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Sonoporation enables high-throughput loading of trehalose into red blood cells

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ABSTRACT

Despite recent advances in biostabilization, clinical blood supplies still experience shortages and storage limitations for red blood cells (RBCs) have not yet been sufficiently addressed. Storing RBCs in a frozen or dried state is an appealing solution to address storage limitations, but many promising cryoprotectants, including the non-reducing sugar trehalose, are impermeant to mammalian cell membranes and cannot be utilized effectively using currently available compound-loading methods. We found that transient pore formation induced by ultrasound and microbubbles (sonoporation) offers an effective means of loading trehalose into RBCs to facilitate long-term storage in a frozen or desiccated state. The protective potential of trehalose loading was demonstrated by freezing processed RBCs at −1 °C/min to −80 °C, then either storing the cells at −80 °C or lyophilizing them. RBCs were either thawed or rehydrated after 42 days of storage and evaluated for membrane integrity and esterase activity to estimate recovery and cell viability. The intracellular concentration of trehalose reached 40 mM after sonoporation and over 95% of treated RBCs were recovered after loading. Loading of trehalose was sufficient to maintain RBC morphology and esterase activity in most cells during freezing (>90% RBC recovery) and to a lower degree after lyophilization and rehydration (>20% recovery). Combining sonoporation with an integrated fluidics device allowed for rapid loading of up to 70 mM trehalose into RBCs. These results demonstrate the potential of sonoporation-mediated trehalose loading to increase recovery of viable RBCs, which could lead to effective methods for long-term stabilization of RBCs.

1. Introduction

Storage of red blood cells (RBCs) for transfusions requires refrigeration at 1–6 °C, and the maximum FDA-approved shelf life for RBCs is only 42 days, which can lead to shortages in supply [1,2]. The ability to store RBCs in a desiccated state at ambient temperatures would halt cellular and metabolic activities and reduce oxidative damage that occurs to RBC membranes in solution when refrigerated, which could significantly increase the shelf life beyond 42 days. In addition, dry preservation of RBCs would facilitate transfusions in places where it is not currently feasible or extremely challenging, including remote medical centers, in the field during military operations, and in the least developed countries where refrigeration may not be available.

The endeavor to lyophilize human blood components first started during World War II, when stabilizing soldiers on the battlefield was a major medical priority [3]. Human plasma was successfully freeze dried and rehydrated for infusion into patients, but cellular blood components proved to be uniquely challenging. Many cryoprotective compounds have been identified and applied to stabilizing blood cells, although the most promising have been sugars, starches, and amino acids [4]. Trehalose, a non-toxic sugar, has exceptional cryoprotective properties and has been shown to protect cellular components very effectively during water stress [5]. In the fully hydrated state, trehalose stabilizes cellular proteins and the plasma membrane through a process of preferential exclusion [6]. This occurs due to the thermodynamically less favorable interactions of trehalose with the surface of these

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biomolecules compared to water. Therefore, any increase in surface area, such as protein denaturation or micelle formation, are unfavorable. At low water contents, this preferential exclusion becomes impossible, and the multiple hydroxyl groups of trehalose interact with the hydrophilic surfaces of proteins and the plasma membrane [7]. These interactions functionally replace water as stabilizer at the hydrophilic surface of proteins and membranes. Finally, in the completely desiccated state, trehalose vitrifies into a glass that physically encases the cell and physically limits movement within the intracellular space. Furthermore, trehalose is biocompatible and used in vaccines and food products, and clinical trials have shown that as much at 54 g of trehalose may be infused over 1 h without toxicity in humans [8,9]. Unfortunately, mammalian cells lack transporters for trehalose uptake into the cytoplasm, but sufficient amounts are needed on both sides of the cell membrane in order to confer protection during desiccation [10]. Therefore, effective methods are needed to load mammalian cells with trehalose for biopreservation purposes [10]. Prior approaches have included passive trehalose loading of RBCs, platelets, and other mammalian cells via fluid-phase endocytosis, genetically engineered pores, chemically modified trehalose derivatives, or electroporation [11].

