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Glycerol-Free Cryopreservation of Red Blood Cells Enabled by Ice-Recrystallization-Inhibiting Polymers

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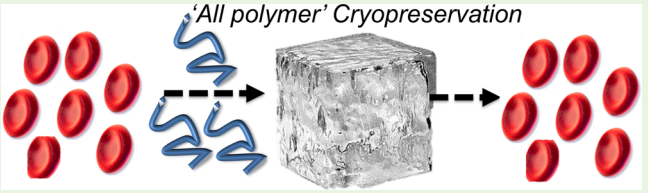
Supporting Information

ABSTRACT: Cryopreservation is fundamental in prolonging the viabilities of cells and tissues of clinical and biotechnological relevance ex vivo. Furthermore, there is an increasing need to address storage at more easily accessible temperatures in the developing world because of limited resources. Here, the cryopreservation of erythrocytes (red blood cells) with storage at −20 °C using hydroxyethyl starch (HES) and the ice recrystallization inhibitor poly(vinyl alcohol) (PVA), which is a biomimetic of naturally occurring antifreeze (glyco)proteins (AF(G)Ps), is described. This strategy eliminates the need for high concentrations of membrane penetrating solvents such as glycerol or dimethyl sulfoxide (DMSO). The addition of only 0.1−0.5 wt % PVA to the polymeric cryoprotectant, HES, significantly enhances cell recovery under conditions that promote damage due to ice recrystallization. The comparative ease with which the addition and removal of both HES and PVA can be attained is an additional attractive quality. Coupled with the benefits attained by the ice recrystallization inhibition activity of PVA, this methodology therefore offers a strategy that could aid the storage and distribution of biological materials.



KEYWORDS: *cryopreservation, poly(vinyl alcohol), blood, erythrocytes, AF(G)P*

INTRODUCTION

Advances in the number and volume of transplantation procedures routinely undertaken[1,2] have necessitated concurrent improvements in the storage and utilization of cells, tissues, and organs ex vivo to improve both storage and logistical problems. Cryopreservation (subzero storage to slow intracellular degradation processes) remains the method of choice for this. Existing strategies focus on maintaining short-term viabilities as opposed to prolonged storage at subzero temperatures demanding a reactive as opposed to proactive approach to transplantation.[3] Blood products are by far the most commonly transplanted material with demand necessitating the donation of 6000 units (470 mL) in the UK and 41 000 units in the USA per day.[4,5] Blood products are typically stored at 4 °C for a period up to 42 days in isotonic solutions, with the notable exception of platelets, which are stored for a period no longer than 7 days.[6,7] Although current rates of turnover dictate this approach usable in most domestic settings, it does not address individuals with rare blood types and storage in areas with logistical difficulties such as combat theaters.[8−10] This can be overcome using glycerol-based strategies developed more than 40 years ago but requires very high concentrations of cryoprotectant (up to 40 wt %). Furthermore, such strategies tend to require very low (−80 °C or −196 °C) storage conditions that are not ubiquitously available.[11,12] Extensive processing prior to transfusion to avoid the cytotoxic effects of glycerol and other low-molecular-weight cryoprotectants (e.g., DMSO) is undesirable. This is due to their membrane-permeable characteristics that can also incite undesirable changes in osmotic pressure.

Ice recrystallization (growth) is a major challenge during the cryopreservation of clinically relevant biological materials (and in food products[13]) and is not inhibited by conventional (solvent-based) cryoprotectants. The rate of ice recrystallization is at its highest 0 °C and −10 °C and therefore its negative impact is most prevalent during the thawing phase of cryopreservation.[14] Nature has evolved elegant solutions to mitigate the effects of ice recrystallization by the production of antifreeze glycoproteins and antifreeze proteins (AF(G)Ps) identified and isolated from polar fish, plant, and insects, commonly.[15−18] AF(G)Ps have the intrinsic ability to inhibit ice recrystallization[19] but their successful application to a variety of cell types ex vivo as cryoprotectants has been limited because of their secondary property, dynamic ice shaping (DIS). The interaction of AFPs and AF(G)Ps with the ice crystal lattice produces a (concentration dependent) effect resulting in a change in ice crystal morphology.[20] These morphological changes create "needle-like" structures inciting extensive mechanical damage that offsets the cryoprotective benefits attained by ice recrystallization inhibition (IRI).[21,22] These side effects have led to mixed results, with failures in improving rat cardiac explant and mouse spermatozoa cryopreservation.[23,24] Conversely,

