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

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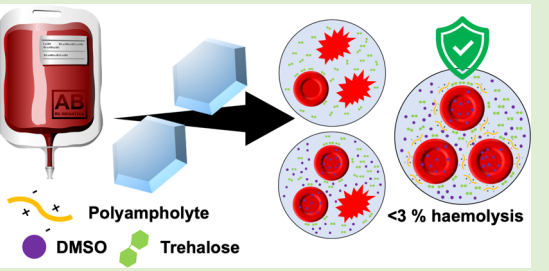
ABSTRACT: From trauma wards to chemotherapy, red blood cells are essential in modern medicine. Current methods to bank red blood cells typically use glycerol (40 wt %) as a cryoprotective agent. Although highly effective, the deglycerolization process, post-thaw, is time-consuming and results in some loss of red blood cells during the washing procedures. Here, we demonstrate that a polyampholyte, a macromolecular cryoprotectant, synergistically enhances ovine red blood cell cryopreservation in a mixed cryoprotectant system. Screening of DMSO and trehalose mixtures identified optimized conditions, where cytotoxicity was minimized but cryoprotective benefit maximized. Supplementation with polyampholyte allowed 97% post-thaw recovery (3% hemolysis), even under extremely challenging slow-freezing and -thawing conditions. Post-thaw washing of the cryoprotectants was tolerated by the cells, which is crucial for any application, and the optimized mixture could be applied directly to cells, causing no hemolysis after 1 h of exposure. The procedure was also scaled to use blood bags, showing utility on a scale relevant for application. Flow cytometry and adenosine triphosphate assays confirmed the integrity of the blood cells post-thaw. Microscopy confirmed intact red blood cells were recovered but with some shrinkage, suggesting that optimization of post-thaw washing could further improve this method. These results show that macromolecular cryoprotectants can provide synergistic benefit, alongside small molecule cryoprotectants, for the storage of essential cell types, as well as potential practical benefits in terms of processing/handling.



INTRODUCTION

Blood banks supply donated blood, primarily consisting of red blood cells (RBCs), to treat patients for blood loss due to injury, surgery, or during chemotherapy. However, blood has a limited refrigerated shelf life and can only be stored for 35 days, according to NHS (UK) guidelines.[1] Cryopreserved blood is just as effective as fresh blood[2] and, with cryopreservation, the storage time of RBCs can be extended indefinitely,[3] allowing the blood to be used in remote areas[4,5] and to mitigate supply during times of restricted stocks/donations or due to disasters.[6,7] RBC cryopreservation is also used in diagnostics[8] and for veterinary blood banking, where blood from a specific species may be difficult to source, making a blood bank challenging to establish.[9−11]

In order to freeze cells of any type, cryoprotectants must be introduced to protect against mechanical ice damage, osmotic stress, and the dehydration that ice growth causes.[12] This must be balanced against the intrinsic cytotoxicity of the cryoprotectants, which are typically organic solvents, with dimethyl sulfoxide (DMSO) widely used for nucleated cells.[13−16] Current cryopreservation practices for RBCs use 20−40 wt % glycerol, which must be added slowly to avoid osmotic shock and then slowly washed out prior to transfusion.[17,18] Washout is ideally performed using an automated system,[19,20] which slowly reduces the concentration of glycerol in the solution, allowing glycerol to diffuse out of the RBCs at a rate that does not cause osmotic stress. This deglycerolization process takes 30−60 min per unit (475 mL) of blood,[21] meaning that, during an unexpected surge in demand for blood, such as during a natural disaster or in a military setting, there could be a significant delay before frozen blood becomes available.[4] Although the use of DMSO to cryopreserve RBCs has been studied (particularly for nonhuman blood),[9,22] it is not widely used for RBC cryopreservation, even though it can be more rapidly washed out (compared to glycerol) due to its greater membrane permeability and lower working concentrations.[22,23] This may be due to cytotoxicity[24,25] or lack of effectiveness compared to glycerol, but it has been shown to be potent for animal RBCs.[9,22]

Trehalose (a nonreducing disaccharide of α-linked glucose) is produced by extremophiles such as tardigrades[26−30] to

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survive in extreme cold environments. Trehalose is noncell permeable and hence acts in the extracellular space, where it reduces ice formation by disrupting hydrogen bonding.[31,32] Trehalose may aid in stabilizing the membrane[33] and can stabilize proteins.[34,35] Trehalose can replace the hydrogen-bonding function of water during desiccation[36] and lyophilization[35] and is known to be at its most effective when it is present on both sides of the cell membrane.[37] For this reason, materials and techniques have been developed to deliver trehalose intracellularly[38] and trehalose-side chain polymers have been reported to protect proteins during lyophilization.[28,39] Trehalose has been shown to enhance RBC cryopreservation[37] and neutralize cryoprotectant toxicity in RBCs.[40]

Another approach evolved by freeze-avoidant extremophiles is the production of ice-binding proteins[41] (also known as antifreeze proteins). These macromolecules can bind to ice, acting to promote ice nucleation[42] or noncolligatively reduce the equilibrium freezing point leading to a thermal hysteresis gap.[41,43−45] One particular property of IBPs is ice recrystallization inhibition (IRI). Ice recrystallization is a source of damage, especially during thawing, in cryopreserved samples and the addition of antifreeze proteins[46] (or polymeric/material mimics)[47−51] has been found to improve post-thaw outcomes by controlling extracellular ice growth.[52] Small molecule IRIs developed by Ben and co-workers have also been shown to be potent ice growth inhibitors.[53] Bromophenyl-β-D-glucopyranoside was shown to enable ∼95% post-thaw membrane integrity of red blood cells with a reduced glycerol concentration of 15 wt %.[54] However, IRIs alone do not always give significant improvements in recovery between different systems and it is clear that all the other mechanisms of damage during cryopreservation should be addressed. Matsumura and Hyon. reported that poly(L-lysine)-based polyampholytes, polymers with mixed positive and negative charges along the backbone,[55] were potent cryopreservation enhancers, especially when used with, for example, DMSO. Polyampholytes have weak IRI activity[56,57] and appear to aid cryopreservation during slow freezing by a distinct mechanism, which could be membrane stabilization.[58] There are also reports of polyampholytes being used to improve vitrification processes (ice-free cryopreservation achieved using high solvent concentrations) potentially by inhibiting ice formation.[59] Polyampholytes have been used for stem (stromal) cell,[60] cell monolayer,[61] and fibroblast[62] cryopreservation. A key point to note is that polyampholytes, when used as the sole cryoprotectant, do not always allow successful cryopreservation. Immediate post-thaw viability values can be false positives (overestimation of function),[63] which is revealed by longer-term post-thaw culture experiments, and supplemental cryoprotectants (such as DMSO) may be required.[62] This evidence suggests that polyampholyte-mediated cryopreservation could be optimized by using it in addition to other cryoprotectants, which each have distinct modes of action, and, more specifically, could be applied as alternatives to glycerol for RBC cryopreservation.

