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Article

Cryopreservation of Avian Erythrocytes Using Macromolecular Cryoprotectants

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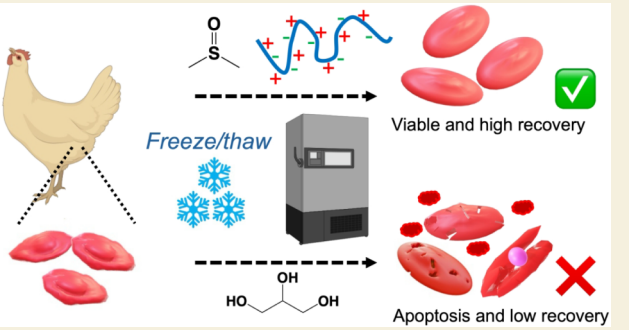
ABSTRACT: Erythrocyte (red blood cell) cryopreservation is an essential biotechnology for healthcare (transfusions), diagnostics, conservation, and fundamental research. Mature mammalian erythrocytes are anucleate, do not divide, and cannot undertake protein biosynthesis. In contrast, avian erythrocytes have a distinct evolutionary pathway, retaining their nucleus, and are capable of other limited functions, such as protein biosynthesis, but have a shorter shelf life than mammalian erythrocytes. Here, we report the successful cryopreservation of avian red blood cells using a polyampholyte (macromolecular cryoprotectant), which can reduce intracellular ice formation. Under optimized conditions with 10% DMSO and 60 mg·mL^{-1} of polyampholyte, ∼90% recovery could be achieved, outperforming a conventional glycerol-based formulation, especially when longer-term storage (up to 6 months) was tested. Scanning electron microscopy confirmed that the cells' morphology was retained. Confocal fluorescence microscopy showed reduced apoptosis compared to glycerol cryopreservation and confirmed increased recovery of actin (cytoskeleton) polymerization post-thaw. Overall, these results show the potential of macromolecular cryoprotectants to be deployed in nonhuman cryobiology for animal health and potentially species conservation.



KEYWORDS: *cryopreservation, polyampholytes, erythrocytes, biomaterials, ice, biobanking*

INTRODUCTION

Cryopreservation enables the long-term storage of cells and tissues while preserving their viability and functionality.[1] Among the diverse cell types that require preservation, avian red blood cells present distinct challenges and opportunities because of their retention of a cell nucleus, unlike mammalian red blood cells which are anucleate.[2] Human blood is most commonly cryopreserved using 40% glycerol.[3] However, prior to the utilization of the blood, the glycerol must be removed through a series of time-consuming washing steps to prevent (fatal) osmotic shock.[4] This additional processing step complicates the overall procedure and poses a risk of cell loss or damage if not performed carefully.[5] Chicken (as a convenient avian model) red blood cells (cRBCs) possess nuclei, which may complicate the cryopreservation process but offer opportunities for studying gene expression, epigenetic regulation, and innate immunity,[6] and chicken blood is an important reagent in veterinary diagnostics, vaccine development, and immunological assays.[7] Techniques such as hemagglutination (HA) and hemagglutination inhibition (HI) use chicken red blood cells (cRBCs) for viral detection and vaccine efficacy evaluation.[7−9] However, the utility of fresh chicken blood is limited by its short shelf life of approximately 1 week at 4 °C, necessitating frequent donor collection, which complicates logistics and compromises standardization. This is in contrast to human RBCs which can be kept >30 days at 4 °C.[10] Effective cryopreservation of cRBCs offers benefits across various domains, including veterinary transfusion medicine, where long-term blood storage addresses donor scarcity, and basic and applied research, where standardized cell banks enhance the reproducibility of experiments. Cryoprotection of cultured red blood cells (cRBCs) could facilitate their use in cultured meat by providing hemoglobin for color, flavor, and iron content while enabling long-term storage and distribution. Despite their potential, the successful cryopreservation of cRBCs remains challenging. The presence of a nucleus necessitates the protection of both cytoplasmic and nuclear structures from freezing-induced injuries. Glycerol, which is effective in mammalian RBCs cryopreservation, performs less

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well in avian erythrocytes. For example, hen RBCs cryopreserved in 20% glycerol + 7.5% HES showed >99% recovery by counting cells, but flow cytometry analysis revealed that 60−80% of these cells were apoptotic or necrotic, indicating that high recovery does not necessarily reflect functional viability.[2] In contrast, 10% DMSO preserved membrane integrity with minimal cell death, offering the best overall balance of viability and structural preservation highlighting DMSO's potential as a base CPA for further optimization.[2] Hen erythrocytes cryopreserved in 10% DMSO and polyvinylpyrrolidone (PVP K15, MW ∼ 1000) were also shown to be effective while glycerol proved unsuitable.[11] These findings demonstrate avian RBC cryopreservation is challenging and requires innovative solutions compared to established techniques for mammalian RBCs.

Recent advancements in cryoprotectant discovery have identified novel agents such as tricine,[12] and proline[13,14] with reduced toxicity compared to traditional cryoprotectants; however, they are rarely suitable on their own and instead synergize with permeating CPAs.[15] Optimization of CPA combinations, concentrations, freezing and thawing rates, and equilibration time[16,17] can all improve post-thaw recovery.[18] To address the challenges associated with conventional CPAs, which do not mitigate all biophysical and biochemical modes of damage during cryopreservation, there has been growth in the "bottom-up" discovery of new molecules and materials.[19] For example, Ben and Acker have explored small molecule ice recrystallization inhibitors to mitigate the stress during transient warming events.[20] Ice binding proteins (also known as antifreeze proteins) have been widely explored due to their ice recrystallization inhibition (IRI) activity, but do not always result in large increases in recovery, depending on the exact cryopreservation conditions and stresses.[21−24] Induced ice nucleation to prevent supercooling can also improve post-thaw recovery,[25] but many ice nucleators are inhomogeneous materials, where the exact active site is not clear,[26] or bacterial lysates.[27] Pollen-washing water has emerged as a leading candidate to prevent water supercooling, and shown to enhance monolayer and spheroid cryopreservation.[28−30] Inspired by ice-binding proteins, several biomimetic macromolecules have emerged,[7] poly(vinyl alcohol)[31] is a potent IRI, as well as oxidized graphene[32] and self-assembled systems such as saffranine-*O*.[33]

