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Freeze-drying, or lyophilization, has shown great promise in addressing many of the logistical challenges of storing and preserving red blood cells (RBCs). A crucial part of any future RBC lyophilization protocol is the primary drying temperature, which affects the sample drying rate and the dried cake's ability to form a stable glassy solid which is critical to long-term RBC survival. The optimal temperature for primary drying is just below the temperature at which the porous structure of the cake begins to collapse, known as the cake collapse temperature. Therefore, this study utilized Freeze-Dry Microscopy of intracellular Trehalose-loaded RBC suspended in a buffer solution containing Human Serum Albumin (HSA) to determine the effect of both RBC and HSA concentration on the measured cake collapse temperature. It was found that increasing RBC concentration from 5% to 20% hematocrit raised the cake collapse temperature from -36.4°C ± 0.3 to -34.8°C ± 0.2, while increasing HSA concentration from 0 to 5% (w/v) raised the cake collapse temperature from -34.8°C ± 0.2 to -20.4°C ± 1.5. This data provides a basis for future study of the relationship between cake collapse and overall cell survival, with the object of building a clinically-viable RBC lyophilization protocol.

The Effect of HSA and Hematocrit on the Cake Collapse Temperature of Lyophilized Red Blood Cells

by

Daniel E. Runyon

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Daniel E. Runyon, Author

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Introduction

Every two seconds, someone in the United States requires a blood transfusion[1]. Red Blood Cells (RBCs) are the most commonly transfused blood product and 15 million units are consumed in the United States annually, serving 300 million people.[2] Despite the critical and constant demand for blood, the longest a red blood cell can be stored safely is 6 weeks[3]. This short shelf life means that blood banks require regular replenishment and cannot be stockpiled effectively. This leads to seasonal shortages, especially in rare blood types, and presents logistical problems in the case of natural disasters. Blood storage is even more problematic in less advanced countries and conflict zones where access to the necessary refrigeration is either limited or nonexistent, greatly decreasing shelf life and dangerously restricting supplies. A better solution to blood storage that circumvents both the need for refrigeration and the limits of traditional shelf life is critical to improving the supply chain of one of the most vital components of medicine.

Freeze-drying, or lyophilization, conceptually provides the ideal solution. By decreasing pressure while the cells are in a glassy state, the cells are completely stripped of their water content without chemically responding to the desiccation stress or altering their functionality. If properly achieved, freeze-dried cells are placed in suspended animation, where normal cell degradation is halted and bacterial infection is almost impossible, allowing cells to be stored at room temperature safely for up to 15 years. This way, blood could be stockpiled and transported like any other medical supply without the additional complexity presented by refrigeration, limiting both the waste from expired blood and the need for continuous resupply. This would also drastically decrease the space and energy requirements for blood storage, since only a minimal amount of reconstituted and refrigerated blood would need to be kept on hand at any

given time. Finally, since the blood would be stored for most of its time in a virtually infection-free state, cases of sepsis due to blood tainted in storage would be drastically reduced.

Freeze-drying has been applied with great success in the preservation of pharmaceutical drugs, allowing their sensitive active ingredients to be stored at room temperature for years without fear of degradation. Advancement has also been made in the freeze-drying of human blood platelets, another critical component of human blood required for transfusions, by intracellular loading of trehalose at 37 degrees, resulting in a reconstituted survival rate of 85%.[4] However, considerable hurdles exist in developing an adequate freeze-drying protocol for RBC which produces acceptable cell recovery upon dehydration. One of the most significant factors is hemolysis, the leakage of hemoglobin from ruptured cell membranes which occurs both during freeze-drying and upon rehydration. This is rupture is due to damage in the cell membrane which occurs when the water is removed in the absence of a protectant[5]. Cells with ruptured membranes cannot survive, which is why percent hemolysis is one of the most important factors in determining RBC viability. Another key factor complicating RBC freeze-drying is hemoglobin oxidation, a reaction by which standard hemoglobin is converted to methemaglobin, which is unable to reversibly bind oxygen, its primary function[6]. Addressing these two issues is critical to any successful RBC freeze-drying process.

Optimizing primary drying temperature is also very important to establishing an effective and efficient freeze-drying protocol for RBC. Primary drying refers to the first phase of the freeze-drying process where ice is sublimated into vapor[7], reducing cell water content to less than 10%. This is the longest and most stressful part of the freeze-drying process for the cells and thus nearly every study on RBC lyophilization focuses on improving cell survivability in this step. Since the lyophilization process is slower and more expensive at lower temperatures,

especially below -40°C, the temperature at which primary drying occurs is important to efficiently dry the cells[8], while ensuring the cells remain in a glassy state, and the dried cake remains intact. The optimal primary drying temperature is found at the highest temperature possible which is both below the solution's glass transition temperature and just below its cake collapse temperature. Thus the determination of cake collapse temperature is the first step in estimating primary drying temperature.

During primary drying, sublimation of the ice crystals leaves behind a porous matrix of dried sample known as the cake. Under a Freeze-dry microscope, this appears as an advancing dark "drying front" which moves radially into the center of the sample as drying progresses, as seen in **Figure 1**. Cake collapse is evidenced when the voids in the dried cake formerly occupied by sublimated ice crystals cannot be supported in a porous structure by cake (**Figure 2**).[9] This weakening of the porous structure occurs at temperatures within 5°C of the glass transition temperature of the dried cake. Unfortunately, the exact nature of physical changes in cake collapse is not well understood and strongly debated due to the complex structural mechanics of glassy materials and thermodynamics involved. However, it has been shown definitively that cell suspensions which have undergone cake collapse have significantly higher water content, are harder to rehydrate, and experience lower cell survival than similar suspensions freeze-dried above the cake collapse temperature.[9]

Figure 1- Stable primary drying of a sample of 20% hematocrit trehalose-loaded red blood cells. The dark section is the dried cake and the frozen sample is the lighter section with dots. The interface between the two is known as the drying front which travels radially in towards the center of the sample. This occurs at temperatures like the one overlaid in the upper left-hand corner which are below the cake collapse temperature, but not so low that the pressure cannot sublimate the ice crystals and dry the sample.

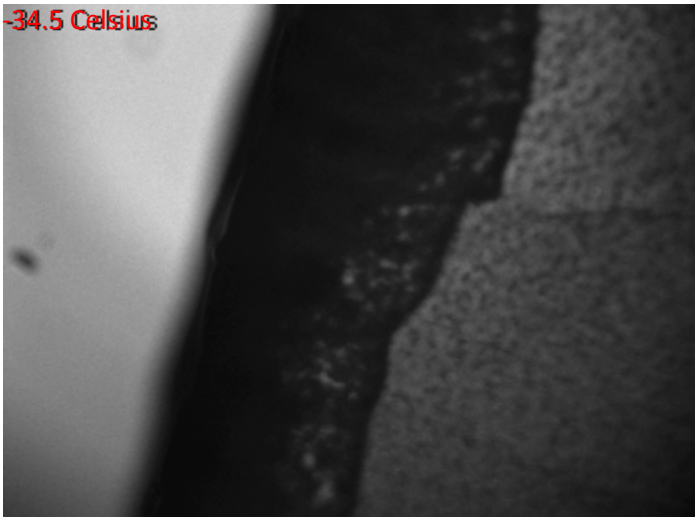


Figure 2- Cake collapse in a sample of 20% hematocrit trehalose-loaded red blood cells. The white voids in the cake, the dark section of the sample, are where the porous structure has collapsed. This occurs at temperatures, like the one overlaid in the top left corner, which are above the overall cake collapse temperature.

