Preservation of Human T Cell Membrane Integrity after Drying and Rehydration

By

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Abstract

The biopharmaceutical industry has reached new frontiers in its ability to produce complex novel modalities for therapeutic application. Novel cell-based therapies offer a promising treatment for cancer by enabling T cells to target tumor antigens through ex vivo engineering of cell receptors and intracellular signaling domains. Development of cell-based therapeutics for clinical and commercial application requires successful manufacturing, stability, and transport. Since significant cell degradation occurs without the use of cryopreservation techniques, cells must be stored at extremely low temperatures (<-130 °C). Successful preservation of human T cells in the dried state provides the opportunity for non-vapor phase storage of the final product. Herein, several key factors are discussed and evaluated for T cell stabilization in the dried state, including the presence of intracellular stabilizer, cell health prior to drying, reconstitution procedure, and formulation components. Residual water content of dried preparations was balanced between increased glass transition temperatures at low moisture and reduced process loss at high moisture. A vacuum-foam drying method demonstrated improved process recovery and increased stability at refrigerated storage compared to other drying techniques. These findings demonstrate the feasibility of drying human T cells with retained membrane integrity after drying and rehydration.

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Introduction

Since the discovery of recombinant DNA technology in the 1970s,[1] the pharmaceutical industry has seen a significant shift from chemically synthesized drugs towards biologics. Some of the earliest FDA-approved biologics include recombinant insulin (Humulin, Eli Lilly) and the first monoclonal antibody muromonab-CD3 (anti-CD3) which were approved in 1982 and 1986, respectively.[2, 3] As disease pathways and cancer targets become better understood and methods in bioengineering improve, the diversity and complexity of biopharmaceuticals evaluated in clinical trials have continued to increase.

One driver for the evaluation of novel therapeutic modalities is the pursuit of improved mechanisms for leveraging the immune system to treat cancer. Anti-CD20 monoclonal antibody (mAb) rituximab was approved by the FDA in 1997 and showed early success for the treatment of lymphoid malignancies. This encouraged development of other innovative therapies, including novel mAbs, bispecific T cell engager antibodies, and chimeric antigen receptor (CAR) T cell therapies.[4] Immunotherapeutic modalities offer new options for treating cancer in addition to those previously available to the health care industry, such as tumor resection, chemotherapy, and radiation therapy.

While there are several immunotherapies on the market and in clinical development with the same end goal of improving cancer treatment for patients, the focus of this work is on novel engineered cellular therapies. CAR T cell therapies enable cytotoxic T lymphocytes to target tumor antigens by engineering cell receptors with an extracellular antigen-binding single chain variable fragment (scFv) coupled to intracellular T cell signaling domains.[5] Maus et al.[6] reviewed the application of CAR T cells as an emerging oncology therapy for hematologic malignancies after 14 clinical trial reports were published. It was noted that the most effective

CAR design resulted in CAR T cells persisting and expanding *in vivo* for several weeks post-infusion. A summation of the trial data showed an 80% response rate in acute lymphatic leukemia.

In 2017, the first CAR T cell therapies were approved by the FDA (Kymriah® and Yescarta®) for treatment of relapsed or refractory B-cell leukemia and lymphoma.[7, 8] Beyond hematologic malignancies, it was reported that CAR T cells targeting interleukin-13 receptor alpha 2 (IL13Rα2) resulted in regression of intracranial and spinal tumors in a patient with recurrent multifocal glioblastoma who had previously, but unsuccessfully, received standard-of-care treatment.[9] This data suggests that T cell therapies can have strong antitumor efficacy against "difficult-to-treat" solid tumors.

Accompanying the promise of novel therapies are new challenges with regards to development and manufacturing. One challenge in particular for the development of biotherapeutic compounds is the stability, or shelf life, of the final drug product during shipping and storage. For many years, the complexity of recombinant protein liabilities (both chemical and physical) led many products to utilize a solid dosage form to extend the shelf life.[10] The traditional means of achieving a dried product has been freeze-drying, as evidenced by the large number of lyophilized products on the market.[11] The freeze-drying process induces new stresses on a compound, such as interfacial (ice-water) stress and cryo-concentration during freezing, dehydration stress from the removal of bulk and bound water, and rehydration stress during reconstitution.[11] Typically, for protein-based therapeutics, these challenges are overcome by the use of lyoprotectants, such as disaccharides and surfactants, and appropriate freeze-drying process design (e.g. product temperature < glass transition/collapse temperature). However, these approaches alone may not be sufficient for successful processing of more complex modalities,

such as cell-based therapies. Currently, stabilization of cell-based therapies is achieved by freezing in the presence of membrane permeable cryoprotectants, such as DMSO, glycerol, and ethylene glycol.[12] These excipients enable the process of vitrification during freezing whereby ice crystal formation is inhibited and cells are stored in the glassy amorphous state. One challenge with the use of these excipients is their extremely low glass transition temperatures (<-83 °C) which require vapor phase cryopreservation (<-130 °C) of the final product.[13]

Several discussions have been presented regarding the stabilization of mammalian cells in the dried state, including a chapter with the same title in the book *Advances in Biopreservation* that is now over a decade old.[14] For drying of mammalian cells, much of the early discussion regarding stabilizing excipients has involved trehalose.[15] Crowe et al.[15] point to lessons from nature as one reason for focus on this particular excipient. Across a wide variety of anhydrobiotic species that can survive extreme desiccation, trehalose has been noted to accumulate at very high concentrations. While trehalose is prevalent in nature during anhydrobiosis, it is often not used during biopreservation since it does not readily diffuse across the cell membrane for stabilization of intracellular components.

Since the presence of stabilizer on both sides of the membrane is critical for cell stabilization during drying, procedures have been developed to load excipients, such as trehalose, into the intracellular matrix. Following an established procedure,[16] Chen et al.[17] introduced intracellular trehalose into mouse fibroblasts using a genetically engineered version of α-hemolysin named H5 that reversibly generates permeable pores in the membrane. First, the authors demonstrated that the presence of high concentrations (0.2 and 0.4 M) of extracellular trehalose did not provide an improvement in post-dried cell membrane integrity in comparison to its absence. However, when intracellular trehalose was loaded by the aforementioned procedure,

the maintenance of membrane integrity post-drying (dried by natural convection and forced laminar flow) was reported to be achieved at and above trehalose concentrations of 0.2 M. This minimum concentration was reported to be in reasonable agreement with an estimate, based on cell diameter (~8 μm) and mass approximations, of 1.3 x 10^{10} trehalose molecules required to fully stabilize each cell. For formulations with sufficient intra- and extracellular trehalose, it was reported that significant membrane integrity was maintained when water content was >~15%, with drying beyond 15% leading to a drastic decrease in membrane integrity. Additionally, significant membrane integrity was maintained after storage at both -80 and -20 °C for several weeks. Over the same time frame, significant losses in membrane integrity were observed at higher storage temperatures (4, 25, and 37 °C), which is likely due to storage above the glass transition temperature (T_g) of dried amorphous trehalose formulations at 15% residual water content. The T_g of amorphous trehalose at 15% (w/w) residual water content was estimated between -20 and -10 °C based on Figure 1 from Ohtake and Wang[18]. It is noteworthy that these water content values are significantly higher than typically utilized for protein and virus stabilization in the dried state.[19-23] This data demonstrates that the presence of intracellular stabilizer is critical for stabilization of cells during drying.

Buchanan et al.[13] reported the stabilization of hematopoietic stem and progenitor cells (HPC) using trehalose loaded via endogenous cell surface receptors. Clonogenic activity was measured by utilizing the colony forming unit (CFU) assay. The cells were prepared in a formulation containing 6.8% trehalose, 2% hydroxyethyl starch and 5% human serum albumin. Trehalose-loaded HPC samples were taken throughout the freeze-drying process and compared to control cells. Through the freezing and primary drying steps, HPC were reported to be stable (i.e. no significant change in CFU). Significant losses in CFU were observed during the

secondary drying step. Evaluation of 5, 10, 15, and 20 °C secondary drying temperatures demonstrated a direct correlation between loss in CFU and drying temperature. Residual water content was decreased as secondary drying temperature was increased and a significant decrease in clonogenic potential was observed when samples were dried below 15% residual water content. These previous examples[13, 17] highlight the need to optimize drying temperatures and residual water content to successfully stabilize cells in the dried state.

Natan et al.[24] reported an optimized freeze-dried formulation of mononuclear cells derived from human umbilical cord blood. A combination of 0.1 M trehalose and 0.945 mg mL^{-1} epigallocatechin gallate (EGCG, an antioxidant) resulted in a post-freeze-dried viability of 91.6% before washing and 62.6% after washing. This was a significant improvement to a formulation with 0.1 M trehalose alone (18.5% and 10.3% before and after washing respectively) and to a formulation with 0.945 mg mL^{-1} EGCG alone (44.8% and 33.4% before and after washing respectively). Interestingly, this study did not evaluate the effect of intracellular trehalose which was previously reported to stabilize mammalian cells in the dried state in the absence of any antioxidant. This example demonstrates the complexity of and potential multi-faceted approach necessary to stabilize mammalian cells in the dried amorphous state.

With the emergence of new therapeutic modalities in pharmaceutical development, it will be necessary for the formulation and drying parameters of each unique product to be adapted/optimized for successful stabilization. As interest in cell-based therapies continues to grow, significant improvements in manufacturing and storage are required. Currently, there is a paucity of literature evaluating the stabilization of T cells in the dried state. One publication evaluated the effect of residual moisture content on Jurkat Clone E6-1 cell viability after microwave-assisted drying in trehalose solutions.[25] Similar to previous studies, dramatic

reductions in post-rehydration cell viability were observed when T cell samples were dried below approximately 20% (wet basis) residual water content.