Matsumura et al. introduced poly(ampholytes):[34,35] polymers with a mixture of cationic and anionic side chains, which improved post-thaw recovery in a range of cell types. Several poly(ampholyte) platforms have now been disclosed, including polysaccharides or those derived from (controlled) radical polymerizations.[36,37] Gibson et al. introduced a synthetically scalable polyampholyte (poly(methyl vinyl ether-*alt*-maleic anhydride)) for cryobiology.[38] This polymer does not interact directly with ice crystals but significantly enhances post-thaw recovery when supplemented into standard CPAs (such as DMSO). For instance, in 2D cell monolayers (A549), supplementing DMSO with this polyampholyte boosted post-thaw viability from ∼24% to >80%.[35] In 3D spheroid models, including hepatocyte,[39] polyampholytes significantly improved cell survival and functional integrity by mitigating membrane damage and preserving the cytoskeletal structure. Recent evidence suggests that polyampholytes reduce intracellular ice formation, as evidenced by Raman cryomicroscopy.[40] Polyampholytes may play a crucial role in preserving protein functionality by stabilizing their native conformations during low-temperature storage.[37,41,42] As they are not cell permeable polyampholytes can be easily removed post-thaw and have been shown to protect mammalian red blood cells.[43,44]

Considering the above, this study aimed to exploit polyampholytes for avian red blood cell cryopreservation. First, the background cryoprotectant system was optimized to transition from glycerol to DMSO, and the polyampholyte was shown to bring post-thaw recovery improvements. The polyampholytes reduced intracellular ice formation which also led to rescue of cytoskeletal polymerization post-thaw. Reduced post-thaw apoptosis was also observed and linked to reduced reactive oxygen species generation, compared to glycerolization. Overall, this shows how avian blood can be effectively cryopreserved using specific macromolecular cryoprotectants which may find use in veterinary and basic sciences.

EXPERIMENTAL SECTION

Materials

Adult chicken red blood cells in Alsever's solution (not defibrinated with a packed cell volume of 15−35%) were purchased from TCS Biosciences. Poly(methyl vinyl ether-*alt*-maleic anhydride) (Mn ≈ 80 kDa), tetrahydrofuran (THF), dimethylamino ethanol, deuterium oxide (D2O), glycerol, dimethyl sulfoxide (DMSO; analytical reagent grade), Triton X-100, and NaOH were purchased from Merck (Gillingham, UK). Dulbecco's phosphate-buffered saline (DPBS), 5(6)-carboxyfluorescein diacetate (CFDA), and annexin V FITC apoptosis detection reagents were purchased from Fisher Scientific (Loughborough, UK). BD FACS flow sheath fluid was purchased from BD Biosciences. L-proline and trehalose (ReagentPlus, >99% purity) were purchased from Sigma-Aldrich.

Red Blood Cell Isolation

Upon receipt, blood was stored at 4 °C and processed within 48 h to minimize cellular degradation. cRBCs are particularly prone to storage-induced damage; therefore, all experiments were performed within 1 week of sample collection. Blood samples were divided into 10 mL aliquots and centrifuged at 2000 rpm for 5 min at room temperature to pellet the red blood cells (cRBCs). The supernatant, which included plasma and Alsever's solution, was carefully removed from the tube. cRBCs were then washed with 8 mL of Dulbecco's phosphate-buffered saline (DPBS) by gentle inversion to remove residual plasma and storage medium components from the cRBCs.

Cryoprotectant (CPA) Preparation

Cryoprotectant solutions were formulated using combinations of glycerol, L-proline, trehalose, dimethyl sulfoxide (DMSO), and polyampholyte (PA) (see Supporting Information for synthesis details). All CPA components were dissolved in sterile 1× PBS and passed through 0.22 μm membrane filters. Cryopreservation solutions were developed using a base formulation of 10% (v/v) DMSO, to which 10−100 mg·mL^{-1} of polyampholytes were added. To further explore the cryoprotective effects, the base was combined with different concentrations of proline and trehalose. Formulations containing 20% (v/v) glycerol were prepared by incorporating different concentrations of proline and trehalose, according to the experimental design.

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Sample Preparation and Cryopreservation Protocols

Washed cRBCs were mixed in a 1:1 ratio with the desired CPA solution (e.g., 500 μL RBC + 500 μL CPA) in sterile cryovials. Following CPA exposure, the samples were incubated at room temperature for durations ranging from 10−15 min to allow for equilibration. Cryopreservation was conducted using a controlled-rate cooling protocol, where samples were cooled at a rate of −1 °C/min to −80 °C using a passive freezing container (CoolCell LX, Corning).[43] For long-term cryopreservation studies, samples were stored at −80 °C for a minimum of 2 weeks before thawing and post-thaw analysis.

Thawing and Post-thaw Processing

Thawing was performed by placing cryovials in a 37 °C water bath for 5 min or at ambient temperature until they were completely liquefied. For samples preserved with a high glycerol content, stepwise deglycerolization was performed using a sequential dilution protocol[45] as follows:

Addition of 200 μL of 12% NaCl, followed by 1 mL of 1.6% NaCl. Subsequently, two washes with 0.9% NaCl supplemented with 0.2% dextrose. Each step involved gentle mixing, incubation for 3−5 min, and centrifugation at 500*g* for 5 min at room temperature. The final cell pellet was resuspended in PBS or the appropriate assay buffer for downstream analysis.

Hemolysis Quantification Using the Alkaline Hematin D-575 (AHD) Assay

Post-thaw hemolysis was assessed by measuring the concentration of free hemoglobin in the supernatant using the alkaline hematin D-575 (AHD) assay.[43,46] Thawed red blood cell (RBC) samples (100 μL) were centrifuged at 2000*g* for 5 min. Subsequently, 40 μL of the supernatant was mixed with 750 μL of AHD reagent, prepared by dissolving 2.5 g Triton X-100 and 0.5 g sodium hydroxide in 100 mL of deionized water. The mixture was vortexed thoroughly, and a 200 μL aliquot was transferred into triplicate wells of a 96-well microplate. Absorbance was measured at 580 nm using a microplate reader (Synergy H1; Agilent BioTek Instruments, Winooski, VT, USA).