The standard method for determining cake collapse temperature is freeze-dry microscopy. A freeze-dry microscope uses a temperature controlled stage to quickly freeze the sample and then slowly heats the sample (~10°C/min) in a low pressure environment until a dried cake is formed. Then the sample is heated slower still (~0.1 °C/min) until visible evidence of cake collapse occurs. The process is recorded in video format and then synced with stage temperature data to determine exact cake collapse temperatures for the sample. From these values, a rough estimate of the most efficient primary drying temperature can be made, generally 1° C below the cake collapse temperature.

There have been many studies into lyoprotectant strategies for freeze-drying RBCs and at least one into the application of freeze-dry microscopy in determining the cake collapse temperature of live cell solutions but so far no study has combined the two in an attempt to assess primary drying temperatures using lyoprotected RBCs. This study intends to act as a bridge between the two, determining the effect of hematocrit and extracellular excipients on cake collapse, and therefore primary drying temperature. These relationships will provide a method to achieve ideal primary drying conditions at whichever temperature other studies discover is best for achieving maximum RBC viability throughout the freeze-drying process.

Literature Review

Lyoprotectant Strategies

Many methods have been considered for stabilizing the RBC membrane during freeze drying. A study by Bakaltcheva et al.[10] treated RBCs with reversible cross-linking reagent dimethyl 3,3-dithiobispropionimidate (DTBP) which significantly decreased deformation and membrane osmotic fragility. In fact, cells treated with 10 mM DTBP for 5 hours or more

showed no hemolysis when rehydrated in distilled water, as opposed to 100% hemolysis for non-cross-linked cells. Most importantly, the cross-linking is reversible with incubation in 10mM dithioerythritol (DTE) for 20 minutes. However, complete reversibility was only seen in RBCs treated with 5 mM of DTBP for 1 hour or less, treatments which did not perform nearly as well in osmotic fragility and deformation tests. Loss of cell deformability in cross-linking process can cause blockages and significant reductions in circulatory flow, so 100% reversibility is critical. Unfortunately, this method of reversible cross-linking has not shown sufficient reversibility without sacrificing its lyoprotective functionality.

Baklatcheva et al. also considered the potential of binding Carbon Monoxide to the RBC hemoglobin prior to freeze-drying to limit oxidative damage to hemoglobin during the freeze-drying process. Cells treated with CO did retain the ability to reversibly bind oxygen after CO was removed, a critical test of its viability as a treatment option. However, this method requires an additional step to remove CO before the blood is available for transfusion, a delay which might not be acceptable.

Another study by Zhou et al.[11] considered intracellular loading of glycerol as a treatment option and found that 53.5% hemoglobin recovery was possible with 40% glycerol. The glycerol also slowed the degradation of important antioxidant enzyme activity during storage, although it could not halt the process. This suggests that intracellular glycerol could greatly decrease the rate of hemoglobin oxidation that occurs in RBCs upon reconstitution. However, no research has yet been performed on the long-term effect of intracellular glycerol on RBC functionality.

Many studies have focused around the lyoprotective potential of different sugars, primarily glucose and trehalose. Glucose and trehalose have the distinct advantage of

metabolizing easily in the blood stream, thus providing no health concerns and therefore no need for an additional step before transfusion like cross-linking reversal or CO removal. Glucose was considered first mainly due to its easy intracellular loading. Monosaccharides are easily diffused into RBC at room temperature, unlike disaccharides like trehalose.

Glucose's membrane stabilization potential has been considered in studies by Han et al.[12], Yu et al.[13], and Crowe et al.[14], as well as in a patent by Goodrich et al.[15] Hemoglobin recovery rates for glucose-loaded RBCs have been reported as high as 70%[12], although extracellular excipients were also credited in this, notably 40 wt% Polyvinylpyrrolidone (PVP) and 25 wt% Human Serum Albumin (HSA). Research by Crowe et al[14] has supported claims made in patents by Goodrich et al.[15] suggesting that glucose-loaded RBCs have potential when freeze-dried in a buffer containing Hydroxyethyl Starch (HES), which lowers the glass transition temperature to ensure the sample remains in the glassy state throughout drying. Both Han et al.[12] and Yu et al.[13] discovered much higher hemoglobin recovery and cell survival in cells loaded with glucose than cells lyophilized with extracellular trehalose, but these studies did not consider intracellular trehalose.

Trehalose has been widely considered as a frontrunner in solving the difficulties caused by freeze-drying RBCs. Trehalose is found in high concentrations (as high as 20% dry weight) in many organisms that survive dehydration in a state known as "anhydrobiosis", life without water. These organisms include ordinary baker's yeast, resurrection plants, cysts of certain crustaceans, many bacteria, and some microscopic animals like Tardigrades, which can survive up to a decade without water[16]. Needless to say, these remarkable properties have sparked considerable interest in intracellular trehalose as a lyoprotectant. Unfortunately, RBC membranes are not permeable to trehalose at room temperature, making the loading of trehalose

into the RBC's cytoplasm a considerable difficulty. Considerable research has gone into finding alternatives to this problem, with methods from high-intensity electrical pulses to genetic engineering.

Zhou et al.'s technique of electroporation, the use of high-energy electrical pulses to reorient phospholipid molecules and increase membrane permeability did improve trehalose uptake significantly, but also caused cells to leak hemoglobin at higher field strengths and pulse durations. Also, electroporation is unlikely to be feasible in preparing large batches of RBC for freeze drying in future commercial and medical applications. Roudolph et al.'s patent applies glycerol as a permeabilizing agent, yielding a cell recovery of around 55%, though no data on intracellular trehalose concentrations is given. Unfortunately, the glycerol would have to be washed from the cells prior to transfusion due to osmotic imbalance which can lyse cells at room temperature, adding a step prior to transfusion. Studies by Oliver et al. and Wolkers et al. have employed endocytosis on blood platelets but unfortunately this method cannot be applied to RBC.