As discussed above, freeze-drying is the most common drying method used for improving storage stability of biopharmaceuticals. However, the freezing step in the freeze-drying process may damage cells during processing without the presence of membrane permeable excipients (e.g. DMSO). Since these excipients pose challenges during drying due to their low glass transition temperature, a drying technology that avoids freezing prior to dehydration may be beneficial for successful cell desiccation. In 2014, Walters et al.[26] reviewed the current state of next-generation drying technologies for biopharmaceutical application and compared them to freeze-drying. There exists an abundance of drying technologies available for industrial applications including; convection drying, fluid bed drying, spray drying, microwave drying, vacuum drying, foam drying, and supercritical fluid drying. However, the need to maintain stability of labile biopharmaceuticals, aseptic processing, and scalability has limited the number of drying technologies available to the biopharmaceutical industry.

A recent evaluation of drying technologies compared the stability of live attenuated influenza vaccine (LAIV)[27] after freeze-drying, spray drying, and vacuum-foam drying. The storage stability of the live attenuated Type-A H1N1 and B-strain influenza vaccines was assessed at various storage temperatures (4, 25, and 37 °C) using a $TCID_{50}$ potency assay. At 4 °C, the foam dried preparation exhibited a rate of titer loss of 0.006 log $TCID_{50}$/mL/week compared to 0.014 log $TCID_{50}$/mL/week and 0.028 log $TCID_{50}$/mL/week for spray dried and freeze-dried preparations, respectively. Foam dried preparations were significantly more stable than spray dried and freeze-dried preparations, while exhibiting low process loss and adequate retention of immunogenicity.

Similarly, Abdul-Fattah et al.[28] compared the stability of a genetically engineered bivalent live attenuated virus vaccine (Medi 534) after freeze-drying, spray drying and vacuum-foam drying. The viral potency process loss of Medi 534 was greatest for freeze-dried material (1.4 $\log_{10}$ $TCID_{50}$/mL) compared to spray dried (0.8 $\log_{10}$ $TCID_{50}$/mL) and foam dried (0.8 $\log_{10}$ $TCID_{50}$/mL). The reason for increased process loss during freeze-drying was suggested to be greater susceptibility of the vaccine to the ice-water interface compared to the air-water interface encountered during foam drying and spray drying. During storage at 25 °C, vacuum-foam dried Medi 534 exhibited a lower rate of loss in potency than spray dried and freeze-dried materials. Abdul-Fattah and coauthors observed that improved storage stability of foam dried preparations was associated with decreased specific surface area and vaccine surface exposure. Based on these data and similar findings in other reports,[29] vacuum-foam drying is considered a promising technique for stabilization of highly sensitive biological materials in the dried state.

Herein, naïve human T cells isolated from primary cells were used as a surrogate system to evaluate the feasibility of preserving engineered therapeutic T cells in the dried state. The intracellular trehalose concentrations in T cells after formulation in trehalose solutions was measured to ensure the presence of stabilizer on both sides of the cell membrane during drying. Vacuum-foam drying was developed and used to evaluate cell stabilization after drying and rehydration. Human T cell health was evaluated based on membrane integrity and cell count measurements. Membrane integrity data from vacuum-foam dried T cell preparations were compared to results from freeze-dried and spray freeze-dried preparations.

Materials & Methods

T cell Sample Preparation

T cells used were primary human pan-T cells cryopreserved at -150 °C at a cell density of 3E7 cells/mL. High purity α, α-trehalose di-hydrate was acquired from Pfanstiehl (Chicago, IL, USA), powder bovine serum albumin (BSA) was acquired from Fisher Scientific (BSA #1; Lot #: 185564) (Waltham, MA, USA) and Sigma-Aldrich (BSA #2; Lot #: SLBX7447) (St. Louis, MO, USA), 25% (w/v) human serum albumin (HSA) (Kenketsu Albumin 25, Lot #: N257N) was acquired from KM Biologics Co., Ltd. (formerly Kaketsuken) (Kumamoto, Japan), sodium chloride and sodium phosphate monobasic monohydrate were acquired from J.T. Baker. To prepare formulations, frozen T cells were thawed by holding vial in hand and diluted to target concentration in phosphate buffered saline (PBS). Then, cells were centrifuged for 10 minutes at ≤1,000 x g, supernatant removed, and resuspended in the appropriate formulation matrix to a target cell density. Incubation of cells prior to formulation was performed by diluting cells to approximately 1E6 cells/mL in complete media (growth media + human serum with IL-6) and statically placing at 37 °C, 5% CO_2 in a culture flask.

Membrane Integrity and Cell Count Assay

Membrane integrity and cell count measurements were obtained using a NucleoCounter® 3000 (NC-3000) (ChemoMetec A/S, Allerød, DK). Neat samples were typically diluted 4:1 in PBS (unless otherwise specified) and cell membrane integrity and count assays were performed using a Via1-Cassette™. The procedure uses membrane penetrating acridine orange (AO) and non-penetrating 4′,6-diamidino-2-phenylindole (DAPI) fluorescent dyes to assess cellular membrane integrity. Dried samples were equilibrated for no less than 30 minutes after

reconstitution prior to analysis. Initial studies showed increased variability when samples were measured immediately after reconstitution (data not shown).

Protein and Trehalose Quantitation

Pierce™ BCA protein assay reagents from Thermo Scientific (Waltham, MA, USA) were used to measure protein concentration. Reagents were added and samples were incubated according to the manufacture's protocol and absorbance was measured at 562 nm. Trehalose concentration was measured following the manufacturer's protocol for a trehalose assay kit from Megazyme (Bray, Ireland). The assay quantitates glucose concentration spectroscopically (indirectly by the reduction of NADP+ to NADPH) before and after incubation with trehalase which hydrolyzes trehalose into two D-glucose molecules. Dilutions were made as needed to test samples in assay linear range of 0.02 to 0.4 mg mL^{-1} trehalose.

Protein Marker Analysis

Cellular suspensions (1 mL) were centrifuged at 1,000 x g for 10 minutes, the supernatant was removed, and pellets were resuspended in water to lyse cells (using 250 or 500 uL). Samples were rapidly frozen in a -150 °C freezer to ensure complete lysis, thawed after 10 minutes and re-centrifuged at 15,000 x g for 5 minutes to remove the cellular debris. The supernatants were diluted and protein concentration was measured using BCA assay.

Vacuum-Foam Drying

Vacuum-foam drying was performed using a LyoStar III lyophilizer (SP Scientific, Warminster, PA). Vacuum-foam drying cycles utilized pressures from 0.05 to 5 Torr and shelf temperatures from 5-30°C. There were two foam drying cycles used in this work. The first cycle ("standard cycle") used a 10 mL vial with 1 mL fill volume. The partially stoppered vials were loaded onto a pre-equilibrated 20 °C shelf, vacuum was pulled to 5 Torr, shelf temperature was

ramped to 30 °C at 1 °C/min and held for 15-20 minutes. Then, the vacuum was pulled to 0.8 Torr and held for varying times depending on the purpose of the experiment. The second cycle ("rapid cycle") used a 2 mL vial with 100 µL fill volume and a nucleation site was added to facilitate rapid boiling (a piece of rubber stopper). The partially stoppered vials were loaded onto a pre-equilibrated 30 °C shelf, vacuum was pulled to 5 Torr and held for 20 minutes. After both cycles were complete, the pressure was increased to approximately 600 Torr and vials were stoppered prior to vacuum release.

Freeze-Drying

Freeze-drying was performed using a LyoStar III lyophilizer (SP Scientific, Warminster, PA) and a 10 mL vial with 1 mL fill volume. The partially stoppered vials were loaded onto a shelf pre-equilibrated at 5 °C, the vials were equilibrated for up to 1 h, the shelf temperature was ramped to -50 °C at 1 °C/min and held for >1 h. The vacuum was pulled to 0.05 Torr and the shelf temperature was ramped at 0.5 °C/min to -30 °C for the primary drying step. The cycle was ended after the Pirani gauge reached equilibrium with the capacitance manometer. After the cycle was complete, the pressure was increased to approximately 600 Torr and vials were stoppered prior to vacuum release.

Spray Freeze-Drying

A Buchi B 290 spray nozzle was configured above a metal tray filled with liquid nitrogen and the product was pumped using a peristaltic pump through the spray nozzle with a nitrogen gas flow rate of 4 L/h for 90 seconds. The frozen material was loaded into a LyoStar III lyophilizer (SP Scientific, Warminster, PA) with a shelf pre-equilibrated at -50 °C. After the liquid nitrogen had completely evaporated, the vacuum was pulled to 0.05 Torr and the shelf temperature was ramped at 0.5 °C/min to -30 °C for the primary drying step. The cycle was

ended (no secondary drying) after 675 minutes (preliminary experiments were performed to determine cycle time required to reach target residual water content). After the cycle was complete, the pressure was increased to approximately 500 Torr and the shelf temperature was ramped to 5 °C. Immediately after the camber pressure reached ambient condition, the tray was transferred into a dry box and product was manually filled into 10 mL vials. A schematic of the spray freeze-drying process is shown in **Figure 6**.

Moisture Analysis

Mettler Toledo (Columbus, OH, USA) Karl Fischer Titration Moisture Analyzer (C30) was used to determine the percent of residual water content in dried samples. Samples were reconstituted using 50% MeOH / 50% formamide diluent and vortexed until dissolved. Titration was performed on reconstituted samples and the total weight of water for each sample was determined. Percent residual water content (wet basis, w.b.) was calculated by dividing the total water content by the total sample weight (solids content + residual water content).