Here, we screened combinations of trehalose (an extracellular cryoprotectant) and DMSO (an intracellular cryoprotectant) with added polyampholytes to find conditions for ovine RBC cryopreservation. This approach identified a cryoprotectant formulation resulting in <3% post-thaw hemolysis, and recovery of intact ovine RBCs that did not show signs of osmotic stress, and was also shown to work on large (400 mL) volumes. Flow cytometry, phase contrast and confocal microscopy, and adenosine triphosphate (ATP) assays confirmed the cell recovery and demonstrated that mixed cryoprotectant strategies can be used to improve post-thaw recoveries.

EXPERIMENTAL SECTION

Materials and Methods. DMSO (analytical reagent grade), poly(methyl vinyl ether-*alt*-maleic anhydride) (average Mn ≈ 80,000 Da, impurities: <2% benzene), Dulbecco's phosphate-buffered saline (1.15 g L^{-1} dibasic sodium phosphate, 0.2 g L^{-1} potassium chloride, 8 g L^{-1} sodium chloride, and 0.2 g L^{-1} monobasic potassium phosphate) (DPBS) were purchased from Sigma-Aldrich. Trehalose dihydrate (purity >98%) was purchased from Carbosynth. Poly(ethylene glycol) (PEG) (average Mn = 4000 g mol^{-1}) was purchased from Sigma-Aldrich. 2-Dimethylaminoethanol (purity 99%) was purchased from Acros Organics. Tetrahydrofuran (laboratory reagent grade) was purchased from Merck. BD FACSFlow sheath fluid was purchased from BD biosciences. Sheep's blood in Alsever's solution (not defibrinated) was from TCS Biosciences. Polyampholyte (poly(vinyl ether-*alt*-maleic acid mono(dimethylamino ethyl)ester)) was synthesized in house as described in the Supporting Information Milli-Q ultrapure water (>18.2 MΩ cm^{-1} 2 ppb) was used throughout.

Blood Preparation. 10 mL sheep's blood in Alsever's solution was transferred into a centrifuge tube and centrifuged for 5 min at 367*g* (2000 rpm). The resulting supernatant was discarded and replaced with 7 mL DPBS and mixed by inversion. The resulting hematocrit was 30%.

High-Throughput Freezing. A liquid-handling system (Pipetmax, Gilson) was used to mix 50 μL of two different cryoprotectants at 2× of final concentration in each well of a 96-well plate. The system then mixed 100 μL of blood prepared in DPBS with the cryoprotectants for a final volume of 200 μL per well. RBCs were incubated with cryoprotectants for 10 min before being frozen in liquid nitrogen vapor. After 20 min, the plate was removed and warmed for 5 min in a 37 °C water bath. The positive and negative controls, DPBS and lysate (100 μL blood, 100 μL lysis buffer, respectively), were added. The plate was centrifuged at 2250 x g (3700 rpm) for 5 min and the AHD assay was conducted as described below. Lysis buffer was 0.32 M sucrose, 5 mM $MgCl_2$, 10 % wt triton X-100, and 10 mM tris HCl pH 7.8.

Washout. The stock washout solution used was 10% DMSO + 100 mM trehalose in Alsever's solution. This was replaced with varying amounts of Alsever's solution for use in each washout step. Blood was transferred into Eppendorf tubes and centrifuged at 2000*g* (6000 rpm) for 1 min. The pellet was resuspended in prewarmed 37 °C 80% washout solution. This was repeated for 40, 20, and 0% washout solutions. Finally, intact RBCs were pelleted to collect remaining lysed blood in the supernatant. The hemolysis from each step was added to calculate post-freeze−thaw−wash survival.

AHD (Hemolysis) Assays. The alkaline hematin D-575 (AHD) assay[64] was used to assess hemolysis after high-throughput freezing; the liquid-handling robot transferred 8 μL of the resulting supernatant from each well to 100 μL AHD solution (100 mL water, 2.5 g triton X100, 0.5 g NaOH) in a 96-well plate. Each well was then manually stirred to ensure any remaining precipitate dissolved. Absorbance of the AHD plate was measured at 580 nm using a plate reader (Synergy HT, BioTek). Recovery was calculated as: (1-(Absorbance-DPBS/(lysate-DPBS)) × 100. For vial freezing, 40 mL of the supernatant from each sample was transferred to 750 mL AHD solution.

Vial Freezing and Large-Volume Assays. For vial freezing, 500 μL blood prepared in DPBS was transferred to Eppendorf tubes and centrifuged for 5 min at 2000*g* (6000 rpm). The resulting pellet was mixed with 1 mL cryoprotectant solution, transferred to cryovials, and incubated for 10 min. Samples were then plunged into LN2, or inserted into a cooler (Coolcell LX Corning) and cooled at −1 °C min^{-1} to −80 °C for 2 h. Samples were warmed in a 37 °C water bath for 5 min or at ambient temperatures until thawed. Finally, samples

