Sucrose was #S-1888. All solutions were prepared in UCV (Unisol Cryoprotectant Vehicle) [33].

2.2. Differential scanning calorimetry (DSC)

Measurements were performed with Perkin-Elmer DSC 7 differential scanning calorimeters equipped with liquid nitrogen cooling baths and using ultra-high purity helium purge gas, running Pyris version 5.00.02 software. Solution samples of approximately 10 mg mass

were sealed in Perkin-Elmer 0219–0062 aluminum sample pans and placed in the DSC sample oven for analysis. An empty sample pan was kept in the DSC reference oven to balance the instrument. The oven temperature was calibrated by measuring the onset of the crystal transition of cyclohexane at -87.06°C and the peak melting temperature of pure water at 0°C . Slow warming rates ($2^{\circ}\text{C}/\text{minute}$) and very small samples ($\sim 1\text{ mg}$) were used for temperature calibration to minimize lag between the oven and sample temperatures. Heat flow was calibrated by measuring the area under the ice melting curve of $\sim 10\text{ mg}$ samples of pure water (334 J/g nominal).

Even though a pure substance such as water melts at a single temperature, a finite sample mass and finite warming rate causes the melting of a pure substance to appear on a DSC thermogram as a peak of finite width. This is caused by the sample temperature slightly lagging behind the DSC oven temperature during melting; the sample stops warming during the brief time required for melting to finish while the oven holding the sample continues warming on the thermogram at the programmed rate. In the case of the 1 mg water sample used for DSC temperature calibration in this study, the maximum rate of melting (melting peak) was observed to occur at a temperature 0.7°C warmer than the onset of melting during warming at $2^{\circ}\text{C}/\text{min}$. Instead of melting onset, the melting peak temperature of water was chosen as the DSC calibration point for 0°C because the events of interest in the warmer temperature range of this study were solution melting peaks that will also exhibit some positive displacement from the actual sample temperature during these events. At the low end of the temperature scale, the onset of the cyclohexane phase transition was used for calibration rather than the peak based on the reasoning that the glass transition temperature measurements made at low temperatures were more similar to onset measurements than peak measurements. The difference between the transition onset and peak of cyclohexane calibration samples was 0.5°C .

2.3. Glass transition temperature determination

Solution samples were “quenched” by cooling from ambient temperature to -150°C at a rate of $100^{\circ}\text{C}/\text{min}$. The DSC was able to maintain this nominal rate in all cases until approximately -120°C , below which the sample temperature slightly lagged the programmed cooling rate. Thermograms were then obtained while warming samples through the glass transition temperature range at $5^{\circ}\text{C}/\text{min}$ (Fig. 1).

Although not necessary for determination of phase-transition temperatures, the thermograms displayed in Figs. 1 and 3 were converted to have specific heat units of $\text{J}/(\text{g}\cdot^{\circ}\text{C})$, by dividing the measured heat flow in mW by the sample mass in mg , and then further dividing by the warming rate in $^{\circ}\text{C}/\text{sec}$, consistent with past practice for comparability [35]. Although specific heat units are used for the comparative study, a reference specific heat has not been established, and the displayed results are indicative of specific heat changes rather than of its intrinsic value. Slope artifacts were removed by manual leveling of thermograms for clarity of the plots, although it bears no effect on transition temperature measurement.

The glass transition temperature, T_g , was measured using the Pyris DSC software, defined as the inflection point [1–4, 7, 9] of the change in specific heat while the material is rewarmed through the glass-transition temperature range at a rate of $5^{\circ}\text{C}/\text{min}$; the measured value

rounded to the nearest degree. The mean of three samples of each solution was documented for extra precaution, as measurements varied by less than one degree between samples. Although the thermograms in Fig. 1 show that some solutions devitrified during warming, the initial cooling rate of 100°C/min was far above the critical cooling rate for all solutions. All samples would therefore have been vitreous (ice free) during warming through glass transition, and all crystallization events are associated with the rewarming phase of the protocol.

