HHS Public Access

Author manuscript

Bioconjug Chem. Author manuscript; available in PMC 2020 January 28.

Published in final edited form as:

Bioconjug Chem. 2018 July 18; 29(7): 2150–2160. doi:10.1021/acs.bioconjchem.8b00271.

The chemistry of lyophilized blood products

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Introduction

Exsanguination can quickly occur in the setting of trauma. In the civilian and combat environment, it is the leading cause of preventable death. The central foci of hemorrhage management are bleeding control, restoration of oxygen carrying capacity, and clotting capability[1]. Concomitant to pre-hospital hemorrhage control, is the implementation of damage control resuscitation (DCR). DCR is characterized by the minimization of crystalloid fluids, hemostatic (balanced) resuscitation, and permissive hypotension. Central to this dogma is the use of hemostatic resuscitation[2].

In the field, resuscitation with conventional blood products proves difficult given the storage requirements and shelf life[3, 4]. Further, storage lesion and functional decay of stored blood products can lead to negative effects on the microcirculation. Five-day shelf lives and stringent storage conditions make traditional blood products impractical in austere military environments that, today, are commonly remote and in the extremes of temperature[5]. The increasing need for blood products in both military and civilian settings has lead to techniques for blood preservation to adapt to these environments. In particular, cryo and lyopreservation techniques have enabled blood product transport and use in these extreme conditions[2].

With the advent of novel bioconjugates, functional biomaterials, and new nanotechnologies with the potential for translation and clinical application, the question of preservation and storage is looming over the field. For many formulations, the lack of effect long term storage could limit or effectively stop translation. In light of this, we have been looking at the issues regarding preservation of complex systems, most notably blood products, to gain insights into the state of the art for preserving the function of bioconjugates.

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A brief history of blood in the field

The issues of remote damage control resuscitation are not new and solutions to overcome the logistical issues of blood product maintenance have been studied for decades. Freeze-dried (lyophilized) plasma (FDP) has been used since World War II (WWII). In addition to FDP, investigators have developed methods for red blood cell (RBC) and platelet (Plt) lyophilization and long-term storage.[3]. Although the risks of transmission curtailed its use in the US, other countries such as France, Germany and South Africa have continued to use FDP in the battlefield.

Today, only lyophilized plasma is available in the military theater and component blood product administration is the standard of care. Other cryo or lyopreserved products are investigated but not currently available in the US. While we continue to separate and cold store our blood products many would agree that the current methods for transfusion and preservation of this limited resource are archaic and in need of modernization.

Principles of Lyophilization

Lyophilization, also known as freeze-drying, is a method used for achieving stable formulations by removing solvent such as water, without disturbing the structure or function of the compound. The method is preferable in comparison to storing therapeutics in aqueous solution at room temperature to avoid degradation pathways such as hydrolysis, cross-linking, oxidation, aggregation and disulfide rearrangements. [6] Lyophilization begins with supercooling for faster freezing of the aqueous solution. Drying then occurs in two steps. In the primary drying phase, sublimation takes place, leading to conversion of ice to water vapor, the driving force being pressure difference in the system. In the secondary drying phase, the unfrozen water is removed due to diffusion of water from higher to lower concentration. [7] In Figure 1, an overview of the lyophilization process is presented.

Ideally, the lyophilized product should be able to retain porosity, avoid structural collapse, and have a high surface area to volume ratio for making rapid dissolution possible. Formulation composition plays a significant role in maintaining the structural integrity, as it determines the collapse temperature depending on individual glass transition temperature of the solute and excipients based on their mass fractions. [8] Cryoprotectants and lyoprotectants are used to prevent deactivation of proteins present during freezing and loss of biological activity during drying. Two proposed mechanisms of how these work suggest either replacement of water by sugar during the drying step due to hydrogen bond formation between protein and sugar, or by vitrification. Table 1 lists common lyoprotectants and cryoprotectants used to stabilize formulations, along with their role in stabilization. These sugars, amino acids and polyols help by providing thermodynamic stability, higher glass transition temperatures, or by stabilizing protein formulations in liquid and freeze-dried states due to fast formation of an amorphous glass phase. [9] Some of these sugars are effective cryoprotectants due to a cell’s capacity to uptake them, leading to higher cryo-survivability. [10] [11–13] Thus, cryoprotectants and lyoprotectants work towards maintaining effective lyophilization without affecting the therapeutic biological activity.