Erglou et al.[17], Chen et al.[18], Gou et al.[19], and Puhlev et al.[20] have all endeavored to apply genetic engineering to the problem. Erglou and Chen utilized a genetically engineered pore-forming protein which increased membrane permeability, allowing intracellular concentrations of up to 0.4 mM trehalose in human 3T3 fibroblasts. Unfortunately, this method has not yet been applied to freeze drying of RBC in particular. Gou and Puhlev genetically engineered human fibroblasts to express the trehalose synthase gene, resulting in significant improvements to cell desiccation tolerance. However, cell viability dropped off significantly over time, making it a poor solution for long term storage, a key feature of freeze-dried RBC. Also, only 0.3-0.4 % dry weight trehalose was ever achieved, much lower than the 20% found in desiccation-resistant

yeasts mentioned in Crowe et al[16]. Genetic engineering of the RBCs themselves will also not be practical for clinical applications, as RBC functionality could be altered by the new gene.

The simplest and most effective solution for trehalose loading, discovered by Satpathy et al.5, is to incubate the cells in an 800 mM trehalose loading buffer at 37° C. This method takes advantage of the cells' increased membrane permeability at this temperature, allowing cytoplasmic concentrations of up to 55mM. The 7 hour incubation required to achieve this concentration is cumbersome, but since it occurs prior to freeze-drying when time is less constrained and demand is not immediate, the time required is less significant. This loading method is used in studies by Torok et al.[21], Lynch et al.[22], and Kheirolomoom et al.[23] to great effect, both as a membrane stabilizer and a hemoglobin oxidation inhibitor.

Significant studies by John Crowe's lab at UC Davis (Crowe et al[24], Torok et al[21], Satpathy et al.5, Kheirolomoom et al[23]) have investigated the potential of intracellular trehalose as both a lyoprotectant and hemoglobin oxidation inhibitor. Crowe et al. found that trehalose protects the membrane by lowering its melting temperature in the dry state, preventing a phase transition caused by the drying which compromises its integrity. Trehalose also raises the cell's glass transition temperature, improving the cell's ability to freeze in the glassy state, another critical factor in cell survival. However, these effects are only significant when trehalose is introduced directly into the cytoplasm of the RBC. This became possible through the work mentioned above by Satpathy et al., whose work became the basis for several future experiments. Torok et al. found that RBC loaded with trehalose in the manner used by Satpathy et al. and lyophilized in a buffer containing 100mOsm ADSOL, 15% HES, 2.5% HSA, and 100mM trehalose yielded a survival rate of around 55%. Optimal residual water contents were found to be between 2 and 4%. Hemoglobin oxidation inhibited by trehalose was further reduced by the addition of 5mM

ascorbic acid in the loading and rehydration buffers, reducing oxidative damage to around 10%. Cell functionality measured by levels of 2,3 DPG and ATP remained stable in lyophilized cells as compared to fresh. Kheirolomoom et al. improved on this technique by adding 0.44 mM unilammelar EPC liposomes to the lyophilization buffer which help support the phospholipid bilayer during lyophilization, preventing hemoglobin leakage, resulting in an unprecedented RBC survival rate of 70%. From these studies, intracellular trehalose, combined with lyophilization buffer solutions containing HES, HSA, and Liposomes and rehydrated in a buffer containing ascorbic acid is likely to produce the most functional, stable cells after freeze-drying.

Effect of Hematocrit on Cell Recovery

The effect of cell concentration of RBC in the lyophilization buffer, hematocrit, has been only sparsely studied, with most studies using a standard 5% hematocrit exclusively. However, research by Arav et al[25] has suggested that, while cell recoveries improve with decreasing hematocrit, the ratio of extracellular trehalose and other lyoprotectants to cell concentration plays a more significant role. The ideal ratio for extracellular trehalose to RBC concentration was found to be 2.83 mg trehalose to 1 mg RBC, corresponding to an extracellular trehalose concentration of 0.1 M in 4% hematocrit. However, Arav's study was limited by a lack of intracellular trehalose loading which may explain why high recovery rates were only found in cells rehydrated in 20% Dextran rather than a more suitable diluted rehydration medium. The effect of this relationship on intracellular trehalose-loaded RBC has not been studied.

Importance of Optimization of Primary Drying Temperature

With all this attention being directed at optimizing the lyoprotectant strategy, surprisingly little research has gone into optimizing the primary drying temperature during lyophilization.

One study by Rindler et al.[26] using 10% hematocrit cells suspended in a solution of hydroxyethyl starch (HES) and maltose found that freeze-drying the sample at too low of a temperature causes the sample to dry slowly and possibly incompletely, even after drying for 72 hours at -45°C. However, drying the cells at too high a temperature and significant structural damage to the cells occurs, causing hemolysis. This may be because the sample experiences cake collapse, a structural breakdown of the dried section of the sample which has been shown to produce lower cell recovery and higher water content in the dried cake. Cake collapse is related to and occurs right around the sample's glass transition temperature, a property which changes with buffer composition. Indeed one of the objects of using excipients like HSA and HES is to raise this glass transition temperature. Unfortunately, this study did not actively measure cake collapse; it only determined RBC hemolysis at different primary drying temperatures to determine an ideal primary drying temperature. No studies have been found that consider the effect of lyoprotectants and excipients on cake collapse to come up with a more informed primary drying temperature which maximizes cell viability while delivering efficient drying.

Use of Freeze-dry microscopy in optimization of Primary Drying

Recent research by Yang et al.[9] has used Freeze-dry microscopy (FDM) to determine a significant influence of extracellular trehalose on cake collapse temperature, especially when concentrations are less than 200 mM in the lyophilization buffer. Lower trehalose concentrations produced lower overall cake collapse temperatures (-30.2°C in 0.05 M trehalose vs. -28.5°C in 0.8 M trehalose). Fetal bovine serum and mannitol were shown to increase cake collapse temperature, but only in solutions with lower trehalose concentrations. Finally, cell density within the range of 10^6 to 10^7 cells/mL was shown to have negligible effect on cake collapse. This study was performed with 3T3 fibroblasts rather than RBCs and did not investigate the

effects of excipients such as HSA or HES, which limits its utility, but its results did confirm that FDM results on cake collapse temperatures are accurate representations of large scale freeze-drying and that the composition of the lyophilization buffer does have considerable effect on cake collapse and, by extension, ideal primary drying temperature.

Object of this study

After extensive research on RBC freeze-drying strategies, this study will use RBCs loaded with trehalose following the procedure devised by Satpathy et al5 to test the effect of varying hematocrit and concentrations of Human Serum Albumin in the lyophilization buffer on cake collapse temperature with the object of optimizing primary drying temperature, an essential part of a comprehensive lyophilization protocol. This study also hopes to call attention to the potential for freeze-dry microscopy in determining ideal primary drying temperatures and the extent to which extracellular excipient concentrations and formulations affect primary drying temperature.

Thesis Statement

The addition of extracellular excipients like Human Serum Albumin, due to their high glass transition temperatures, causes a statistically significant increase in the cake collapse temperature of trehalose-loaded RBC in a manner which is concentration dependent, while increasing the hematocrit of RBC increases the cake collapse temperature by raising the average particle size, reducing the entropy of the sample and supporting greater stability in the glassy state.