Differential Scanning Calorimetry

Prior to differential scanning calorimetry (DSC) scans, dried samples were ground, mixed and packed into a 40 μL aluminum pan in a dry box and hermetically seal with an aluminum lid. Heat flows were acquired using a Mettler Toledo (Columbus, OH, USA) DSC822e at 2.0 °C/min with temperature modulation amplitude of 0.25 °C and a period of 15-30 seconds. Heat flows were transformed and analyzed using the Mettler-Toledo STARe software.

Results & Discussion

Chapter 1: Trehalose Uptake

Maintaining cellular membrane integrity after cells are exposed to drying and rehydration stress requires the presence of stabilizer on both sides of the membrane. Efficient delivery of trehalose across the cell membrane can be challenging due to poor permeability and transport of polar compounds across the lipid bilayer. Several strategies have been developed to efficiently transport trehalose across mammalian cell membranes, including electroporation,[30] fluid-phase endocytosis,[31] and microinjection.[32] For example, Satpathy et al.[33] utilized osmotic imbalance and incubation above the phospholipid phase transition to increase trehalose uptake in red blood cells. The authors reported low trehalose uptake at 4 °C, moderate uptake at 23 °C and the highest uptake at 37 °C; however, increasing incubation temperature directly correlated with rate of hemolysis. A different strategy employed by Abazari et al.[34] was to increase the lipophilicity of trehalose by acetylation of hydroxyl groups. This strategy was used for rat hepatocytes and significant trehalose was found to accumulate intracellularly. The highest intracellular trehalose concentration was observed after incubation with trehalose hexaacetate which possessed the highest logP value. Trehalose quantitation showed that trehalose hexaacetate is deacetylated into trehalose inside the cell by intracellular esterases. Based on this mechanism, trehalose levels (combination of free and acetylated trehalose) reached 10-fold higher intracellularly compared to the extracellular environment.

The above methods demonstrate efficient transport of trehalose inside the cell. Since the primary drying process utilized in this work, namely vacuum-foam drying, requires notably high solids content, it was proposed to evaluate trehalose uptake using osmotic imbalance to force

sufficient amount of stabilizer across the cell membrane. The amount of intracellular trehalose taken up by human T cells at extracellular concentrations up to 300 mg mL^{-1} was evaluated.

Since bulk analyte concentration measurements are not able to distinguish extracellular and intracellular concentrations, intracellular components had to be separated from the extracellular matrix to accurately quantitate intracellular concentration. Additionally, accurate measurement of the cell concentration in the final sample preparation (i.e. after the extracellular matrix and lysed cells have been removed) required an analyte marker that correlated to cell density.

Jansen et al.[35] evaluated both DNA and protein as analyte markers for peripheral blood mononuclear cell counting. The authors concluded that DNA was better suited as a cell counting marker; however, this was due to hemoglobin contamination (from red blood cells) in the whole blood samples impacting the protein marker results. Since protein contamination was not expected for the T cell samples used in this work, a protein analyte marker for cell counting was evaluated (same approach used by Abazari et al.[34]).

Cell count was measured using the NC-3000 cell count assay and samples were diluted in PBS to three different cell densities (1E6, 5E6 and 1E7). The protein marker concentration (after lysis) was measured and plotted against cell density (before lysis) in **Figure 1**. There was a strong correlation (>0.99 r^2) between protein marker concentration and cell density. Based on these data, a protein marker was used as an indirect measure of cell density during trehalose uptake experiments. Measured protein concentrations at all cell densities were compared to an expected protein concentration value that assumed a cellular composition of 18% protein (Table 2-3 in Alberts et al.[36]). The cellular protein concentration values were between 58 - 74% of the expected amount. The lower protein content per cell values than expected may be due to loss of

membrane and structural proteins during lysis and cell debris removal. The cellular protein content (µg protein/cell) was determined by dividing the micrograms of protein/mL preparation by the number of cells/mL. The average cellular protein content was 6.5E-5 ± 2.8E-6 µg protein / cell (calculated using protein marker preparations at 5E6 and 1E7 cells/mL, n = 7).

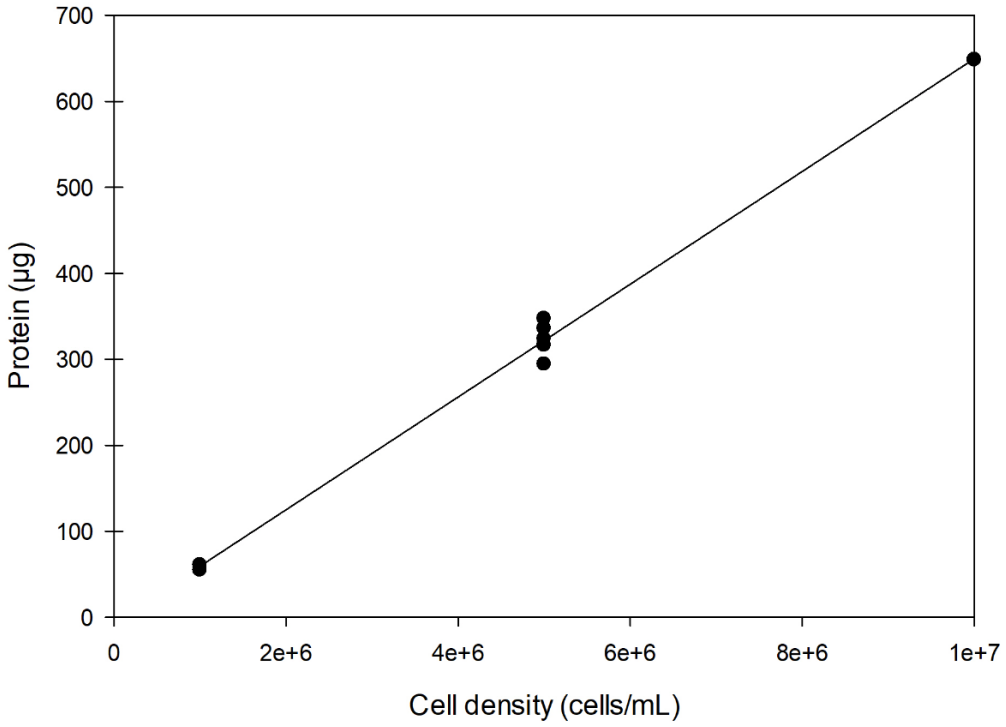

Figure 1. Protein marker concentration (after lysis) and cell count (before lysis).

The rate and amount of intracellular trehalose uptake in human T cells at varying concentrations was evaluated. Cells were prepared at 5E6 cells/mL in 10 mM phosphate, 150 mM NaCl at pH 7.4 (PBS) with trehalose concentrations ranging from 0 to 300 mg mL^{-1}. Cell preparations were incubated at ambient conditions and 1.5 mL aliquots were taken at defined time intervals (0, 1, 2, 4, and 6 hr). Aliquots were washed twice by centrifuging for 5 minutes at 3,000 x g, removing the supernatant and resuspending with 1.5 mL of 10 mg mL^{-1} sucrose in PBS (for osmotic balance). After the second wash, samples were centrifuged, the supernatant was removed, pellet was resuspended in 250 µL of water and then samples were frozen in a -150

°C freezer to lyse the cells. Preparations were thawed and centrifuged for 15 minutes at 15,000 x g to remove cellular debris. The supernatant was analyzed for trehalose and protein marker concentration. The cellular protein content from **Figure 1** was used to estimate the intracellular trehalose concentration on a cellular basis. In order to determine the final milligrams of intracellular trehalose per milliliter, cellular volume was calculated using a cell diameter of 10 µm (estimated from cell count data) and assuming spherical geometry.

Figure 2 shows concentration dependent trehalose uptake in human T cells. While the exact mechanism of uptake is not fully understood, fluid-phase endocytosis and facilitated diffusion were two of the mechanisms proposed. Oliver et al.[31] demonstrated the fluid-phase endocytosis mechanism of trehalose uptake for human mesenchymal stem cells when stored above their first membrane phase transition temperature (15 °C).

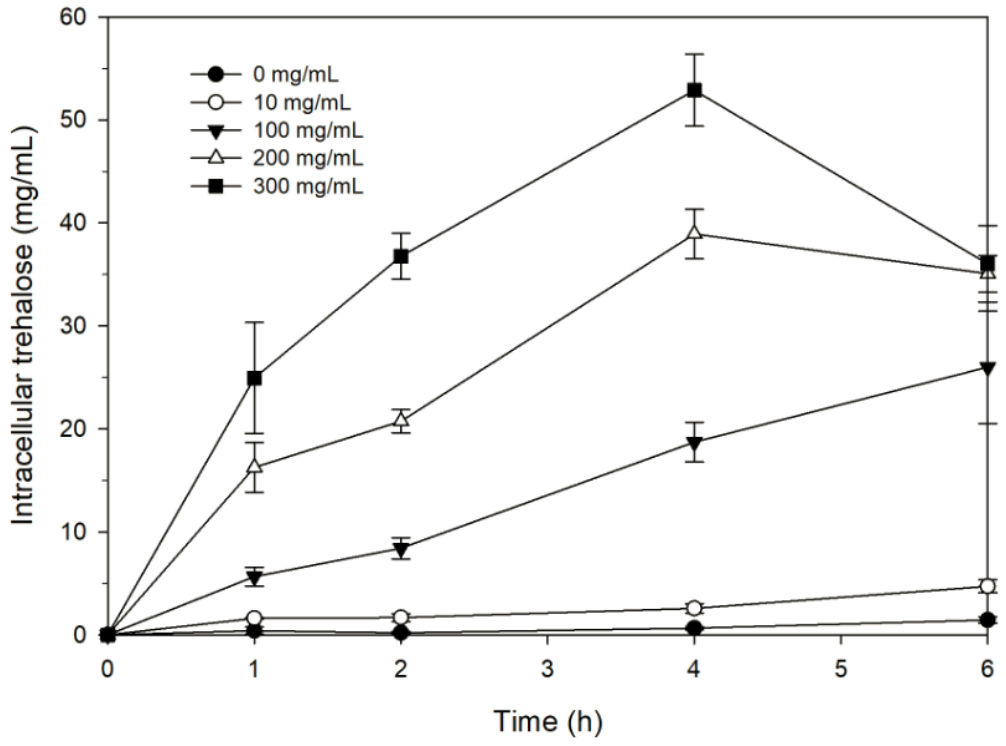

Figure 2. Kinetics of intracellular trehalose uptake in human T cells. Data presented as average ± standard deviation (n = 3).