2.4. Melting temperature determination

Solution samples were quenched to -150°C as described above and then warmed at 5°C/min to allow devitrification (rewarming-phase crystallization) followed by ice melting as seen in Fig. 1. The solution melting temperature, T_m , was taken as the mean melting peak temperature of three different samples rounded to the nearest degree. Variability between samples was less than a degree. Due to its crystallization suppression tendency, DP6 + 6% 1,3-cyclohexanediol had to be warmed at 2°C/min for devitrification to occur and permit measurement of a melting temperature.

To better understand the magnitude of instrument lag effects on phase transition measurements, a 10 mg sample of pure water was warmed at 2, 5, 10, 20, 40, 80, and 160°C/min. Relative to the melting onset temperature during warming at 2°C/min, the melting peak temperatures were displaced by +2.5°C, +3.8°C, +5.7°C, +8.4°C, +13°C, +21°C, and +32°C at these respective warming rates. The melting peak temperature difference of 1.3°C between warming at 2°C/min and 5°C/min is larger than the expected lag error of any melting temperature measurements done at 5°C/min in this study. This is because the quantities of ice melted in the cryoprotectant solutions of the study were one to two orders of magnitude less than the amount of ice that melted in this 10 mg sample of pure water. Smaller lag during melting smaller quantities of ice is evident from the thermograms of Fig. 3. In Fig. 3, DP6, which formed the most ice of any solution in this study, shows a melting temperature lag of only +15°C during warming at 160°C/min compared to +32°C for the same mass of water at this warming rate.

DP6 + 12% PEG 400 did not devitrify even while warming at the very slow rate of 2°C/min. To generate some ice in this solution so that its melting temperature could be measured, it was necessary to resort to a method that has been previously used for ice nucleation and ice growth temperature mapping in vitrification solutions [26, 38]. First, the solution was cooled at 80°C/min and held at a temperature near the glass transition temperature for a period of time for ice to nucleate. Then the solution was warmed at 80°C/min and held at a warmer temperature below the anticipated melting temperature to grow the nucleated ice. The solution was then warmed back to ambient temperature to detect a melting peak, if present. What finally succeeded for DP6 + 12% PEG 400 was a temperature hold at -105°C for 32 minutes for ice to nucleate, followed by a second temperature hold near or at -65°C for 128 minutes for ice to grow. Upon subsequent warming at 5°C/min, it was possible to detect and measure a melting peak.

It should be noted that the solution melting temperature is not the temperature at which ice begins to melt during warming. Unlike pure water, melting can begin in solutions at very

low temperatures if significant amounts of ice are present. This is because conversion of liquid water to ice during freezing increases the solute concentration in the remaining liquid, which reduces the melting temperature of ice in contact with that liquid. Consistent with cryobiology convention, the solution melting temperature, T_m , is defined as the highest temperature at which ice is thermodynamically stable in the solution. It is the temperature at which the last bit of ice melts during warming of a frozen solution, which ideally corresponds with the endotherm peak on a warming thermogram.

2.5. Critical cooling rate determination

The critical cooling rate of a solution is the cooling rate necessary to keep ice formation during cooling at or below a threshold amount that is deemed negligible. Critical cooling rates can be determined by the method developed by Boutron [1, 5–7, 35]. DSC thermograms are obtained for samples of a particular solution while cooling from 0°C to –150°C at various rates, typically ranging from 5°C/min to 80°C/min. If an exothermic freezing peak appears in a thermogram, the corresponding area under the curve, H (J/g sample mass) is calculated. The amount of ice that forms is expressed as the dimensionless parameter $q = H/(334 \text{ J/g}) \times 100$. It is approximately equal to the percentage of the sample that freezes. Figure 2 displays q as a function of cooling rate, and a least-squares fit of experimental data to the Fourth Model of Boutron [5]. In this Boutron model, the quantity of ice formed during cooling follows the sigmoidal relation:

$$\frac{k_4}{|v|} = -\ln(1-x^{1/3}) + \frac{1}{2}\ln(1+x^{1/3}+x^{2/3}) + \sqrt{3}\arctan\left(\frac{\sqrt{3}x^{1/3}}{2+x^{1/3}}\right) \quad (1)$$

where x is the ratio ($0 \leq x \leq 1$) of ice crystallized during cooling, q , to the maximum quantity of ice that can crystallize during very slow cooling, $q_{\max}$, v is the cooling rate, and k_4 is a constant. k_4 and $q_{\max}$ are the fitted parameters using the least-squares approximation technique. Theoretical critical cooling rates v_{crr} are calculated from:

$$v_{\text{crr}} = \frac{k_4}{3 \times \left(\frac{0.2}{q_{\max}}\right)^{1/3}} \quad (2)$$

This is the cooling rate at which freezing is limited to approximately 0.2% of the solution mass ($q=0.2$, deemed to be negligible) [9].