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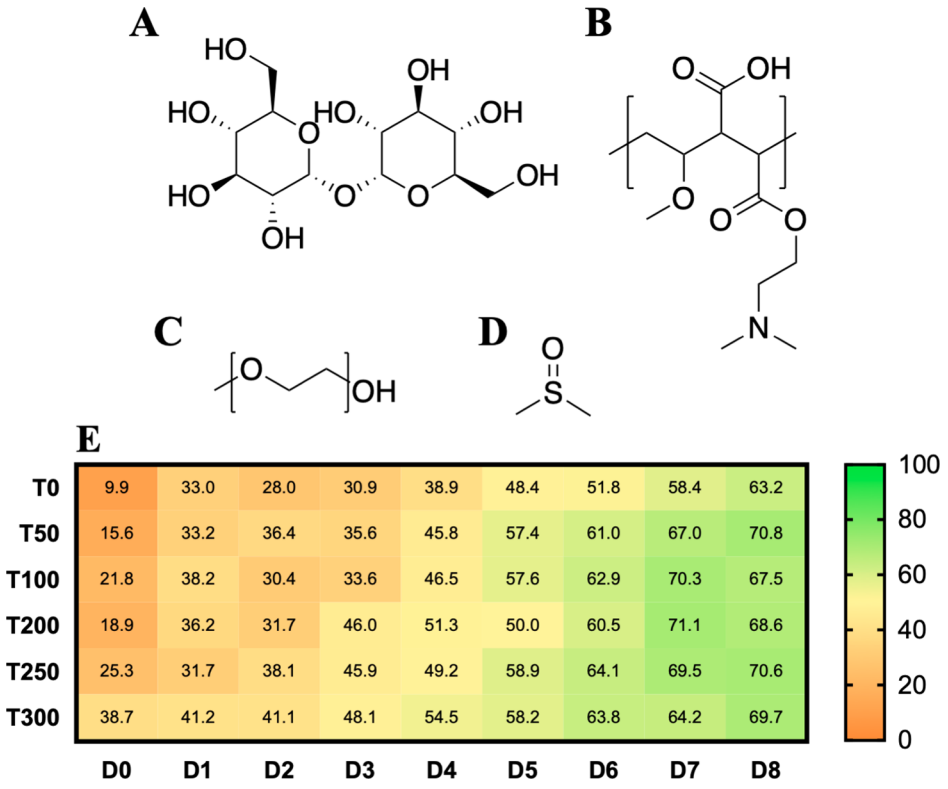

Figure 1. Cryopreservation screening using mixtures of trehalose and DMSO. Chemical structures of (A) trehalose; (B) polyampholyte (poly(vinyl ether-*alt*-maleic acid mono(dimethylamino ethyl)ester)); (C) PEG Mn = 4000 g mol^{-1}; (D) DMSO; and (E) heat map showing RBC recovery using different cryoprotectant mixtures. DMSO concentrations are on the *x*-axis (D_x v/v %) and increasing trehalose concentrations down the *y*-axis (T_x mmol). Total volume of 200 μL in each well (100 μL of ovine RBCs suspended in DPBS). Solutions were frozen in liquid nitrogen vapor and thawed in a 37 °C water bath. Hemolysis was assessed using the AHD assay and recovery is reported as 100%—hemolysis.

were centrifuged at 2000*g* (6000 rpm) for 5 min. For high-volume freezing, 200 mL 2× cryoprotectant was mixed with 200 mL blood and transferred to PVC blood bags (TRO-DONEX, Praxisdienst), cooled in a −80 °C freezer to −80 °C, and thawed in a 37 °C water bath. Washing and the AHD assay were carried out as above, using 1 mL aliquots from the bag for the high-volume assay.

Cytotoxicity Assay. 500 μL blood was transferred to centrifuge tubes and centrifuged at 2000*g* (6000 rpm) for 5 min. The pellet was mixed with 1 mL cryoprotectant solution, transferred to cryovials, and incubated for the desired time. Blood was then centrifuged for at 2000*g* (6000 rpm) for 5 min. The AHD assay was carried out as above.

Confocal Microscopy. Live red blood cell confocal imaging, before and after freezing with cryoprotectants, was undertaken using a Zeiss LSM 710 inverted microscope, equipped with three photomultiplier detectors (GaAsP, multialkali, and BiG.2) and a multichannel spectral imaging detector. Red blood cells (diluted 500-fold) were placed on MatTek glass bottom dishes (no. 1.5, 35 mm) and brightfield images were taken using a C-Apochromat 63x/1.20 W Korr M27 objective lens and ×3.0 zoom. Z-stacks were taken every *z* = 0.3 μm until the whole cell was imaged. Zeiss ZEN (black edition) 2.3 lite was utilized for image collection and maximum intensity projection processing.

ATP Luciferase Assay. 5 μL of the recovered blood (not normalized to cell count or recovery, using the cryopreservation/washing procedure described above) was added to 95 μL Alsever's solution in a 96-well plate. 100 μL CellTiter-Glo 3D Reagent (Promega) was added, and the solution was thoroughly mixed, then incubated at ambient temperatures for 30 min. Luminescence (visible light ~740 to 380 nm) was then measured using a plate reader (Synergy HT, BioTek). Background noise was controlled by subtracting luminescence from samples that were incubated with Alsever's solution instead of the CellTiter-Glo 3D Reagent. To make the standard curve, 0.011 g adenosine 5′-triphosphate disodium salt hydrate (Thermo Scientific) was added to 1 L of distilled water and diluted via serial dilatation in a 96-well plate. The luciferase assay was conducted as above. The initial 20× dilution of the original samples was accounted for when calculating the final ATP concentration.

RESULTS AND DISCUSSION

The core hypothesis for this work was that the cytotoxicity associated with many cryoprotectants could be mitigated by combining multiple cryoprotectants (small molecule and macromolecular) at concentrations lower than they would normally be used individually. As each cryoprotectant has a unique mechanism of action (or toxicity), a synergistic cryoprotectant outcome could potentially be achieved. This approach would allow additional benefits from polyampholytes (a versatile and diverse class of macromolecular cryoprotectants)[55,65] to be realized. This is crucial as, at present, polyampholytes function as supplemental, rather than as the sole, components.[62] Our polyampholyte of choice, poly(vinyl ether-*alt*-maleic acid mono(dimethylamino ethyl)ester), was synthesized from commercially available poly(methyl vinyl ether-*alt*-maleic anhydride) undergoing a single-step ring-opening reaction with 2-dimethylaminoethanol.[61] This synthetic route is scalable and can be conducted using high-grade materials, and hence is suitable for scale-up and translation. As glycerol is challenging to remove from RBCs post-thaw and has already been widely investigated,[66] DMSO and trehalose were chosen as alternative cryoprotectants. These are not normally used for blood as DMSO is hemolytic at the high concentration, where it can be used as the lone cryoprotectant