Materials and Methods

Composition of Loading and Buffer solutions

The 1X Dulbecco's Phosphate-buffered saline (Invitrogen) used contains 800mg/L sodium chloride, 200mg/L potassium chloride, 100mg/L calcium chloride, 100 mg/L magnesium chloride, 200 mg/L monopotassium phosphate, and 2160 mg/L monosodium phosphate.

The loading buffer contains 100 mOsm ADSOL diluted from a 462 mOsm stock solution (111mM glucose (Mallinckrodt), 2mM adenine (Sigma) , 154 mM sodium chloride (Macron), and 41 mM mannitol VWR), 800 mM trehalose (Alfa Aestar), and 6.6 mM k-phosphate.

The lyophilization buffer contains 100 mOsm ADSOL, 100mM trehalose, 6.6 mM k-phosphate (Mallinckrodt), and up to 10% Human Serum Albumin (MP Biomedicals), except in control runs in which PBS was used.

All buffers were prepared and added to the RBCs using sterile methods under a laminar flow hood (The Baker Company Steril Gard III Advance).

Blood collection and separation

Whole human blood was collected from healthy adult volunteers with informed consent and stored at 4°C for less than 2 weeks. Erythrocytes were separated from the platelet-rich plasma by centrifugation in an Eppendorf 5415d centrifuge at 2000 rpm for 14 minutes. The erythrocytes were then washed three times in 1X PBS for 10 minutes at 2600 rpm before being stored at 65% hematocrit in 1X PBS for use within 2 days.

Trehalose Loading

PBS was removed from the blood using centrifugation at 2600 rpm for 10 minutes. Following the procedure set forward by Satpathy et al.5, the cells were then suspended in the loading buffer

at 30% hematocrit, gently mixed, and incubated in a VWR 2310 MINI CO_2 Incubator at 37° C for 7 hours.

Lyophilization Sample Compositions

After incubation, the cells were centrifuged at 5000 rpm for 1 min to remove the loading buffer, and the lyophilization buffer was added to produce solutions of varying hematocrit and Human Serum Albumin concentrations. Samples of 0, 5%, 20% and 40% hematocrit were tested in triplicate in buffers without Human Serum Albumin to test the effect of RBC concentration on cake collapse. An experimental control was also performed at 20% hematocrit with PBS as the buffer solution to determine the effect of extracellular trehalose, mannitol, glucose, and the other excipients in the buffer on cake collapse. The effect of Human Serum Albumin was then assessed with 2.5%, 5%, and 10% (w/v) HSA added to the lyophilization buffer solution of 20% hematocrit.

Freeze-Dry Microscopy Protocol

5µL samples were auto-pipetted onto slides placed on the temperature-controlled silver block of a Linkam FDCS 196 Cryostage in a Lecia DM 2500 Freeze-dry microscope. A cover slip was placed on top of the sample separated by a spacer, carefully working to avoid air bubbles, and the sample area was sealed. The sample was then cooled to -50°C at 50°C/min by a Linkam liquid nitrogen pump controlled by a Linkam CI 94 controller using the Linksys 32 computer interface. After confirmation that the sample was completely frozen, a vacuum pump was turned on and the sample temperature was raised to -40°C at 4°C/min or until a drying front began to form and then raised slowly at 0.1 °C/min until the cake collapsed. Temperatures of micro and overall collapse were noted, as well as sample pressure. Microcollapse was recorded when voids

first began to form in the cake and overall collapse was noted when voids became more prevalent and began to expand and connect to other voids, leading to a total loss of structure. This definition of collapse conforms to those used by Fonseca et al[27] and Yang et al[9]. Results were confirmed by a QImaging Qicam whose image-sequenced video was overlaid with temperature control data. Images of the equipment setup can be seen below in **Figure 3**.

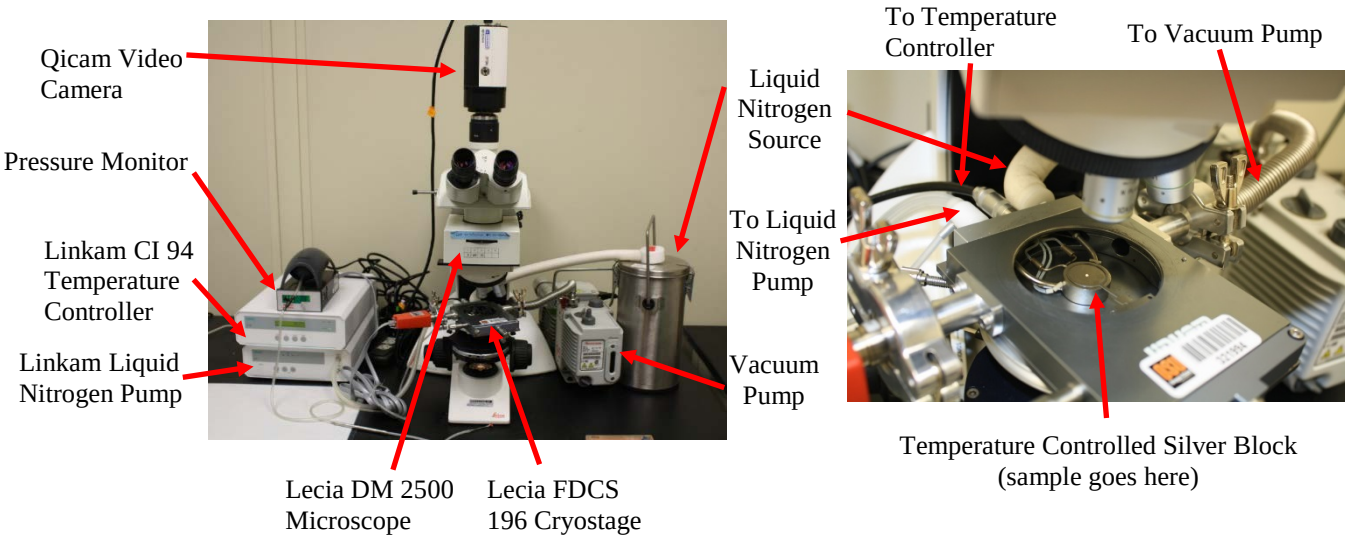


Figure 3- Equipment setup with a close-up view of the cryostage in which the freeze drying takes place.

Assay Controls

Several assay control tests were conducted to ensure accurate temperature control reading from the Freeze-Dry Microscope. Instead of the lyophilization buffers mentioned above, solutions of 5% (w/v) sucrose, 10% (w/v) saline, and pure deionized water were prepared. Cake collapse temperature recorded of the sucrose solution, eutectic point of the saline, and melting point of the deionized water were all compared to literature values to determine Freeze-Dry Microscope systemic error.

Statistical analysis

Standard error was used for all error bars and significance between data sets was determined by 2-tailed t-test (n=3 for all data).

Results

Assay Controls

Three assay control tests were performed to judge the accuracy and precision of the microscope's temperature control system. The first control test assessed the cake collapse temperature of a 5% (w/v) sucrose solution in pure deionized water, yielding a mean overall cake collapse temperature of -32.7 °C ± 0.6, shown in **Figure 4**, which compares well with the reported value in literature, -33 °C[27], falling within the standard error. A 2-tailed t-test confirmed that there was no significant difference between the cake collapse temperature and the literature value ($p<0.05$).