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Plasma Preservation

In trauma patients with massive exsanguination, freshly-frozen plasma (FFP) provides a large load of coagulation factors to aid hemorrhage control. [14–17] Freeze-dried plasma is of interest as it is possible to store the formulation at room temperature, and hence makes it a suitable choice in combat situations. The freeze-dried plasma manufactured by the French army is compatible with any blood type, as it is from pooled blood of more than ten donors, followed by selective neutralization of anti-A and anti-B hemagglutinins. [18] A photochemical inactivation step has been made part of the process to further reduce risk of transfusion related diseases. [19] Table 2 summarizes several studies performed on lyophilized plasma, characterizing the coagulation factors. Based on the studies, long-term storage at elevated temperatures can reduce the activity of coagulation factors and inhibitors compared to fresh frozen plasma although the efficacy of lyophilized plasma was similar to fresh frozen plasma. [20–22]

An emerging concept is using spray drying to dry the plasma into powdery form and reconstitute before administering. While coagulation factors were slightly lower compared to fresh frozen plasma for spray dried plasma resuspended in deionized water, resuspension in 1.5% glycine showed similar profile for coagulation factor compared with fresh frozen plasma. Optimum reconstitution methods and potential immunogenicity need to be evaluated for future studies. [23] Dried plasma products are not yet approved by Food and Drug Administration for use in the United States, however, the Department of Defense (DoD) and Biomedical Advanced Research and Development Authority are working on developing dried plasma along with clinical trials to establish their efficacy and similarity to fresh frozen plasma. [24, 25]

Erythrocyte Preservation

Lyophilized blood substitutes eliminate freezing requirements and offer extended shelf life. Much work has been directed towards developing lyophilized blood products having same efficacy as fresh blood products, but at the same time for making these available in austere environment with limited storage and transport conditions. Key factors to be considered during lyophilization of erythrocytes include determining methemoglobin level, preservation of metabolic pathways and oxygen carrier capacity. Numerous studies have been performed on that end and a summary is presented in table 3. Optimized freezing temperature and freeze-drying temperature can lead to formulations with unaltered metabolic pathways and prevent hemoglobin loss. [26–28]

Addition of cryopreservatives such as trehalose have been used and compared to formulations without any cryopreservatives, these have lower methemoglobin level, slightly altered quantity of metabolic enzymes and secondary structure of hemoglobin remains unaltered. [29] During freezing, irreversible structural change can happen due to displacement of water adjacent to cells. Disaccharides such as trehalose can replace the displaced water and form hydrogen bond with cells, slowing down damages. Moreover, while freezing, due to changes in membrane, cells can uptake trehalose. Zhang et al. found that for mammalian cells, DNA content remains least affected for lyophilized formulations with trehalose however recommended storage temperature was 4°C. [30] Among methods

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for increasing trehalose uptake, electroporation has been used, however, hemolysis rate is even higher compared to lyophilized erythrocytes with trehalose without electroporation.[31] Synthetic biopolymer, PP50, was also used to bind to phospholipid cell membrane, bending hydrocarbon chains, and increasing trehalose uptake. This method showed decrease in hemoglobin oxidation with increase in trehalose uptake, i.e. PP50 attachment to phospholipid membrane.[32] All these suggest that optimized formulations with lyoprotectant can produce stable lyophilized erythrocytes with least affected hemoglobin content and metabolite content.

Platelet preservation—Platelets are primarily responsible for the cessation of bleeding through the formation of an organized clot. This occurs by the sequential binding to exposed receptors on the damaged endothelium and the release of the many coagulation factors they store. In order to achieve local hemostasis the platelet must remain intact and thus researchers have sought ways to preserve the procoagulant properties of the platelet following lyophilization or cryopreservation.

