Frozen Storage of Proteins: Use of Mannitol to Generate a Homogenous Freeze-concentrate

Jayesh Sonje[a,1], Carly Fleagle Chisholm[b] and Raj Suryanarayanan[a]

[a] Department of Pharmaceutics, College of Pharmacy, 308 Harvard St. SE, University of Minnesota, Minneapolis, MN 55455, USA

[b] Drug Product Development, BioTherapeutics Development & Supply, Janssen Research & Development, Malvern, PA 19355, USA

Correspondence to: Raj Suryanarayanan, PhD

Telephone: (612) 624-9626 Fax: (612) 626-2125

Email: surya001@umn.edu

[1] Current affiliation: Pfizer Biotherapeutics, Pfizer Inc., Andover, MA 01810, USA

ABSTRACT

Therapeutic proteins may be subjected to several freeze-thaw cycles throughout manufacturing and storage. The protein solution composition and the freezing conditions may lead to incomplete ice crystallization in the frozen state. This can also result in freeze-concentrate heterogeneity characterized by multiple glass transition temperatures and protein destabilization. The overall objective was to investigate the potential advantages of including a crystallizing excipient (mannitol) along with a sugar (sucrose or trehalose) for frozen storage. This study showed that the addition of mannitol, a readily crystallizing excipient, facilitated ice crystallization. Inclusion of an isothermal hold during cooling (annealing) maximized the mannitol crystallization and resulted in a homogenous freeze-concentrate of a constant composition characterized by a single glass transition temperature. The role of freezing rate and annealing on both mannitol and ice crystallization were discerned using high intensity synchrotron radiation. The addition of sucrose or trehalose, at an appropriate concentration, stabilized the protein. The mannitol to sugar ratio (3:1 or 1:1, 5% w/v) was optimized to selectively cause maximal crystallization of mannitol while retaining the sugar amorphous. Human serum albumin (1 mg/mL) in these optimized and annealed compositions did not show any meaningful aggregation, even after multiple freeze-thaw cycles. Thus, in addition to a sugar as a stabilizer, the use of a crystallizing excipient coupled with an annealing step can provide an avenue for frozen storage of proteins.

Keywords: Homogenous freeze-concentrate, matrix, mannitol, sucrose, trehalose, frozen storage, freeze-thaw, protein

1. INTRODUCTION

Manufacturing of macromolecules is often divided into two steps – Drug Substance (DS) and Drug Product (DP) manufacturing. The DS is often stored in the frozen state and thawed for DP manufacturing. Occasionally, intermediate solutions and drug product (DP) are also stored frozen (Rathore and Rajan, 2008). The frozen state substantially enhances the shelf life of the DS by minimizing its mobility and hence slowing down reaction rates. It also reduces the risk of microbial growth and helps overcome transport-related stress (e.g. shaking, agitation etc.) (Kolhe and Badkar, 2011; Singh et al., 2009). However, destabilization of macromolecules can occur during freezing and thawing and freeze-thaw related issues can be challenging to overcome. Successful frozen storage of macromolecules requires careful consideration of the biophysical principles dictating stability (Singh et al., 2009).

Protein formulations commonly contain several excipients including a buffer, a sugar or sugar-alcohol, and a surfactant to prevent protein degradation which can occur through various mechanisms. Freeze concentration or cold denaturation induced aggregation remains of concern during freezing and frozen storage (Arsiccio and Pisano, 2020; Authelin et al., 2020). Freezing is initiated by ice nucleation followed by ice crystal growth. Due to supercooling, ice nucleation is often observed substantially below the equilibrium freezing point (Carpenter et al., 1997). Most of the water then separates into ice crystals. The excipient (also referred to as solute) may either crystallize or be retained as a freeze-concentrate. Ice formation is influenced by numerous factors including shelf temperature, freezing ~~and thawing~~ rate, formulation composition and concentration, as well as container shape and size. Once ice nucleates, crystallization and phase separation of the ice continues until a maximally freeze concentrated solution is achieved (assuming the solute is retained amorphous). During the freezing process, proteins are exposed to

stresses including adsorption at the ice-water interface , pH changes due to freeze-concentration and phase separation causing unequal distribution of the stabilizer and protein (Bhatnagar et al., 2007; Connolly et al., 2015; Piedmonte et al., 2007). The cooling rate and the type and concentration of excipients are important factors governing protein stability.

The role of stabilizers (also referred to as cryoprotectants) is to prevent protein denaturation including aggregation during processing and storage. Non-crystallizing excipients (stabilizers) are known to prevent protein denaturation by preferential exclusion and viscous glass formation (Chang and Pikal, 2009). The most important role of these stabilizers is to prevent the unfolding of the protein both during freezing and thawing. According to the preferential exclusion mechanism, the amorphous excipient is selectively excluded from the immediate vicinity of the protein surface thereby enabling its stabilization in the native state. A second mechanism is the decrease in protein mobility brought about by the increase in viscosity of the freeze-concentrate (Wang, 2005). In order to exert their protective action, these stabilizers should remain amorphous.