After 6 hours of incubation, the 10 mg mL^{-1} extracellular formulation reached approximately 4.7 mg mL^{-1} intracellular trehalose. This data is in reasonable agreement with work by Abazari et al.[34] which showed approximately 2 - 4 mg mL^{-1} (6 - 11 mM) trehalose were loaded intracellularly (for rat hepatocytes) using approximately the same extracellular trehalose concentration (10 mg mL^{-1}). When extracellular trehalose was increased 10-, 20-, and 30-fold, a significant increase in trehalose uptake was observed. After 2 hours of incubation, intracellular trehalose concentrations of 8.4, 20.7 and 36.7 mg mL^{-1} were observed for 100, 200, and 300 mg mL^{-1} extracellular concentration formulations, respectively. The maximum intracellular trehalose observed was in 300 mg mL^{-1} extracellular trehalose; although, there was not a significant difference between the intracellular trehalose concentrations at 200 and 300 mg mL^{-1} after 6 hours of incubation.

During red blood cell loading experiments, Satpathy et al.[33] reported the lack of trehalose uptake below 600 mM (~205 mg mL^{-1}) which suggested facilitated diffusion was not the mechanism of trehalose uptake. Sufficient energy to drive trehalose across the membrane was achieved at extracellular trehalose levels above 600 mM (~205 mg mL^{-1}). When trehalose was loaded at nearly 300 mg mL^{-1} (274 mg mL^{-1}) extracellular trehalose at 37 °C for 16 hours, intracellular trehalose levels were able to reach 65 mM (~22 mg mL^{-1}). These data for red blood cells are in reasonable agreement (approximately 60% difference) with the results for T cells where, 200 and 300 mg mL^{-1} extracellular trehalose levels resulted in approximately 35 mg mL^{-1} intracellular levels after 6 hr incubation at ~23 °C (ambient). In contrast to red blood cells, the results show that extracellular concentrations below 600 mM (i.e. 300 mM) are able to drive trehalose across the T cell membrane at ~23 °C (ambient) which may be due to differences in membrane composition of the two cell types. Additional variability in the intracellular

concentration results between these two studies may be due to differences in the assumptions (e.g. cell volume) required for the intracellular calculation.

T cell membrane integrity of preparations with 0 and 100 mg mL^{-1} extracellular trehalose dropped from approximately 86-88% to 82% following 6 hours of incubation at ~23 °C (ambient) during loading. The 300 mg mL^{-1} extracellular trehalose preparations had lower initial and 6 hours membrane integrity, which ranged between 78-80% at both time points (initial and after 6 hours).

These data demonstrate that a significant amount of trehalose can be loaded intracellularly in a relatively short period of time when formulated in extracellular trehalose concentrations ranging from approximately 100 to 300 mg mL^{-1}. The cell stabilization data in the following sections was collected using 30% (300 mg mL^{-1}) extracellular trehalose formulations due to the high success rate of vacuum-foam drying at this concentration. When lower trehalose formulations were evaluated, a combination of either freezing during vacuum-foam drying, low vacuum-foam drying success (discussed below) or poor membrane integrity results was observed (data not shown). These outcomes made it difficult to draw conclusions at lower concentrations of trehalose. Further studies would be required to optimize loading at reduced trehalose concentrations with improved liquid stability and vacuum-foam drying success.

Chapter 2: Drying Process Development

In 1913, Charles Campbell invented a process for drying milk that resulted in a light and porous material that dissolved more rapidly than powders produced from earlier processes.[37] The process involved whipping or foaming a milk concentrate prior to drying the material by exposure to hot air. In 1943, Wilbert Heyman designed another foam drying process that utilized a high vacuum to boil a lemon extract and corn syrup mixture at lower temperatures until the bubbles began to solidify.[38] The final product resulted in a dried foam structure which could be further dehydrated and ground to a desired particle size. Over the next half century, the process of drying a foamy material with the use of a vacuum (i.e. vacuum-foam drying) would be adapted to stabilize bacteria,[39] live viruses,[40] and other biologically active materials.[41] This section describes the process development performed to utilize vacuum-foam drying, as well as freeze-drying and spray freeze-drying, in this work.

Vacuum-foam drying transforms a solution or suspension into a dried static foam through vacuum-induced evaporation and boiling. This differs from the primary dehydration process during freeze-drying, which is sublimation. Vacuum-foam drying enables removal of water by evaporation and boiling at low processing temperatures (e.g. <10 °C) through the application of a vacuum. For pharmaceutical development and manufacturing, this method can be performed using a lyophilizer capable of pressure control at a higher range than a typical freeze-drying cycle (e.g., 1-10 Torr). The vacuum-induced boiling process gradually solidifies the wet material resulting in a final dried foam with an expanded volume compared to a typical freeze-dried cake. Development of a foam dried biotherapeutic material often utilizes high concentration sugar formulations which depress the freezing point and increase the viscosity of the solution.[40]

Figure 3 shows that increasing sucrose concentration from 15 to 40% (w/v), while maintaining a 1 mL fill, correlates with increasing volumes of the final dried foam.

Figure 3. Vacuum-foam dried (VFD) preparations of 15%, 30%, and 40% sucrose (left to right).

An example of data from a laboratory vacuum-foam drying cycle used in this work is presented in **Figure 4** (the cycle is similar to the standard cycle described in Materials & Methods). Partially stoppered vials were loaded onto a pre-equilibrated shelf at 20 °C, the pressure was decreased from ambient to 5000 mTorr (based on the capacitance manometer), the shelf temperature was ramped to 30 °C at 1 °C/min and held for 15 minutes. When the vacuum reached 5 Torr, the product temperature decreased below 10 °C due to the vapor pressure of water at this pressure. This decrease in pressure can either result in evaporation at the solution surface (i.e. vacuum dry) or a combination of boiling and evaporation (i.e. vacuum-foam drying). The boiling/foaming process during vacuum-foam drying increases the solution surface area and facilitates efficient removal of water. This process results in a lower residual water content of the final dried product compared to vacuum drying alone (data not shown). Therefore, development work is required prior to processing biological materials to ensure that the selected formulation is suitable for foam drying.

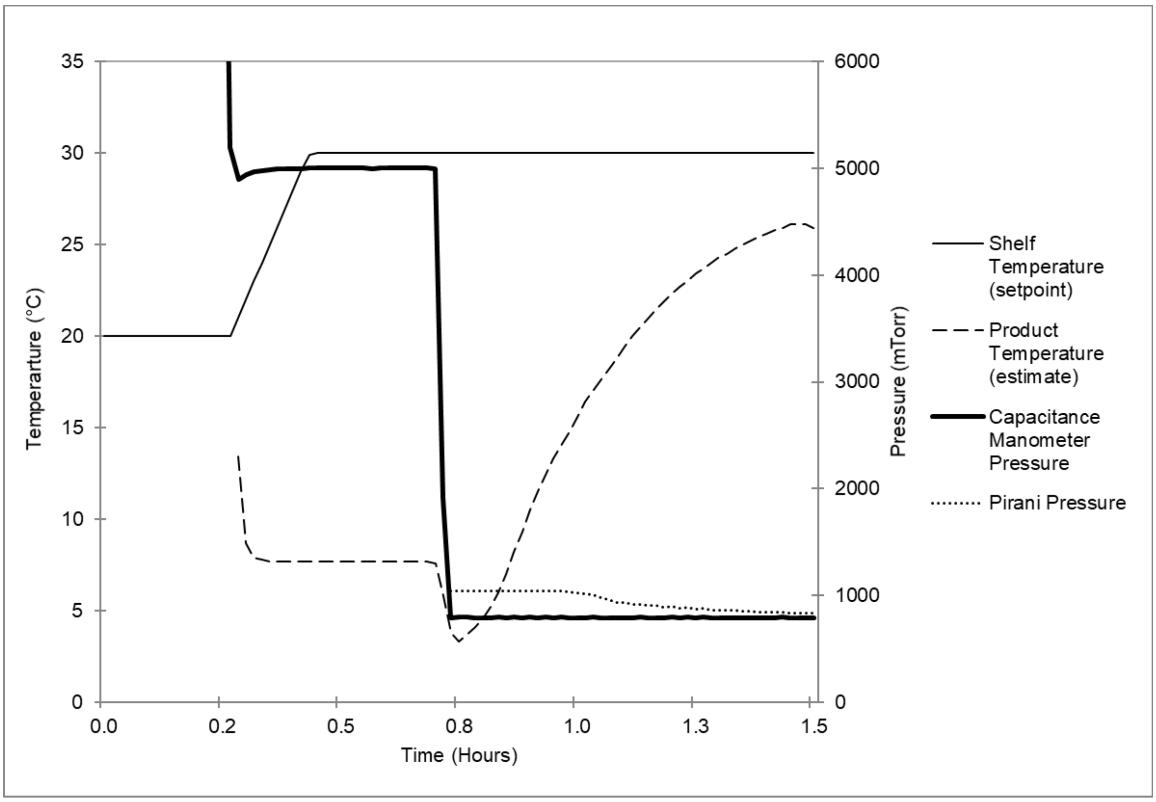

Figure 4. Vacuum-foam drying cycle data

After the 15-minute hold, the pressure was decreased from 5 Torr to 0.8 Torr and water continued to be removed by a combination of boiling and evaporation until it gradually solidified. As the rate of water removal decreased, evaporative cooling was reduced and the product temperature increased until it reached equilibrium near the shelf temperature (**Figure 4**). The initial foam drying phase is deemed complete once the Pirani pressure gauge reached equilibrium with the capacitance manometer (**Figure 4**). After the initial foam drying phase, residual water content can be reduced further through the desorption of water during a secondary drying step which is typically performed at a higher shelf temperature. The rate of water desorption is slower for a foam-dried material compared to a freeze-dried material and, therefore, longer secondary drying times may be required in order to achieve comparable residual water

content.[42] A secondary drying step was typically not used in this work since low residual water content resulted in reduced membrane integrity of dried T cell preparations.