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however, beneficial effects have been attained with rat islets and rat hepatocytes.[25,26]

A further challenge of applying AF(G)Ps is the expense of isolating them from natural sources, and in the case of AF(G)Ps, designing synthetic or biosynthetic tools to enable glycosylation remains challenging. Therefore, synthetic mimics of AF(G)Ps are highly desirable that may additionally limit any potential immunogenic issues associated with xenobiotics.[27,28] Ben and co-workers have developed small peptides with definite IRI activity, but these still require multistep synthesis.[29−32] Recent findings by Capicciotti et al. have shown the benefits of such peptides in cryopreserving red blood cells with reduced levels of glycerol, but could not remove it completely.[33] Remarkably, considering its simple structure, the synthetic polymer PVA has been found to have IRI activity comparable to the AF(G)P type 1 proteins.[34−37] No other synthetic polymer has been described with equivalent activity, although poly(ampholytes) are emerging as candidates.[38−40] PVA is particularly appealing as it is available to GMP standards (good manufacturing practice) and is FDA approved in food, cosmetic, and pharmaceutical preparations, lowering the barrier for translation.[41] Recent work has described the use of PVA in the (nonvitrifying) cryopreservation of erythrocytes (red blood cells) where the ice growth inhibition activity was shown to improve cell recovery, over short freezing periods.[42] The levels of blood recovery, however, were still below that achieved with glycerolization and what is required clinically—although it should be highlighted again that glycerol itself is not a desirable system due to processing challenges. HES is a polymeric cryoprotectant that functions extracellular and unlike glycerol (or DMSO) does not induce vitrification.[43] The high concentrations of HES required (up to 20 wt %) lead to highly viscous solutions, which are not easy to process or handle. However, its low osmolarity means that it incites less osmotic stress on red blood cells than glycerol and is easy to remove by washing. HES has previously also been used clinically as a plasma expander.[44,45]

Considering the above, the aim of this study was to probe whether the cryoprotective effects of a traditional cryoprotectant (HES) could be improved synergistically by addition of an ice recrystallization inhibitor (PVA). This manuscript describes an evaluation of their combined cryoprotective role, and seeks to apply this system to "high-temperature" freezing, which may remove the need for liquid nitrogen cooling.

RESULTS AND DISCUSSION

The main aim of this study was to determine if synthetic polymers, which can slow the rate of ice recrystallization, can be used to enhance glycerol-free erythrocyte cryopreservation. PVA is a potent ice recrystallization inhibitor (Figures S1 and S2) and hence has the potential to enhance cryopreservation in a similar context to AF(G)Ps and associated analogues without the detrimental effects of DIS. To demonstrate the activity of PVA, we conducted "splat" tests. Briefly, this assay measures the rate of ice crystal growth at sub zero temperatures, and example micrographs of ice wafers grown in phosphate buffer saline (PBS) alone and with addition of 2 mg mL^{-1} PVA (9 kDa) are shown in Figure 1. Ten milligrams per milliliter HES (used in the cryopreservation system below) was also tested. Higher concentrations could not be used because of its viscosity, which interfered with the assay.

Figure 1 clearly shows the potent ice growth inhibiting activity of PVA, in contrast to HES (at 10 mg mL^{-1}) and most other synthetic polymers. HES has been shown to be a useful cryoprotectant for red blood cells, and has the advantage over

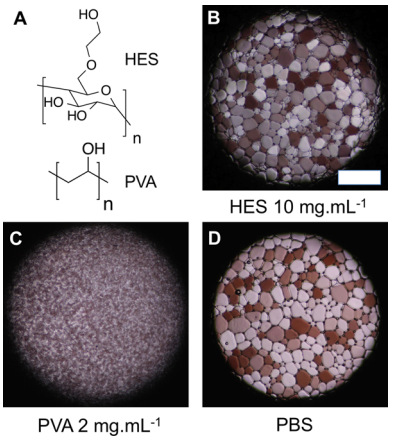

Figure 1. Ice recrystallization inhibitors. (A) Chemical structures of polymers used here. Ice crystals grown for 30 min at −6 °C in (B) PBS + HES, (C) PBS + PVA, (D) phosphate buffer saline (PBS) alone. HES degree of substitution = 0.6. Scale bar = 500 μm.