Hemolysis recovery was calculated using the following equation:

$$\text{Recovery (\%)} = \left[1 - \frac{A_{\text{Sample}} - A_{\text{DPBS}}}{A_{\text{Lysate}} - A_{\text{DPBS}}}\right] \times 100$$

Fluorescence Microscopy Analysis

Fluorescence microscopy was used to examine the cytoskeletal organization and nuclear morphology of the chicken erythrocytes.[47] Whole blood samples were diluted 1:5 (v/v) with phosphate-buffered saline (PBS). The diluted samples were smeared onto D-polylysine-coated glass slides (Polysine Adhesion Microscope Slides, Epredia, Netherlands), air-dried, and fixed with 4% paraformaldehyde for 10 min at RT. The cells were then permeabilized with 0.2% Triton X-100 for 10 min. All steps were followed by three gentle washes with cold phosphate-buffered saline (PBS) to minimize the loss of cells.

To visualize filamentous actin (F-actin), the slides were incubated with FITC-conjugated phalloidin (1:1000 in PBS) for 40 min at room temperature. Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 μg/mL for 10 min. After staining, the slides were washed three times with PBS.

Images were acquired using a spinning disk confocal microscope (IXplore SpinSR Super Resolution Microscope System, model IX83; Evident Corporation, Tokyo, Japan) equipped with appropriate filter sets for FITC (excitation/emission: 488/520 nm) and DAPI (excitation/emission: 358/461 nm) detection. Nuclear staining was performed using DAPI to visualize the cell nuclei and facilitate cell identification.

Detection of Apoptosis Using Annexin V-FITC Staining and Confocal Microscopy

Apoptotic changes in post-thaw chicken erythrocytes were assessed using annexin V-FITC, which binds to phosphatidylserine residues that are externalized on the outer leaflet of the plasma membrane during the early stages of apoptosis. After cryopreservation and thawing, the samples were washed with phosphate-buffered saline (PBS) to remove residual cryoprotectants and incubated for 1 h at 37 °C to allow for post-thaw recovery. The cells were then resuspended in annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4), and 5 μL of annexin V-FITC (Thermo Fisher Scientific) was added to 100 μL of the cell suspension (approximately 150,000 cells per well). The samples were then incubated in the dark at room temperature for 15 min before analysis. Following incubation, the cells were gently washed and smeared onto poly D-lysine-coated glass slides. After air-drying, the smears were fixed with 4% paraformaldehyde for 10 min at RT. Fluorescence imaging was performed using a spinning disk confocal microscope (Olympus) equipped with the appropriate filter sets for FITC detection (excitation/emission: 488/520 nm). Nuclear staining was performed using 4′,6-diamidino-2-phenylindole (DAPI; 1 μg/mL, 10 min incubation; excitation/emission: 358/461 nm) to visualize the nuclei and assist with cell identification. All samples were thoroughly washed with PBS after each staining step to minimize the background fluorescence. Apoptotic cells were identified by positive annexin V-FITC staining, which was localized to the cell membrane. Imaging data were processed and analyzed using ImageJ (NIH) to determine the fluorescence intensity and cellular localization.[48]

Scanning Electron Microscopy (SEM)

Fresh, thawed, and postwash red blood cells (cRBCs) that had been stored in either freezing medium or phosphate-buffered saline (PBS) were prepared for ultrastructural analysis using scanning electron microscopy. Samples were fixed in 2.0% glutaraldehyde prepared in 0.1 M PBS (pH 7.2) for 1 h at room temperature, followed by three washes with the same PBS to remove residual fixative.[49]

Dehydration was performed using a graded ethanol series (10−100%), and the samples were subsequently dried using critical-point drying. The dried specimens were sputter-coated with a 10 nm gold−palladium layer (Au:Pd, 80:20) using a Quorum Q150T ES sputter coater (Quorum Technologies Ltd., UK).

Imaging was performed using a Quanta 250 field emission scanning electron microscope (FEG-SEM, Thermo Fisher Scientific) operated at 10 kV. Cell morphology was observed at magnifications of 1,000×−2,000×.

Raman Spectroscopy and Sample Preparation

Raman spectra were acquired using a Renishaw inVia confocal Raman microscope in backscattering configuration, coupled with a Linkam cryo-stage. The Linksys 32 software was used to

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control the cryostage parameters. A 532 nm diode laser (~20 mW) was focused onto the samples through a 50× objective lens and a 600 grooves/mm diffraction grating. Spectral data were recorded between 50 and 3800 cm^{-1} and processed using ImageJ software.

cRBCs were prepared by centrifugation at 300*g* for 5 min, washed twice with DPBS, and resuspended in cryoprotectant solutions containing 10% DMSO alone or in combination with various other CPAs, such as glycerol, trehalose, or proline, each prepared in DPBS without FBS to avoid artifacts. After 10 min of incubation in the cryoprotectant, 1−3 μL of the cell suspension was deposited onto the sapphire glass of the cooling stage and covered with a quartz crucible to prevent its evaporation. The samples were cooled from room temperature to −50 °C at a rate of 1 °C/min. Raman imaging was performed after a 5−10 min equilibration. Bright-field and Raman images were simultaneously captured, and the spectra of each CPA component were identified and mapped. Ice formation was identified by examining the OH stretching band (3087−3162 cm^{-1}), where the absence of a signal indicated the absence of intracellular ice.[18,50] Deuterated dimethyl sulfoxide ($DMSO_{d_6}$) was used as the solvent in Raman spectroscopy to reduce spectral interference from solvent vibrations.[51]

Statistical Analysis

Data were plotted and analyzed using Graphpad Prism 9 using Tukey's post hoc test comparison of experimental groups with the appropriate control group.