When a solution containing a crystallizing solute is cooled, at the eutectic temperature, complete crystallization of the solute and ice should ideally occur. However, most of the solutes used in protein formulations do not crystallize readily when frozen. When a solute is retained amorphous, an important attribute of the frozen system is the glass transition temperature of the freeze-concentrate (Tg'). Stabilizers used in protein formulations including sugars, surfactants, and amino acids contribute to formation of an amorphous matrix. Their utility comes from their ability to remain amorphous with the protein in the freeze concentrated phase. Ideally, storage of the frozen mass in the deeply glassy state, for example at approximately 50°C below the glass transition temperature (Tg' - 50), is assumed to inhibit mobility sufficiently to prevent mobility-induced protein degradation or excipient crystallization (Hancock and Zografi, 1997). However, given the

nature of supply chain logistics, it can be challenging to store the frozen bulk at very low temperatures. There are examples of several systems stored at -70ºC which can be practically difficult, expensive, and pose significant challenges with maintaining the cold chain. The freeze-concentrate composition dictates the Tg' and decreases with an increase in unfrozen water content. From a processing and storage perspective, we desire compositions with the highest possible Tg' to avoid the need for storage at very low temperatures.

Sugars such as trehalose and sucrose are used as stabilizers in protein solutions due to their ability to serve as cryoprotectants and resist crystallization. In lyophilized formulations, a combination of stabilizer and a bulking agent has been used as a successful strategy to prevent protein aggregation as well as provide necessary mechanical strength to the final cake (Johnson et al., 2002). Mannitol, a popular bulking agent, has been used due its high propensity to crystallize during freezing along with its high eutectic melting temperature which results in short drying cycles (Kim et al., 1998). Sucrose-mannitol-water ternary solutions exhibit two Tg' (~ -48 and -34ºC) during freezing. The multiple Tg' values reflect heterogeneity in the freeze concentrate. The composition with a lower glass transition temperature has a higher amount of unfrozen water ("water rich" phase) while the composition with the higher glass transition temperature is a "solute rich" phase). However, irrespective of composition and processing (annealing), sucrose is consistently retained in the amorphous state. Annealing is typically conducted to facilitate solute crystallization in frozen systems (Searles et al., 2001). In ternary mannitol-sucrose-water systems, complete mannitol crystallization, and hence its phase separation, will result in a frozen matrix which will resemble sucrose-water binary system. Since mannitol has a strong propensity to crystallize, this can be achieved with the judicious selection of processing steps.

Trehalose-mannitol-water ternary solutions also exhibit two Tg' (~ -45 and -32ºC) reflecting heterogeneity in the freeze concentrate. Investigation of different weight ratios of mannitol to trehalose (R) revealed that when R=1, at annealing temperatures higher than Tg', mannitol crystallization led to trehalose crystallization, whereas at R = 3 only mannitol crystallized (Jena et al., 2017). Crystallization of trehalose may compromise its cryoprotectant function. Connolly et al. investigated the impact of cooling rate, storage temperature, and formulation composition on mAb aggregation in the presence of trehalose during lyophilization. The trehalose crystallization in this case was ensured either by seeding or by controlled ice nucleation. The mAb showed highest aggregation after 12-months storage at a temperature of -20°C as opposed to no aggregation in samples stored at -40°C. The study highlights that if the storage temperature is below the Tg' of the frozen matrix, protein aggregation can be significantly prevented (Connolly et al., 2015). Furthermore, it is necessary to identify trehalose to mannitol ratios which will lead to selective crystallization of only mannitol and not trehalose. It is also instructive to recognize that the protein, in a concentration dependent manner, will inhibit the crystallization of both trehalose and mannitol. The process parameters for annealing may be designed to promote the crystallization of only mannitol.