glycerol in that it can be directly transfused (although it is still preferable to remove it).[43] To achieve cryopreservation, relatively large amounts of HES are required, sometimes as high as 22.5 wt %,[43,46] which gives viscous solutions that are hard to process and cannot be transfused without dilution. However, HES is appealing, as it functions in an entirely extracellular context and is unable to penetrate cells, unlike low-molecular-weight cryoprotectants such as glycerol. We therefore reasoned that the combination of HES and PVA collectively could provide a "polymer-only" cryoprotective solution.

To highlight the benefits of HES over glycerol in this role, a series of trial cryopreservation experiments were performed involving the addition of cryoprotectant with variable equilibration times. Samples were then frozen by immersion in liquid N_2 for 10 min prior to thawing rapidly (42 °C) to limit the impact of ice recrystallization (Figure 2).

Using glycerol (20 wt %), it was possible to achieve cryopreservation with 80% cell recovery but only if a precise equilibration time, 1200 s, was chosen. Too short of a time, and

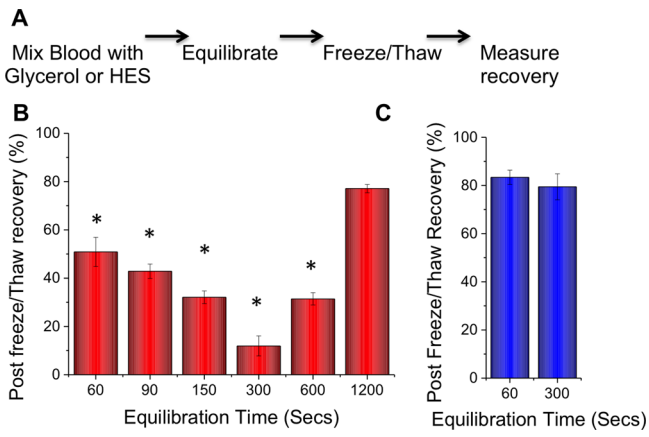

Figure 2. Effect of equilibration period on the postcryopreservation recovery of erythrocytes. (A) Process used for cryopreservation. Freezing time was 10 min at −196 °C; (B) cell recovery (from hemolysis assay) upon addition of 20 wt % glycerol; (C) cell recovery (from hemolysis assay) upon addition of 175 mg mL^{-1} HES. * Indicates a statistically significant ($p < 0.005$) difference relative to 1200 s. In b, the two values are not statistically different.

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the cryopreservation failed. This is because glycerol must diffuse across the cell membrane into the cells to achieve its protective benefit. In the early stages (short times), there is osmotic stress due to the high molarity of the glycerol solution. Equilibration for longer periods (hours) leads to cell death due to the intrinsic cytotoxicity of the glycerol toward RBCs. This highlights a key challenge associated with glycerolization of blood, in that extremely controlled protocols are required to both add and remove the glycerol, and any deviation in the process, results in nontransfusable blood. As a comparison, HES (175 mg mL^{-1} (14.8 wt %)) was also used. In Figure 2C, there was no observable effect of equilibration time—the HES is a passive additive, neither binding to the cells, nor entering them. Control experiments also confirmed that HES does not lead to hemeagglutination. Because of its extracellular nature, HES can also be removed by simple washing.

With this encouraging data to hand, the impact of the addition of PVA on red blood cell recovery could be investigated using both slow (23 °C) and fast (42 °C) thawing temperatures for short freezing times (as a screening approach). Slow thawing exacerbates cell damage due to ice growth and is more representative of large-volume cryopreservation. A HES concentration of 175 mg mL^{-1} was selected to provide a balance between manageable viscosity and cell recovery (Figure S4). A higher concentration of HES does improve cell recovery, but the viscosity of the solution presents a processing barrier. Lower concentrations of HES give reduced cell recovery, but enabled us to probe the additive effect of the PVA. PVA only needs very low concentrations for IRI activity, thereby lowering the total cryoprotectant concentration relative to a HES solution alone. The results of this are shown in Figure 3.