The most common methods of trehalose loading into platelets and RBCs were established by incubating the cells in hypertonic solutions containing trehalose, which stimulated a significant uptake of trehalose into the intracellular space [12]. Early passive incubation methods for loading this sugar into platelets reached intracellular trehalose concentrations of up to 17 mM when incubated for 4 h at 37 °C in a solution containing 35 mM trehalose. At this trehalose loading efficiency of 50%, platelets could be lyophilized and rehydrated with up to 85% recovery. This technique has been further refined to the point where lyophilized platelets are currently undergoing clinical trials [13–15]. Despite the success in freeze-drying platelets, efforts to preserve functional RBCs have proven to be more challenging. Similar incubation-based loading techniques used for platelets are capable of loading trehalose into RBCs, but hemoglobin oxidizes during the 7 h incubation period at 37 °C which is needed to reach sufficient amounts of trehalose in the cytoplasm [16]. Several other loading strategies have shown to allow loading of trehalose into human RBCs to confer protection during freezing and lyophilization, including but not limited to electroporation, trehalose-loaded liposome fusion, and apatite nanoparticle-facilitated membrane permeation. Electroporation loaded up to 63 mM trehalose into RBCs using four 1 ms pulses over 1 min at 1.5 K v/cm in an 800 mM trehalose solution. Excitingly, 71% of these RBCs were recovered after being freeze-dried and rehydrated [17]. Intracellular trehalose concentration of 15 mM were reached using trehalose-loaded liposomes and even at this relatively low concentration protection of RBC during freeze-thaw was observed with 66 ± 5% of cells maintaining membrane integrity compared to 29 ± 4% of cells without sugar loading [18]. With the use of apatite nanoparticles trehalose loading into RBCs of up to 63 mM was achieved when incubated with 350 mM trehalose at a pH 6.5 for 7 h at 37 °C. Approximately 90% of these RBCs were recovered when thawed rapidly at 37 °C after being frozen in liquid nitrogen. Samples were stored at −80 °C for 24 h prior to thawing to keep storage conditions similar to those used for glycerolized RBCs [19].

To address the challenge of efficient loading of trehalose into RBCs, we investigate the application of a loading method termed “sonoporation” which was previously only used in applications not related to cell stabilization. Sonoporation occurs when ultrasound drives oscillation and collapse of microbubbles near cell membranes to induce formation of transient pores, which enables uptake of impermeant compounds including trehalose [20]. This process also induces microstreaming in the surrounding fluid that can enhance transport of compounds through formed pores, which typically reseal within several seconds in a calcium dependent pathway [21]. Thus, sonoporation can rapidly load compounds into cells with higher efficiency compared to methods that depend on passive diffusion [22]. Furthermore, microbubbles are biocompatible and approved for clinical use as intravenous ultrasound contrast agents to image cardiac blood flow [23] and are in development for targeted gene and drug delivery applications *in vivo* [24]. To characterize the feasibility of sonoporation as a reliable method for loading trehalose into RBCs we first investigated the use of sonoporation in bulk samples with volumes of 500 μL. Encouraged by the results an acoustofluidic device that exposes RBCs and microbubbles to ultrasound while constantly flowing through a channel was developed to improve trehalose loading and sample throughput (Fig. 1). Our criteria for a promising new loading method were: (1) RBCs loading with trehalose at comparable efficiencies to previously described methods and (2) avoidance of cellular stresses that require high trehalose concentrations and long incubation periods.

Development of an effective method to load human RBCs with trehalose in order to allow for storage in a dried state has the potential to fundamentally change transfusion medicine. Thousands of people around the globe die each year due to lack of a stable blood supply and any approach to store viable transfusion units at ambient temperatures would have a significant impact on human health care [25]. Furthermore, dry preservation of RBCs would allow for building strategic reserves for natural disasters such as the COVID-19 crisis. Finally, shelf-stable RBCs would improve transfusion capabilities in far-forward military settings, during long-duration space missions, for patient stabilization during emergency responses, and in other situations during which current transfusion units cannot be employed due to their cold-storage requirement and short shelf life. The development of rapid and effective technologies for loading of protective compounds across the plasma membrane is crucial step towards shelf-stable RBCs for transfusion medicine.

2. Material and methods

2.1. Chemicals

Low endotoxin α,α-trehalose dihydrate was obtained from Pfanstiehl Inc. (Waukegan, IL). All other compounds were obtained from VWR (Radnor, PA) or Sigma Aldrich (St. Louis, MI) and were of the highest purity commercially available. Water for solution preparation was purified with a Milli-Q Reagent Water System (Billerica, MA).