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for RBCs, and trehalose is noncell penetrative and hence has limited benefits on its own. Therefore, combining these three very different cryoprotectants may allow for high recoveries in a nontraditional RBC cryopreservation formulation. Using a robotic liquid-handling system, combinations of DMSO (0−8% v/v) and trehalose (0−300 mM) were prepared, applied to ovine red blood cells (a model for human RBCs)[67] and screened for cryopreservation in 96-well plates. It should be noted that the 96-well plates (unless using a specialized freezer)[68] are not optimal conditions for freezing RBCs (in terms of total recovery) but allow rapid screening to find candidate solutions for further testing.[69] Cryopreservation success was determined by the alkaline hematin D-575 (AHD) assay, in which hemoglobin is converted to AHD and the concentration is determined relative to a control using UV−Vis spectroscopy at 575 nm.[64] In total, 54 conditions were screened and the results of this are shown in the heat map in Figure 1 (data are also tabulated in Figure S2). Note that the concentrations indicated are the units most commonly used for those cryoprotectants, to allow comparison with the diverse literature that do not use identical units: solvents such as DMSO are denoted using percentage by volume, osmolytes (trehalose) using molarity, and polymers by weight per mL. Unless otherwise stated, all cryoprotectants were dissolved in DPBS (8 g L^{-1} sodium chloride, 0.2 g L^{-1} potassium chloride, 0.2 g L^{-1} monobasic potassium phosphate, and 1.15 g L^{-1} dibasic sodium phosphate). Trehalose alone gave a maximum recovery of 39% at 300 mM. DMSO at 8% v/v (the highest concentration in the screen, due to dilution factors within the mixing protocol) gave an average (mean) recovery of 63%, which increased to 70% when combined with 300 mM trehalose. While 70% recovery for a nucleated cell line would be a significant post-thaw result, for RBCs glycerol can give >85% recovery post-thaw.[19] This highlights the challenge of discovering innovative solutions, which can reach the high level of protection offered by glycerol, while being comparatively easy to washout. As a control, betaine was explored by this matrix strategy with trehalose, but there was no evidence of synergy, which is potentially due to their likely similar mechanisms of action (Supporting Information).

Encouraged by the screening results, vial-based freezing (to ensure consistent cooling rates for each sample) was undertaken. The data in Figure 1 showed that trehalose had less impact at higher DMSO concentrations than at lower concentrations. Similarly, the overall difference in recovery between 100 mM trehalose and higher concentrations (300 mM) was minimal, hence 100 mM was chosen to ensure the total osmotic pressure (explored later) was not too high. Cryopreservation was evaluated under fast freeze/fast thaw conditions (frozen in liquid nitrogen and thawed in a 37 °C water bath) and the results are shown in Figure 2. As might be expected for these conditions, the trehalose and polyampholyte alone gave moderate recoveries (~40%). The polyampholyte has been reported (as the sole cryoprotectant) to give above 50% recovery in RBCs[61] but, as can be seen here, the recovery varied between samples when used as a sole cryoprotectant. However, previous reports used PBS, not DPBS, as the carrier (which has additional ions), which may explain this variance. When the polyampholyte was combined with DMSO, the post-thaw recovery was increased to 98%. Without the trehalose, recovery was slightly lower, with a larger spread of results, suggesting that the mixed cryoprotectant strategy helps mitigate different modes of damage, leading to an exceptional

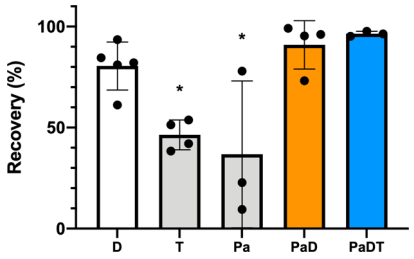

Figure 2. Recovery of RBCs after being rapidly frozen in liquid nitrogen and thawed for 5 min in a 37 °C water bath. Prior to freezing, RBCs were incubated for 10 min with the cryoprotectant. Pa = 100 mg mL^{-1} polyampholyte, *D* = 10% DMSO, and *T* = 100 mM trehalose. Data represent the mean ± SD of at least three independent experiments (*N* = 19, *P* = 0.0008, and **P* < 0.05 from 10% DMSO).

recovery with almost no batch-to-batch variance. This supports our hypothesis that the combination of extracellular (trehalose) and intracellular (DMSO) cryoprotectants, at lower concentrations than conventionally used (to reduce hemolysis), provides a synergistic benefit. Furthermore, the polyampholyte helps mitigate damage further, which is explored more below.

To test the system further, a more challenging slow-thaw approach (which enables recrystallization to occur, causing significant damage)[48,54] was employed. The results were in agreement with the fast thaw data from Figure 2, with the mixed cryoprotectant solution again leading to < 2% hemolysis (Figure 3). This polyampholyte is not a potent IRI, but at the

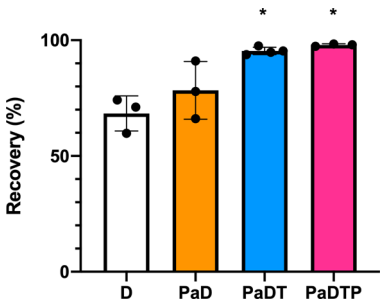

Figure 3. Recovery of RBCs after being rapidly frozen in liquid nitrogen and thawed in air at ambient temperatures. Prior to freezing, RBCs were incubated for 10 min with the cryoprotectants. Pa = 100 mg mL^{-1} polyampholyte, *D* = 10% DMSO, *T* = 100 mM trehalose, and *P* = 100 mg mL^{-1} PEG. Data represent the mean ± SD of at least three independent experiments (*N* = 13, *P* = 0.0012, and **P* < 0.05 from 10% DMSO).

concentration applied here it can inhibit ice growth, which may be mitigating some of the damage[56,61] (although any macromolecule at sufficiently high concentration can slow ice growth). However, the extent of cell recovery here is greater than using poly(vinyl alcohol), which is a potent IRI,[48] and hence the primary mode of action of the polyampholyte would seem to be distinct.