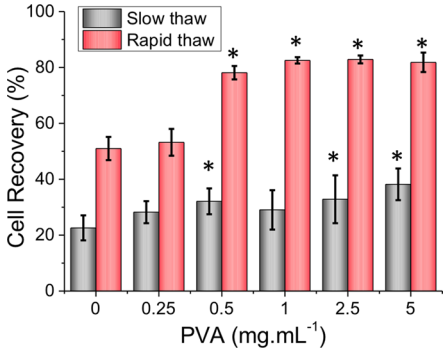

Figure 3. Recovery of red blood cells following cryopreservation at −196 °C for 30 min and then thawed either rapidly (42 °C) or slowly (23 °C). Solutions contain HES (175 mg mL^{-1}) with indicated concentration of PVA. Error bars represent ± standard deviation from a minimum of 3 independent experiments. * Indicates a statistically significant difference relative to control with no PVA. ($p < 0.05$).

Under the conditions used (again note that minor changes in freezing conditions have major impact on cell recovery), the HES alone lead to around 22% cell recovery. Under fast thaw conditions this increased to around 52% recovery. At both thawing rates, addition of PVA at concentrations up to 5 mg mL^{-1} had a dramatic effect on the cell recovery. Under slow conditions 10−15% increases in cell recovery was observed, and with fast thawing up to 30% additional recovery. The differences due to thawing rate can be attributable to both the IRI activity, but also differences in solution viscosity, which changes with temperature, especially with macromolecular additives.

With the obvious enhancement in cell recoveries associated with adding PVA, we set out to investigate cell storage over longer times. Additionally, it was also desirable to probe the effect of storage temperature on cell recovery. Storage at −196 °C (liquid nitrogen) is very efficient and used for example to store spermatozoa in the UK.[47] For applications in the developing world, or in conflict zones, liquid nitrogen is not convenient. Therefore, storage at −20 °C was investigated, which is far easer to achieve without mains electricity supply. A significant advantage of using all polymeric cryoprotectants, is that due to their high molecular weight, the total molarity of the solutions is far lower than in glycerol-based mixtures, and hence the freezing point is depressed to a lesser extent (the freezing point is directly proportional to the concentration of dissolved solutes). Figure 4

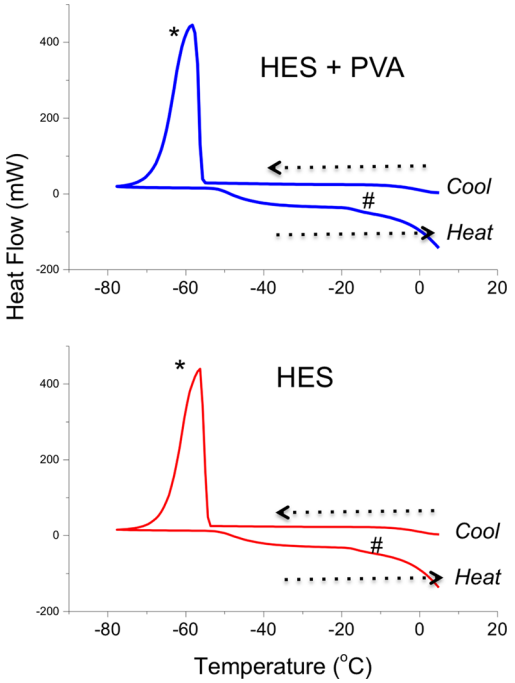

Figure 4. DSC analysis of the freeze−thawing of HES/PVA solutions. Samples were cooled and heated at 40 °C.min^{-1} and 2 °C min^{-1} respectively. * Indicates formation of ice and # the onset of melting. [HES] = 175 mg mL^{-1} and [PVA] = 1 mg mL^{-1}.

shows DSC (differential scanning calorimetry) analysis of solutions of HES with/without PVA as it is cooled and thawed. In both cases crystallization can be observed by the exotherm at approximately −60 °C. While this does not rule out any glassy (vitrified) regions, especially intracellular, the large value confirms that ice is present.[48] Upon heating, both solutions begin to melt just above −20 °C confirming that −20 °C storage should be possible. There is an endotherm at −50 °C in both solutions, possibly indicative of a glass-transition for the partially vitrified intracellular components.