Lyophilized platelets were first developed to evaluate the effectiveness of von Willenbrand factor in clinical assays [33]. Platelets are exquisitely sensitive to chilling. When stored below 20°C they change their shape with filamentous actin reorganization, increase intracellular calcium and can undergo secretion of alpha granules mimicking physiologic activation[34–36]. Preservatives such as Me_2SO, hydroxyethyl starch, glycerol-glucose, and Me_2SO with second messenger effectors have been studied[37, 38]. Me_2SO emerged as superior in preservation but had poor in-vivo recovery and high clearance by the reticuloendothelial system [39, 40]. Trehalose, as mentioned above, is capable of increasing cell tolerance to dehydration and is used as a cryo and lyoprotectant [12, 41]. The mechanism by which trehalose allows for platelet preservation is still unknown. The “water replacement hypothesis”, as mentioned above, states that trehalose replaces water in forming hydrogen bonds with proteins and membrane lipids during dehydration [42]. An alternative hypothesis suggests that trehalose forms an amorphous glassy matrix instead of the formation of ice crystal during the preservation. When compared to another small carbohydrate such as sucrose, trehalose has a higher glass transition and its dehydrate crystalline structure may explain its improved lyophilized stability[42, 43].

Preservation of biologic function is paramount in the investigation of platelet preservation. Platelets lyophilized with trehalose as a protectant have not only been shown to survive with a survival rate of 85% but maintain thrombogenic capabilities in response to thrombin, collagen, and ristocetin [12]. These properties have played out in vivo where species specific lyophilize dplatelets were shown to be capable of clot formation and decrease of blood loss [44]. These functional properties make lyophilized platelets an attractive alternative to the current standard of care.

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Biomimetic Biosynthetic Alternatives to Resuscitation in Austere Environments

Hemostatic Nanoparticles

Nanomaterials have emerged as promising alternatives to current blood products or even their lyophilized counterparts. Several groups utilize the specific component interactions of the coagulation system for developing targeted hemostats. Lyophilized polymeric nanoparticles conjugated with peptide containing arginine-glycine-aspartic acid (RGD) mimic fibrinogen activity, leading to reduced bleeding times in rodents by up to 50% following femoral artery injury. Moreover, poly-lactic acid core instead of previously used poly-lactic-co-glycolic acid makes it suitable for storing at room temperature without decreasing hemostatic efficacy. [45, 46] Platelet like nanoparticles of discoidal shape decorated with ligands have been reported to reduce bleeding time by 65%. [47] Liposome nanovesicles with PEG linker joining peptides that can bind to activated platelet integrins GPIIb-IIIa and P-Selectin have been assessed for clot formation. However, these require storage at 4°C in aqueous solution, and the presence of complex ligands complicate long-term storage limiting clinical translation. [47, 48] Another emerging synthetic material is the POLYSTAT, that acts like FXIIIa and crosslinks with fibrin for increasing clot stiffness. The synthetic polymer is lyophilized, hence can be stored long-term and administered by first responders. Once patient is stabilized, though, the clot must be surgically removed. Compared to human FXIIIa, POLYSTAT can reduce hemorrhage volume. [49] There are several topical hemostatic materials utilizing fibrinogen, fibrin, chitin, chitosan, or mineral zeolites for concentrating clotting factors and continuing the healing process. [50] Mentioned studies mainly focused in synthetic blood products directed towards intravenous administration. As these are administered intravenously, size of these are of higher importance, as aggregation or degradation of biomolecules during lyophilization process can provoke adverse biological reactions in immune system as well as reduce efficacy of the treatments.

Artificial Oxygen Carriers

The primary function of hemoglobin (Hb) is to transport and deliver oxygen to the tissues. Artificial oxygen carriers have been investigated as alternatives to RBCs, given the aforementioned difficulties in austere environments for blood maintenance. Several carriers have been developed and can be grouped broadly into -based materials, synthetic Fe^{2+} porphyrin-based materials, and hemoglobin (Hb) based materials [51–54].