In ternary mannitol-sugar-water systems, substantial if not complete mannitol phase separation (crystallization) can be accomplished if it is initiated early in the freezing process. Complete mannitol phase separation (crystallization) in a frozen matrix is a desirable attribute. Mannitol crystallization will also result in the crystallization of the associated unfrozen water. This has the potential to reduce the phase separation. We hypothesize that in frozen systems, mannitol crystallization *during* freezing can result in a homogeneous freeze concentrate (characterized by a single Tg'). Complete mannitol crystallization can be accomplished by (i) annealing the solution

during cooling and (ii) using specific mannitol to sugar ratios. In other words, the goal of our work was to use a crystallizing excipient to promote ice crystallization and obtain a freeze-concentrate of consistent and constant composition. Baseline thermal characterization of mannitol-sucrose and mannitol-trehalose mixtures of different compositions was performed using DSC. In order to identify the phases crystallizing from solution, *in situ* (during cooling as well as heating) low temperature X-ray diffractometric measurements (synchrotron source) was performed. To achieve maximum mannitol and ice crystallization, process conditions including (i) cooling at slow rates, and (ii) isothermally holding the optimized mannitol-sugar compositions at desired subambient temperatures during cooling were investigated. In the context of this work, we refer to the isothermal hold during cooling step as 'annealing'. Human serum albumin (HSA) was the model protein used in this study. Aggregation of HSA in solutions that contained histidine, mannitol, and either sucrose or trehalose was monitored by SE-HPLC after multiple freeze-thaw cycles.

2. MATERIALS AND METHODS

2.1. Materials

Mannitol ($C_6H_{14}O_6$), sucrose ($C_{12}H_{22}O_{11}$), trehalose ($C_{12}H_{22}O_{11}$), L-histidine, histidine monohydrochloride and human serum albumin (HSA, $\geq$ 99.0% purified) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aqueous solutions of mannitol and either sucrose or trehalose were prepared in 1:1, 2:1 and 3:1 ratio with a total solute concentration of 5% w/v in 10 mM histidine buffer solutions.

2.2. Differential Scanning Calorimetry (DSC)

A differential scanning calorimeter (model Q2000 TA instruments, New Castle, DE, USA) equipped with a cooling system was used. The instrument was calibrated with tin and indium. Dry

nitrogen at 50 mL/min was used as the purge gas. In the first set of studies, approximately 15 mg of solution was weighed in an aluminum pan, sealed hermetically, cooled to -40°C and held for 15 minutes and warmed to 10°C at 10, 5 and 0.5°C/min.

In another set of studies, about 20 mg of the aqueous solutions were weighed into an aluminum pan, sealed hermetically, and cooled from room temperature to -60°C at 1°C/min, held isothermally for 10 min, and heated at 1°C/min to room temperature, under a stream of nitrogen. Annealing/temperature cycling was performed to achieve maximum solute and ice crystallization. Samples were annealed at -20°C for 2 to 16 hours above Tg' of the system during warming. The solutions were cooled to -60°C and rewarmed, the Tg', heat capacity associated with Tg' (Δ Cp) and the enthalpy of ice and solute melting endotherms in the final warming curve were recorded.

2.3. Synchrotron XRD (Transmission Mode)

Phase transformations during freezing and warming were also characterized at the synchrotron X-ray beamline 17-BM-B (sector 17; Advanced Photon Source, Argonne National Laboratory, IL, USA). A monochromatic X-ray beam (λ=0.45452 Å, beam size 300 µm x 300 µm) and a two-dimensional (2D) area detector (XRD-1621, Perkin Elmer) were used. More details with respect to the experimental setup can be found in our earlier publication (Bhatnagar et al., 2020).

The aqueous solutions were placed in a custom-made copper sample holder with a Kapton® window, polyether ether ketone (PEEK) base and a thermocouple. A T-thermocouple (Omega) was used to record the real-time temperature using a temperature input device (NI USB-TC01, National Instruments, TX). Freezing and warming of 100 µL of sample placed in the V-shaped copper sample holder was carried out with the aid of Cryostream 700 plus (Oxford Cryosystems Ltd, Oxford, UK) adjusted 3 to 5 cm above the sample holder. The temperature difference between the cryostream and sample holder was determined by cooling an aqueous sodium chloride (23%

w/w) solution and determining the sodium chloride - water eutectic temperature. Background signal was collected by exposing the sample holder without any solution. The 2D X-ray patterns collected were converted to 1D 2θ scans using GSAS-II software (Edgewall Software) (Toby and Von Dreele, 2013). The crystalline phases were identified, and the integrated peak intensities were determined using commercial software (JADE 2010, Material Data, Inc.).

2.4. Size Exclusion High Performance Liquid Chromatography (SE-HPLC)

Protein aggregation in formulations was monitored by determining the % monomer and % high molecular weight species (HMWS) using SE-HPLC. An ACQUITY UPLC Protein BEH 200 SEC column was used on an ACQUITY UPLC H class system with UV detection (Waters Corporation, Milford, MA). The mobile phase solution contained 0.2 M Sodium Phosphate at pH 6.8. Injection volume for each sample was 20 µL and the flow rate was 0.5 mL/min. Absorbance of the eluent at 220 nm and 280 nm was measured and chromatograms were analyzed using Empower® 3 software (Waters Corporation, Milford, MA).