DP6 was more resistant to ice formation than most cryoprotective solutions previously reported in the literature. At typical DSC cooling rates of 5°C/min to 80°C/min, virtually no ice formed in DP6. This made DSC analysis difficult since the signal associated with freezing became of the same magnitude as the noise and other artifacts at scan rates below 2°C/min. This difficulty necessitated the development of a modified method to measure small amounts of ice formation during cooling.

While the measurement of the critical cooling rate, v_{crr} , in the current study followed the Boutron method, as described above, the quantity of ice formed, q , as a function of the

cooling rate was measured as follows. Each sample was first cooled from 0°C to -90°C at a particular cooling rate (studied rates being 1, 2, 5, 10, 20, 40, or 80°C/min), but no measurements were taken during this step. Samples were then immediately warmed from -90°C to +30°C at the rapid rate of 160°C/min. This fast scan rate required larger changes in DSC heater power during shorter times spent traversing phase transitions. This is because a specific amount of energy, which is the product of power and time, must be supplied to melt a specific amount of ice. The fast scan therefore amplified the signature of phase transition events on the recorded thermogram, making small melting peaks easier to detect and quantify. The fast warming rate also minimized any further growth of ice so that the melting peak observed during warming resulted primarily from ice that formed during cooling. Any formed ice during the initial cooling to -90°C was thus captured as an area under the melting peak portion of the thermogram during the following rapid warming. The average values of repeated runs under the same conditions ($n=3$) are plotted as q in Fig. 2 for cooling rates from 1°C/min up to 10°C/min. Values of q were corrected upward by 13% to account for under-measurement of melting peak area observed to occur in pure water samples melted at 160°C/min as a calibration check. A constant background melt of 0.3 J/g ($q = 0.09$) was observed after cooling at even the highest cooling rates (20, 40, and 80°C/min). This was attributed to devitrification (growth of nucleated ice during warming) and subtracted from q before plotting in Fig. 2.

Some explanation is needed as to why a study of the critical cooling rate necessary for vitrification can be done by cooling to only -90°C, which is more than 20°C warmer than the glass transition temperature of DP6. It was hypothesized that all ice that could grow during vitrification would grow before reaching -90°C because the high viscosity at lower temperatures would practically arrest further ice growth. If true, cooling below -90°C would only result in additional ice nucleation rather than ice growth, creating a larger devitrification effect in the background during warming.

The adequacy of -90°C as a minimum temperature for DP6 cooling studies was confirmed by repeating selected runs using nadir temperatures of -100°C and -80°C instead of -90°C. Cooling to only -80°C before warming resulted in less ice observed to melt during warming than after cooling to -90°C. Cooling to -80°C was therefore inadequate to replicate the ice growth that occurred if cooling was continued to lower temperatures. Cooling to -100°C resulted in a larger devitrification effect observed during warming after cooling at all rates, but the same amount of melting ice as observed with -90°C after subtraction of the larger background devitrification effect. Cooling to -90°C was therefore deemed adequate to replicate the extent of ice growth that would occur during vitrification of DP6, while minimizing devitrification effects to maximize measurement sensitivity to small amounts of ice. In a previous nucleation mapping study of the vitrification solution M22 and a more dilute version of it, -90°C was also found to lie within a quiescent temperature zone that is below temperatures where ice growth is rapid and above temperatures where ice nucleation is rapid [38].