The addition of 100 mg mL^{-1} PEG was explored as an additional additive for this process. The rationale was that many uncharged water-binding macromolecules can benefit RBC cryopreservation to an extent, as exemplified by poly(hydroxyethyl starch).[70] PEG has proven to be effective in other cell types[71] and, when used in combination with polymeric IRIs, for protein/bacteria storage.[72,73] Addition of PEG was not detrimental to cryopreservation and may have

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yielded a small (but statistically insignificant) improvement. However, additional benefits of using PEG were realized when variable freeze/thaw rates were tested (see below).

These results are important, as they show that minimal hemolysis occurs in these mixed cryoprotectant solutions and that the formulation is tolerant to changes in freezing/thawing rates, which are challenging to control with precision in larger volume samples (addressed later in the article). To explore conditions that might be considered least optimal (slow freeze + slow thaw), but the most practical for red blood cell storage, a panel of cryoprotectant formulations were further tested (Figure 4). Under these conditions, the weak IRI activity of the

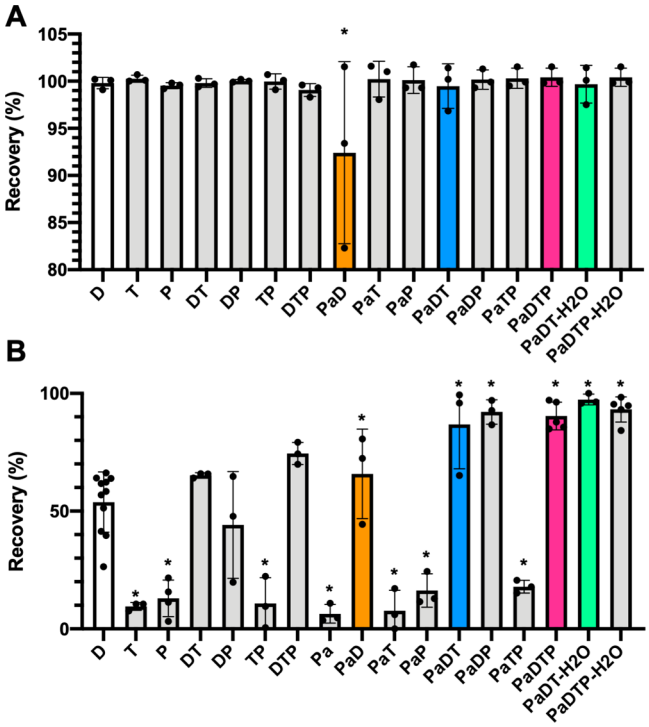

Figure 4. Vial-based cryopreservation of cryoprotectant formulations. (A) Recovery of RBCs after being incubated with cryoprotectants for 1 h. Data represent the mean ± SD of three independent experiments (N = 51, P = 0.1582, and *P < 0.05 from 10% DMSO). (B) Recovery of RBCs after being cooled at −1 °C min^{-1} to −80 °C and thawed in air at ambient temperatures. Prior to freezing, RBCs were incubated for 10 min with the cryoprotectants. Data represent the mean ± SD of at least three independent experiments (N = 19, P = 0.0008, and *P < 0.05 from 10% DMSO). Pa = 100 mg mL^{-1} polyampholyte, D = 10% DMSO, T = 100 mM trehalose, and P = 100 mg mL^{-1} PEG.

polyampholyte will provide some benefit by reducing ice growth in slow thawing, even though this property does not appear to be its primary mode of cryoprotection.[65] It was also important, from a practical perspective, to explore the use of water rather than DPBS as the carrier solution for the cryoprotectants. The rationale behind this was that the use of high molar concentrations of extracellular cryoprotectants will increase the osmolarity of the solutions beyond optimal physiological levels. Therefore, by removing the noncryoprotective ions found in DPBS, the osmolarity of the solution can be reduced to be closer to the optimal level. We found that when blood was cryopreserved with 300 mM trehalose in H_2O, the resulting recovery was higher than blood cryopreserved in 300 mM trehalose in DPBS (Supporting Information). The measured osmolarity of 100 mg mL^{-1} polyampholyte + 100 mM trehalose in DPBS was 563 mOsm, whereas 100 mg mL^{-1} polyampholyte + 100 mM trehalose in H_2O has a combined osmolarity of 239−276 mOsm. This is closer to the physiological osmolarity of blood, which is around 288 mOsm.[74] These measurements exclude DMSO because DMSO enters the cell and equilibrates, canceling out its effect on osmotic stress once equilibrium is reached. The panel was designed to highlight the importance of each individual component and to determine how essential each component is. To ensure any reduction in cell recovery post-thaw was not due to intrinsic toxicity (i.e., lysis), all solutions were first incubated with red blood cells for 1 h and hemolysis was determined, shown in Figure 4A. The only condition that showed appreciable hemolysis was 10% DMSO + 100 mg mL^{-1} polyampholyte. However, the addition of trehalose and/or PEG to the cryoprotectant solution reduced hemolysis to negligible levels, suggesting that they neutralize the combined toxicity of DMSO and polyampholyte. In the case of trehalose, its ability to neutralize cryoprotectant toxicity has been previously reported.[36] PEG has the ability to stabilize proteins, which might be beneficial here,[75] but it also replaces water and hence reduces the total ice fraction formed. Ultimately, these hemolysis data show that any reduction in recovery during the process of cryopreservation can, therefore, be attributed to freeze-induced damage, not cytotoxicity.

Figure 4B shows the results of cryopreservation screening using the above formulations. The results after cooling blood at a rate of −1 °C min^{-1} and slowly warming at ambient temperature, until thawed, demonstrate that the most effective formulation was PaDT-H_2O (100 mg mL^{-1} polyampholyte, 10% DMSO, and 100 mM trehalose, in water), giving a post-thaw recovery of 97% (Figure 4B). DMSO was essential in these, highlighting the need for an intracellular cryoprotectant. The addition of trehalose and/or polyampholyte was able to enhance the cryoprotective effect of DMSO to enable near-zero hemolysis.

Our approach of using increasingly challenging freeze/thaw rates is supported by the reduction in the effectiveness of 10% DMSO when used alone, across all three freezing experiments, from 81% (fast freeze/thaw) to just 54% when slow freeze/thaw was used. By only using optimized conditions, which cannot be replicated with clinically relevant volumes of blood, overestimation of recovery is possible. Our unique polymer-containing formulations clearly mitigate these problems. The formulation giving the highest recovery, PaDT-H_2O, was singled out for further testing; PaDT-H_2O was not hemolytic at the 1 h time point and did not show any progressive hemolysis when measured at the 15, 30, 45, and 60 min time points (Supporting Information).