2.2. Bulk sonoporation treatment

Human red blood cells were obtained from clinical samples collected at the University of Louisville medical center with informed consent and institutional approval. RBCs were stored in CPD/AS-1 solution at 4 °C until use. RBCs were diluted to 5 million cells/mL in 500 μL of phosphate buffered saline (PBS) containing 200 mM trehalose (PBS/Tr) or SLB (200 mM trehalose, 60 mM sodium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EGTA, 20 mM HEPES-NaOH, pH 7.1) immediately prior to treatment. Lipid-coated decafluorobutane gas-filled microbubbles were synthesized as described previously [26]. This microbubble stock solution has a concentration of approximately $1.3 \bullet 10^9$ microbubbles/mL with a diameter distribution of 2.2 ± 1.1 μm and a zeta potential of 21.1 ± 0.7 mV in PBS diluted 100-fold with deionized water. Microbubbles were added to RBC solutions in 15 mL conical tubes, then the tube were partially submerged in water positioned 4 cm away from an ultrasound transducer (Fig. 1A and B). To avoid separation of the positively buoyant microbubbles from the negatively buoyant RBCs, the samples were treated with ultrasound pulses immediately upon mixing. Ultrasound pulses were generated using an ultrasound imaging system (ATL P4-1 probe on Vantage 64LE, Verasonics, Kirkland, WA, USA) with a 2.5 MHz center frequency, a pulse duration of 2 μs, and 0.25 MPa peak negative pressure pulses for 60 s followed by 0.90 MPa peak negative pressure pulses for 10 s at 21 °C. The acoustic pressures were calibrated using a 0.2 mm needle hydrophone at the focal distance (Precision Acoustics, Dorchester, UK).

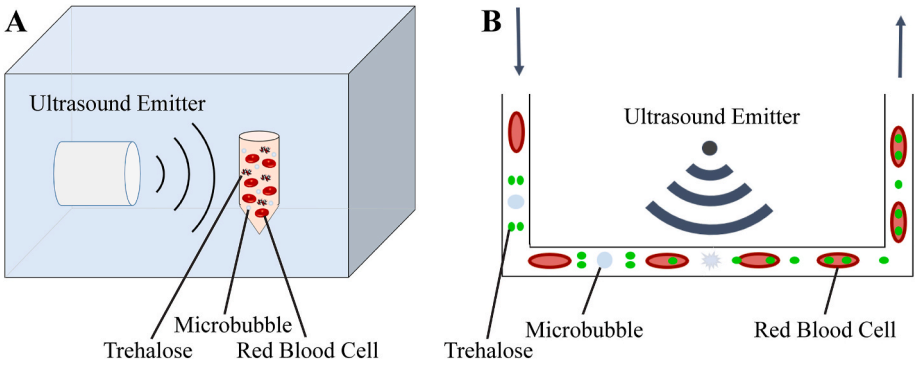

Fig. 1. Bulk and Acoustofluidic Loading Systems: A) Sample tubes containing trehalose, RBCs, and microbubbles in 1xPBS were submerged in a water tank and exposed to ultrasound to induce cavitation for 1 min. B) Samples of RBCs in 1xPBS with trehalose and microbubbles were flown through a microfluidic channel. Ultrasound waves induced microbubble cavitation to induce loading.

2.3. Acoustofluidic treatment

An acoustofluidic device containing a fluidic channel and integrated ultrasound transmitter was used to process volumes of RBCs required for transfusion experiments *in vivo*, as previously described [27]. The acoustofluidic system enables increased throughput and better consistency of trehalose loading into RBCs compared to the bulk treatment system. RBCs were diluted to 50 million cells/mL in 5 mL of PBS/Tr with added decafluorobutane microbubbles immediately prior to treatment, then loaded into a 15 mL falcon tube (Corning, New York, USA) which was fitted with a pressure-based pumping reservoir cap (Darwin Microfluidics, Paris, France) and stored on ice. An automated syringe pump was used to pressurize the tube chamber, creating a flow rate of 50 mL/h through a concentric spiral channel with a diameter of 500 μm × 200 μm. RBCs were exposed to ultrasound in the system using an Arduino-driven ultrasound piezo transducer (5 V, 8 MHz) positioned directly above the fluidic channel. After sonoporation, the RBC solution was pumped into a separate conical tube that was stored on ice until the entire sample was processed. The device was cleaned between runs with 10 mL of 20% ethanol, then 20 mL ultrapure water, then equilibrated in PBS/Tr.

2.4. Intracellular trehalose measurements

Trehalose concentrations were measured using a commercially available trehalose assay kit (Megazyme, Chicago, IL) following the instructions provided by the manufacturer. Briefly, extracellular trehalose was washed from the RBCs with three 10 mL PBS washes using centrifugation at 500×*g* for 5 min each before lysing the cells in pure water. To remove endogenous glucose, the enzymes hexokinase and glucose-6-phosphate dehydrogenase and the substrates ATP and NADP were added to the sample. Next, the enzyme trehalase was added and the molar amount of trehalose was calculated as half of the amount of produced NADPH, which was calculated using an extinction coefficient of $\varepsilon = 6300$ L x mol^{-1} x cm^{-1}. The resulting amount of trehalose was scaled to the intracellular volume of all RBCs in the sample, assuming 50fL/RBC on average, and the intracellular trehalose concentration is reported in mM [28].