DP6 is so stable against ice formation that even cooling at only 1°C/min resulted in an amount of ice that was still far from the maximum possible ice formation at equilibrium conditions. As can be seen in Fig. 2, the amount of ice formed at 1°C/min and higher rates is

very small. Consequently, q_{max} , which is the maximum amount of ice that can form at very slow cooling rates, as the system approaches thermodynamic equilibrium, was poorly determined by curve fitting to ice observed to form at 1, 2, 5 and 10°C/min cooling rates for DP6. Therefore, an independent estimate of q_{max} was made by measuring the amount of ice that devitrified in three DP6 samples warmed at only 2°C/min from -150°C. This slow warming rate (very much less than the critical warming rate of DP6 measured elsewhere in this study) resulted in a near-equilibrium amount of ice forming in DP6 during devitrification. The resulting estimate of $q_{max}=11.3\%$ was entered as the presumed low-cooling-rate limit for q at the 0.12°C/min position on Fig. 2. The 0.12°C/min position was used rather than the lowest rate of 0.1 °C/min on the graph for better visibility of the vertical error bars. The rate has no other significance.

2.6. Critical warming rate determination

Samples were rapidly cooled to -150°C to vitrify them as described above, and then warmed at 2, 5, 10, 20, 40, 80 or 160°C/min. As seen in Figs. 3 and 5 for DP6, the amount of ice forming and subsequently melting decreases with the increasing warming rate (i.e., devitrification and subsequent melting). The temperature difference between the peak of devitrification, T_d , and the peak of melting, T_m , also decreases as warming rate increases. The warming rate at which the amount of devitrified ice becomes negligible is the critical warming rate.

Boutron defined the critical warming rate as the rate that results in less than $q = 0.5$ ice formation during warming, and showed that this corresponds to $T_d/T_m=0.95$ when T_d and T_m are expressed in Kelvins [8]. This condition corresponds approximately with less than 0.5% of the solution mass freezing due to devitrification during warming. Since T_m was measured to be 238K for DP6 in the present study, it implies that $T_d=226$ K and that $T_m - T_d = 12$ K at the critical warming rate of DP6. The linear dependency [6, 7, 35] of $T_m - T_d$ (which conveniently cancels temperature measurement lag effects by subtraction) upon the logarithm of the warming rate was used to infer the warming rate at which $T_m - T_d = 12$ K, as shown in Fig. 4. The thermogram obtained at a warming rate of 10°C/min was excluded from the analysis because the distorted devitrification peak that can be seen in Fig. 3 prevented an adequate measurement of T_d at this specific warming rate.

For the solutions that included additives with DP6, devitrification at all warming rates studied was either non-existent or too small to permit meaningful analysis. Critical warming rates for these solutions were determined by direct examination of quantities of ice plotted in Fig. 5 or by inference.

3. Results

3.1. Glass transition and melting temperatures

Measured glass transition and melting temperatures are listed in Table 1. The first four solutions formed ice by ordinary devitrification during slow rewarming after vitrification, permitting identification and quantification of a melting peak on the thermogram.

Table 2 lists the various ice nucleation and growth parameters used in attempt to form ice in DP6 + 12% PEG 400. The most successful protocol combined cooling to -105°C and holding for 32 minutes to nucleate ice, then warming to -65°C or -60°C and holding for 128 minutes to grow the ice. The sample was then re-cooled at $80^{\circ}\text{C}/\text{min}$ to -90°C , and finally warmed from -90°C to $+10^{\circ}\text{C}$ at $5^{\circ}\text{C}/\text{min}$ to track the melting peak. This protocol yielded approximately 1 J/g of ice around the melting peak in four out of six samples. With the hindsight knowledge that the melting temperature of DP6 + 12% PEG 400 was -45°C , ice would probably have grown faster in the solution while holding a growth temperature somewhat warmer than -60°C .

No ice could be grown in DP6 + 0.5 M sucrose even when using the extreme nucleation and growth conditions listed in Table 2. A melting temperature therefore could not be determined.

3.2. Critical cooling rate

DP6 was the only solution that formed enough ice during cooling at practical rates to determine a critical cooling rate by fitting the Fourth Model of Boutron. The fitted values of q_{max} and k_4 were respectively 11.3 and $2.12^{\circ}\text{C}/\text{min}$, yielding a critical cooling rate of $v_{ccr} = 2.7^{\circ}\text{C}/\text{minute}$.