With this information to hand, longer term cryopreservation was conducted. Figure 5 shows the results of 3-day cryopreservation studies using 175 mg.mL^{-1} HES with varying concentrations of PVA. The blood/cryoprotectant mixtures were rapidly frozen in liquid nitrogen for 10 min to ensure rapid freezing and the formation of small ice crystals. It is crucial to note that there is no vitrification in this system. After freezing, the samples were subsequently transferred to either a liquid nitrogen storage tank, or into a conventional −20 °C freezer. As confirm in

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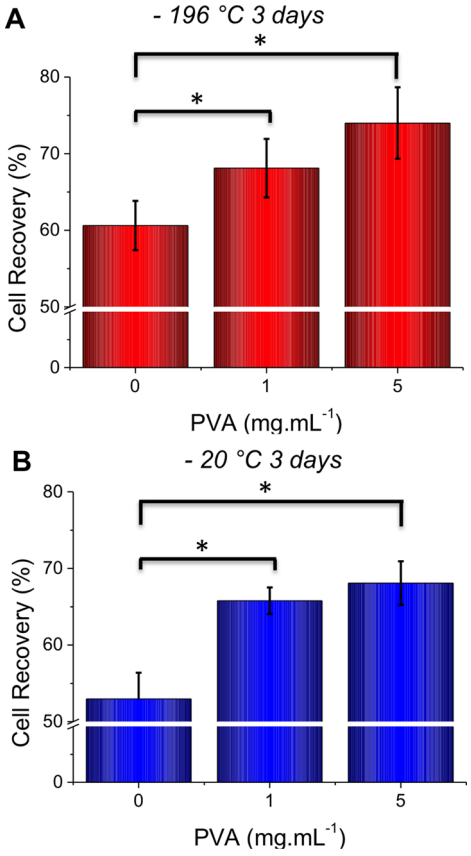

Figure 5. Red blood cell recovery after 3 days cryopreservation at the indicated temperature. All samples were initially flash frozen at −196 °C prior to storage at indicated temperature and were thawed slowly (23 °C). Solutions contain HES (175 mg mL^{-1}) with indicated concentration of PVA. Error bars represent ± standard deviation from a minimum of 3 independent experiments. * Indicates a statistically significant difference relative to control with no PVA. ($p < 0.005$).

Figure 4, the melting point of the HES/PVA solutions was ∼ −20 °C, which along with visual inspection confirm a solid was formed. After 3 days, the samples were removed and allowed to thaw slowly at 23 °C. The use of slow thawing makes this a more rigorous assessment of the cryoprotective properties and enables the effect of PVA as a recrystallization inhibitor to be shown.

At −196 °C, recovery rates of 60% were observed, which dramatically increased to 70 and 75% by addition of 1 and 5 mg mL^{-1} PVA, respectively. This is a significant increase in recovery levels, considering the extremely low concentration of PVA being applied, and is in agreement with earlier reports on PVA only cryopreservation solutions. In the HES solution alone, only 50% cell recovery was obtained. This is as expected, as at −20 °C in this solution, extensive ice growth would be expected over 3 days, due to the relatively high temperatures. Adding PVA at 1 and 5 $mg.mL^{-1}$ gave a dramatic increase in recovery to 70%. This was almost as good as what was achieved using liquid nitrogen freezing, and represents a positive result. There are very few examples of such high-temperature cryopreservation, and this is enabled by the use of low molarity solutions. The addition of PVA will act to slow ice growth, not just during the sample thawing process, but also during the act of storage itself. Other cryoprotectants such as glycerol, DMSO, and HES show little or no ice recrystallization inhibition activity, enabling this to be directly correlated with the PVA activity.

Taken together, these results show that polymeric recrystallization inhibitors have real potential to enhance cellular cryopreservation, which in turn can have a dramatic effect on healthcare services where easy access to primary cells is required.