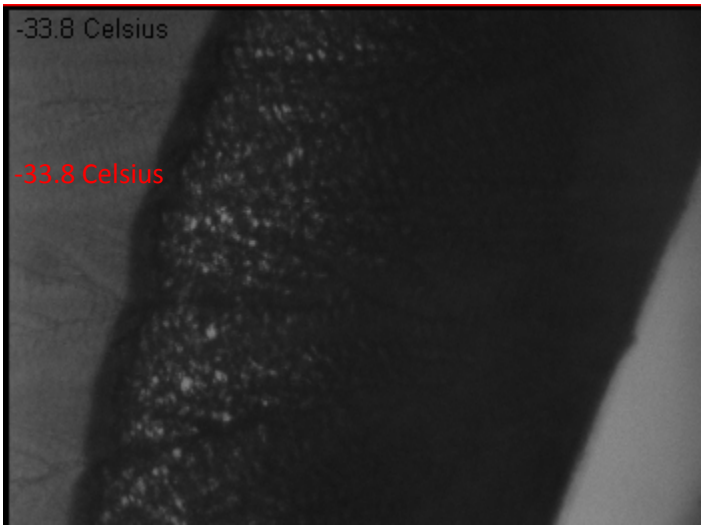


Figure 4- Overall cake collapse of 5% (w/v) sucrose solution. Measured collapse temperature is overlaid in the upper right hand corner. Due to some delay between the temperature control data

and the video imaging, there may be up to a 0.3°C difference between overlay and recorded data points values.

A second control test assessed the melting point of deionized water, which was measured at -0.333 ° C ± 0.033. Since it is well known that water melts at 0°C, this result was quite troubling given it's significant difference ($p>0.05$) and a calibration adjustment was considered. However, a third test was devised to see if this systematic error occurred at the temperatures of projected cake collapse, hypothesized to be somewhere between -40 and -20 °C. This test measured the eutectic point of a 10% saline solution in DI water, which has a well-established value of -21.1 °C[28]. The microscope measured a eutectic point of -20.9 °C ± 0.088, shown in **Figure 5**, which was not significantly different from the literature value ($p<0.05$). Therefore, given that the temperature performed accurately at the temperature range of interest, no calibration adjustment was deemed necessary. However, the wide confidence interval of the first measurement suggests a limit to precision in future cake collapse temperatures.

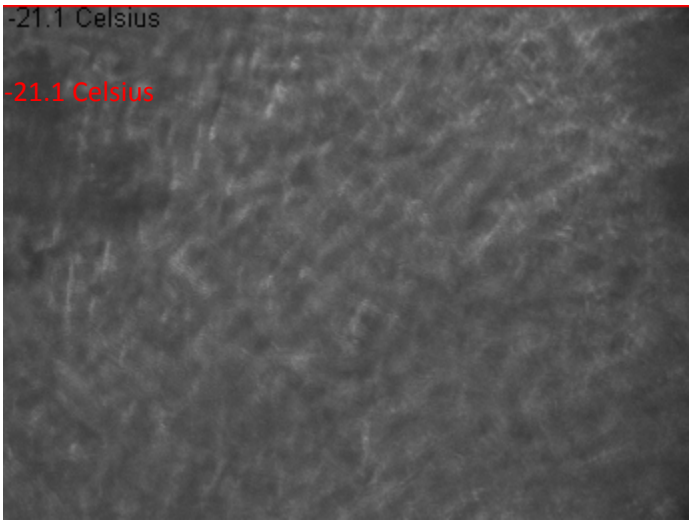


Figure 5- Eutectic Point of 10% (w/v) saline solution. Measured eutectic transition temperature is overlaid in the upper right hand corner. Note the blurring over the crystalline structure. This is

caused by a brine solution which has begun to melt and rests over the ice, denoting the eutectic point has been reached.

Effect of Hematocrit on Cake Collapse

After observing cake collapse temperatures at hematocrits of 0, 5, 20, and 40%, increasing RBC concentration significantly increased overall collapse temperatures ($p<0.05$) from -37.8°C ± 0.1 in the pure lyophilization buffer to -34.8 °C ± 0.2 in the 20% hematocrit solution, as seen in graph and visual form in **Figures 6-9**. Unfortunately, the 40% hematocrit solution was too opaque to determine visually whether a cake had formed or collapsed using the current microscopy method. Further studies using alternative techniques will need to be performed to analyze cake collapse for hematocrits higher than 20%.

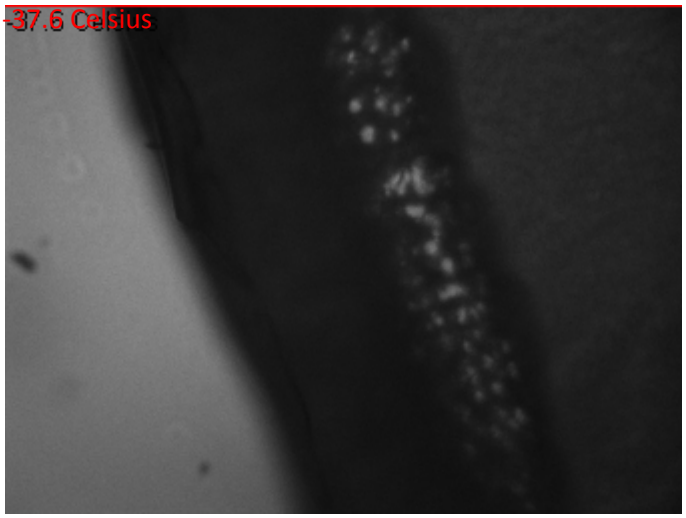


Figure 6- Overall Collapse of Lyophilization Buffer without RBCs (0% hematocrit). Measured collapse temperature is overlaid in the upper left hand corner.