RESULTS AND DISCUSSION

To screen for avian RBC cryopreservation a range of formulations containing DMSO and/or trehalose and/or proline were initially screened, informed by our previous studies on mammalian RBCs.[43] Post-thaw recovery was quantified by the alkaline hematin D-575 (AHD) assay, to quantify the fraction of hemoglobin released relative to unfrozen controls, to give a percentage recovery. From this initial screening 5% DMSO + 0.2 M proline +0.4 M trehalose showed the highest recovery (Figure S11) giving recovery of ~30%. The combination of DMSO with either proline or trehalose alone (Groups H and I) yielded moderate to low recovery levels. Guided by this, the impact of polyampholyte (PA) supplementation was assessed with the results shown in Figure 1. The polyampholyte was synthesized by ring opening of (poly(methyl vinyl ether-*alt*-maleic anhydride)), Figure 1A, using a previously reported method (see Experimental Section). This method gives a regioregular polymer with precise control over the cationic/anionic groups which is essential for function and can be conducted on 100s gram scale using low cost starting materials. Addition of PA increased cRBC post-thaw recovery, particularly with the CPA formulation containing 5% DMSO + 0.25 M proline + 0.4 M trehalose (designated as formulation C). This formulation showed recovery values closely matching those of 10% DMSO, with minimal variance between replicates, although no statistical difference was observed. Reducing the total DMSO from 10 to 5% is expected to be desirable to speed post-thaw washing and to reduce any potential complications from the cytotoxic nature of DMSO. Elevated concentrations of polyampholytes (100 mg·mL^{-1}) were less effective, possibly due to osmotic or chemical stress on the RBC membranes, highlighting the importance of this initial screening step.

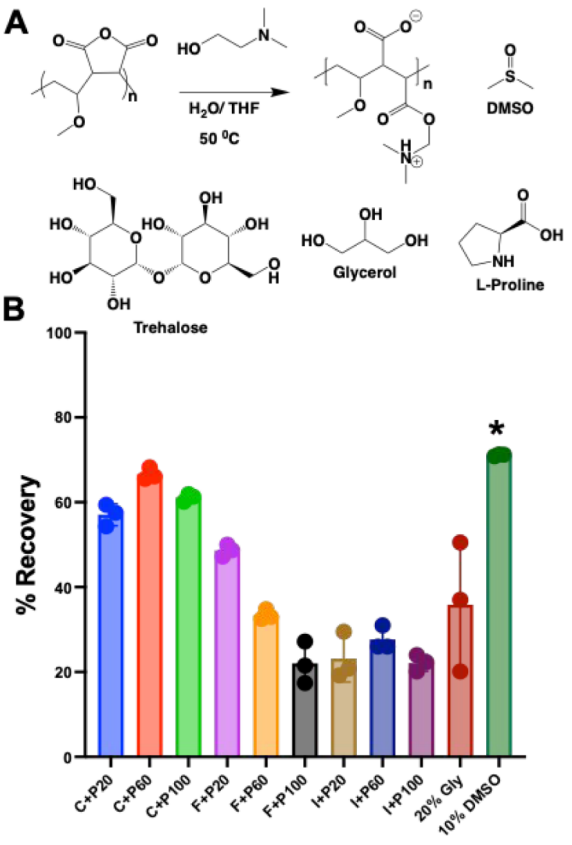

Figure 1. Post-thaw recovery of cRBCs. (A) Cryoprotectants used in this study; (B) post-thaw cRBC recovery reported from hemolysis measurements. Formulations: F (5% DMSO + 0.25 M proline + 0.35 M trehalose), I (5% DMSO + 0.4 M trehalose), and formulation C (5% DMSO + 0.25 M proline + 0.4 M trehalose). Polyampholytes was also added: P20 (20 mg·mL^{-1}), P60 (60 mg·mL^{-1}), and P100 (100 mg·mL^{-1}) as indicated. Post-thaw recovery is mean ± standard deviation (SD) from three biological replicates (n = 12). Differences in recovery among all tested conditions compared to 10% DMSO controls, are considered statistically significant using a threshold of $p < 0.05$.

The above showed that PA supplemented into DMSO was a potent cRBC cryoprotective solution, but it was also important to compare to glycerol, which is the most commonly used CPA for mammalian (including human) RBCs. cRBCs were cryopreserved using glycerol with and without PA and the results are shown in Figure 2. As seen with DMSO, post-thaw recovery was increased when polyampholytes were added to 20% glycerol. These results suggest that to use glycerol, higher concentrations are required (20% versus 10%) compared to DMSO, which would subsequently require more extensive washing post-thaw. Hence, DMSO was taken forward in this study, but the potential for glycerol-based formulations cannot be excluded.

To evaluate the impact of polyampholytes on DMSO-based cryopreservation further, cRBCs were cryopreserved using 5% DMSO (v/v) in combination with polyampholytes (PA) at a wider range of concentrations from 100 to 10 mg·mL^{-1} as shown in Figure 3A. The data show a concentration-dependent protective effect of PA. Maximum recovery was observed at 80 mg·mL^{-1} PA, whereas the performance decreased at lower concentrations. The 10 and 20 mg·mL^{-1} PA combinations provided minimal protection, with hemolysis levels similar to

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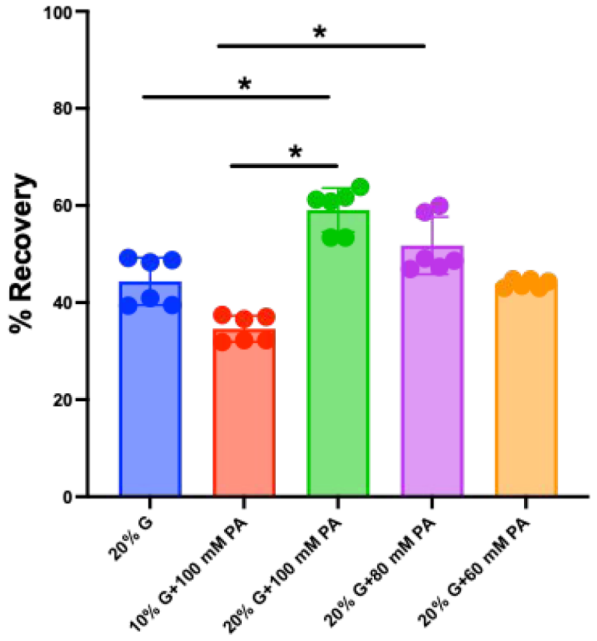

Figure 2. Post-thaw recovery of red blood cells (cRBCs) using glycerol. Twenty % G = 20% glycerol and 10% G = 10% glycerol, in addition to P60, P80, and P100, which are polyampholytes at 60 mg·mL^{-1}, 80 mg·mL^{-1}, and 100 mg·mL^{-1}, respectively. Data are presented as the mean ± SD from at least three biological replicates (n = 12). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) with statistically different outcomes indicated by *.

those observed with suboptimal cryoprotectant base formulations.