2.5. RBC freezing

The in PBS/Tr trehalose-loaded RBCs were washed once and then frozen in PBS/Tr or SLB (200 mM trehalose, 60 mM sodium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 3 mM $MgCl_2$, 0.5 mM EGTA, 20 mM HEPES-NaOH, pH 7.1) at a concentration of 1 million cells/mL using cryovials at a rate of −1 °C/min to −80 °C in a passive controlled-rate freezing device (BioCision, LLC, Larkspur, CA). Samples were stored for 42 days at −80 °C to determine freeze-recovery and allow for comparison to storage success of glycerolized RBC. After 42 days, cells were thawed rapidly in a 37 °C water bath and recovery was quantified by manual counting with a hemocytometer. Calcein-AM viability assays were conducted by adding to 500 μl of RBCs a concentration of 5 μM calcein, then these cells were incubated at 37 °C for 30 min [29]. The calcein fluorescence intensity was then quantified using flow cytometry (MACSQuant Analyzer, Miltenyi Biotec, Germany).

2.6. Rat blood collection and processing

Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA, USA). Donor rats were anesthetized using 5% isoflurane and 5–8 mL of blood was collected into a sodium citrate containing syringe via cardiac puncture into the left ventricle with a 25-gauge needle. RBCs were separated from the whole blood by centrifugation at 600×*g* for 10 min and the platelet-rich plasma was removed using a sterile pipette. Packed RBCs were washed three times in CPD-AS1 and passed through a 45-μm membrane filter, then stored at 4 °C until used for experiments. Rat RBCs were frozen and thawed as described above, then concentrated via centrifugation to a final concentration of 5 billion cells/mL for transfusion into rats.

2.7. Rat blood transfusion and analysis

Rats were anesthetized using 2–3% isoflurane, then 25% of the total blood volume of each rat was removed through a jugular vein cannula. An equivalent volume of saline, control RBCs (washed and stored at 4 °C), or frozen/thawed trehalose-loaded RBCs was transfused through the jugular vein cannula at a rate of 150 μL/min. The rats were sacrificed 24 h after transfusion and blood samples were collected for analysis using a VetScan HM2 hematology analyzer (Abaxis, Union City, CA, USA) to determine RBC concentrations and hemoglobin levels. Blood samples were also collected prior to transfusion to assess baseline levels using the hematology analyzer.

2.8. RBC lyophilization

RBCs were washed in SLB and aliquoted into 1 mL cryovials as described above for the freeze/thaw studies but, after reaching −80 °C, were sublimated under an atmosphere of 1 mbar for 48 h to ensure complete drying using a Labconco FreeZone 4.5 plus freeze dryer (Labcono, Kansas City, MO). Due to the external flask design of the freeze dryer, samples slowly warmed up to room temperature during lyophilization. Dried RBCs were sealed and stored in a desiccator cabinet over anhydrous $CaSO_4$ (Hammond Drierite, Xenia, OH) for 42 days at room temperature. Cells were rehydrated slowly with 1 mL of purified water by gently rotating the tube. Complete rehydration of the RBCs was achieved in under 20 s. RBC recovery was quantified by manual

counting with a hemocytometer. Calcein-AM based viability assays were conducted by adding to 500 μL of RBCs in suspension calcein at a concentration of 5 μM followed by incubation at 37 °C for 30 min [29]. Calcein fluorescence intensity was quantified using flow cytometry (MACSQuant Analyzer, Miltenyi Biotec, Germany).

2.9. Scanning electron microscopy

RBC samples were fixed with 2.5% glyceraldehyde and dehydrated with consecutive ethanol resuspensions starting at 50% v/v and increasing by 10% until the cells were resuspended in 100% ethanol before imaging with a Zeiss Supra 35 scanning electron microscope (Zeiss, San Diego, CA). Cells were plated onto aluminum stages and allowed to settle on the stage before excess ethanol was removed with a micropipette. The samples were then allowed to completed dry over anhydrous calcium sulfate for 1 h before imaging. The samples were then sputter coated with palladium and gold to prevent charging artefacts before viewing. Images were taken at an EHT of 5 KV and a working distance of 2.6 mm under a vacuum.

2.10. Statistical analyses

Data were analyzed with one-way ANOVA tests using SigmaPlot 14.5 (Systat Software Inc., San Jose, CA) using a Holm-Sidak post-hoc analysis.