Hemoglobin is comprised of four polypeptide chains with Fe^{2+} protoporphyrin IX as a prosthetic group. Logically, the isolation and encapsulation of cell-free Hb was sought as a potential solution. Unfortunately, cell-free quatnary Hb was found to dimerize in the absence of a cell and be rendered inactive. In order to overcome this, bioconjugative methods were sought. Polyethylene glycol conjugation, polysaccharide conjugation, and glutaraldehyde polymerized Hb have been studied but fraught with in vivo complications such as renal toxicity or the binding of nitric oxide or carbon monoxide, inducing vasoconstriction and paradoxical lowering of oxygen delivery to the tissues[54–56]. To avoid this, these free Hbs were encapsulated to preserve the oxygen carrying activity and prevent undo monoxide

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binding. Liposomal Hb carriers emerged as promising encapsulation agents but because of their inherently unstable nature required stabilization [57]. Polymerization of phospholipids with 1,2–dioctadecadienoylsn-glycero-3-phosphotidalcholine, for example, was capable of generating stable liposomes resistant to macrophage attack up to 30 days in vivo [55, 58]. Alternatives to liposomes have also been investigated because of their increased stability.

PEG conjugation to a lipid backbone has been synthesized and proposed as a more stable alternative to liposomal Hb carriers. PEG conjugation offers the ability to load large amounts of Hb into vesicles because of the volume exclusion effect of the outer surface PEG[54]. Complement activation is an unfortunate complication of the injection of liposomal Hb seen in previous studies. In order to overcome this issue a minimal amount of a negatively charge phospholipid is required. PEG-modification and a negatively charged lipid DHSG have been able to maintain compatibility with blood and not cause thrombocytopenia or pulmonary hypertension, commonly seen reaction to other encapsulated Hbs [54, 59]. PEG modification of phospholipids has enabled a prolonged circulation time through decreased reticulo-endothelial system uptake. These approaches have ushered this innovative solution closer to reality in the clinic.

Stabilizing Polymeric Nanomaterials through Lyophilization

Freeze-drying or lyophilization of polymeric nanomaterials and bioconjugates can result in concentrated formulation, and hence would be beneficial in determining dosages. Moreover, it has higher shelf life and can be stored at elevated temperatures. However, it is possible to lose stability of suspensions after freeze drying due to van der Waal's interaction between particles, depending on its surface charge. [60] Surface modification by adding ionic surfactants can prevent aggregation, undesired protein adsorption on surface or macrophage clearance that can cause lower biodistribution. [61, 62] Common destabilization phenomena that can occur in nanoparticles include the displacement of steric stabilizing surfactant layer from polymeric nanoparticles, or flocculation due to bridging in a dilute dispersion if a high molecular weight polymer is added. [63] Moreover, type of polymer, pH, entrapped drugs and storage temperature can influence chemical stability of lyophilized nanoparticles. [63] Lyophilization of polymeric nanoparticles is not much different from general lyophilization process, where an amorphous glassy state goes through sublimation to remove frozen water. Cryo and lyoprotectants are added to either bulk up concentration for instances when concentration is very low, or for adjusting pH, protecting against drying or freezing stresses, or to increase collapse temperature of formulation. [63] There is much debate whether a fast freezing or slow freezing condition is better for nanoparticles suspensions. Increase in cryoprotectants molecular weight also can increase nanoparticles' redispersion. As most of the example mentioned in this document are in lab-scale, scaled up nanoparticle lyophilization systems are still lacking. [64] Table 4 presents a list of cryo and lyoprotectants used for freeze drying polymeric nanomaterials and bioconjugates. Sugars like fructose, glucose, trehalose, lactose, sorbitol etc. form amorphous glassy layer preventing sticking between particles. [65] For Insulin loaded in poly-lactic-co glycolic acid nanoparticles, using sugars as cryoprotectants resulted in particle size similar to initial size after lyophilization. [66] Use of surfactants such as Polyacids (PVA, Pluronics, Tween) can also reduce aggregation by stabilizing the suspensions as these are adsorbed on the surface of