To improve post-thaw recovery, cRBCs were cryopreserved using 10% DMSO in combination with polyampholytes, Figure 3B. The data demonstrated a significant improvement in recovery compared to that of the 5% DMSO formulation, Figure 3B. The highest recovery was observed with 10% DMSO + 100 mg·mL^{-1} PA, yielding >92% recovery. A gradual decline in efficacy was observed with decreasing PA concentrations, although 10% DMSO + 80 or 60 mg·mL^{-1} PA still provided substantial protection (~90%). The use of lower concentrations of PA might be preferable in situations where total cryoprotectant concentration was more crucial than total post-thaw recovery.

The synergy of the PA and DMSO with trehalose (0.4 M), and proline (0.2 M) was further evaluated, Figure 4. The use of 60 mg·mL^{-1} polyampholyte in the absence of DMSO yielded lower recovery (mean: 38.0% ± 14.0%, $p < 0.001$ vs 10% DMSO), confirming that polyampholytes alone are insufficient for membrane stabilization under cryogenic stress. The addition of trehalose (0.4 M) to 10% DMSO improved post-thaw viability (mean: 71.6% ± 6.7%), although not to the extent achieved with DMSO and polyampholytes. Notably, combining all three agents (10% DMSO, 60 mg·mL^{-1} polyampholyte, and 0.4 M trehalose) did not further enhance recovery indicating an antagonistic or nonadditive effect when all three components were used in combination. This is in contrast to what is seen with mammalian RBCs, where proline/trehalose mixtures are beneficial and highlights the physiological differences between mammalian and avian blood.[43] The use of proline (0.2 M) in combination with 10% DMSO (mean: 65.3% ± 7.9%) resulted in only a marginal

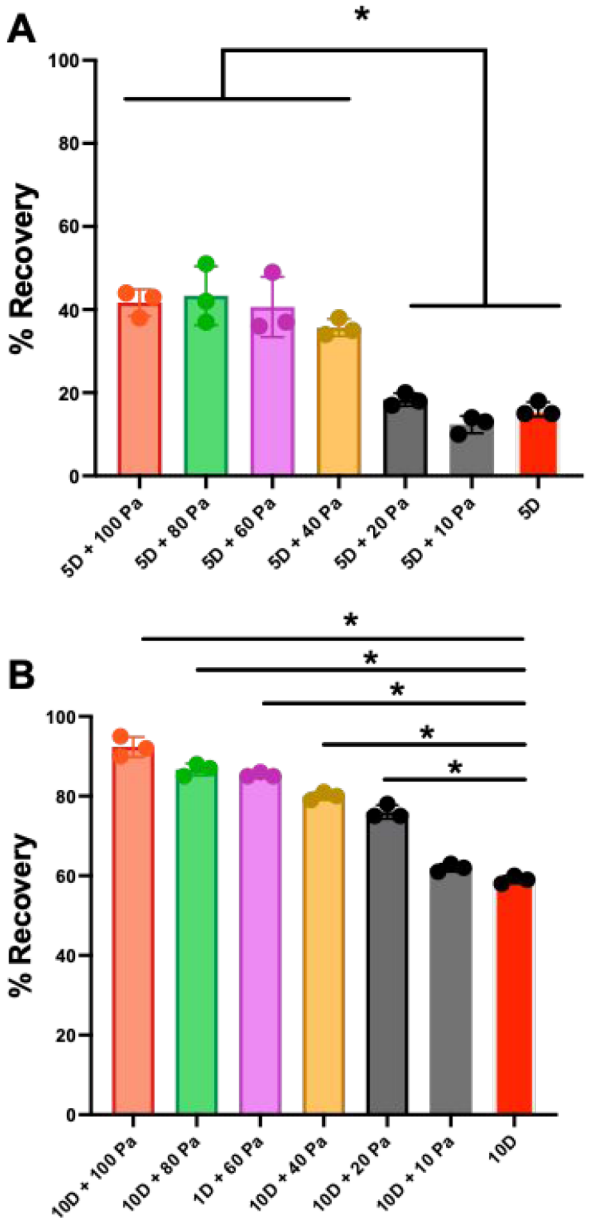

Figure 3. Post-thaw recovery of cRBCs using different DMSO and polyampholyte concentrations. (A) Recovery of cRBCs using 5% DMSO (5D) supplemented with polyampholytes (Pa): 10, 20, 40, 60, 80, and 100 Pa (corresponding to 10, 20, 40, 60, 80, and 100 mg·mL^{-1}, respectively). (B) Recovery of cRBCs using 10% DMSO (10D) with the same series of polyampholyte concentrations (10−100 Pa). Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$) with statistically different outcomes indicated by *. Post-thaw recovery was assessed using a hemolysis assay and is presented as the mean ± standard deviation (SD) from a minimum of three biological replicates (n = 12).

increase in recovery compared to 10% DMSO alone, and this difference was not statistically significant. Therefore, proline was not pursued further in subsequent experiments. Taken together, these results demonstrate that the incorporation of 60 mg·mL^{-1} polyampholyte into a 10% DMSO formulation significantly improves the post-thaw recovery of cRBCs.