The formulation composition itself plays a significant role in the ability of the foam drying cycle to produce a dried foam. Depending on the formulation of the material being processed, the application of vacuum (e.g. from ambient to 5 Torr) may yield a dried thin-film (**Figure 5a**) instead of a foam structure. Some proteins, such as BSA, are known to promote foam formation[43] and were hypothesized to improve foamability during the vacuum-foam drying process. The impact of BSA protein on the foamability of a 30% sucrose formulation during vacuum drying was evaluated (**Figure 5**). The drying cycle used a shelf temperature starting at 0 °C during the initial pressure decrease from ambient to 5 Torr prior to ramping to 30 °C. The reduced temperature differential between shelf and product was previously shown to reduce foamability (data not shown) and, therefore, these conditions challenged the robustness of the formulations to foam formation. **Figure 5** shows the final product after foam drying 300 mg mL^{-1} sucrose solution with and without BSA at 30 mg mL^{-1} (10 mL vials with a 1 mL fill volume). While the sucrose solution alone did not result in foam formation, the addition of BSA successfully produced a dried foam structure in all vials.

Figure 5. Comparison of product appearance for 30% sucrose solution (a) and 30% sucrose, 3% BSA solution (b) after vacuum drying cycle.

These data show that formulations containing high concentration of sucrose can be robustly foam dried with the addition of 30 mg mL^{-1} BSA. The heterogeneity in vial-to-vial appearance from a vacuum-foam drying process is a challenge that should be considered during formulation optimization and process development.[44] Additionally, vial-to-vial variability in foam success can be observed where some vials (containing the same formulation) within a given batch will successfully boil/foam while others within the same batch will not. During initial evaluations of this next-generation technology, formulations that consistently result in a foam dried product should be used and future work can be performed to address these additional challenges.

Freeze-drying and spray freeze-drying cycles were developed to target higher residual water content (~6-10% w.b.) than typically utilized for biotherapeutics. A freeze-drying cycle

with a conservative primary drying step and no secondary drying step was used to evaluate process loss and stability for freeze-dried preparations. A schematic overview of the laboratory scale spray freeze-drying process is shown in **Figure 6**. Spray freeze-drying cycle parameters are described in Materials & Methods. Similar to the freeze-drying cycle, the secondary drying step was not performed for the spray freeze-drying process.

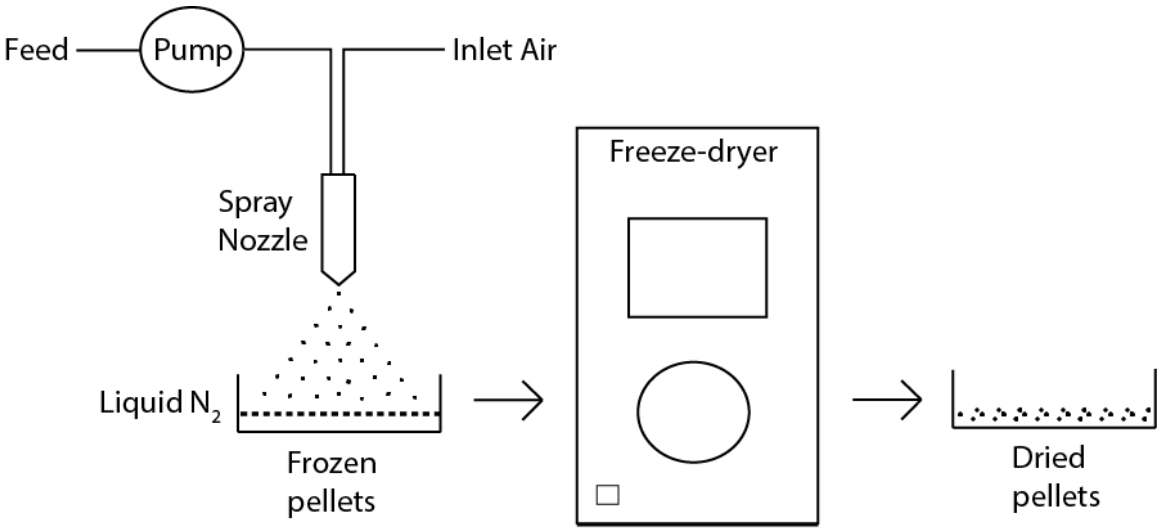

Figure 6. Laboratory Scale Spray Freeze-Drying Schematic

Chapter 3: Membrane Integrity after Drying and Rehydration

Membrane integrity of pre- and post-dried cell preparations was measured using a NC-3000 Via1-Cassette™ membrane integrity and cell count assay. The procedure uses membrane penetrating acridine orange (AO) and non-penetrating 4′,6-diamidino-2-phenylindole (DAPI) fluorescent dyes to assess cellular membrane integrity (see Materials & Methods). After drying and rehydration, cells possessed varying levels of DAPI penetration. **Figure 7** shows images of cells with varying AO to DAPI area ratios. Cells without DAPI observed were quantified as possessing membrane integrity. Cells with DAPI observed, even at significantly lower levels than AO, were quantified as not possessing membrane integrity. The varying levels of DAPI penetration may suggest variability in the structural damage induced on the cell population.

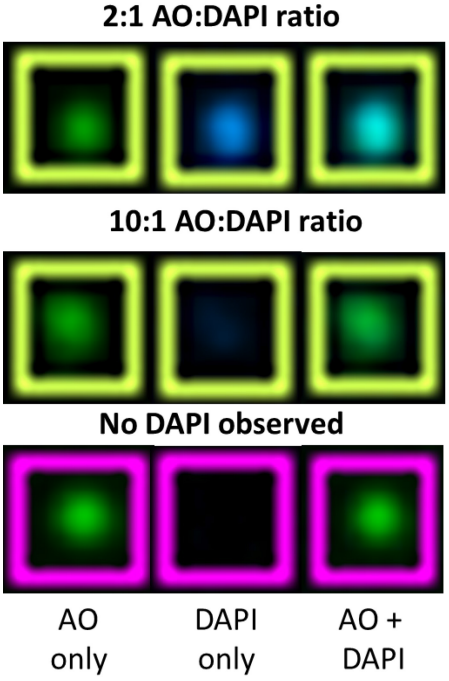

Figure 7. Images from the NC-3000 membrane integrity and cell count assay. Membrane penetrating acridine orange (green) and non-penetrating 4′,6-diamidino-2-phenylindole (blue) are used to assess membrane integrity.

Preserving membrane integrity can be challenging due to the many factors that play a role in maintaining an intact phospholipid bi-layer through drying and rehydration. Since cryopreserved cells were not in an optimal state immediately after thaw, the impact of subsequent recovery (i.e. incubation in media at 37 °C, 5% CO_2) after the cells were thawed was evaluated. After recovery, cells were formulated at 5E6 cells/mL, 30% trehalose, 3% BSA #1 in PBS and membrane integrity was measured. While the primary purpose of trehalose in the formulation is cell stabilization, BSA was predominantly used for its favorable foaming properties (see Drying Process Development). It is noteworthy that albumin (human) is found in multiple approved CAR T cell therapy formulations[45, 46] that are stored in the frozen state.

Membrane integrity of all 5E6 cells/mL cell preparations prior to drying was approximately 90% (after centrifugation and resuspension in the formulation matrix). However, prior to formulation, the number of cells per mL after 3 days of incubation in the culture flask was approximately double the initial (0 day) density, demonstrating cell proliferation during incubation. Using the standard foam drying cycle, 1 mL aliquots were dried to 10.4 ± 0.3% (average ± std, n = 2) residual water content (w.b.). After rehydration with water and 4:1 dilution in PBS (pH 7.4), samples were held for approximately one hour and membrane integrity was measured. While pre-drying membrane integrity was comparable for all samples, there was a significant improvement in membrane integrity for cells that were incubated for at least 1 day as opposed to dried immediately after being thawed (**Figure 8**). This data suggests that beyond pre-drying membrane integrity, the overall health and growth phase of the cells prior to drying plays a significant role in their ability to withstand drying and rehydration stresses.