2.5. Freeze-thaw

Freeze−thawing was carried out in a benchtop freeze-dryer (VirTis AdVantage, Gardiner, NY). Each formulation was filled (3 mL) in 10 mL glass vials (DWK Wheaton, IL). For unannealed samples, the vials were first cooled from 25°C to 5°C, held for 30 min, and further cooled to -40°C and held for 30 minutes. The frozen samples were thawed back to 20°C with an isothermal hold step at 5°C for 30 minutes. Both cooling and thawing rates were 1°C/min. After thawing the vials were swirled. The process of freeze-thawing was repeated 5 times. Similar protocol was used for annealed samples with an additional annealing (isothermal hold) step at -20°C for 2 hours.

3. RESULTS AND DISCUSSION

3.1. Mannitol-sucrose systems

Thermal characterization of different ratios of mannitol to sugar were performed using DSC. The solutions were cooled from RT to -40 ºC and then heated to RT at cooling and heating rates of either 10 or 5 ºC/min. A higher ramp rate was chosen to screen the thermal behavior of with different mannitol to sugar ratios with the goal of identifying compositions in which mannitol crystallization was observed during cooling. However, only the final heating curves are shown (Figure 1 and 3). At M:S ratios of 1:1, 1:2 and 1:4, there was no evidence of mannitol crystallization, both during cooling (not shown) and heating (Figure 1, panels A (10 °C/min) and C (5 °C/min)). At a M:S ratio of 2:1, an exotherm was observed during heating, attributable to mannitol crystallization (Figures 1 A and C). The DSC curve of this composition has been expanded in panels B and D. The system was characterized by two glass transition events, Tg" (lower temperature transition) at ~-32 ºC and Tg' at ~ -27 ºC and these seemed to be unaffected by the cooling rate. However, at the lower cooling rate of 5 ºC/min, there was an increase in the fraction of mannitol crystallizing from solution (enthalpy of exotherm at ~ 10 ºC; panels B and D). When the cooling rate was decreased to 0.5º C/min, there was evidence of mannitol crystallization during cooling (exotherm at ~-23 ºC; Figure 2A). The inset is an expanded view. When the solution was heated, again a crystallization exotherm was observed at ~ -25 ºC. Thus, crystallization of mannitol was not complete during cooling. This amorphous fraction crystallized during heating, immediately above the Tg'.

3.2. Mannitol-trehalose systems

Qualitatively similar results were obtained in M:T systems with different ratios (Figure 3, panels A and C). Though this system also exhibited two glass transition events (Tg' and Tg"), the cooling

rate influenced their values. However, this was not investigated further. The enthalpy of mannitol crystallization was much higher in presence of trehalose (Figure 3 B and D). It is known that trehalose facilitates mannitol crystallization (Jena et al., 2016). The influence of trehalose was further evident at a slower cooling rate of 0.5 ºC/min (data not shown). There was pronounced but incomplete crystallization of mannitol during cooling. However, the amorphous fraction crystallized during heating (Figure 4). Thus, the crystallization behavior of mannitol was similar in the presence of the two sugars, sucrose and trehalose. When the cooling rate was further decreased to 0.1 ºC/min, mannitol appeared to crystallize completely during cooling (supplementary information; Figure S1). An exotherm, attributable to mannitol crystallization, was not observed during heating and the system was characterized by a single glass transition event at ~ -32 ºC (Figure 5 B).

To evaluate in greater detail the crystallization behavior of mannitol during cooling, the mannitol: trehalose ratio was increased (3:1). At a cooling rate of 0.1°C/min, ice crystallization occurred earlier (exotherm at ~ -5 °C) and was followed by a second crystallization exotherm at ~ -16 °C with an enthalpy of crystallization of ~9 J/g (supplementary information; Figure S2). Interestingly, when this sample was heated from -40 °C to 10 °C (at 0.1 °C/min), the Tg' as well as the mannitol crystallization exotherm were not seen (data not shown). This suggests that the crystallization exotherm post ice crystallization during cooling can be attributed to mannitol crystallization. The high enthalpy value (~9 J/g), coupled with the absence of crystallization exotherm during heating, suggests complete mannitol crystallization during cooling. The absence of glass transition during heating also supports this contention.