Before cryopreserved blood can be used in a medical or research scenario, the cryoprotectants are ideally washed out. To ensure that it was possible to remove the cryoprotectants post-thaw, RBCs cryopreserved with PaDT-H_2O were pelleted and resuspended in 80% washout solution (stock washout solution is 10% DMSO + 100 mM trehalose in Alsever's solution). This was repeated for 40% then 20% washout solutions before being resuspended in Alsever's solution. The washout process was achieved in under 10 min. The final post-freeze−thaw−wash recovery was 80% (Figure 5). Although this was not a statistically significant difference from the 97% recovery obtained immediately post-thaw, this does represent a small loss during washout. Hemolysis during washout could be

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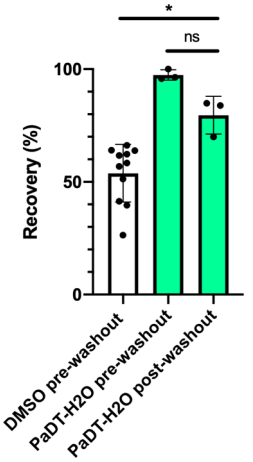

Figure 5. Recovery of RBCs after washing-out cryoprotectant solutions. RBCs were cooled at −1 °C min^{-1} to −80 °C and slowly thawed in air at ambient temperatures. Prior to freezing, RBCs were incubated for 10 min with the cryoprotectants (10% DMSO, 100 mM trehalose, and 100 mg mL^{-1} polyampholyte in water). Prewashout indicates immediate post-thaw recovery and postwashout indicates recovery after cryoprotectant removal and reconstitution in Alsever's solution. Recovery is 100%—hemolysis. Data represent the mean ± SD of at least three independent experiments. ($N = 17$, $P < 0.0001$, $^*P < 0.05$, ns = not significant).

further reduced by using a cell-processing device.[17,19] For comparison, Briard et al. reported up to 80% recovery when using small molecule ice recrystallization inhibitors in cryopreservation mixtures containing 15 wt % glycerol, but these were prewashout values.[76] Hence, our macromolecular approach can match, or even outperform, current methods and may allow for faster washing-out processes, which would need to be validated in automated systems in the future.

To assess the post-freeze−thaw−wash integrity, flow cytometry was used to assess the RBC morphology (Figure 6A−D). RBCs were cryopreserved in the optimal PaDT-H_2O formulation under slow-freeze/slow-thaw conditions and washed using the method described above. Size (forward scatter) would be increased in swollen RBCs, whereas optical complexity (side scatter) would be increased in crenated (shrunken) cells. These analyses would indicate if cells are damaged in such a way as to alter the shape of their surface membrane, have internal damage resulting in granule/inclusion body formation, or blebbing that indicates apoptosis or cytoskeletal damage.[77,78] Compared to fresh RBCs (Figure 6A), those cryopreserved in our formulation (after washout) showed similar profiles (Figure 4B). For comparison, RBCs were also suspended in hypotonic (0.125 M) and hypertonic (0.5 M) saline solutions showing how forward/side scatter profiles would change in response to osmotic stress. The RBC's morphology was also investigated by optical microscopy (Figure 6E−G), ruling out any crenation due to osmotic stress, and hence our solutions clearly lead to intact RBCs post-thaw. Osmotic fragility assays (Figure S5) also suggest the RBCs are intact post-thaw and washout.

To further investigate the post-thaw integrity of the RBCs, live confocal microscopy was used (Figure 7A). Cells were cryopreserved in PaDT-H_2O, thawed, and washed as described above and compared to nonfrozen controls. Compared to the control, a higher proportion of the cells appears to be crenated, which may reflect the minor differences observed in the forward and side scatter shown by flow cytometry (Figure 6). The shrinkage suggests that the exact osmolarity of the washout solutions could be tuned further and that replacement of some salts into the cryopreservation buffer (which to control osmolarity used water not saline) may provide opportunity to optimize this. To investigate the function of the cells, ATP levels post-thaw were compared. ATP depletion results in a reduction of the cell's ability to maintain homeostatic processes such as ion transport maintenance of the cytoskeleton and membrane, which could lead to crenation.[79,80] A fluorescence-based luciferase assay: CellTiter-Glo 3D Cell Viability Assay (Promega), was used to measure the concentration of ATP. RBCs were cryopreserved in PaDT-H_2O or 10 wt % DMSO, thawed, and washed as described above. The RBCs were stored for 24 h at 4 °C prior to the assay to allow any delayed leakage to be observed. The ATP concentration in post-freeze−thaw−wash RBCs frozen with PaDT-H_2O was similar to that of the fresh control (3.1 vs 3.7 μM). Both were significantly higher than cells cryopreserved with DMSO-alone. ATP loss in cells (Figure 7B) cryopreserved with PaDT-H_2O does not exceed average hemolysis, (20% hemolysis vs 16% loss of ATP), therefore the loss of ATP is likely due to cell loss during cryopreservation and washout, rather than indicating a loss of viability. This is in agreement with previous research, which shows that cryopreservation of RBCs does not result in significant depletion of ATP[81] and indicates good metabolic health.

To test our cryoprotectant solution under more relevant conditions, a larger volume of RBCs (400 mL) was incubated with PaDT-H_2O for 10 min and frozen in poly(vinyl chloride) blood bags. This is essential, as thermal gradients upon both freezing and thawing are minimized in small-volume experiments, such as above, but RBCs are required in larger volumes for medical settings and hence is a robust challenge. The blood bags were cooled at an uncontrolled rate by being placed directly in a −80 °C freezer before thawing in a 37 °C water bath. Post-thaw recovery was 84.5%, and there was very little hemolysis during washout (using 1 mL aliquots, not the whole sample) with a post-freeze−wash−thaw recovery of 83.4% (Figure 8). This recovery value is higher than the 80% minimum standard according to the American Association of Blood Banks[82] and U.S. military.[83] The slightly lower post-thaw recovery obtained with large volumes, compared to vials, is to be expected due to thermal gradients (outside thawing fast than inside), which may promote ice recrystallization. The process used here was not optimized and could be enhanced through more control of freezing/thawing rates or the use of heat transfer devices to improve contact. However, the high postwashout recoveries are already in the range for application and the present method is easy, based on simply placing in a freezer and thawing in a water bath, which is an appealing process.