Note that $q = 11.3$ at $0.12^{\circ}\text{C}/\text{min}$ of Fig. 2 was the only data point at cooling rates below $1^{\circ}\text{C}/\text{min}$, which guaranteed that the best fit value for q_{max} would converge to that same value. As explained in Materials and Methods, the value $q = 11.3$ that was inserted near the slow cooling limit of Fig. 2 is actually the amount ice observed to devitrify and then melt during slow warming rather than a measurement of ice freezing during cooling at $0.12^{\circ}\text{C}/\text{min}$. The use of a devitrification measurement in place of a freezing measurement is justified because ice will grow to reach the same thermodynamic maximum (q_{max}) during long dwell times below T_m while warming or cooling. Even though this estimate of q_{max} is consistent with a lower bound of 10.2 ($q = 10.2$ being the maximum amount of ice observed in one of the $1^{\circ}\text{C}/\text{min}$ cooled samples), and appears to be approaching an equilibrium maximum amount of ice when compared to quantities of ice observed to devitrify in DP6 at higher warming rates in Fig. 5, the estimate of $q_{max} = 11.3$ for DP6 in this work must be considered approximate. Very tedious measurements might reveal a value for q_{max} slightly larger than 11.3, perhaps up to 12. However, the value of $2.7^{\circ}\text{C}/\text{min}$ as the critical cooling rate for DP6 is much more certain because the value of k_4 and the associated critical cooling rate is tightly bounded by surrounding measured data points, and relatively insensitive to the value of q_{max} .

No attempt was made to directly measure the critical cooling rate of the other solutions that contained additives to DP6. Since the critical cooling rate of DP6 itself is of the order of $1^{\circ}\text{C}/\text{min}$, all additives were present at concentrations of 6% or greater, and the critical warming rate of the least stable solution with additives was only $15^{\circ}\text{C}/\text{min}$ (critical cooling rates generally being orders of magnitude lower than critical warming rates), it was concluded without measurement that the critical cooling rates of the remaining solutions were necessarily lower than $1^{\circ}\text{C}/\text{min}$.

3.3. Critical warming rate

The amount of ice that was observed to form by devitrification during warming of vitrified samples, as a function of warming rate, is shown in Fig. 5. For DP6, the critical warming rate necessary to avoid more than $q = 0.5$ (1.67 J/g) of a vitrified sample from freezing during warming was calculated to be 189°C/min. For DP6 + 0.175M sucrose, the amounts of ice observed to devitrify during warming at 10°C/min and 20°C/min were respectively $q = 0.69$ and 0.30 . By interpolation, the critical warming rate expected to yield $q = 0.5$ is 15°C/min. For DP6 + 6% 2,3-butanediol, the mean amount of ice observed to form by devitrification during warming at 5°C/min was $q = 0.53$. The critical warming rate of DP6 + 6% 2,3-butanediol is therefore 5°C/min by direct measurement. For DP6 + 6% 1,3-cyclohexanediol, the mean amount of ice observed to form by devitrification during warming at 2°C/min was $q = 0.23$. Observing from Fig. 5 that the amount of ice tends to vary inversely with warming rate, the critical warming rate at which DP6 + 6% 1,3-cyclohexanediol is expected form $q = 0.5$ ice is approximately 1°C/min. Since no ice crystallized by devitrification in DP6 + 12% PEG 400 while warming at 2°C/min, indicating that it is even more stable than the preceding solution, the critical warming rate of DP6 + 12% PEG 400 is less than 1°C/min. Since no ice could be grown in DP6 + 0.5 M sucrose under conditions that did grow ice in DP6 + 12% PEG, the critical warming rate of DP6 + 0.5 M sucrose must be even less than that of DP6 + 12% PEG. These results are summarized in Table 1.

4. Discussion

Not surprisingly, glass transition temperatures tended to be higher for solutions with greater stability against ice formation during warming (devitrification). Both higher glass transition temperatures and greater stability against ice formation are expected as water content of solutions decreases. However DP6 + 12% PEG 400 was notable in having the same glass transition temperature as the solutions with 2,3-butanediol and 1,3-cyclohexanediol despite ~6% less water content. This may be due to the ether linkages of the PEG polymer having weaker self-interaction than the hydroxyl groups of the other additives.