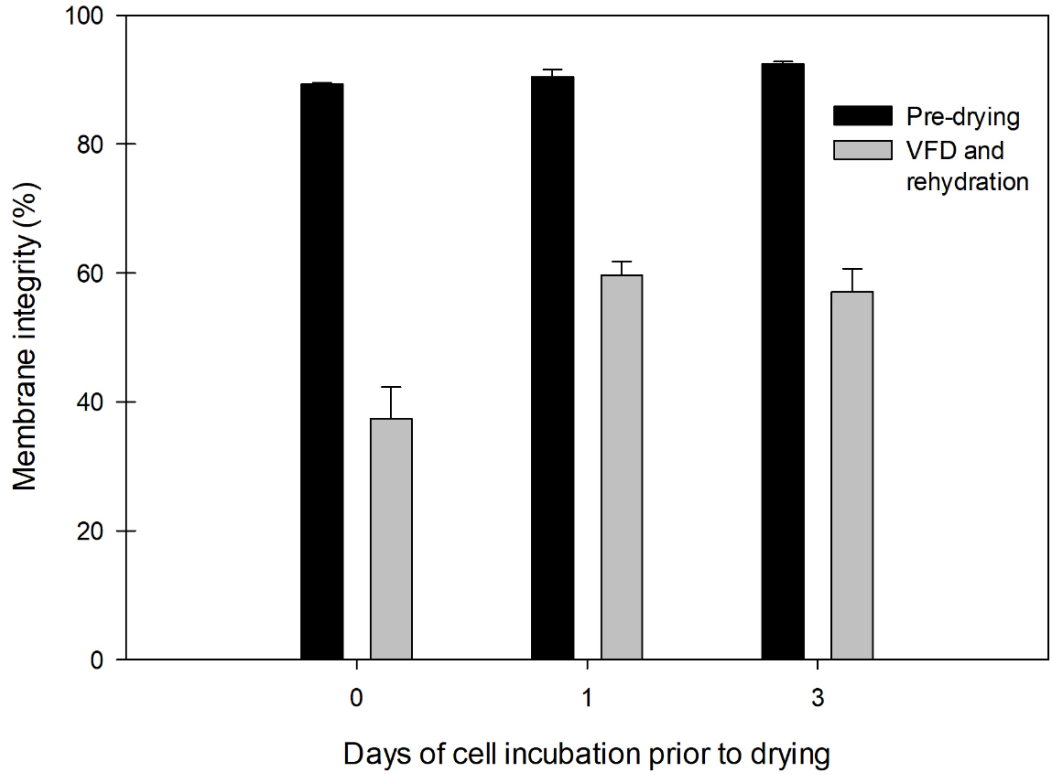

Figure 8. Impact of cell incubation time (prior to drying) on T cell membrane integrity after vacuum-foam drying (VFD) and rehydration. Data presented as average ± standard deviation (n =4) and compared to pre-dried samples (n = 2).

The drying time and kinetics were expected to play a significant role in preservation of membrane integrity throughout drying. To evaluate these parameters, cells were incubated for 1 day, prepared in the same formulation (5E6 cells/mL, 30% trehalose, 3% BSA #1 in PBS) and dried using a rapid foam drying cycle (20-minute drying time). After the rapid drying cycle, the membrane integrity of the human T cells decreased 29.8% (89.0 ± 2.8% to 59.2 ± 3.5%) (**Table 1**). Interestingly, the membrane integrity results were not significantly different from the previous data using a standard foam drying cycle (100-minute drying time), which resulted in a decrease of 30.7% (90.4 ± 0.9% to 59.7 ± 0.9%). This data suggests that the cell recovery in a

high concentration trehalose-based formulation was not significantly improved by reducing drying time.

Since at high trehalose concentrations a significant amount of trehalose is loaded into the intracellular space, the rehydration/dilution method was expected to have an impact on osmotic shock, swelling and potentially lysis of the cells. In 1959, Leach and Scott discussed the surprising fact that many researchers aiming to dry micro-organisms did not discuss the impact of rehydration method. Leach and Scott showed that the survival of dried bacteria was impacted by rehydration rate and concentration of solutes in the rehydration medium.[47] Several later studies have also demonstrated the importance of rehydration medium and procedure on cell viability.[48-50] Therefore, the impact of reconstitution procedure on cell membrane integrity after rehydration was evaluated.

Table 1 shows the impact of various reconstitution methods on the membrane integrity of dried cell preparations. The cells were dried at 5E6 cells/mL, 30% trehalose, 3% BSA #1 in PBS using the rapid drying cycle and a 100 µL fill volume. Method 1 was expected to result in the most significant osmotic shock (i.e. the largest differential in solute concentration across the membrane) since the matrix is rehydrated and diluted (4:1) with PBS during the initial reconstitution step. Methods 2 through 4 differ in the diluent type (PBS or match buffer) and timing of the dilution (concurrently with reconstitution or after a 1-hour post-reconstitution hold). Method 5 used a partial reconstitution with water and a 10-minute hold prior to diluting in match buffer. These methods were hypothesized to improve the membrane integrity of rehydrated cells by reducing the osmotic shock compared to method 1. All the subsequent methods outperformed method 1 and method 5 (i.e. partial reconstitution method) resulted in the highest post-rehydration membrane integrity (84.0 ± 0.2%).

Reconstitution Method	Water (uL)	PBS dilution (uL)	Match buffer dilution (uL)†	Membrane Integrity (%)‡
Pre-drying: Sample diluted prior to measurement	N/A	400	N/A	89.0 ± 2.8
Method 1: Water and PBS added during reconstitution, viability measured after ≥1 h hold	70	400	N/A	59.2 ± 3.5
Method 2: Water and match buffer added during reconstitution, viability measured after ≥1 h hold	70	N/A	400	80.2 ± 2.6
Method 3: Water added during reconstitution, held for 1 h, diluted with PBS, viability measured	70	400	N/A	78.7 ± 5.6
Method 4: Water added during reconstitution, held for 1 h, diluted with match buffer, viability measured	70	N/A	400	77.3 ± 2.1
Method 5: Water added during reconstitution, held for 10 min, diluted with match buffer, viability measured after ≥50 min hold	45	N/A	425	84.0 ± 0.2

† Match buffer = 30% trehalose, 3% BSA #1 in PBS (pH 7.4); ‡ n = 2; Vial fill volume of 100 µL (with approximately 30% solids content) in a 2 mL vial, Dilutions targeted a final volume of 500 µL

Table 1. Comparison of various reconstitution methods and impact to membrane integrity after rehydration.

A sample prepared at 1E6 cells/mL was compared to 5E6 cells/mL to test the impact of cell concentration on membrane integrity after drying and rehydration (**Figure 9**). The 1E6 cells/mL preparation possessed lower membrane integrity after reconstitution using method 1. The 5E6 cells/mL preparations exhibited improved tolerability to osmotic shock during reconstitution per method 1. However, when dried samples were reconstituted with water and diluted with match buffer (30% trehalose, 3% BSA #1 in PBS), there was not a significant difference in membrane integrity.

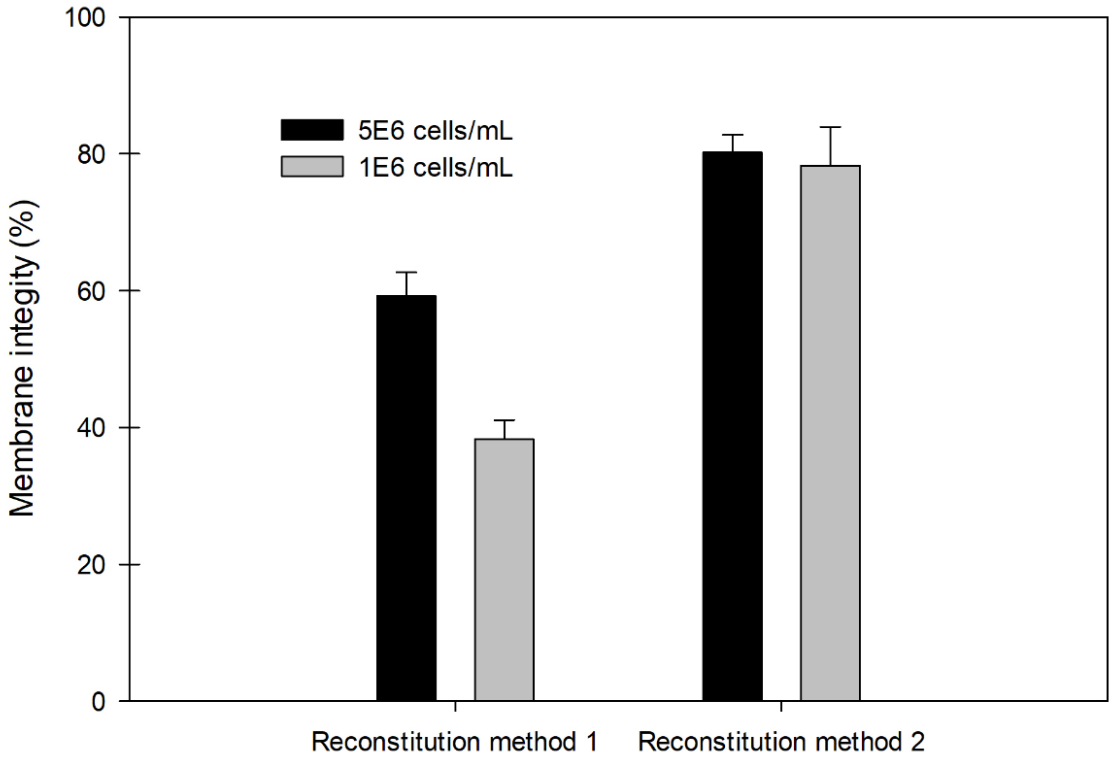

Figure 9. Impact of cell density and reconstitution method on membrane integrity after vacuum-foam drying. Data presented as average ± standard deviation (n = 2).