The above data demonstrate the benefit of mixed macromolecular cryoprotectant solutions. The mode of action of polyampholytes is still under investigation,[65] but it appears they do not have a significant impact on the formation/growth of ice. To ensure that the cryopreservation process used here was not leading to vitrification (ice-free state), solutions were analyzed using differential scanning calorimetry (DSC). Latent heat of cooling during the phase change from liquid water to ice corresponds to the amount of ice formed. Figure 9 shows that in each formulation, ice was indeed forming, ruling out vitrification. The total ice fraction was reduced upon addition

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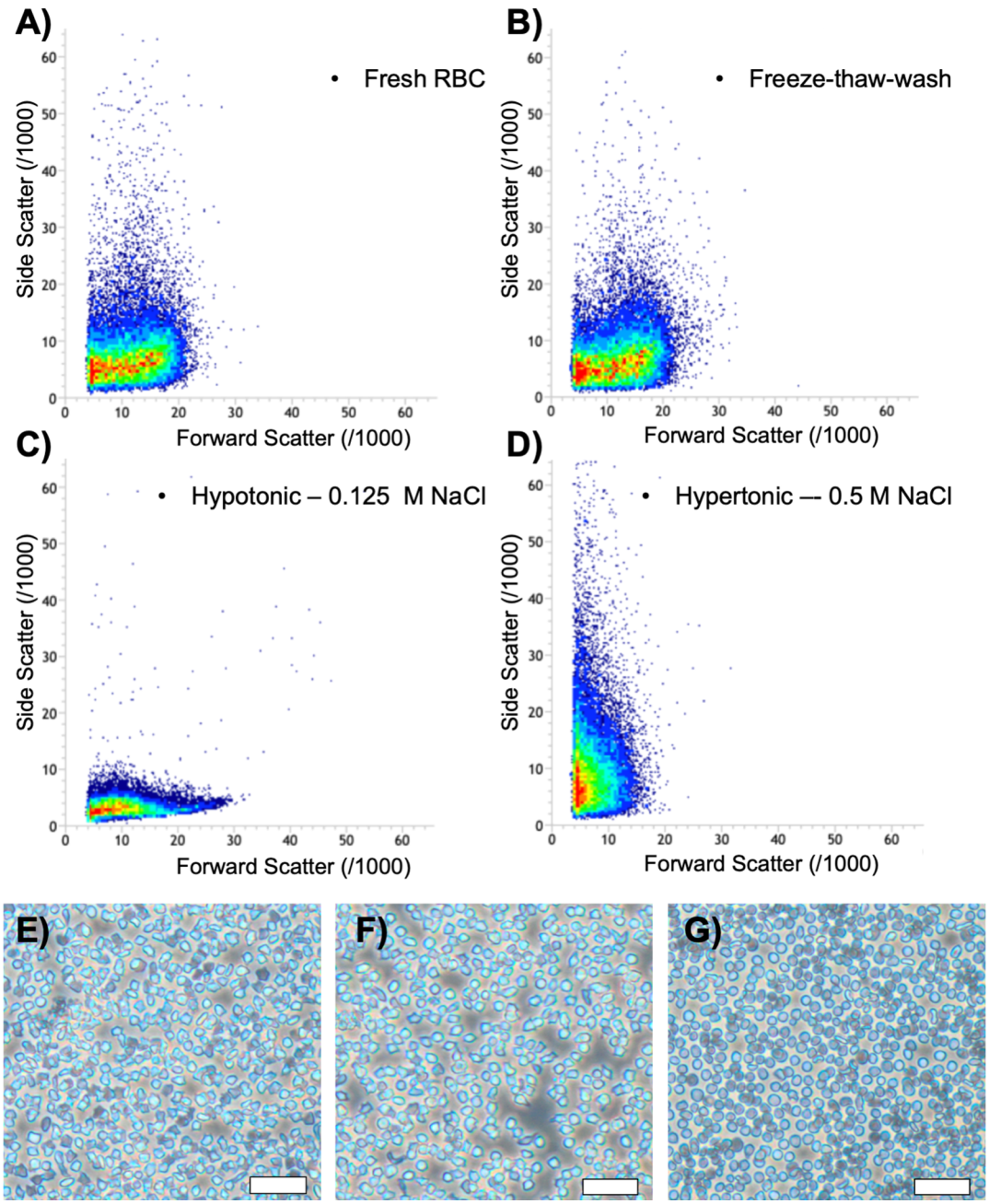

Figure 6. Top: Flow cytometry plots showing forward scatter and side scatter of (A) fresh and (B) post-freeze−thaw−wash RBCs in Alsever's solution. For comparison are fresh RBCs under (C) hypotonic and (D) hypertonic conditions. Bottom: Phase contrast microscopy images of RBCs. (E) Fresh in Alsever's solution. (F) Fresh in DPBS. (G) Post-freeze−thaw−wash in Alsever's solution. Scale bar represents 20 μm.

of each additive, as would be expected. The top performing conditions, which also had the largest overall additive concentration, had the lowest ice fraction. Both the polyampholyte and trehalose have (relatively weak) ice recrystallization inhibition activity[29,65] but are not reported to impact ice nucleation (or inhibition of nucleation). The ability to reduce ice crystal size during freezing has been proposed as a mechanism of action of DMSO.[84]

CONCLUSIONS

Synergy in mixed cryoprotectant formulations may overcome the intrinsic toxicity limits associated with high concentrations of any individual cryoprotectant and take advantage of their multiple mechanisms of action. Here, the impact of mixing trehalose, DMSO, and a polyampholyte, which all have distinct modes of cryoprotective function, was evaluated for red blood cell cryopreservation. The cryoprotectants were first screened using liquid-handling systems to identify optimal mixtures in a 96-well plate format. A synergistic effect was identified and then studied further using vial-based freezing. It was found that all three components were essential to achieve the best cryopreservation results, enabling 97% recovery, even with nonoptimal slow-thawing conditions. The formulation was nonhemolytic after a 1 h exposure. A particular benefit of this system was the fast washout of cryoprotectants, which did not lead to significant cell loss, with 80% of RBCs recovered intact after washing. This was crucial as deglycerolization of RBCs can be a time-consuming process, even with automated