The measured glass transition temperature of -115°C for DP6 is higher than the value of -119°C that has been previously reported [29]. This could be due to different but common definitions of the glass transition, where the current study uses the inflection point of the thermogram rather than the halfway point of the heat capacity change. The melting point of -35°C is nearly the same as the -34°C that has been previously reported [29], a difference that is within the uncertainty in measurements.

The critical cooling rate of 2.7°C/min measured for DP6 is considerably lower than the value of ~40°C/min previously reported [29], and less than the 7.2°C/min observed to be insufficient to prevent ice crystallization in DP6 (prepared in pure water rather than vehicle solution) [30]. However these previous studies were based on visual observation of absence of ice in volumes of several milliliters. For consistency with past DSC studies in cryobiology, the present study defines the critical cooling rate as the cooling rate calculated to prevent more than 0.2% of the solution from freezing. While this is a quantitatively small amount ice, it could be deemed as a failure to vitrify in visual assays that are sensitive to the

presence of small volume fractions of ice particles within an otherwise clear vitreous volume. On the other hand, the problem of accounting for this discrepancy in living tissues may be partly offset by the fact that the stability of the amorphous state in tissues that are equilibrated with vitrification solutions may be greater than the stability of the solutions themselves, as determined by DSC measurements [28]. This may be even more true when the cells within the tissue are shrunken prior to vitrification, concentrating intracellular proteins [31].

Nevertheless, it remains a limitation of DSC investigation of solutions with strong vitrification tendency and low critical cooling rates, such those in the present study (on the order of 1°C/min or less), that failure of such solutions to vitrify is a stochastic process dependent upon sparsely-distributed heterogeneous ice nucleating contaminants in the solution and sample pan. The random presence or absence of such nucleators in a 10 mg DSC sample has a large effect on individual sample measurements, as evidenced by the large error bars in Fig. 2 for the 1°C/min cooling rate.

The measured 189°C/min critical warming rate for DP6 agrees well with the previously reported value of 200°C/min [29]. This critical warming rate and the measured ≈3°C/min critical cooling rate of DP6 can be compared with a critical cooling rate of <1°C/min and critical warming rate of 50°C/min reported for the vitrification solution VS55 (VS41A) [26]. The DP6 plus SIMs and sucrose solutions were even more stable against devitrification during warming, with the most stable solutions comparable to or exceeding the stability of the kidney vitrification solution, M22 [19], reported to have a critical warming rate on the order of 1°C/min [37].

Remarkably, 1,3-cyclohexanediol conferred even greater resistance to devitrification than the same mass of 2,3-butanediol added to DP6. Meso-depleted 2,3-butanediol is known as a very good glass forming solute in cryobiology [7, 9]. Either 1,3-cyclohexanediol is also a very good glass former by virtue of strong interaction with water molecules, or as a larger molecule it is increasing solution viscosity more than 2,3-butanediol, or it may be acting directly against ice [16, 34].

Sucrose was the least effective additive on a per mass basis for suppressing ice formation in DP6. Paradoxically, that property may make it the most useful additive in practice because previous studies have found that vitrification solutions composed of solutes that must be present at higher concentrations to achieve vitrification (“poor glass formers”) have favorable toxicity properties [20], and in fact, the sucrose solutions studied here have been shown to have relatively low toxicity (unpublished results). This advantage may or may not convey to whole organs, however, since for whole organs, high viscosity may make it difficult to perfuse such solutions [23], and the high tonicities of 0.175–0.5M sucrose (>1.6–2.7 times isotonic) may exacerbate chilling injury in some organs [19]. More research on these points is therefore needed.

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Fig. 1.

Typical thermograms obtained while warming at 5°C/min after rapid cooling at a rate of 100°C/min. While the thermogram curve is normalized to have specific heat units, the vertical shift of each thermogram is arbitrary, due to the lack of a reference point. Thermogram curvature at higher temperature, above the peak-melting temperature, is an instrumental artifact.

Fig. 2.

Quantity of ice formed during cooling of DP6 samples as a function of cooling rate. The dimensionless unit, q , is percent phase transition heat relative to what would be measured if a mass of pure water equal to the sample mass completely froze. Error bars are $\pm$ standard error of the mean of four samples. Boutron's Fourth Model was approximated by a variance-weighted least-squares curve fitting of Eq. (1) to measured data. The parameter q_{max} is an estimation of the maximum amount of ice that can form in DP6 during quasi-steady freezing.