An unexpected change in membrane integrity results after drying was observed during follow-up experimentation (data not shown). Based on these data, a study was executed to determine if the different source of BSA (BSA #2, manufactured by Sigma-Aldrich, see Materials & Methods) used in these experiments had a significant impact on cell stabilization. To evaluate whether an impurity in one of the BSA sources was leading to stabilization/destabilization during drying, cells were dried using a 30% trehalose, 3% albumin (in PBS at pH 7.4) formulation with different albumins (BSA #1, BSA #2, and HSA). Formulation buffers containing BSA and HSA were dialyzed (Thermo Scientific Slide-A-Lyzer Dialysis Cassettes, 7,000 MWCO) against 30% trehalose in PBS to remove any small molecule impurities and results were compared to non-dialyzed buffer preparations. All formulations were

vacuum-foam dried using the rapid foam drying method to a final residual water content of 13.4-15.4% (w.b.).

Figure 10 shows the membrane integrity results after reconstituting dried foam preparations with water and concurrent dilution in match buffer (reconstitution method 2 from **Table 1**). Dialyzed and non-dialyzed BSA #1 formulations possessed significantly higher membrane integrity after reconstitution than BSA #2 formulations (dialyzed and non-dialyzed). Dialysis of BSA #2 improved final membrane integrity suggesting that an impurity in BSA #2 may have contributed to cell degradation during drying. However, even dialysis of BSA #2 did not yield membrane integrity results comparable to BSA #1, therefore, the small molecule impurity was not likely the only factor contributing to instability. The replacement of BSA #1 with HSA did not have a significant impact on foam drying success or membrane integrity suggesting that the common pharmaceutical excipient may be a replacement for BSA. This experiment showed similar results between BSA #1 and HSA as well as improved membrane integrity of dialyzed BSA #2, thus no further investigation was performed using BSA #2.

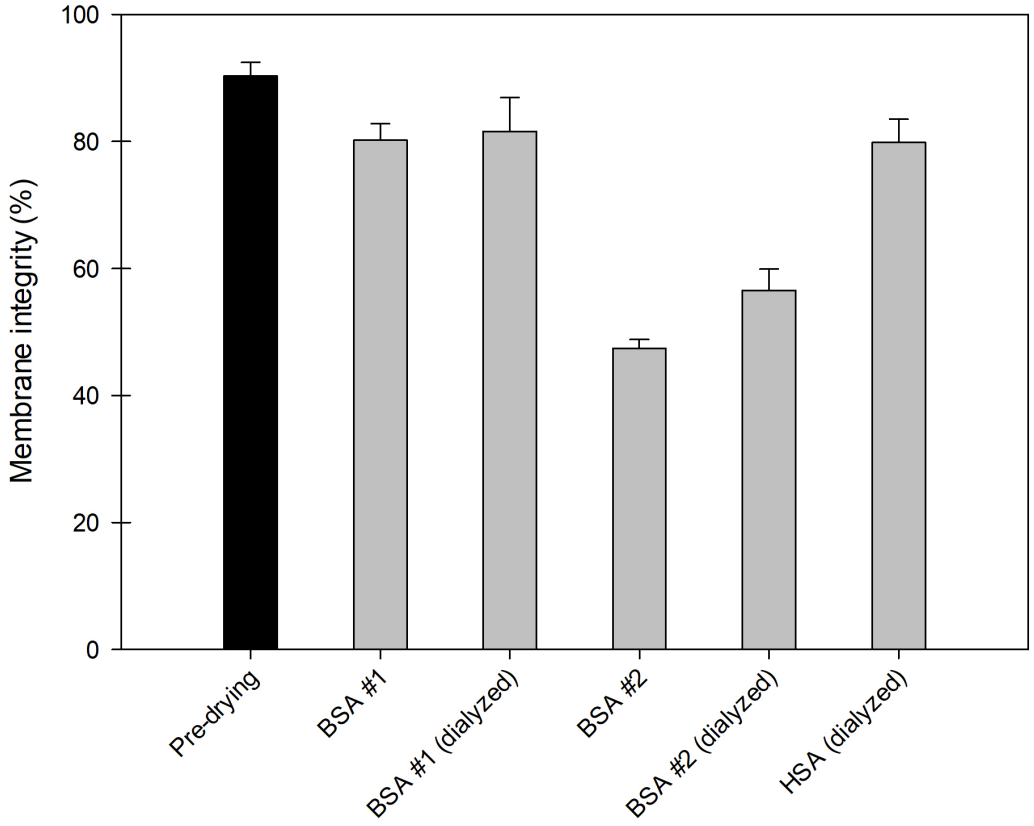

Figure 10. Impact of albumin type on T cell membrane integrity after drying for 30% trehalose, 3% albumin in PBS (pH 7.4) formulations. Membrane integrity of all formulations was measured prior to drying and the average (± std, n =2) membrane integrity (%) is presented (black) for comparison to results after drying and rehydration (grey). Rehydration was performed using reconstitution method 2 (see Table 1).

The next step towards producing a stable amorphous human T cell solid was to evaluate the impact of residual water content on membrane integrity and glass transition temperature of the amorphous matrix. Human T cells incubated for 2 days were formulated with 30% trehalose, 3% BSA #1 in PBS and dried using the standard foam drying method in a 10 mL vial with a 1 mL fill volume (approximately 1E6 cells/mL). A reconstitution method similar to method 5 from

Table 1 (10 times larger reconstitution/dilution volumes used due to 10 times larger fill volume) was used to rehydrate dried foams for membrane integrity testing.

Table 2 shows the change in residual water content, glass transition temperature, and membrane integrity throughout the drying process. The glass transition temperature of a dried product must be higher than the intended storage condition to reduce degradation during storage.[51] The glass transition temperature of the foam dried material was increased through reducing the residual water content.

The pre-dried T cell formulation possessed 94.0% membrane integrity. After 45 minutes of drying, a sample was removed from the freeze-dryer possessing a residual water content of 20% (w.b.) and a rehydrated membrane integrity of 88.5%. After 90 minutes of drying, the residual water content was reduced to 11.7% (w.b.) and the rehydrated membrane integrity to 86.5% (less than 1 $\log_{10}$ loss compared to starting formulation). The glass transition temperature (midpoint) of the 90-minute sample remained lower than ambient storage (T_g = 12.3 °C). As the residual water content was reduced below 10%, the glass transition temperature increased above ambient storage, however, the membrane integrity continued to decrease (82.3%). It is possible that the reconstitution procedure may have a greater impact to membrane integrity as samples become drier; therefore, further optimization of the rehydration method may be needed to improve membrane integrity results for lower residual water content samples.

Overall, this data demonstrates the need to optimize residual water content to balance degradation during drying and a glass transition temperature high enough for convenient storage. **Figure 11** presents a visualization of this balance by plotting cellular membrane integrity and glass transition temperature as a function of residual water content. The data show that high levels of membrane integrity can be retained after drying and rehydration.

Attempts to re-grow the cells after rehydration by diluting them in cell growth medium and placing in the incubator were unsuccessful. A significant number of cells aggregated and/or lysed and no cell growth was observed. One potential reasons for this could be that the cells are going through a lipid-phase transition when placed in the 37 °C incubator leading to a leaky membrane and cell degradation. Another possible reason is that during dilution, the cells are placed in a hypotonic solution (i.e. more solutes such as trehalose inside the cell compared to the extracellular matrix) leading to uptake of water and cell lysis.

Drying time (hr)	Residual water content (%, w.b.)	T_g (°C) (onset, midpoint)	Log_{10} loss in membrane integrity	Membrane integrity (%) ± std (n=6)
0	N/A	N/A	N/A	94.0 ± 0.4[‡]
0.75	20.0	not detected	0.74	88.5 ± 2.4
1.5	11.7	7.8, 12.3	0.88	86.5 ± 2.7
3	9.4	21.4, 25.8	1.07	82.3 ± 2.7
6	7.9	28.8, 32.6	1.15	79.9 ± 4.8
12	6.1	36.7, 42.3	1.36	71.3 ± 6.2

[‡] n = 3

Table 2. Impact of drying time and residual water content on T cell membrane integrity and T_g.

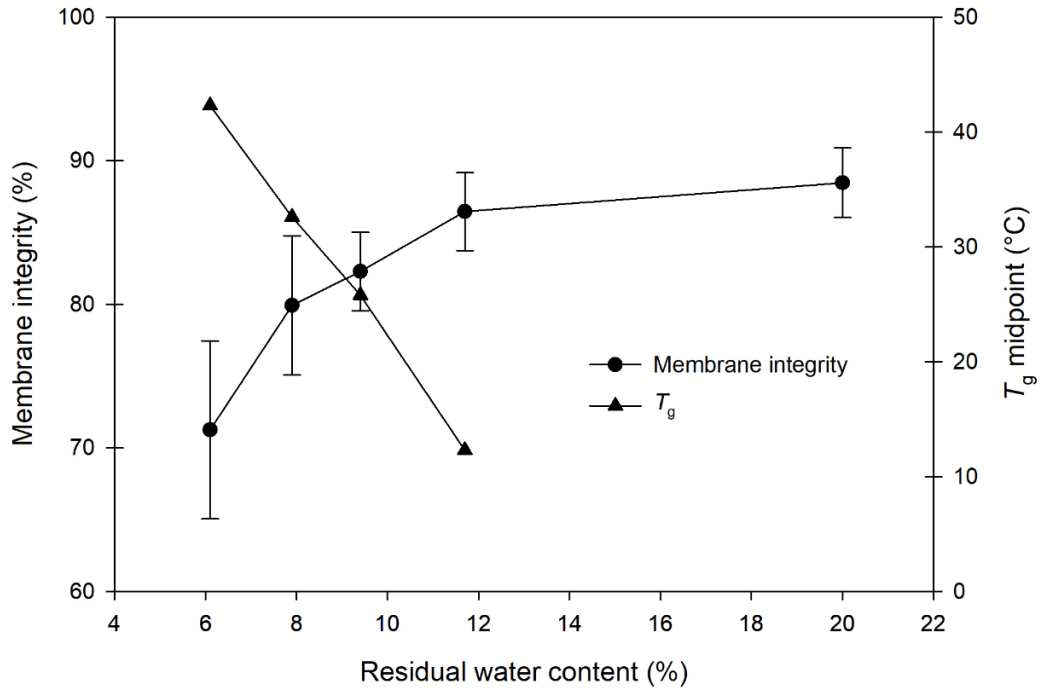

Figure 11. Impact of residual water content on membrane integrity and T_g. Membrane integrity data presented as average ± standard deviation (n = 2).