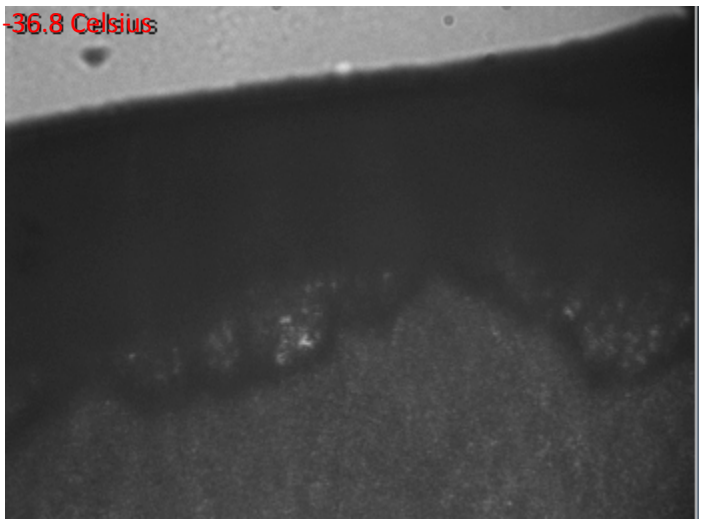
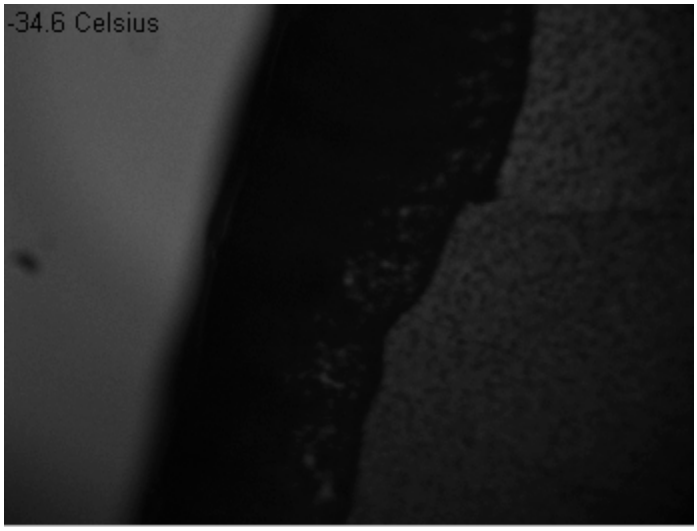


Figure 7- Overall Collapse of 5% hematocrit in lyophilization buffer. Measured collapse



temperature is overlaid in the upper left hand corner.

-34.6 Celsius

Figure 8- Overall Collapse of 20% hematocrit in lyophilization buffer. Measured collapse temperature is overlaid in the upper left hand corner.

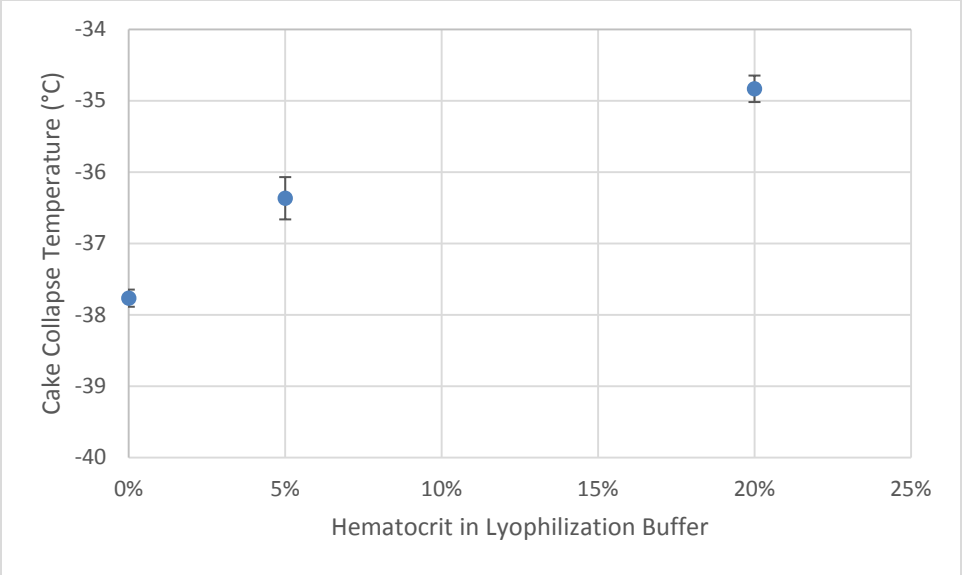


Figure 9- Mean overall collapse temperature at varying hematocrit in the lyophilization buffer (No HSA). 40% hematocrit is absent due to the opacity of the sample at high cell concentrations interfering with observation of cake collapse. Error bars represent the standard error of all 3 replicates.

Effect of Extracellular HSA on Cake Collapse

Cake collapse was then measured at 2.5, 5, and 10% (w/v) of Human Serum Albumin (HSA) in the lyophilization buffer, keeping hematocrit constant at 20%. Even adding minimal amounts of extracellular HSA significantly increased the sample's overall cake collapse temperature ($p<0.5$) from -34.8 ± 0.186 °C without HSA to -25.4 ± 1.2 °C with just 2.5% (w/v), as seen in **Figure 10**. Further addition of HSA provided a smaller, yet significant increase ($p<0.5$), to -20.4 ± 1.5 °C. However, additional HSA up to 10% (w/v) did not significantly affect the overall collapse temperature ($p>0.5$). No significant morphological differences were visible between the cake collapse of RBCs lyophilized with HSA and that of RBCs lyophilized without HSA (**Figures 11-**

12). Other excipients in the buffer also appear to play a role in cake collapse as well. Cells suspended in a 1X PBS buffer at 20% hematocrit experienced cake collapse at -38.6 ± 0.4, more than 5° C below that of 20% hematocrit RBC in the ADSOL-trehalose buffer (**Appendix I**).

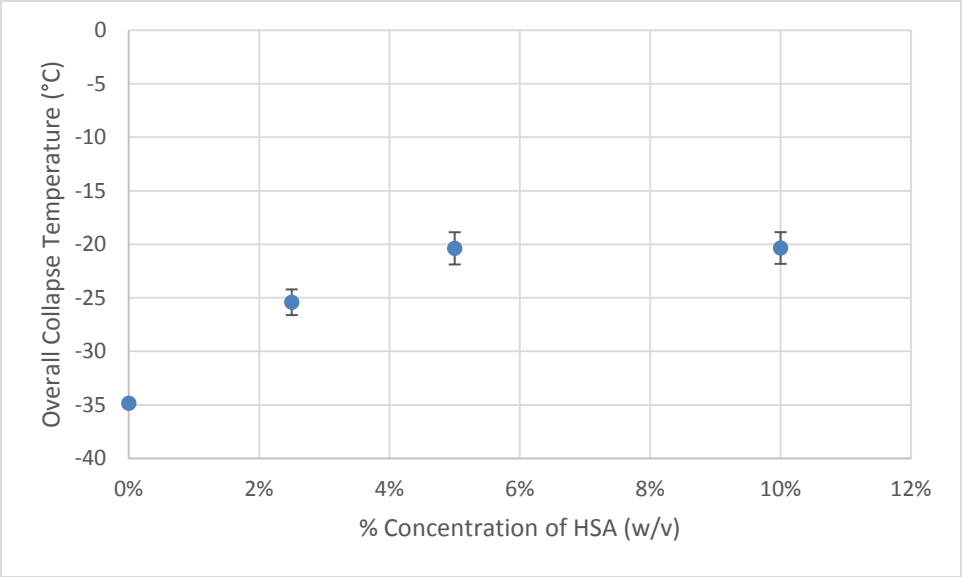


Figure 10- Overall Cake Collapse Temperatures of RBCs treated with varying concentrations of Human Serum Albumin. Error bars represent standard error of 3 replicates. Each HSA-treated sample contained one outlier which greatly expanded the error. This is likely due to difficulty achieving good mixing, thus increasing the possibility of uneven concentrations of HSA throughout the sample.