Fig. 3.

Thermograms of DP6 warming at various rates after cooling to -150°C . Vertical scale placement for each thermogram is arbitrary. Heat flow changes within individual thermograms, normalized to heat capacity units, are to scale. Progressively less ice forms during devitrification and melts at higher warming rates. Glass transitions and ice melting peaks are shifted to the right at higher warming rates due to increased time lag between the sample and the temperature chamber of the DSC. Since the solution melting temperature is a thermodynamic constant, the rightward shift of the melting peak is a direct measure of this instrumental lag at different warming rates. The even greater rightward shift of the devitrification peak as warming rate increases is due to this instrumental lag plus an actual shift in the real temperature that devitrification peaks at with higher warming rates.

Fig. 4.

Peak temperature difference between melting and devitrification (T_m and T_d , respectively) for DP6 versus warming rate. The temperature difference was measured directly from the thermograms on Fig. 3 without time lag compensation, since both measured values lag at the same rate for a specific run. The curve represents a linear regression fit to the measured temperature differences. The critical warming rate for DP6 is the extrapolation of the fitted line to $(T_m - T_d) = 12^\circ\text{C}$, which corresponds to $T_d/T_m = 0.95$ at which point 99.5% of the solution mass is expected to remain free of ice during warming.

Fig. 5.

Relative quantity of ice formed due to devitrification during warming after vitrification at a cooling rate of 100°C/min. The dimensionless unit q represents the ratio of melted devitrified ice H to the latent heat of pure water ice 334 J/g in percentage, which represents an approximate measure of the percent of the sample that freezes (devitrifies) during warming. Error bars are $\pm$ standard error of the mean of three samples. Tests on pure water showed that at warming rates faster than 20°C/min, the amount of ice measured to melt was under-measured by increasing amounts up to 12% at 160°C/min. This error, smaller than the random error of measurements plotted here, is not corrected for.

Table 1.

Phase-transition temperatures, and critical cooling and warming rates for the solutions tested.

Solution	Glass Transition Temperature T_g	Melting Temperature T_m	Critical Cooling Rate	Critical Warming Rate
DP6	−115°C	−35°C	2.7°C/min	189°C/min
DP6 + 6% 2,3-butanediol	−110°C	−45°C	< 1°C/min	5°C/min
DP6 + 6% 1,3-cyclohexanediol	−110°C	−43°C	< 1°C/min	≈ 1°C/min
DP6 + 12% PEG 400	−110°C	−45°C	< 1°C/min	< 1°C/min
DP6 + 0.175 M (6%) sucrose	−113°C	−39°C	< 1°C/min	15°C/min
DP6 + 0.5 M (17.1%) sucrose	−107°C	unobtainable	< 1°C/min	< 1°C/min

Table 2.

Quantity of ice formed in DP6 + 12% PEG 400 as a function of the holding time at a specific ice nucleation temperature, followed by ice growth during a second hold time at a warmer temperature. Each row represents one trial.

Nucleation Temperature	Nucleation Time	Growth Temperature	Growth Time	Ice Formed
-110°C	16 minutes	-70°C	64 minutes	0 J/g
-110°C	16 minutes	-65°C	64 minutes	0 J/g
-110°C	16 minutes	-60°C	64 minutes	0 J/g
-100°C	16 minutes	-70°C	64 minutes	0 J/g
-100°C	16 minutes	-65°C	64 minutes	0 J/g
-100°C	16 minutes	-60°C	64 minutes	0 J/g
-105°C	32 minutes	-70°C	128 minutes	0.31 J/g
-105°C	32 minutes	-65°C	128 minutes	1.24 J/g
-105°C	32 minutes	-60°C	128 minutes	1.53 J/g
-105°C	32 minutes	-65°C	128 minutes	1.10 J/g
-105°C	32 minutes	-65°C	128 minutes	1.12 J/g
-105°C	32 minutes	-65°C	128 minutes	0 J/g
-105°C	32 minutes	-65°C	128 minutes	0 J/g
-105°C	32 minutes	-65°C	128 minutes	1.11 J/g
-105°C	32 minutes	-65°C	128 minutes	1.12 J/g