With a successful cryopreservation formulation, longer term cryopreservation outcomes, could be tested. Selected formulations were used to cryopreserve cRBCs for 2 weeks at

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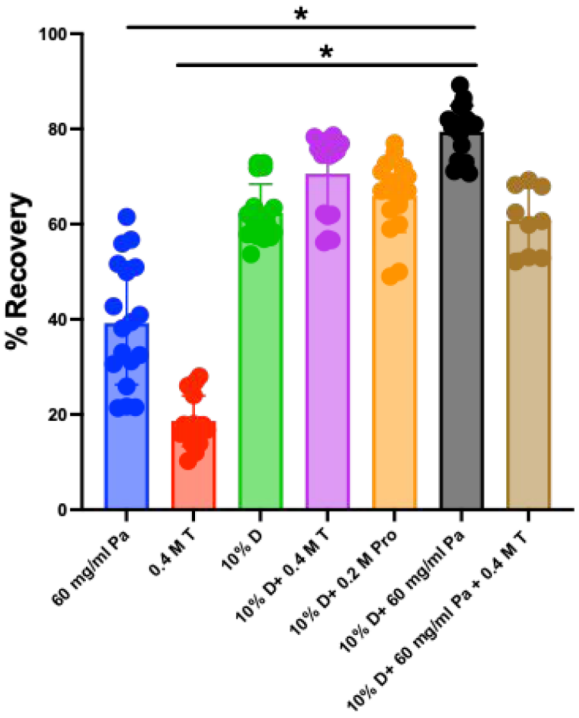

Figure 4. Post-thaw recovery of cRBCs. Cryoprotectant formulations tested included polyampholytes (Pa), trehalose (T), and proline (Pro). Data represent the mean post-thaw recovery from n = 16 independent replicates per condition. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test; differences between 60 mg·mL^{-1} Pa, 0.4 M T and 10% D + 60 mg·mL^{-1} Pa were statistically significant using a threshold of $p < 0.05$.

−80 °C. The formulations included 20% glycerol with 60 mg·mL^{-1} PA; 10% DMSO; 10% DMSO with either 0.2 M proline, 0.4 or 0.6 M trehalose, and 10% DMSO supplemented with 60 mg·mL^{-1} PA. Post-thaw cRBC recovery (Figure 5) showed a clear distinction between the different formulations. The highest recovery was observed with 10% DMSO + 60 mg·mL^{-1} PA, achieving a mean recovery of 82.7%, significantly outperforming all other groups ($p < 0.01$, one-way ANOVA with Tukey's post hoc test). Recovery from 10% DMSO + 0.4 M trehalose was significantly higher (~70.6%) than that from 10% DMSO alone ($p < 0.05$), indicating a beneficial osmoprotective effect of trehalose. Increasing trehalose to 0.6 M or adding 0.2 M proline did not yield further improvements, suggesting saturation or potential osmotic stress at higher additive levels. Conversely, the formulation containing 20% glycerol + 60 mg·mL^{-1} PA resulted in significantly lower recovery (~55.2%) than DMSO-based formulations, indicating that although it was partially successful for short-term freezing it does not work for long-term. Overall, the results confirmed that supplementation with 10% DMSO and 60 mg·mL^{-1} polyampholytes provides the most effective long-term cryoprotection for chicken red blood cells, with statistically superior recovery compared to other tested CPA strategies.

To examine the effects of the polyampholytes on cellular morphology prior to freezing, cRBCs were incubated for 10 min in phosphate-buffered saline (PBS) containing either 20% glycerol or 10% dimethyl sulfoxide (DMSO), supplemented with 60 mg·mL^{-1} polyampholytes (PA) to match the conditions used in cryopreservation, and then imaged by confocal microscopy, Figure S10. This analysis confirmed no

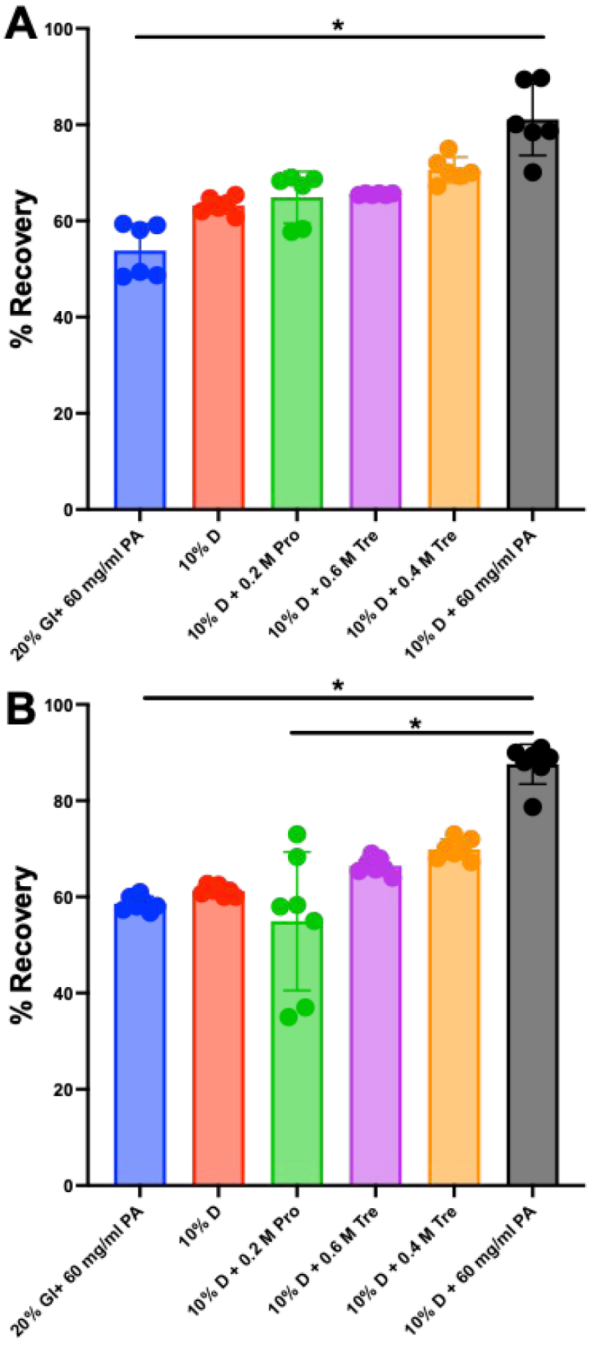

Figure 5. Post-thaw recovery of cRBCs after extended storage at −80 °C. Cryoprotectant formulations included PA = polyampholytes, Tre = trehalose, Pro = proline alone or in combination with glycerol or DMSO. (A) 14 days; (B) 172 days (~6 months). Each data point is the mean recovery from n = 6 independent replicates. Significant differences ($p < 0.05$) are denoted, based on one-way ANOVA and Tukey's post hoc test.

significant impact on cellular morphology suggesting that short-term exposure to these CPAs did not substantially alter the cRBC size or shape, agreeing with the hemolysis assays.