Synchrotron XRD experiments performed during cooling from room temperature to -45°C followed by heating the frozen solution back to room temperature at 1°C/min helped in further

understanding of the mannitol-trehalose 3:1 (5% w/v) system. During cooling, ice peaks first appeared at ~ -9°C followed by appearance of mannitol hemihydrate peaks at ~ -18°C (Figure 7 A). Further cooling to -45°C resulted in a slight increase in the hemihydrate and ice peak intensities. It was evident from the shape of the peaks that ice crystallization was substantially incomplete, indicating that a large fraction of the solute was in the freeze-concentrate along with unfrozen water. In addition, the heterogeneity of the freeze-concentrate was evident from the two glass transitions observed in the DSC. During heating, until ~ -23°C, the hemihydrate and ice peaks did not reveal any change in peak intensity. Further heating resulted in (i) increase in the hemihydrate as well as ice peak intensities, and (ii) transition of the hemihydrate to δ-form of mannitol prior to eutectic melting at ~ 0°C. A pronounced and sharp increase in mannitol peak intensities was observed around Tg'. Thus, the pronounced crystallization propensity of mannitol at T > Tg', observed earlier in the DSC (Figures 1 and 3; panels B and D) was supported by the XRD results.

The propensity of an excipient to crystallize during thawing can have implications on protein stability. The effects would be exacerbated if a system undergoes multiple freeze-thaw cycles. Since proteins are stored frozen, often for prolonged time periods, freeze-thaw studies provide an avenue to understand the impact of long term frozen storage. These results have some important practical implications. Desai et al showed in a glycine mAb formation, protein aggregation increased when the thawing rate was decreased (Desai, 2017). They attributed aggregation during thawing to the crystallization of glycine. The perturbations at the ice-liquid interface brought about by the crystallization of the unfrozen water associated with solute could be a major destabilizing factor. Thus, the existence of amorphous solute in the frozen state, which can then recrystallize during heating can be a source of protein instability. It is instructive to note that during slow

thawing, it is not the solute crystallization per se, but the formation of these "new" ice interfaces (at T > Tg') that is the major destabilizing factor (Figure 7B).

3.3. Mannitol-trehalose (3:1) systems – effect of isothermal hold

The next objective was to determine the effect of holding at -20 °C for different time periods on the phase behavior of excipient mixtures. Mannitol and trehalose individually are known to exhibit two characteristic glass transition temperatures. The glass transition temperatures of mannitol (5% w/w) were ~ -32 (Tg'') and ~ -25°C (Tg') and of trehalose were ~ -45°C (Tg'') and ~ -31°C (Tg') (Pyne et al., 2002). The Tg''' value of -31°C for the mannitol-trehalose (3:1) is close to the Tg'' values of mannitol (-32°C). However, the Tg' value (-32°C) of the mixture was closer to the Tg' of trehalose (Figure 6A). The two glass transitions likely reflect the existence of two slightly different compositions in the freeze concentrate. The composition with the lower glass transition (Tg'') is expected to contain a higher amount of unfrozen water and this can be thought of as a "water-rich phase". The composition with the higher glass transition (Tg') is likely the maximally freeze-concentrated phase. The ΔCp value can be an approximate measure of the "amount" of amorphous phase. The ΔCp values associated with Tg'' and Tg' were 0.19 and 0.09 J/g°C respectively (Table 1). Based on this, the composition characterized by Tg" would constitute the larger amorphous fraction.

After the cooling rate was optimized in section 3.2, the effect of annealing during cooling, on the crystallization behavior of mannitol was investigated. The solutions were cooled to -20° C at 0.5 °C/min and held for up to 16 hours. They were then further cooled to -60°C and then heated back to RT. In the absence of isothermal hold, Tg" and Tg' were observed at ~ -41 and -32 ºC respectively (Figure 6A). When held for ≥ 2 hours at -20°C, the Tg" disappeared while the Tg' remained unaffected (Figure 6B). Thus, holding at -20 ºC even for only 2 hours seems to cause complete crystallization of the composition with the higher water content. There was also a

pronounced reduction in ΔCp associated with Tg' suggesting substantial crystallization of this composition (Figure 6B). The holding time was progressively increased up to 16 hours. The Tg' remained unaffected confirming that this was the maximally freeze-concentrated system of constant composition (confirmed in the synchrotron XRD studies in the next section). There appeared to be a small decrease in the magnitude of ΔCp at the longer annealing times. However, these results should be viewed with caution in light of the low ΔCp values. Finally, the enthalpy of fusion (mannitol-ice eutectic + ice melting) also provided evidence of amorphous phase crystallization due to the isothermal hold at -20 ºC. Holding for 2 hours, caused an appreciable increase in the enthalpy value (Table 1). This could be a consequence of the crystallization of mannitol and the associated unfrozen water. Holding for a longer time period did not have any noticeable effect on the observed enthalpy value. This result in not surprising in light of the small change in ΔCp (Tg') at holding times > 2 hours (Table 1). Annealing the samples at -37°C - a temperature above Tg'' (- 41°C) but below Tg' (-32°C) for 2 hours, resulted in disappearance of the Tg". On reheating, a single glass transition was observed at -34°C followed by an exotherm attributable to mannitol crystallization. Thus, when annealed at a temperature below Tg', mannitol was retained amorphous (supplementary information; Figure S3).