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nanoparticles. [66–69] However, for surfactants such as poloxamer that have higher solubility in lower temperatures of water, during freezing, destabilization can occur. [62] The level of aggregation leading to an increase in nanoparticle size depends on the cryo or lyoprotectant used and in many cases, requires sonication to retain the initial size. Sonication uses ultrasonic sound waves to disrupt agglomerates but leads to increase particle collisions and paradoxically can lead to agglomeration. Combining surfactants with cryo and lyoprotectants can enhance the freeze-drying process resulting in better redispersion, and thus preservation of particle radius pre and post-lyophilization. [62, 69] Depending on materials properties, pressure and temperature gradients and shock waves can result in aggregation as well. Specifically, for bioconjugates that have proteins conjugated, the organic molecule can go through irreversible degradation leading to adverse biological reactions. [70] Taurozzi et al. have outlined a number of considerations for standardized reporting of sonicating applications for engineered nanoparticles. [70]

The aim in lyophilizing polymeric formulations with biomolecules conjugated is to concentrate the formulation for easier dosages, better shelf lives and higher storage temperatures. Combining cryoprotectants and surfactants can help in achieving final and initial radius of particle ratios much closer to 1. [62, 69] An optimized lyophilization process with rightly chosen protectants and surfactants can help in overcoming some of the difficulties associated with aggregation post-lyophilization, help preserve bioactivity and physical characteristics for the formulation as well as avoid adverse reactions in the body.

Conclusions

The transport and maintenance of blood products in the military theater has been limited by the portability of blood components and their ability to tolerate. As early as WWII, the use of lyophilization emerged as a suitable method for blood product preservation and almost 80 years later we continue to utilize this method. Advancements in conjugation chemistry have made long-term cryo- and lyopreservation a reality. Likewise, continued progress in nanomaterial synthesis carries a significant potential for alternatives to conventional blood product preservation. While many of these advancements have yet to make it into the clinical armamentarium the possibilities are vast and greatly anticipated.

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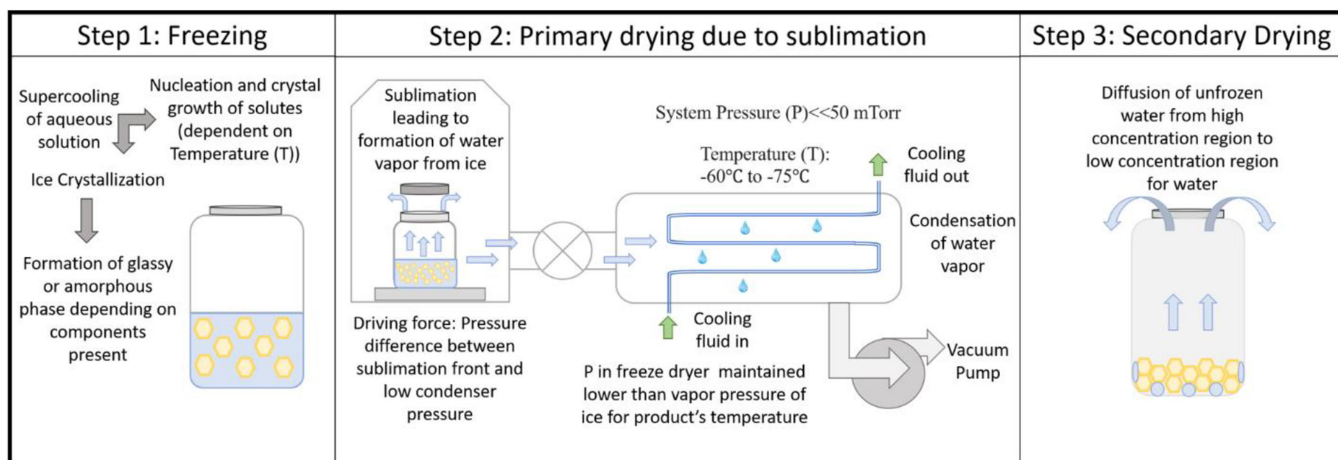

Figure 1:

Lyophilization begins with freezing of products and after the sample is supercooled, removal of water happens due to sublimation occurring during primary drying and desorption of water during secondary drying. This leads to formation of porous product cake.