Figure 6 shows confocal fluorescence microscopy images and quantitative analysis of cRBC cytoskeleton integrity post-thaw with different cryoprotectants (CPA). The cytoskeleton was stained with a fluorescent marker, and the nuclei were counterstained with DAPI (blue), Figure 6. Control (without cryopreservation) samples exhibited well-preserved, continu-

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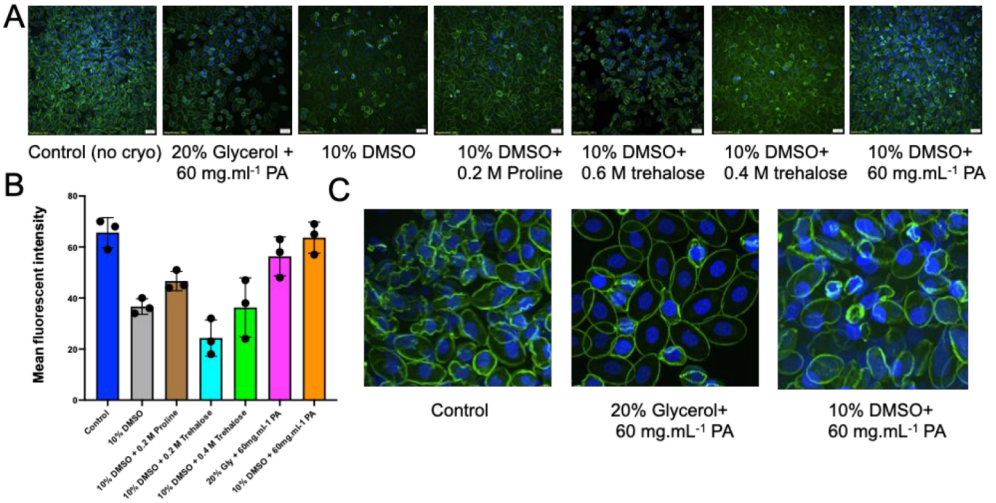

Figure 6. Effect of cryoprotectant formulations on the cytoskeletal integrity of cRBCs postcryopreservation. (A) Representative immunofluorescence images of cRBCs stained to visualize cytoskeletal filaments (green) and nuclei (blue, DAPI), post-thaw; (B) quantitative analysis of mean fluorescence intensity representing cytoskeletal integrity across different treatment groups (n = 3); (C) zoomed views of representative fields for control, 20% glycerol + 60 mg·mL^{-1} PA, and 10% DMSO + 60 mg·mL^{-1} PA.

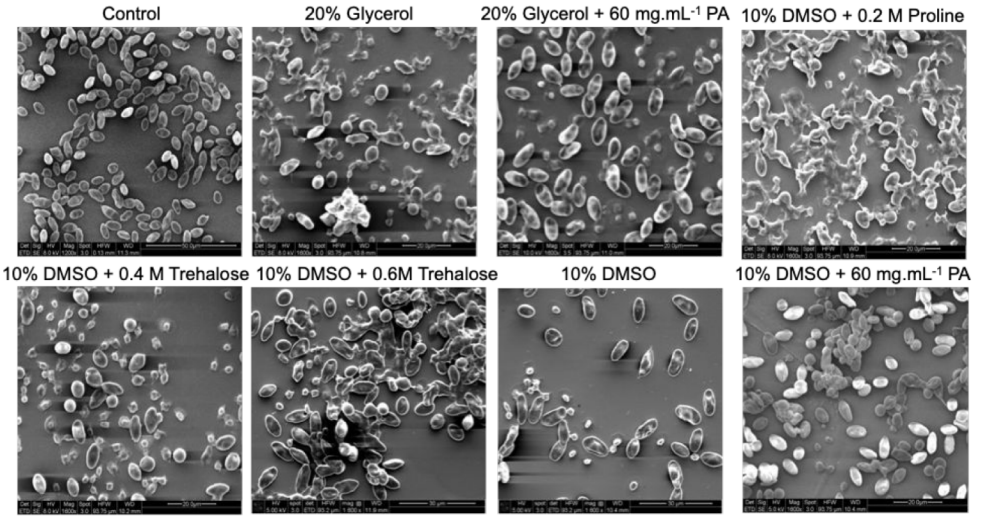

Figure 7. Scanning electron microscopy (SEM) images of post-thaw cRBC using the indicated cryoprotectants. Control are unfrozen cRBC.

ous, and evenly distributed cytoskeletal structures around cRBCs, reflecting the native membrane and cytoskeletal integrity. The 20% glycerol + 60 mg·mL^{-1} PA-treated cRBCs showed some disruption in cytoskeletal staining, with slight irregularities and reduced fluorescence intensity, suggesting partial damage or remodeling of the cytoskeleton due to cryopreservation stress. The 10% DMSO + 60 mg·mL^{-1} PA group demonstrated cytoskeletal preservation that closely resembled that of the control group, with clear, defined cytoskeletal outlines and minimal disruption, suggesting superior protection of the membrane and cytoskeletal components during freezing and thawing. Other treatments (10% DMSO alone and 10% DMSO combined with proline or trehalose) maintained intermediate levels of cytoskeletal integrity, with some fragmentation or reduced fluorescence intensity, consistent with moderate cryoprotective efficacy. Other DMSO-based treatments with proline or trehalose showed intermediate fluorescence intensities, which were statistically different from those of the control and 10% DMSO + PA groups ($p < 0.05$), indicating partial cytoskeletal preservation. These results demonstrate that cryopreservation with 10% DMSO supplemented with 60 mg·mL^{-1} polyampholytes most effectively preserves cRBC cytoskeletal integrity. This is consistent with the beneficial effort of polyampholytes which protect the intracellular environment during cryopreservation by reducing intracellular ice growth, despite being an extracellular cryoprotectant.[37,40] We also tested the polyampholyte for ice nucleation activity (as extracellular ice nucleation is known to have beneficial cryopreservation outcomes[28,52]), using a bespoke nucleation testing apparatus. This showed that the polyampholyte did not act as nucleator supporting our hypothesis (supported in other cell-based models)[40] that the polyampholytes reduce intracellular ice formation potentially be aiding dehydration/CPA penetration.