The effect of annealing on the crystallization behavior of mannitol and ice could be discerned from synchrotron XRD experiments performed during cooling (Figure 8A). Ice crystallization was the first event observed (~ -10°C) followed by the appearance of β-mannitol peaks at -12°C. During the isothermal hold at -12°C, there was a pronounced increase in the intensities of both mannitol and ice peaks. This was in line with the earlier report of Mehta et al that MHH formation was completely prevented when crystallization occurred between -10 and -15°C (Mehta et al., 2013). Once β-mannitol and ice peaks formed during annealing at -12°C, the peak intensities increased,

both during annealing and further cooling until -15°C (Figure 8A). On further cooling to -40°C, there was negligible change in the peak intensities.

On heating the frozen system from -45°C to -2°C, there was no increase in ice and mannitol peak intensities up to ~ - 5°C. There was then a progressive decrease in the peak intensities until they disappeared at ~ 1°C (Figure 8B). Thus, the behavior of the annealed system during heating was very different from that of the unannealed solutions (compared Figures 7B and 8B).

The XRD results revealed that annealing during cooling enabled us to: (i) promote selective crystallization of the anhydrous form of mannitol, and (ii) maximize ice crystallization during cooling and thereby minimize the residual unfrozen water crystallization during heating (post Tg').

However, there is one potential challenge. It is well known that mannitol crystallization can promote trehalose crystallization (Sundaramurthi and Suryanarayanan, 2010). The crystallization of trehalose would be highly undesirable since it could compromise its cryoprotectant function. In order to minimize the risk of trehalose crystallization, its concentration was low (1.25%). Moreover, in spite of the high sensitivity of the technique, there was no evidence of trehalose crystallization (Figure 8). In frozen aqueous systems, trehalose crystallizes as a dihydrate with characteristic peaks at 8.8 and 12.6°2θ (Sundaramurthi et al., 2010). These peaks were absent in the frozen solutions (Figure 8). DSC provided additional evidence of the potential for trehalose to be retained in the amorphous state. Even when the annealing time was increased up to 24 hours, the Tg' was invariant (- 31°C) suggesting the retention of a freeze concentrate of constant composition.

Thus, with the judicious selection of the concentrations of trehalose and mannitol, while substantial crystallization of mannitol can be accomplished the trehalose is retained amorphous. Thus, the risk

of trehalose-crystallization induced loss in cryoprotection would be minimized while causing substantial (if not complete) crystallization of mannitol in the desired anhydrous state

Thus, annealing during cooling enabled us to: (i) promote the selective crystallization of the anhydrous form of mannitol, and (ii) obtain a single maximally freeze-concentrated phase of constant composition (Tg').

3.4. Mannitol-sucrose (3:1) systems – effect of isothermal hold

The behavior of mannitol-sucrose (3:1) systems was substantially similar to that of mannitol-trehalose (3:1). While the DSC curves are not shown, the results are summarized in Table 2. The Tg" and the Tg' values were virtually identical for the unannealed mannitol-trehalose and mannitol-sucrose systems (Tables 1 and 2). The one notable difference was the ΔCp at Tg" which was much less (0.08 J/g°C) for the sucrose containing systems. When this "population" was removed by annealing, presumably by crystallization of mannitol and the associated unfrozen water, the attendant increase in the enthalpy of fusion (overlapping of mannitol-ice eutectic and ice melting) was small (from 328 to 333 J/g; Table 2).

3.5. HSA aggregation: Effect of processing (annealed versus unannealed) and composition

Using HSA (1 mg/mL) as a model protein, in selected mannitol-sucrose and mannitol-trehalose buffered solutions, we evaluated the impact of processing on protein aggregation. Selected compositions were also subjected to DSC, at a cooling rate of 1°C/ min, with and without an isothermal hold step. HSA, at a concentration of 1 mg/mL, had no significant impact on mannitol phase behavior (data not shown). Buffered protein solutions in mannitol, sucrose, or trehalose alone and in mannitol-sugar (1:1 and 3:1) mixtures were subjected to multiple freezing and thawing cycles (5 F/T). The 5 freeze-thaw cycles were conducted at a ramp rate of 1°C/min. One set of samples were frozen with an annealing step at -20°C for 2 hours during cooling, while the

other set did not include annealing. The protein stability before and after freezing and thawing was evaluated by SE-HPLC.