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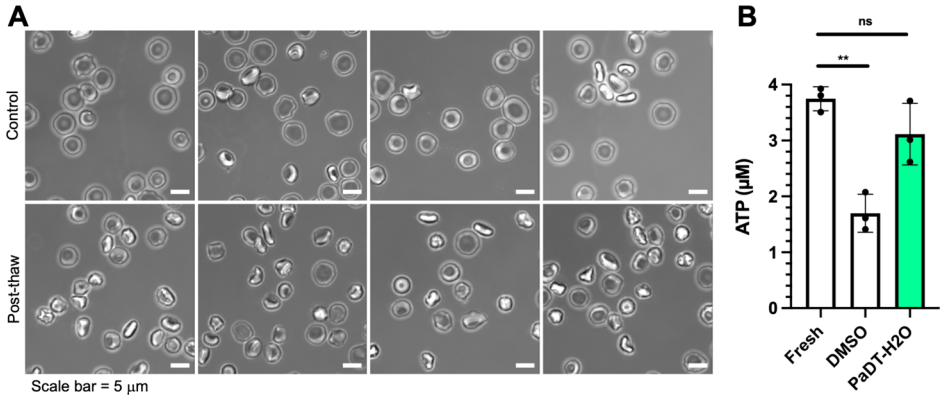

Figure 7. Post-thaw RBC integrity. (A) Confocal microscopy of freeze/thawed RBCs (using an optimal cryoprotectant mixture) compared to fresh RBCs. (B) ATP quantification comparing fresh (nonfrozen) with DMSO, or optimal cryoprotectant mixture post-thaw. RBCs were cooled at −1 °C min^{-1} to −80 °C and slowly thawed in air at ambient temperatures before cryoprotectant washout and storage at 4 °C for 24 h. Prior to freezing, RBCs were incubated for 10 min with the cryoprotectants (10% DMSO or 10% DMSO, 100 mM trehalose and 100 mg mL^{-1} polyampholyte in water). Data represent total ATP in the samples, not normalized to cell recovery (discussed in the text). Data represent the mean ± SD of three independent experiments (N = 9, P < 0.0019, *P < 0.05, ns = not significant).

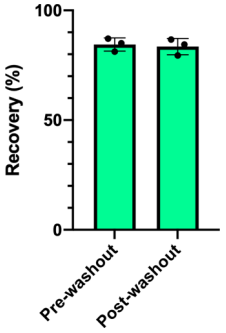

Figure 8. Large volume (400 mL) RBC cryopreservation. 400 mL of RBCs was placed into blood bags and cooled at an uncontrolled rate to − 80 °C, and warmed in a 37 °C water bath. A sample was taken from each bag, the cryoprotectant removed, and resuspended in Alsever's solution. Prior to freezing, RBCs were incubated for 10 min with the cryoprotectant (10% DMSO, 100 mM trehalose, and 100 mg mL^{-1} polyampholyte in water). Data represent the mean ± SD of three independent experiments (N = 6, P = 0.75).

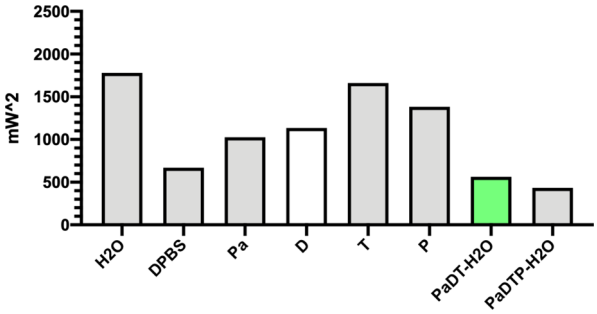

Figure 9. DSC analysis of cryoprotectant solutions. 40 μL samples were analyzed and the data are the integration of the peak corresponding to ice formation during cooling at 10 °C min^{-1}. The formulation used for large volume freezing, PaDT-H_2O, is indicated in green, 10% DMSO is indicated in white. Pa = 100 mg mL^{-1} polyampholyte, D = 10% DMSO, T = 100 mM trehalose, and P = 100 mg mL^{-1} PEG.

facilities, and simplifying the process without stressing the cells is crucial for any innovative formulation. A large volume (400 mL) study was conducted—this was essential as vial-based freezing does not replicate the thermal gradients that blood bags are exposed to during freezing and thawing. The cryoprotectant formulation performed well, allowing 84% postwashout recovery. Flow cytometry and optical microscopy confirmed that the blood cells were intact, and a luciferase assay showed that there was no significant loss of ATP indicative of successful recovery. However, confocal microscopy did reveal some morphological changes and shrinkage, which suggests that optimization of the washout solution and the ion-balance could further improve this cryoprotectant formulation, which was formulated in water, not saline, to control the osmolarity. The exact cryoprotective mechanism of polyampholytes is not yet clear, but this mixed formulation benefits from intracellular (DMSO) and extracellular (trehalose) protection, which along with the polymer lead to reduced hemolysis. While these cryoprotectants are not currently used for blood banking, the results clearly show that combining cryoprotectants with different modes of action can lead to high post-thaw recovery, comparable to glycerolization, with the macromolecular cryoprotectant acting synergistically with the small molecules. The fast and easy washout procedures may also find use in reducing the time from thaw to application.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.1c00599.

This inlcudes additional materials and methods s, and tabulated data. Addition RBC recovery data is also included. physical and analytical methods; statistics; flow cytometry; microscopy; nuclear magnetic resonance spectroscopy; synthesis of poly(vinyl ether-alt-maleic acid mono(dimethylamino ethyl)ester); differential scanning calorimetry; high-throughput cryoprotectant screening; recovery of R (PDF).

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Notes

The authors declare the following competing financial interest(s): MIG is a named inventor on a patent application using the materials described here. MIG and TC are share holders and directors of Cryologyx Ltd.
Background data are available at wrap.warwick.ac.uk.

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