3. Results

3.1. Sonoporation toxicity and loading efficiency

Cellular toxicity of the sonoporation process was assessed by exposing RBCs to various microbubble (MB) doses and ultrasound using two different solutions, PBS/Tr or SLB. As data in Table 1 show, cell recovery was consistently above 95% for MB doses of up to 10% (v/v), and no significant decrease in cell numbers was observed immediately after sonoporation in either solution. Encouraged by the low cytotoxicity of the process, we next investigated the impact of MB concentration on the amount of trehalose loaded into RBCs. Not surprisingly, even without sonoporation, we observed low levels of trehalose uptake of 19 ± 4 mM ($n = 3$) after simply incubating RBCs in PBS/Tr for 1 h, which is likely due to the hypertonicity of the incubation solution that causes leakage of trehalose across the plasma membrane which is known to occur at ~4 °C [30]. However, significant higher trehalose delivery occurred during sonoporation using MB concentrations ranging from 2.5% to 10% and sugar delivery increased with increasing MB concentrations (Fig. 2). To increase sample throughput to volumes suitable for transfusion experiments, we next utilized an acoustofluidic platform for trehalose loading. High throughput processing of rat RBCs using this platform did not reduce loading efficiency of trehalose into the RBCs and loading was substantially higher than the bulk treatment method in conical tubes (Supp. Fig. 1).

Table 1
Microbubbles are non-toxic using two different buffer systems (bulk treatment method).

Microbubble Concentration (v/v)	[a]PBS/Tr ***Recovery (%)***	[a]SLB ***Recovery (%)***
0%	96.4 ± 3.09	98.6 ± 2.58
2%	97.7 ± 2.39	97.7 ± 3.09
5%	96.2 ± 2.62	96.5 ± 1.38
10%	95.2 ± 6.46	96.1 ± 1.75

[a] Cells were exposed microbubbles and ultrasound pulses at room temperature for 70s and membrane integrity was assessed using trypan blue staining (n = 9–15; ±SD).

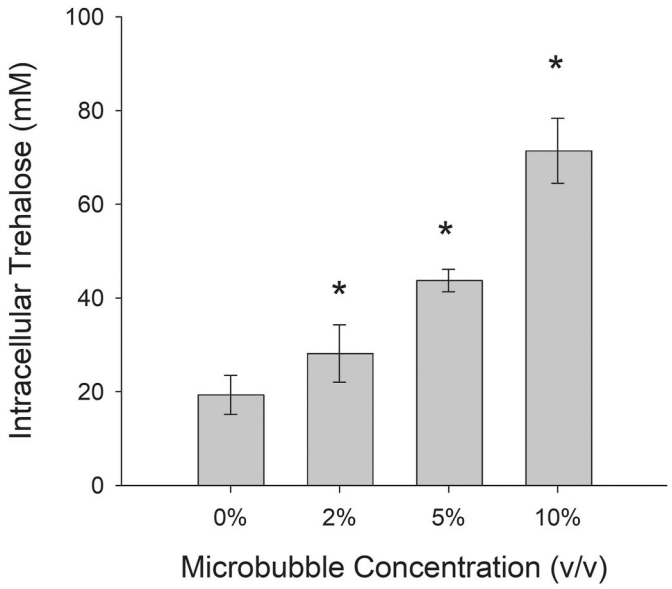

Fig. 2. Compound loading into RBCs. Trehalose uptake correlates with microbubble dose using PBS/Tr and bulk processing ($n = 4$, ±SD) *significant increase in trehalose uptake above samples treated without microbubbles.

3.2. RBC frozen storage

Injury conveyed by the freezing process, was assessed by analyzed membrane integrity and intracellular esterase activity after freezing and thawing of RBCs. Cells processed with MBs and ultrasound treatment were recovered in significantly greater numbers than untreated cells after 42 days of storage at −80 °C using PBS/Tr for trehalose loading and PBS/Tr or SLB for freezing (Fig. 3A). Untreated RBCs were recovered in highly inconsistent quantities with an average recovery below 40%, whereas over 80% of treated RBCs were recovered at microbubble doses ranging from 2% to 10%. Cells loaded with trehalose and frozen in SLB were recovered in higher quantities than those treated and frozen in PBS/Tr ($p < 0.05$, $n = 12$). However, no statistically significant difference in cell recovery was founded for untreated cells frozen in SLB or PBS. Esterase activity of recovered RBCs after thawing was independent of the differences in RBC recovery and not significantly different for samples processed in PBS/Tr or SLB, where nearly 100% of recovered RBCs were scored as viable in all samples (Fig. 3B).