Figure 11 demonstrates that the dried trehalose-based formulation (30% trehalose, 3% BSA in PBS) exhibits a glass transition temperature above refrigeration storage (i.e. 2-8 °C) at and below approximately 12% residual water content. Previous work showed that storage stability of vacuum-foam dried T cells decreased when stored above their glass transition temperature compared to below (data not shown).[52] To evaluate the use of various drying techniques, human T cells (approximately 2.5E6 cells/mL) were dried using three different drying methods; vacuum-foam drying, freeze-drying, and spray freeze-drying. Prior to drying, the cells were diluted in complete media (growth media + human serum with IL-6) and incubated at 37 °C, 5% CO_2 in a culture flask for 3 days. The drying cycles were designed to target between approximately 8% to 10% residual water content (see Drying Process Development). The residual water content of the dried preparations was 6.5% for freeze-drying, 9.2% for

vacuum-foam drying, and 7.6% for spray freeze-drying. The freeze-dried preparations were noted to have about 1.5% lower residual water content than the target range and, therefore, expected to possess lower membrane integrity after rehydration.

Figure 12 shows the appearance of freeze-dried, vacuum-foam dried and spray freeze-dried T cell preparations in a 10 mL vial. The freeze-dried preparation exhibited typical cake appearance (i.e. no collapse or shrinkage) with minor cracking which is not expected to impact product quality.[44] Some splashing was observed during the vacuum-foam drying process and is visible on the side of the foam dried vial. The atomization step of the spray freeze-drying process generates an aerosol that is then frozen and freeze-dried into spherical particles resulting in a semi-flowable powder. There were not any significant challenges with reconstitution for any of the products. The reconstitution procedure used a partial water reconstitution followed by dilution in match buffer after 10 minutes and a 50-minute hold (reconstitution method 5 in **Table 1**). Foaming was observed initially after reconstitution of the spray freeze-dried powder that dissipated after a few minutes. The reconstitution volumes for the spray freeze-dried preparation was adjusted since the powder was filled manually into the vial resulting in powder weight that was not identical to the freeze-dried and vacuum foam-dried preparations.

Figure 12. Freeze-dried (left), vacuum-foam dried (center) and spray freeze-dried (right) preparations of dried human T cells (Formulation: 30% trehalose, 3% BSA in PBS, pH 7.4).

The membrane intergity of each sample was measured and compared to the pre-dried control (**Figure 13**). The membrane integrity of the pre-dried T cell formulation was 91.2±1.5%. Vacuum-foam drying resulted in a lower process loss (6.5%) than the freeze-drying (20.4%) and spray freeze-drying (64.9%) processes, although, as previously noted, the residual water content of the vacuum-foam dried preparation was 1-3% higher. The freeze-dried process loss was consistent with the loss observed when vacuum-foam dried preparations were dried to similar residual water content (**Figure 11**). Based on **Figure 11**, 6-7% residual water content of vacuum-foam dried preparations resulted in post-rehydration membrane integrity between approximately 70-75%.

The membrane integrity results after 12 weeks of storage at 5 °C demonstrate that the vacuum-foam dried material (89.7±1.7%) was significantly more stable than the freeze-dried (42.9%) and spray dried (16.3%) material. The improved stability of vacuum-foam dried preparations was demonstrated even though all materials were stored below (i.e. >~20 °C) their glass transition temperature (T_g estimated based on Figure 11 and residual water content values).

Figure 13 demonstrates that T cell membrane integrity can be retained through the vacuum-foam drying process and after storage at refrigeration temperature. These data demonstrate improved storage stability in foam dried preparations compared to freeze-dried and spray freeze-dried preparations. The results are consitent with findings from studies evaluating a monoclonal antibody[53] and a live attenuated vaccine[54] demonstrating the versatility of the process to stabilize various therapeutic compounds. The improved storage stability of macromolecules in a dried amorphous foam has previously been correlated to reduced specific surface area and molecular mobility.[42, 53]

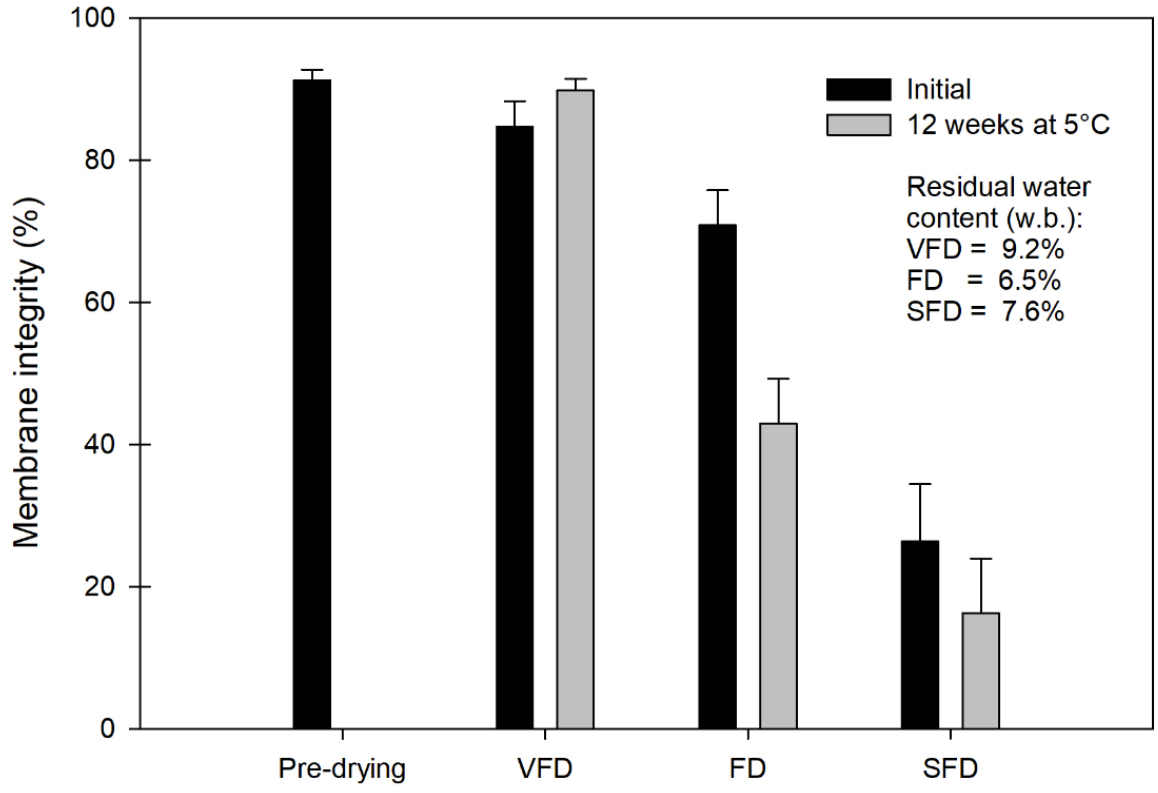

Figure 13. Membrane integrity of human T cells in 30% trehalose, 3% BSA in PBS, pH 7.4 formulation after drying and 12 weeks of storage at 5 °C compared to pre-dried control. Data presented as average ± standard deviation (n = 3-5). (VFD = vacuum-foam dried; FD = freeze-dried; SFD = spray freeze-dried).

Conclusion

The preservation of human T cell membrane integrity after drying in the amorphous state and rehydration required utilization of a systematic approach that evolved as additional data/learnings were acquired. Kinetic studies demonstrated the uptake of trehalose into the intracellular matrix of T cells when formulated at high trehalose concentrations. Recovering post-thawed cells in a 37 °C, 5% CO_2 incubator for at least 1 day prior to drying was shown to significantly improve post-drying and rehydration membrane integrity. Several studies demonstrated that the reconstitution medium and procedure used to rehydrate dried T cell preparations have a significant impact on membrane integrity. The residual water content in the dried amorphous solid was shown to be a critical factor that needs to balance improved process recovery at higher residual water content and increased glass transition temperature at lower residual water content. The technique used to dry the cells was also shown to have an impact on the process recovery and storage stability of the cells. Vacuum-foam dried preparations exhibited reduced process loss and improved storage stability at 2-8 °C compared to freeze-dried and spray-freeze dried preparations when formulated in a high concentration trehalose-based solution. These findings and fundamental learnings can be used to improve T cell stabilization during drying and rehydration in future work.

There may be stabilizing excipients not evaluated in this work that are able to improve membrane integrity results after drying to lower residual water content values. Drying procedures should continue to be optimized to evaluate the use of lower trehalose concentration formulations and additional stabilizing excipients. Since membrane integrity is vital to cell structure, it was the main attribute assessed in this work to evaluate cell health, however, additional work will be required to enable cell re-growth and maintenance of cell functionality

(e.g. antigen recognition). Learnings from this work regarding drying procedure and reconstitution method can be leveraged in future studies in order to achieve functional mammalian cells capable of proliferation after drying and rehydration. The ability to preserve human T cells in the dried amorphous state will enable new options for storing and delivering these life-saving therapies to patients.

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