We will first look at the effect of freeze-thaw stress on unannealed HSA formulations that contained either mannitol, sucrose or trehalose individually. Generally, increased protein aggregation after multiple F/T cycles is not uncommon due to the stresses proteins encounter throughout freezing and thawing. All the formulations that were subjected to 5 F/T cycles showed an increase in high molecular weight species (HMWS) compared to their respective controls (Figure 9). The most pronounced increases in %HMWS ($\geq$ 1.5.%) were observed in the freeze-thawed formulations containing mannitol or trehalose. In the former case, this is attributed to phase separation brought about by crystallization of mannitol, and the consequent lack of cryoprotection. It is important to note that there was pronounced mannitol crystallization during thawing (Figure 7b) which explains aggregation in mannitol alone formulations. While trehalose remained amorphous, HSA aggregation was observed. We expected the %HMWS to be lower in this formulation since trehalose is a commonly used cryoprotectant. Additional studies would be needed to understand this result.

There was a favorable effect of annealing in the HSA formulation that contained mannitol. In case of mannitol, there are two possible stabilization mechanisms. Substantial crystallization of mannitol during annealing, resulting in a homogenous freeze-concentrate may have facilitated protein stabilization. Secondly, there was no evidence of mannitol crystallization during thawing (Figure 8b). As a result, there was no generation of "new" ice interfaces during thawing – hence the potential for reduced protein aggregation. While the effect of annealing was less pronounced in the presence of sucrose, the process resulted in a single Tg' again indicating a homogenous freeze-concentrate while monomer was substantially retained. While annealing also resulted in a

single Tg' in the trehalose system, there was no reduction in % HMWS compared to the unannealed formulation.

Next, the effect of mannitol-sucrose combination (1:1) was compared with the individual solutes. The %HMWS after 5 freeze-thaw cycles in mannitol-sucrose combinations was much lower than in mannitol or sucrose alone for both annealed and unannealed samples. This composition appears to be better than all the other systems investigated. Similarly, the mannitol-trehalose combination (1:1) performed better than the individual solutes and annealing resulted in pronounced stabilization.

Increasing the mannitol concentration, either with sucrose or with trehalose (3:1) did not have an evident impact on HSA aggregation compared to respective 1:1 compositions. The stabilizing effect of sucrose was readily evident.. The 3:1 mannitol-sugar combinations also had less aggregation than respective formulations composed of mannitol, sucrose, or trehalose alone. However, annealing did not appear to have any additional stabilizing effect. This result is not surprising because mannitol is present at a much higher concentration than the sugar (3:1), it crystallized readily during cooling (Table 1). Thus, the annealing step is not expected to bring about additional mannitol crystallization and the consequent stabilization.

Micro-flow imaging (MFI) was used to characterize sub-visible particles. However, no meaningful trends were observed. The subvisible particles counts for all solutions before and after freeze-thaw were low and were less than 6,000 particles >10 micron and less than 600 particles > 25 micron (supplementary information; Table S1). In other words, the systems met the USP specifications (USP 787/788).

As we compare the two sugars, over a range of compositions as well as the effect of the annealing step, the superiority of sucrose is consistently evident. The underlying mechanism of

cryoprotection was outside the scope of this work. Interestingly, the mannitol-sugar combination with annealing performed better or equivalent to the sugar alone formulations in terms of protein stabilization. Although limited to a single protein (HSA), the most pronounced stabilization effect following annealing was observed at a 1:1 mannitol-sugar ratio. This supports our hypothesis that inclusion of mannitol along with sugar and causing complete mannitol crystallization during freezing can generate a homogeneous matrix where protein stability can be maintained or even improved.

4. Conclusion

Freezing and thawing are critical unit operations in biotherapeutic manufacturing. A lack of control in these steps, and specifically, fast freezing and slow thawing appeared to facilitate protein aggregation. The addition of mannitol and facilitating its crystallization through annealing enabled us to obtain a homogenous freeze-concentrate with a single glass transition (Tg'). Thus, annealing during cooling enabled us: (i) promote the selective crystallization of the anhydrous form of mannitol, and (ii) obtain a single maximally freeze-concentrated phase of constant composition (Tg'). Including an annealing step during freezing can be a practical approach to maximize excipient, as well as associated unfrozen water crystallization, early in the freezing process. This approach can minimize, if not eliminate, the need for precise control of the freezing and thawing rates. This protocol can also be applied for drug substance storage, wherein the storage temperature < Tg'.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Arsiccio, A., Pisano, R., 2020. The Ice-Water Interface and Protein Stability: A Review. J Pharm Sci.https://doi.org/10.1016/j.xphs.2020.03.022

Authelin, J.R., Rodrigues, M.A., Tchessalov, S., Singh, S.K., McCoy, T., Wang, S., Shalaev, E., 2020. Freezing of Biologicals Revisited: Scale, Stability, Excipients, and Degradation Stresses. J Pharm Sci 109, 44-61.10.1016/j.xphs.2019.10.062