3.3. Rat transfusion model

To broadly assess the safety of sonoporated RBCs, a rat model of acute blood loss was utilized, and the rats were assessed for up to 24 h after transfusion. All rats survived the infusion of RBCs that were processed with our fluidics device and infused immediately or stored at −80 °C for a minimum of 24 h before infusion. No adverse effects were observed during the 24 h post-transfusion period in any experimental group. Furthermore, hematology analysis determined that rats transfused with either frozen/thawed or refrigerated RBCs had significantly higher RBC counts and hemoglobin concentrations compared to rats infused with saline only (Fig. 4, $p < 0.05$). No significant difference in these metrics between rats transfused with processed/thawed RBCs compared with transfusion of refrigerated RBCs were found.

3.4. RBC freeze-dried storage

To investigate the potential for lyophilization of RBCs after trehalose loading, cell membrane integrity was investigated by SEM after sample rehydration. Virtually no morphological intact cells were recovered without trehalose loading, regardless if cells were lyophilized in SLB or

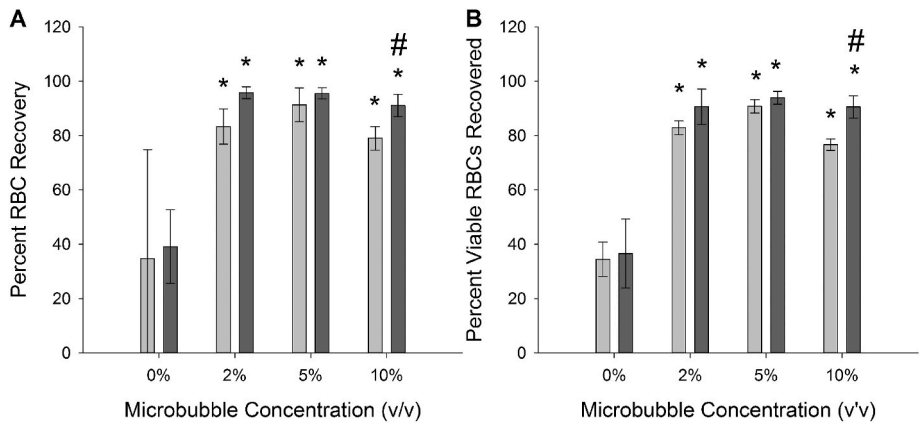

Fig. 3. Number of intact cells recovered after freezing and thawing (bulk treatment method). A) Recovery of intact RBCs after freezing and thawing in PBS/Tr (light) and SLB (dark) at different microbubble concentrations. B) Recovery of viable RBCs determined by calcein AM staining ($n = 3$, ±SD; $p < 0.05$); *significant increase relative to 0%; #significant increase relative to PBS/Tr at the same microbubble concentration.

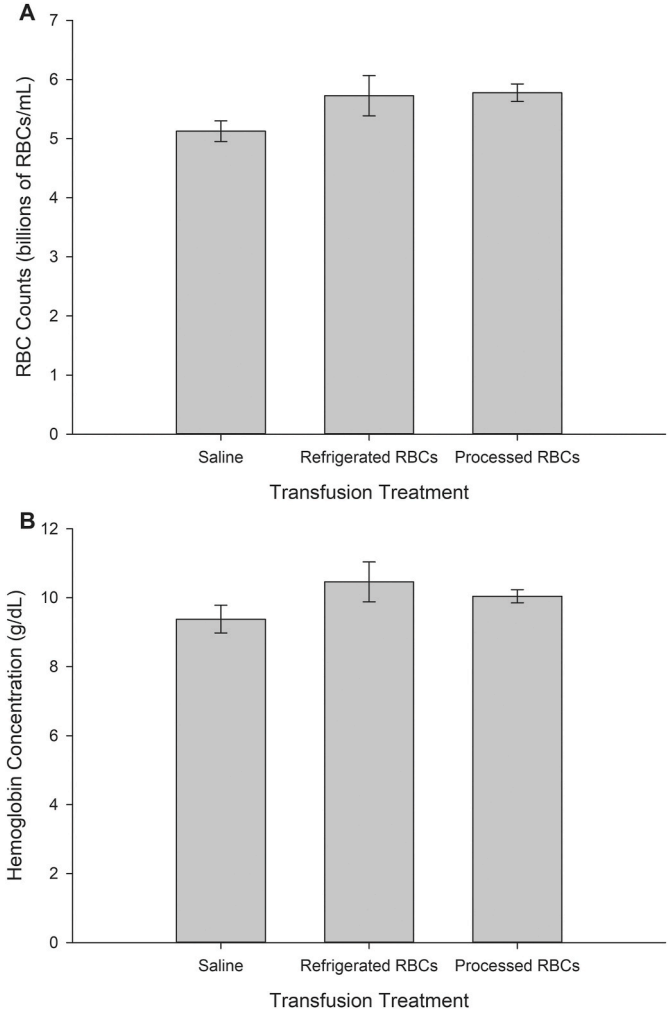

Fig. 4. Rat blood 24 h after transfusion. A) The RBC concentration in the rats was significantly higher for rats infused with frozen and thawed RBCs compared to saline. B) Rat hemoglobin levels were higher in rats infused with frozen and thawed RBCs compared to saline.