Scanning electron microscopy (SEM) was used as an additional method to assess the morphological integrity of cRBCs following cryopreservation, Figure 7. Fresh cRBCs (control) presented a uniform population of biconvex elliptical cells with distinct central bulges indicative of intact nuclei, and

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also smooth and uncompromised surfaces. Following cryopreservation with 20% glycerol (20% G), the cells showed morphological distortion, characterized by extensive cell shrinkage, irregular shapes, and compromised surface integrity, significantly deviating from the native cRBC morphology. Similarly, 10% DMSO alone damaged many cells, leading to highly shrunken and misshapen cells with rough surfaces and considerable aggregation. Although the addition of 0.2 M proline, 0.4 M trehalose, or 0.6 M trehalose to 10% DMSO partially mitigated the damage, they did not fully retain the native elliptical, nucleated morphology, and some degree of irregular shaping and surface irregularities persisted, in an inhomogeneous population. In stark contrast to the conventional cryoprotectants, formulations incorporating 60 mg·mL^{-1} polyampholyte (PA), revealed the recovery of more homogeneous cRBCs with retained elliptical morphologies, smooth surfaces, and were by visual inspection near identical to fresh cells. This underlines the potency of the polyampholytes in cryobiology.

To gain a more in-depth biochemical analysis of post-thaw function, apoptosis (programmed cell death) was measured using confocal microscopy and the annexin V FITC (green, indicative of early apoptosis) and DAPI (blue, nuclear stain) staining protocol, Figure 8. The negative control, representing

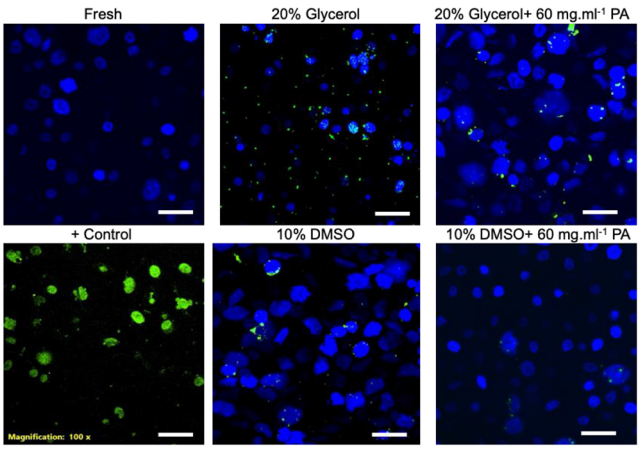

Figure 8. Representative fluorescence confocal microscopy images of DAPI (blue) and annexin V-FITC (green) dual fluorescent staining showing apoptosis in cRBCs, 6 h post-thaw, after cryopreservation in with the indicated conditions. Fresh = cRBCs without treatment; Control = cRBCs treated with staurosporine to induce apoptosis. Scale bar is 20 μm.

noncryopreserved cRBCs, exhibited a healthy population characterized by uniformly stained blue nuclei and negligible green annexin V signal. Following cryopreservation with 20% glycerol or 10% DMSO resulted in a widespread and statistically significant increase in green annexin V fluorescence indicating extensive membrane compromise and induction of apoptotic or necrotic pathways within 6 h of thawing. Supplementation with 60 mg·mL^{-1} polyampholyte (PA) gave statistically significant improvement in post-thaw viability (less green stain). Crucially, the mean fluorescence intensity values, as shown in Supplementary Figure S12, for both PA-containing groups were statistically comparable to those of the non-cryopreserved control, closely mirroring the low background observed in the negative control and maintaining a high density of intact, blue nuclei.

CONCLUSIONS

In this work we demonstrate that chicken red blood cells, cRBCs, can be effectively cryopreserved by supplementing DMSO with polyampholytes, which function as extracellular cryoprotectants to mitigate intracellular ice formation: a primary cause of cryoinjury which can otherwise lead to apoptosis and cytoskeleton depolymerization. Avian blood is nucleated, unlike mammalian, and hence the cryopreservation processes required are unique and not predictable based on previous evidence on mammalian red blood cells. Here we identified that DMSO plus polyampholytes enabled post-thaw recovery rates of approximately 90% after storage at −80 °C for up to 6 months. This was an improvement over the conventional approach using glycerol. In short-term storage glycerol cryopreservation was also improved by the polyampholyte, but after 7 days the glycerol performance diminished significantly justifying the switch to DMSO. Further optimization including fine-tuning the osmolarity might improve these, however, so a glycerol-based solution should not be fully excluded. Confocal fluorescence microscopy demonstrated that addition of the polyampholyte lead to increased cytoskeleton polymerization post-thaw, which has previously been linked to reduced intracellular ice formation and is a key component for a functioning cell. Confocal fluorescence and scanning electron microscopies demonstrated that the pre- and post-thaw cRBCs were intact and had morphology comparable to fresh, which is a crucial measure for RBC function. Collectively, these findings emphasize the potential of macromolecular cryoprotectants to enable the storage of avian blood which may find application in conservation and veterinary science and also provide basic cryobiology insight into how different species' blood is impacted by cryopreservation stresses. It also highlights the importance of identified new macromolecular cryoprotectants, including those which might be suitable for *in vivo* use.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acspolymersau.5c00151.

Additional cryopreservation data and polymer synthesis details (PDF)

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Notes

The authors declare the following competing financial interest(s): MIG is an inventor on a patent relating to the polymers used in this study.

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