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Table 1:

List of commonly used lyoprotectants and cryoprotectants in lyophilization [71]

<table>
<tr><th>General Classification</th><th>Examples</th><th>Role of lyoprotectants and cryoprotectants</th></tr>
<tr><td>Sugars</td><td>Lactose, maltose, trehalose, sucrose</td><td rowspan="4">I. Minimizing changes in conformation
II. Formation of glassy state to immobilize proteins and solvent to prevent degradation
III. Increase in chemical potential of proteins and additives due to cryoprotectants lead to unfavorable thermodynamic state.
IV. Leading to preferential exclusion of solutes from protein surface
V. Strong correlation between increase in preferential exclusion and effectiveness of cryoprotectants</td></tr>
<tr><td>Amino acids</td><td>Glycine, histidine, arginine</td></tr>
<tr><td>Polyols</td><td>Glycerol, sorbitol, mannitol</td></tr>
<tr><td>Polymers</td><td>Polyethylene glycol, dextran</td></tr>
</table>

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Table 2:

A summary of studies done on freeze dried plasma and its results

Description of study	Parameters considered	Results
Study on use of freeze dried plasma derived from patients receiving oral anticoagulants as means of calibrating thromboplastin	Thromboplastins derived from human, bovine and rabbits used in the study. Clotting times, and prothrombin time ratios were determined, with normal plasma used as control.	Prothrombin time ratio remained unaffected as seen in the control plasma. However, one characteristic of freeze dried plasma was longer time taken to form a clot. In temperature exposure at 37 °C, factor V and VII were affected, and had an impact on clotting times [20]
Study on efficacy of freeze dried plasma containing high titer of anti-lipopolysaccharide (anti-LPS)	A double blinded study on 60 consenting adult burn patients. Control constituted of no plasma, while remaining groups were given freeze dried plasma containing either high titer anti-LPS or low titer anti-LPS. Blood culture, endotoxin levels, bacterial swabs were checked for the three groups.	The results did not indicate high titer anti-LPS influencing mortality rate, but lower gram-negative bacterial infection was reported from the results. High titer anti-LPS also decreased endotoxin level in the first week. [72]
Comparison of freezing, freeze drying and storage duration of plasma on fibrin network formation	The study was continued for four consecutive months. Fresh plasma was used as positive control in the study.	Freeze-dried plasma had more similarity with fresh plasma in terms of permeability, compaction and mass-length ratio. While fibrinogen content was like fresh plasma, freeze dried plasma had lower fibrin content. Both freezing, and freeze-drying altered the activity of plasma compared to fresh plasma. [73]
Evaluating various sugars, and amino acids as lyoprotectants in freeze dried plasma	Plasma containing 60mm sucrose, trehalose, mannitol, Sorbitol or glycine was freeze-dried. After allowing forced degradation to occur, clotting times were determined for each scenario. As control, freeze dried plasma without any additives was used.	Prothrombin time, activated partial thromboplastin time, and thrombin time, indicators for time required to form a clot, were highest for the control plasma, and lowest for freeze dried plasma containing glycine as observed post lyophilization phase. [13]
Evaluating impact of residual moisture and formulation on factor VII and factor V in lyophilized plasma	Either Glycine, or Hepes or a combination of both were studied as stabilizers. Accelerated degradation studies at 37 °C and 45 °C were performed.	For freeze dried products, activity of factor V and VII was lowest in freeze dried plasma without any stabilizer. However, the formulation with glycine and Hepes had least change in activity compared to other formulations studied. [74]
Evaluating effect of solvent/ detergent treatment and lyophilization in proteome of plasma	2D gel electrophoresis and mass spectrometry were used to identify proteins.	Even after 24 months storage, coagulation factors where within normal range in lyophilized blood. In case of inhibitors, other than plasmin inhibitor and protein S, all others tested where within normal range. S/D treatment had effect on proteome in 38 points, while scenario in which only lyophilization was applied, no changes in proteome observed. [21]
Study on use of freeze-dried plasma in combat setting in Afghanistan	Biological tests performed include hemoglobin concentration, platelet count, fibrinogen level, prothrombin time and data from thromboelastography	While efficacy was observed to be similar to fresh frozen plasma, prothrombin time, factor indicating clotting time increased by 3.3 seconds for lyophilized plasma. [75]
Managing bleeding in hemophilia patient with freeze dried plasma	FVIII is administered generally for hemophilia patients. For a location with limited medication options, freeze dried plasma was used as a substitute for a case of acute bleeding in a pediatric case.	To reach the optimum FVIII dosage a large bolus had to be administered, however the efficacy was similar to that of fresh frozen plasma. The study highlights benefits of using freeze dried plasma in locations where low temperature storage can be challenging, as well as medication and therapy choices are limited. [76]
Assessing changes in coagulation factors and inhibitors after lyophilization in plasma	The coagulation factors quantified were compared to fresh frozen plasma. Moreover, the data mentioned were also collected after 24-month storage at 2–8 °C and 23–27°C.	Coagulation factors were least affected when stored at 4°C. At room temperature storage, protein S and FVIII activity decreased over time during long term storage. Reported data on clinical efficacy mentions similar activity of freeze dried plasma when compared to fresh frozen plasma. [22]
Determining efficacy of lyophilized plasma when administered during early resuscitation in cases of traumatic brain injury and hemorrhagic shock	A randomized study performed with one group receiving fresh frozen plasma and other group receiving lyophilized plasma. The study was undertaken in large animals.	Other than difference in lesion size, both groups showed similar cognitive functions without any treatment related complications.[77]