PBS/Tr (Fig. 5A). In contrast, morphological intact RBCs were recovered after rehydration of trehalose-loaded samples stored desiccated for 42 days, and significant increases in recovery were observed in samples using SLB and MB doses between 2% and 10% (Fig. 5A). RBCs treated in SLB showed levels of esterase activity similar to the values found after freeze-thawing, with approximately 99% of the recovered cells retaining enzymatic activity (Fig. 5B). RBCs that were loaded with trehalose using ultrasound and 10% MBs, lyophilized in SLB, and rehydrated 42 days later, maintained their biconcave discoidal membrane structure and showed minimal membrane fusion. Cells were of similar size to refrigerated control samples and showed no surface abnormalities (Fig. 6).

4. Discussion

Ultrasound and microbubbles were employed as a method to actively transport trehalose across the plasma membrane of RBCs in a process termed sonoporation. Effective methods with low cytotoxicity for loading membrane impermeant protectants, such as the sugar trehalose, into the intracellular space are a necessary step towards improved long-term storage protocols for RBCs. We have demonstrated the feasibility of sonoporation for trehalose loading and morphological intact RBCs were recovered after freezing and thawing of processed cells. Despite the absence of glycerol, a commonly utilized RBC cryoprotectant which needs to be removed stepwise before transfusion, 95% of viable cells were recovered after thawing without the need of a trehalose removal steps. Furthermore, an acute model of blood loss in rats demonstrated that the thawed RBCs did not induce any adverse transfusion reactions during 24 h post transfusion.

Two different sonoporation-based methods for trehalose loading were employed. Bulk sonoporation induced the uptake of approximately 71 mM intracellular trehalose after only 1 min of treatment, whereas a high throughput acoustofluidic system loaded approximately 150 mM intracellular trehalose in approximately the same amount of time. This represents a 36% and 75% loading efficiency based on the 200 mM extracellular trehalose solution used, which is competitive with other loading methods. These values were determined based on a 50 fL cytoplasmic volume of osmotically active water in a solution of approximately 500 mOsm [28].

High loading efficiency makes sonoporation a competitive method for loading trehalose into RBCs. Hypertonicity-induced leakage, for example, allows RBCs to accumulate up to 40 mM intracellular trehalose after a 7 h incubation at 37 °C in ADSOL containing 800 mM trehalose [30]. However, this long incubation period limits the throughput of samples and may cause irreversible hemoglobin oxidation [16]. Freezing-induced trehalose uptake in fibroblasts, which occurs when the plasma membrane is permeabilized by the localized phase transitions from a liquid crystal phase to a gel phase, results in over 100 mM intracellular trehalose from a 250 mM extracellular trehalose solution [31]. During freezing, the nucleation of ice crystals reduces the available

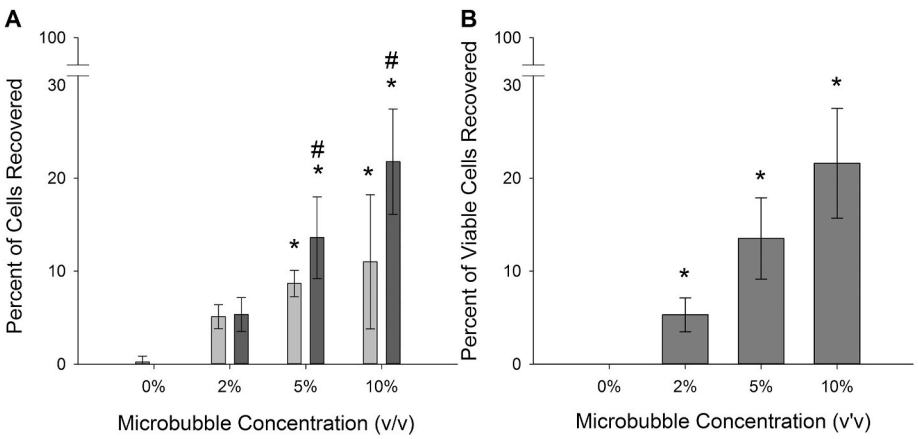

Fig. 5. Number of intact cells recovered after freeze drying. A) Recovery of intact RBCs after freezing and thawing in PBS/Tr (light) and SLB (dark) at different microbubble concentrations. B) Recovery of viable RBCs determined by calcein AM staining. *significant increase relative to 0% microbubbles. #significant difference between PBS/Tr and SLB at same microbubble concentration ($n = 3$, ±SD; $p < 0.05$).