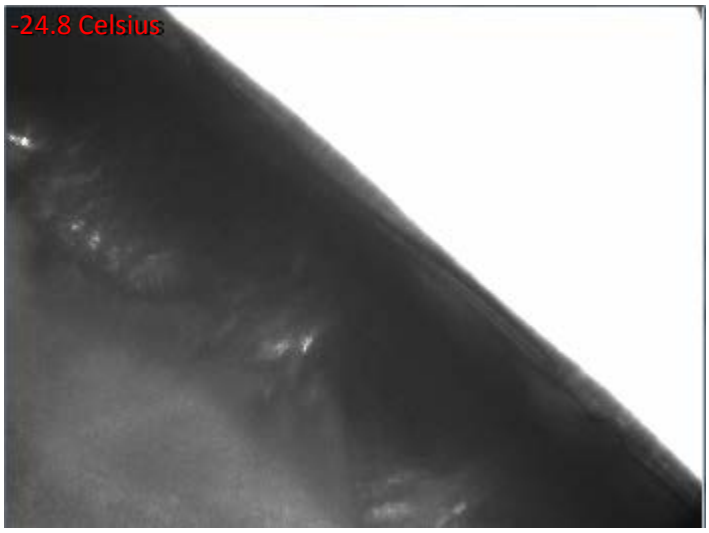


Figure 11- Overall Collapse of 20% Hematocrit RBC Lyophilized with 5% HSA in the buffer. The measured Collapse Temperature noted in the upper left corner is a few degrees high due to overlay syncing problems.

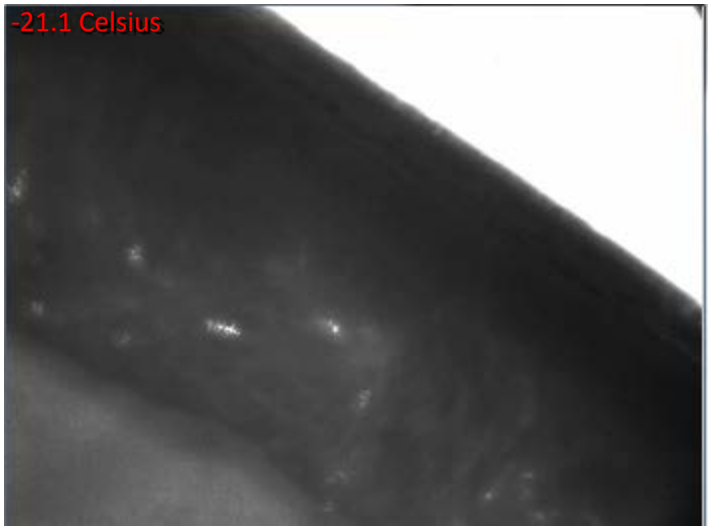


Figure 12- Overall Collapse of 20% Hematocrit RBC Lyophilized with 10% HSA in the buffer. Measured Collapse Temperature noted in the upper left corner.

Discussion

This study used Freeze Dry Microscopy to determine the effect of increasing concentrations of excipients such as Human Serum Albumin in the lyophilization buffer, as well as hematocrit levels, on the cake collapse temperature of trehalose-loaded RBC. As most studies in RBC lyophilization focus on measuring survivability metrics, measuring cake collapse temperatures may seem a secondary concern affecting only the optimization of a process not yet proved viable. In fact, cake collapse temperature has significant impact on these very studies. Since cake collapse has not been thoroughly investigated, these studies perform primary drying at a semi-arbitrary temperature, generally below -35 °C. While this decision will certainly guarantee formation of a solid cake with most buffer formulations, the drying process is drawn out considerably due to the inefficiency of freeze-drying at a lower temperature than necessary. Since it is unlikely that any final RBC lyophilization process will occur at such inefficiently low temperatures, further proof of concept studies should consider the effects of higher primary drying temperatures and shorter drying times on hemolysis and hemoglobin oxidation. This study intends to provide a basis for such studies by characterizing the upper temperature limits of one particular RBC freeze-drying formulation currently in the literature.

For this reason, cells loaded with intracellular trehalose were used in this study, one of the more successful and promising methods of enhancing RBC survivability pioneered by Satpathy et al[5] and used by Torok et al.[21], Lynch et al.[22], and Kheirolomoom et al.[23] to produce high survival rates. The effect of HSA, a key extracellular excipient used in many of these studies, was also considered. Unfortunately, due to time constraints and acquisition difficulties, the effects of other excipients such as Hydroxyethyl Starch and phospholipid vesicles on optimal primary

drying conditions were not considered and should be investigated in a future study. Still, by copying a validated lyophilization protocol, these cake collapse temperatures will have significant relevance to any study wishing to examine this process at higher primary drying temperatures.

The first element of the lyophilization protocol investigated in this study, hematocrit, is one of the least studied components of RBC lyophilization, with most research in literature content with keeping it at a constant 5%. However, any future lyophilization protocol will have to operate at higher hematocrits to be efficient enough to be commercially-viable and generate a product that can, with minimal concentration, be administered in a clinical setting for a blood transfusion, which typically require hematocrits of between 55 and 65%[29]. If RBCs were lyophilized at 5% hematocrit, the minimum concentration ratio would be 11:1, making it an extremely inefficient process. If any reasonable RBC lyophilization process is to be suggested, it must occur at a much higher hematocrit. This study characterized the effect of cake collapse temperature, a definitive metric in determining the temperature at which the RBCs go through the critical primary drying phase, on hematocrit and discovered that with increasing hematocrit, cake collapse temperatures also increased.

This trend was surprising, because it was initially believed increasing the concentration of RBC would lower the solution's glass transition temperature, and thus its cake collapse temperature. However, the idea that RBCs would lower the cake collapse temperature was formed mostly on the logic that, if researchers had tried adding excipients with high glass transition temperatures to the buffer, why would they not consider using higher cell concentrations if they did increase cake collapse. This logic failed to account for the possibility that glass transition temperatures for cell suspensions were unknown or not considered in these studies.

The rise in cake collapse temperatures at higher hematocrits may be a function of increased amounts of trehalose in the solution located in the cytoplasm of the RBCs present at higher cell concentrations which raises the mean glass transition temperature of the sample. That would help to explain why this result does not directly contradict the work of Yang et al.[9], who determined that increasing cell concentration of 3T3 Fibroblasts from 10^6 to 10^7 cells/mL had no effect on cake collapse temperature. Since the 3T3 Fibroblasts did not contain trehalose, increasing cell concentration would not have affected the glass transition temperature significantly, especially at the low cell concentrations considered in that study. It is also possible that the addition of high molecular weight solids like cells would decrease the net entropy of the solution, providing improved stability to the glassy state. Many large molecules like HSA and HES have high glass transition temperatures and work by Carpenter et al.[8] has suggested that increased concentrations of proteins, another large molecule, can also increase the solution glass transition temperature which would naturally yield higher collapse temperatures.