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Table 3:

Summary of studies done on lyophilized erythrocytes

Description of study	Parameters considered	Results
Evaluating stability of lyophilized lipid encapsulated hemoglobin (LEH)	Stability of frozen LEH, lyophilized LEH and lyophilized LEH containing trehalose as excipient was compared over a time of 6 months	For lyophilized LEH particle size increased during rehydration, and hemoglobin content decreased, while size for formulation with trehalose remained unchanged, and initially slight hemoglobin oxidation was observed but it remained unchanged over time. [78]
Effect on metabolic activity of erythrocytes due to lyophilization	The activity of hemoglobin is regulated by ATP and 2,3-diphosphoglycerate, produced during glycolysis through Embden-Meyerhof pathway and pentose-phosphate pathway. Whether any of the enzymes in these pathways were affected was studied. Results were compared against that of fresh non-lyophilized erythrocytes.	A part of glycolytic enzymes remains unaffected, while 3 enzymes decreased significantly in lyophilized erythrocytes. Lyophilized erythrocytes had unaffected Methemoglobin reduction pathway, hence usual oxygen transport would not be hampered, and even with slight alterations in enzymes produced, it can be considered as a viable option. [26]
Study on determining effective freezing rate for freeze drying erythrocytes	The recovery rate for hemoglobin and stability were determined and compared against thawed erythrocytes	Cooling rate of 200K/min was found to be effective and as cooling rate was increased before lyophilization, recovery rate for hemoglobin also increased, suggesting higher freezing rate leads to better cryopreservation. While for thawed sample increasing cooling rate lead to lower stability. [27]
Evaluating effect of freeze drying of erythrocytes at ultra-low temperature	As cryoprotectants, hydroxyethyl starch and disaccharide D-maltose was used and temperature for −5 to −65°C was used.	Highest stability, i.e. Recovery rate for hemoglobin, after resuspension was observed at −35°C and the authors suggest the reason is higher temperature leading to damaging effects and colder temperature leading to insufficient driving force for water transport. [28]
Preservation of erythrocyte activity with trehalose	Formulation prepared with trehalose in presence of hydroxyethyl starch, and human serum albumin. Rate of hemolysis, methemoglobin formation and metabolites of glycolysis were tracked in the lyophilized RBC	Secondary structure of hemoglobin and major metabolic pathways remain unchanged, with 50% hemolysis during rehydration. Hence trehalose loaded formulations could be stable lyophilized erythrocytes. [29]
Using phospholipids with trehalose for freeze drying erythrocytes	Phospholipids were hypothesized to protect membrane from damage during drying. Hemolysis (%) and methemoglobin level were compared in formulations with trehalose, with trehalose and phospholipid, with phospholipids only and for cases without either trehalose or phospholipid.	Trehalose and phospholipid loaded erythrocytes had lowest amount of methemoglobin formation and least percent hemolysis, suggesting a stable formulation for lyophilized erythrocytes. [79]
Determining stability of lyophilized hemoglobin for DCIP test	Lyophilized hemoglobin and variant hemoglobin E were used in the test and storage duration of 12 months was used	Lyophilized hemoglobin could be used as reliable control in the test due to its stability over the storage duration [80]