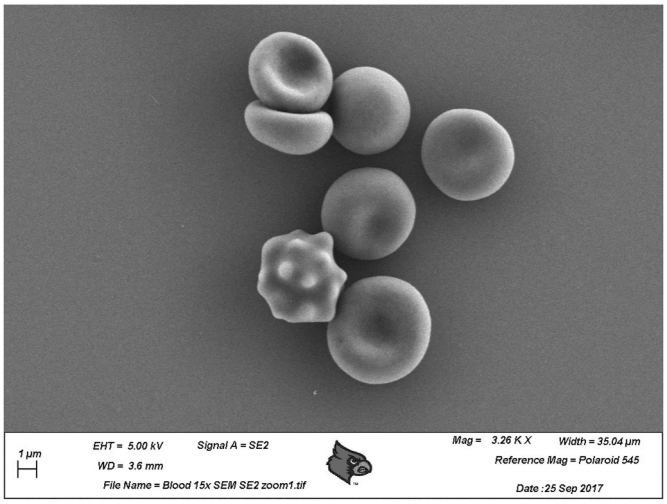

Fig. 6. RBC Membrane and cytoskeletal integrity after rehydration after 42 days at ambient temperatures. Cells were imaged at 5Kv electron high tension (EHT) in a near-vacuum. The results are representative of 3 independent experiments.

water, resulting in an increase of trehalose concentration in the remaining liquid water [31]. However, this loading mechanism occurs during ice nucleation and is therefore less compatible with methods such as spray drying or snap freezing of RBCs. The majority of commonly utilized loading approaches, including electroporation, rely on chemical shifts in the phospholipid bilayer to allow diffusion to transfer trehalose into the cytoplasm [17]. As previously demonstrated, these methods can load sufficient amounts of trehalose into RBCs to confer protection during both freezing and drying. However, most rely on diffusion, show low rates of loading and require substantially higher concentrations of extracellular trehalose than 200 mM. Although electroporation loaded RBCs within the same amount of time as sonoporation, the cells did not maintain their biconcave discoidal shape [17]. Therefore, the main advantage of sonoporation over most methods appears to be the fluid streaming that occurs during microbubble cavitation and the more active nature of the loading process.

Other strategies, such as biopolymer-mediated trehalose uptake, can load up to 120 mM trehalose at relatively low osmolarities [32]. However, these biopolymers still require a 9 h incubation at up to 37 °C in 350 mM trehalose solutions. Furthermore, these polymers must be removed prior to use of RBCs in a clinical setting, whereas the lipid microbubbles used in sonoporation are already infused as ultrasound contrast agents [33]. One benefit of biopolymer-induced trehalose loading is its relatively low hemolysis rate at approximately 25% at the highest treatment conditions, which are generally in excess of the requirements for cryopreservation. Further research is ongoing to characterize retention of hemoglobin and other cellular components during sonoporation.

Sonoporation has the advantage that trehalose loading can be achieved in under 1 min and combined with a fluidics device the process can be easily scaled to the desired throughput. Furthermore, the ability to modulate trehalose loading by modifying the microbubble dose allows for precise loading of the desired trehalose concentrations, which may prove critical for striking a balance between loading sufficient trehalose for effective cryopreservation while minimizing the risk of osmotically induced hemolysis upon infusion. Additional ways to affect loading, such as adjusting the lipid composition of the microbubbles, temperature of the device as the cells flow through, ultrasound frequency and pressure, flow rate and cell concentration, and the dimensions and shapes of the acoustofluidic channel allows for optimizing compound delivery for a large variety of cells.

In summary, the application of sonoporation to increase loading of cellular protectants into RBCs is a promising avenue to improve both cryopreservation and lyopreservation of human blood cells for long-term storage [34]. This study demonstrates that the sonoporation technique increases intracellular trehalose delivery and processed RBCs maintained their typical morphology and esterase activity after freezing and drying and were well tolerated in a hemorrhagic shock model in rats. However, extensive characterization of RBC biology, such as hemolysis, before and after loading and storage are critical next steps for verifying the usefulness of this method for clinical blood stabilization. Further studies are needed prior to clinical translation of these results, which nevertheless represent an important step forward in the quest to develop shelf-stable RBCs for long-term storage at ambient temperatures. In this context, experiments using biotinylated RBCs to determine the period of time processed cells remain in circulation after transfusion are currently being performed.

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Declaration of competing interest

Some of the authors (B.R.J., J.A.K., M.A.M.) are co-inventors on a US

patent application (62/519,638) and have a controlling interest in a startup company focused on commercializing the technology presented here.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2020.12.005.

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