CONCLUSIONS

This contribution describes the benefit of adding polymeric recrystallization inhibitors to cryopreservation solutions. The polymer, PVA, mimics the function of antifreeze (glyco)proteins in slowing ice crystal growth, particularly during thawing, reducing cellular stress. PVA was combined with another, known, cryoprotectant HES. HES is an ideal partner to PVA as it does not enter the cells, and due to its high molecular weight does not lead to osmotic stress, when compared to solvent based cryoprotectants such as glycerol. Addition of just 1−5 mg mL^{-1} of PVA (0.1−0.5 wt %) was found to increase cellular recovery, post thaw, by over 30% in some cases. Using liquid nitrogen freezing and slow thaw methods, cell recoveries of ∼75% were achieved, despite this being a nonoptimal thawing strategy, representative of a low resource environment. Finally, due to the low molarity of the "all polymer" cryoprotection solution it was shown to be possible to store red blood cells frozen, at −20 °C. This is particularly significant, as a −20 °C can be possible using minimal electricity, or portable generation, such as solar power. These findings demonstrate that by taking a bioinspired approach it is possible to enhance cryopreservation with the aim of removing (or reducing) the need for organic solvents, and enabling higher temperature (−20 °C) cryopreservation, which may be of use in developing countries or for logistical purposes.

EXPERIMENTAL SECTION

Materials and Methods. *Materials.* Nine kilodalton poly(vinyl alcohol) (PVA) (80% hydrolyzed) was obtained from Sigma-Aldrich Company Ltd. (Dorset, UK). Two hundred kilodalton hydroxyethyl starch (HES) with 50% degree of hydroxyethyl group substitution was purchased from Carbosynth Ltd. for use in all cryopreservation experiments (Berkshire, UK). 130 kDa Hydroxyethyl Starch (HES) with 40% degree of hydrolysis was sourced from Fresenius Kabi Ltd. (Cheshire, UK) and used in "splat" assay and DSC experiments. All polymers were exhaustively dialyzed (3 kDa MWCO) against Milli-Q water prior to use to remove any low molecular weight contaminants. Ovine erythrocytes in Alsevers solution were purchased ad hoc from TCS Biosciences Ltd. (Buckinghamshire, UK) when required and used within 7 days. When not in use, erythrocytes were stored as supplied at 4 °C. All other reagents were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK).

"Splat" Assay for Determining Ice Recrystallization Inhibition. The ice recrystallization inhibition properties of HES and PVA were exemplified using a modified "splat" assay.[35,36] The "splat" assay entails dropping an ∼10 μL droplet from a height of approximately 2 m onto a No.0 (0.085−0.13 mm thick) coverslip, precooled (CO_2(s) ≈ −50 °C) on an aluminum surface forming an instant polycrystalline wafer comprising very small ice crystals (<10 μm Ø) of a thickness typically less than 50 μm. The coverslip is then annealed at −6 °C for 30 min on a N_2(l) cooled Linkam BCS 196 cryostage (Linkam Scientific Instruments Ltd., Surrey, UK) coupled to an Olympus CX41 microscope equipped with UIS-2 20*x*/0.45/∞/0−2/FN22, UIS-2 4*x*/0.1/∞/-/FN22 and UIS-2 10*x*/0.2/∞/-/FN22 lenses (Olympus Ltd., Essex, UK) through cross polarizers. At least three fields of view (1.33 mm^2) were photographed for each compound and concentration. The largest dimension of the 10 largest ice crystals from each image were then measured using the freely available image analysis software imageJ (http://rsbweb.nih.gov/ij/index.html) and the mean (μm) and standard deviation calculated. Data were plotted in Origin 8.5 (OriginLab Corp, MA, USA), allowing the mean largest grain size (MLGS (μm)) with a resolution of up to 0.2 μm to be defined.

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Processing of Erythrocytes Prior to Cryopreservation. As-supplied Ovine erythrocytes in Alsevers solution were centrifuged at 5000 rpm at 23 °C for 10 min. The supernatant (containing plasma) was removed and discarded. Samples were then prepared by the addition of 6% (w/v) mannitol (limits osmotic induced swelling in vitro),[49] 1.3% (w/v) NaCl, and as indicated, 2-fold the required final concentration of glycerol or HES and/nor PVA to an equivalent volume (250 μL) of prepared ovine erythrocytes. This yielded a mixture of 3% (w/v) mannitol, 0.65% NaCl, and up to either 20 wt % glycerol or 175 mg.mL^{-1} HES with or without up to 5 mg mL^{-1} PVA in accordance with previously published cryopreservation strategies.[50] Samples were vortexed rigorously immediately prior to equilibration and/or freezing in order to ensure a homogeneous suspension of erythrocytes in solution in 1.8 mL cryovials (Fisher Scientific UK Ltd., Leicestershire, UK) with a total volume of 500 μL.