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Table 4:

Surfactants, Lyoprotectants, and Cryoprotectants used for bioconjugates and nanomaterials

Surfactants, lyoprotectants and Cryoprotectants	Mechanism/purpose	Outcomes
Polyacids (polyacrylic acid, citric acid)	• Coacervate precipitation due to hydrogen bonding between oxygen in surface peg group and hydroxyl ion in carboxylic part of polyacid. • This method is of interest due to possibility of using heterobifunctional peg in for binding with biomolecules of interest.	Since bonding is reversible, the hypothesis was that neutralizing ph would resuspend the nanoparticles after lyophilization on plga-peg nanoparticles. Sucrose was used as a control to check redispersion in its presence or absence. In presence of high amount of sucrose, the nanoparticles resuspended, while for nanoparticles without sucrose, 15 minutes sonication was required. [67]
High molecular weight polyethylene glycol	• PEG between 0.4–20 kDa was used as cryoprotectant at different freezing rates. • The rate of aggregation depended on change in particle size. • Higher particle size compared to initial size represents higher order of aggregation.	Aggregation was highest in low molecular weight peg in comparison to suspension with high molecular weight peg. Freezing rate did not affect the rate of aggregation. [81]
Surface stabilization with quaternary ammonium groups	• As a second cryoprotectant lactose, microcrystalline cellulose or calcium phosphate was used. • Four model polymeric nanoparticle were investigated. • The surface stabilization through ammonium group caused low contact angles.	Higher wettability of polymer surface and high minimum film formation were attributed for better redispersion of polymeric nanoparticles released from granular, pelleted or tablet formulations. [68]
Polyvinyl alcohol (pva)	• Freeze drying of polycaprolactone nano-capsules in presence of pva • Comparing observations against sugars (glucose, mannitol, trehalose) used for cryoprotection	Using 5% w/v pva resulted in almost complete resuspension of nanoparticles (ratio of final to initial size was 1.008), and the trend remains similar for freezing in liquid nitrogen, in pre-chilled freezer, as well as freeze dryer shelf. Moreover, combining sugars can bring the ratio close to 1. Hence, a combination of cryoprotectants can offer better redispersion of nanosuspensions. [69]
Pluronics	• Role of poloxamer 188 was investigated • Surfactants such as these are supposed to be adsorbed in surface of polymer nanoparticles	Enhanced cake formation and combining with sugar lead to easily dispersible formulations. 5% poloxamer solution used lead to ratio of final and initial radiuses close to 1.1, while combining glucose resulted in ratio close to 1. [62]
Trehalose	• Using trehalose to modify solvation layer around biomolecules due to larger hydrated volume. • Designed polymers were formed from diazide-trehalose comonomer and dialkyne comonomer • Flocculation levels were studied in media containing serum proteins	Presence of trehalose in the lyophilized nanomaterials resulted in lower flocculation and higher efficacy for delivering biomolecules (pdna into hela cell lines in mentioned study) [82]
Lactose and sucrose	• Formation of glassy layer around particles to prevent aggregation. • Studying change in physical properties of lyophilized cake with variation in amount of sugar cryoprotectants for cationic nanoparticles	In absence of cryoprotectants, higher freezing temperature can lead to higher flocculation. As amount of cryoprotectant increases, the effect of freezing temperature on size of nanomaterials started decreasing However, redipersion at 5% w/v of sugar used resulted in size closest to initial particle size, and as amount of sugar was increased, particle size went up as well. [65]

Surfactants, lyoprotectants and Cryoprotectants	Mechanism/purpose	Outcomes
Dextran	• Formation of stabilizing layer on particles, resulting in an interface between water and the latex particle	As amount of dextran increases, layer thickness on particles increase, and size when re-dispersed after freeze drying becomes closer to initial size. As molecular weight of dextran increases, lesser amount of dextran (w/w) is required for better resuspension of latex particles. [83]

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