This relationship in trehalose-loaded RBCs is important to consider for optimizing primary drying temperature in future RBC freeze-drying applications which may wish to freeze-dry RBCs at higher, more economically-efficient and clinically-relevant hematocrits than those currently studied in the field.

This study also considered the effect of Human Serum Albumin on cake collapse temperatures, a common excipient in several studies[12, 21, 23] focused on RBC survivability. However, none of these studies investigated its link to cake collapse temperature, only glass transition. In this study, HSA was shown to significantly raise cake collapse temperature, especially at low concentrations, in a manner that was concentration-dependent, producing cake collapse temperatures as high as -20.4 ± 6.48 °C with only 5% (w/v) HSA in the lyophilization buffer.

It is important to note the considerable variation in HSA-related cake collapse temperatures, resulting in standard error of nearly 1.5 °C. This is the product of single outliers likely caused by non-uniform HSA concentrations due to poor mixing **(see Appendix I for raw data)**. Future studies should increase replicates and ensure sufficient mixing of HSA into the lyophilization buffer prior to microscopy.

Despite this variance in the data, a clear increase in cake collapse temperature is seen at increasing HSA concentrations. Yang et al.[9] suggests that HSA's high glass transition temperature may considerably raise the glass transition of the lyophilization buffer, causing this corresponding increase in cake collapse temperature by increasing stability of the glassy state. It is likely that cake collapse temperatures level off at higher concentrations of HSA because the stabilizing effect of its high glass transition temperature are overshadowed by other limiting factors at temperatures above -21 °C. Further experimentation will be required to understand role of these physical phenomena on cake collapse if higher cake collapse temperatures are desired.

As this relationship between HSA concentration and cake collapse allows for primary drying to occur efficiently at much higher temperatures, it will likely lead to a significant reduction in drying time and cooling requirements which could impact future research into the development of a commercially-viable lyophilization process. The decrease in drying time alone has a number of potential benefits. Firstly, it reduces the amount of time for hemolysis and hemoglobin oxidation to occur, potentially limiting these significant challenges to RBC survivability. However, the rates of hemolysis and hemoglobin oxidation may go up significantly at higher primary drying temperatures, so further research is required to assess this possibility. Research by Rindler et al[26] suggests that hemolysis increases at temperatures beyond

an optimal temperature. However, since this study did not consider cake collapse temperature, it is unknown if this trend begins at temperatures above or below the cake collapse temeprature. Shortened drying time will also reduce all variable costs associated with the process, savings that will be further enhanced by the higher lyophilzation temperature which will require less coolant to sustain. Finally, shortening drying time will reduce the delay between blood donor and recipient, which for rare blood types can be especially critical.

The results of this study clearly confirm that the addition of HSA in the buffer solution increases the cake collapse temperature of the solution in a concentration-dependent manner, but that at concentrations beyond 10% (w/v), no significant change is detected. In contrast, measurements of collapse temperatures at increasing the hematocrit of the RBC solution also displayed an increase, contrary to initial conclusions drawn from the literature. More importantly, the significance of this increase in cake collapse temperature suggests that future lyophilization protocols should consider primary drying at higher temperatures and cell concentrations than those currently used in literature. While additional research with increased replicates and resolution, examining the effects of HES, phospholipid membranes, and other excipients on cake collapse, and correlating the effect of higher primary drying temperatures on RBC survival rate must be done before a true lyophilization protocol can be recommended, this study has laid the preliminary groundwork for a promising new field of inquiry that may lead to an effective, optimized, clinically-viable RBC freeze-drying method.

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Appendix I

Control Data

5% Sucrose in DI water

Trial	Microcollapse Temperature (°C)	Total Collapse Temperature (°C)	Mean	Standard Deviation	95% CI	Standard Error
1	-33	-31.5	-32.6667	1.040833	2.585573	0.600925
2	-33.6	-33				
3	-34.1	-33.5				

DI water melting point

Trial	Melting Point onset (°C)	Melting Point overall (°C)	Mean	Standard Deviation	95% CI	Standard Error
1	-0.8	-0.4	-0.33333	0.057735	0.143422	0.033333
2	-0.5	-0.3				
3	-0.4	-0.3				

10% saline eutectic point

Trial	Eutectic Point (°C)		Mean	Standard Deviation	95% CI	Standard Error
1	-20.9		-20.9333	0.152753	0.379458	0.088192
2	-20.8					
3	-21.1					

20% hematocrit RBC in PBS

Trial	Microcollapse Temperature (°C)	Total Collapse Temperature (°C)	Mean	Standard Deviation	95% CI	Standard Error
1	-38.4	-38	-38.625	0.684957	1.701529	0.39546
2	-39	-38.4				
3*	-39.7	-39.6				
4	-39.2	-38.5				

Lyophilization Buffer only

Trial	Microcollapse Temperature (°C)	Total Collapse Temperature (°C)	Mean	Standard Deviation	95% CI	Standard Error
1	-38.3	-38	-37.7667	0.208167	0.517115	0.120185
2	-37.9	-37.6				
3	-38	-37.7				

5% Hematocrit, No HSA

Trial	Microcollapse Temperature (°C)	Total Collapse Temperature (°C)	Mean	Standard Deviation	95% CI	Standard Error
1	-36.5	-35.8	-36.3667	0.51316	1.27476	0.296273
2	-37.1	-36.8				
3	-37.1	-36.5				

20% Hematocrit, No HSA

Trial	Microcollapse Temperature (°C)	Total Collapse Temperature (°C)	Mean	Standard Deviation	95% CI	Standard Error
1	-35.3	-34.7	-34.8333	0.321455	0.798539	0.185592
2	-35.5	-35.2				
3	-35.4	-34.6				

HSA Trials- All at 20% Hematocrit; 2.5, 5, and 10% (w/v) HSA

20% Hematocrit, 2.5% HSA

Trial	Microcollapse Temperature (°C)	Total Collapse Temperature (°C)	Mean	Standard Deviation	95% CI	Standard Error
1	-27.1	-26.6	-25.4	2.078461	5.163183	1.2
2	-24	-23				
3	-27.2	-26.6				

20% Hematocrit, 5% HSA

Trial	Microcollapse Temperature (°C)	Total Collapse Temperature (°C)	Mean	Standard Deviation	95% CI	Standard Error
1	-23.4	-22.6	-20.3667	2.60832	6.479426	1.505914
2	-18.6	-17.5				
3	-21.4	-21				

20% Hematocrit, 5% HSA

Trial	Microcollapse Temperature (°C)	Total Collapse Temperature (°C)	Mean	Standard Deviation	95% CI	Standard Error
1	-23.6	-22.5	-20.3333	2.565801	6.373802	1.481366
2	-18.6	-17.5				
3	-21.4	-21				