Freezing, Storing, and Thawing of Erythrocytes. Freshly prepared erythrocyte solutions as aforementioned were frozen by immersion in liquid N_2 (−196 °C) for a period of 10 min prior to thawing (Figure 2) or were kept in liquid N_2 for a specified period of time of up to 3 days (Figures 3 and 5) or transferred to −20 °C for up to 3 days (Figure 5). Thawing of samples was undertaken either slowly or rapidly regardless of the storage strategy (−20 °C or −196 °C) utilized. Rapid thawing was defined by the full immersion of samples in a 42 °C water bath for a period of 2 min (red blood cells are able to tolerate temperatures of 42 °C). This ensured that samples were fully thawed but not heated above 37 °C. Slow thawing was undertaken by transferring samples to 23 °C in air for a minimum of 60 min. This promoted extensively ice recrystallization while ensuring samples were fully thawed. A minimum of three replicates were used per data point.

Processing of Erythrocytes Post Cryopreservation and Quantification of Cell Recovery. Four hundred microliter aliquots of each cryopreserved sample were transferred to 1.5 mL eppendorf tubes individually and centrifuged at 6000 rpm for 5 min at 23 °C. Intact erythrocytes will therefore be pelleted and the resulting supernatant containing the hemoglobin of lysed cells. Ten microliters of supernatant was then added to 90 μL of PBS in duplicate in a 96-well plate. The ordering of this process is important to ensure that no radical changes in osmolarity occur that could incite hemolysis and therefore lower the perceived cell recovery. Absorbance measurements at 450 nm using a BioTek Synergy HT plate reader (BioTek UK, Bedfordshire, UK) were taken to gauge the extent of hemolysis. 100% cell recovery (0% hemolysis) was defined as the absorbance of erythrocytes in 3% mannitol and 0.65% NaCl but in the absence of glycerol, HES and/or PVA not subjected to any cryopreservation treatment. 0% cell recovery (100% hemolysis) was defined by the cryopreservation of erythrocytes in H_2O under identical freeze and slow thaw conditions. The combination of osmotic stress induced lysis due to H_2O and cryopreservation in absence of cryoprotectants ensured complete hemolysis. 0 and 100% cell recovery values were delineated for each experiment to minimize the slight discrepancies in hematocrit for each as supplied ovine erythrocyte solution.

Differential Scanning Calorimetry. Samples were prepared by weighing standard 40 μL aluminum crucibles (Mettler Toledo, Leicestershire, UK) and adding 15 μL of analyte before sealing (hermetically) and reweighing in order to quantify the exact mass of sample. Each sample was then transferred to a liquid nitrogen cooled DSC 1 STAR system (Mettler Toledo, Leicestershire, UK) differential scanning calorimeter. The mass of the aluminum crucible and sample mass was inputted into the complementary STAR^e thermal analysis software to retain a digital record and aid analysis. Each DSC sample was individually cooled from +5 °C to −78 °C at a rate of 40 °C/min while concurrently monitoring the heat flow (mW) of the system to detect any endothermic or exothermic transitions. When samples were cooled to −78 °C, each sample was then immediately warmed slowly at a rate of 2 °C/min.

Data Analysis. All statistics and calculations for (%) cell recovery were determined using Microsoft Excel 2011 for Mac. Significance determination for cryopreservation data in Figures 3 and 5 used a two-tailed homoscedastic Student's *t* test with a 95% confidence interval.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.5b00162.

Quantitative ice recrystallization inhibition activity of the polymers and variable thawing temperature data (PDF)

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Author Contributions

R.C.D. undertook the experiments; M.I.G. conceived the project; and R.C.D., M.V., D.A.M.; and M.I.G. planned the experiments. R.C.D. and M.I.G. analyzed the data. M.I.G. and R.C.D. wrote the paper. All others have read the manuscript and commented on it.

Notes

The authors declare the following competing financial interest(s): R.C.D. and M.I.G. have a patent filed that relates to this work, PCT/GB2013/050277.

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