Bhatnagar, B.S., Bogner, R.H., Pikal, M.J., 2007. Protein stability during freezing: separation of stresses and mechanisms of protein stabilization. Pharm Dev Technol 12, 505-523.https://doi.org/10.1080/10837450701481157

Bhatnagar, B.S., Sonje, J., Shalaev, E., Martin, S.W., Teagarden, D.L., Suryanarayanan, R., 2020. A refined phase diagram of the tert-butanol–water system and implications on lyophilization process optimization of pharmaceuticals. Phys Chem Chem Phys

Carpenter, J.F., Pikal, M.J., Chang, B.S., Randolph, T.W., 1997. Rational design of stable lyophilized protein formulations: some practical advice. Pharm Res 14, 969

Chang, L.L., Pikal, M.J., 2009. Mechanisms of protein stabilization in the solid state. J Pharm Sci 98, 2886-2908

Connolly, B.D., Le, L., Patapoff, T.W., Cromwell, M.E.M., Moore, J.M.R., Lam, P., 2015. Protein Aggregation in Frozen Trehalose Formulations: Effects of Composition, Cooling Rate, and Storage Temperature. J Pharm Sci 104, 4170-4184.10.1002/jps.24646

Desai, K.G., W. Aaron Pruett, Peter J. Martin, James D. Colandene, and Douglas P. Nesta, 2017. Impact of manufacturing-scale freeze-thaw conditions on a mAb solution. BioPharm Int 30 no. 2, 30-36

Hancock, B.C., Zografi, G., 1997. Characteristics and significance of the amorphous state in pharmaceutical systems. Journal of pharmaceutical sciences 86, 1-12

Jena, S., Horn, J., Suryanarayanan, R., Friess, W., Aksan, A., 2017. Effects of excipient interactions on the state of the freeze-concentrate and protein stability. Pharm Res 34, 462-478

Jena, S., Suryanarayanan, R., Aksan, A., 2016. Mutual influence of mannitol and trehalose on crystallization behavior in frozen solutions. Pharm Res 33, 1413-1425
Johnson, R.E., Kirchhoff, C.F., Gaud, H.T., 2002. Mannitol-sucrose mixtures--versatile formulations for protein lyophilization. J Pharm Sci 91, 914-922.10.1002/jps.10094
Kim, A.I., Akers, M.J., Nail, S.L., 1998. The physical state of mannitol after freeze-drying: effects of mannitol concentration, freezing rate, and a noncrystallizing cosolute. J Pharm Sci 87, 931-935
Kolhe, P., Badkar, A., 2011. Protein and solute distribution in drug substance containers during frozen storage and post-thawing: A tool to understand and define freezing–thawing parameters in biotechnology process development. Biotechnology progress 27, 494-504
Mehta, M., Bhardwaj, S.P., Suryanarayanan, R., 2013. Controlling the physical form of mannitol in freeze-dried systems. Eur J Pharm Biopharm 85, 207-213
Piedmonte, D.M., Summers, C., McAuley, A., Karamujic, L., Ratnaswamy, G., 2007. Sorbitol crystallization can lead to protein aggregation in frozen protein formulations. Pharm Res 24, 136-146.10.1007/s11095-006-9131-1
Pyne, A., Surana, R., Suryanarayanan, R., 2002. Crystallization of mannitol below Tg' during freeze-drying in binary and ternary aqueous systems. Pharm Res 19, 901-908
Rathore, N., Rajan, R.S., 2008. Current perspectives on stability of protein drug products during formulation, fill and finish operations. Biotechnology progress 24, 504-514
Searles, J.A., Carpenter, J.F., Randolph, T.W., 2001. Annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determine Tg' in pharmaceutical lyophilization. Journal of pharmaceutical sciences 90, 872-887
Singh, S., Kolhe, P., Wang, W., Nema, S., 2009. Large Scale Freezing of Biologics, A Practitioner's Review, Part One: Fundamental Aspects. Bioprocess Int. 7, 32-44
Sundaramurthi, P., Patapoff, T.W., Suryanarayanan, R., 2010. Crystallization of trehalose in frozen solutions and its phase behavior during drying. Pharm Res 27, 2374-2383
Sundaramurthi, P., Suryanarayanan, R., 2010. Trehalose crystallization during freeze-drying: implications on lyoprotection. J Phys Chem Lett 1, 510-514
Toby, B.H., Von Dreele, R.B., 2013. GSAS-II: the genesis of a modern open-source all purpose crystallography software package. J. Appl. Crystallogr 46, 544-549
Wang, W., 2005. Protein aggregation and its inhibition in biopharmaceutics. Int J Pharm 289, 1-30.10.1016/j.ijpharm.2004.11.014

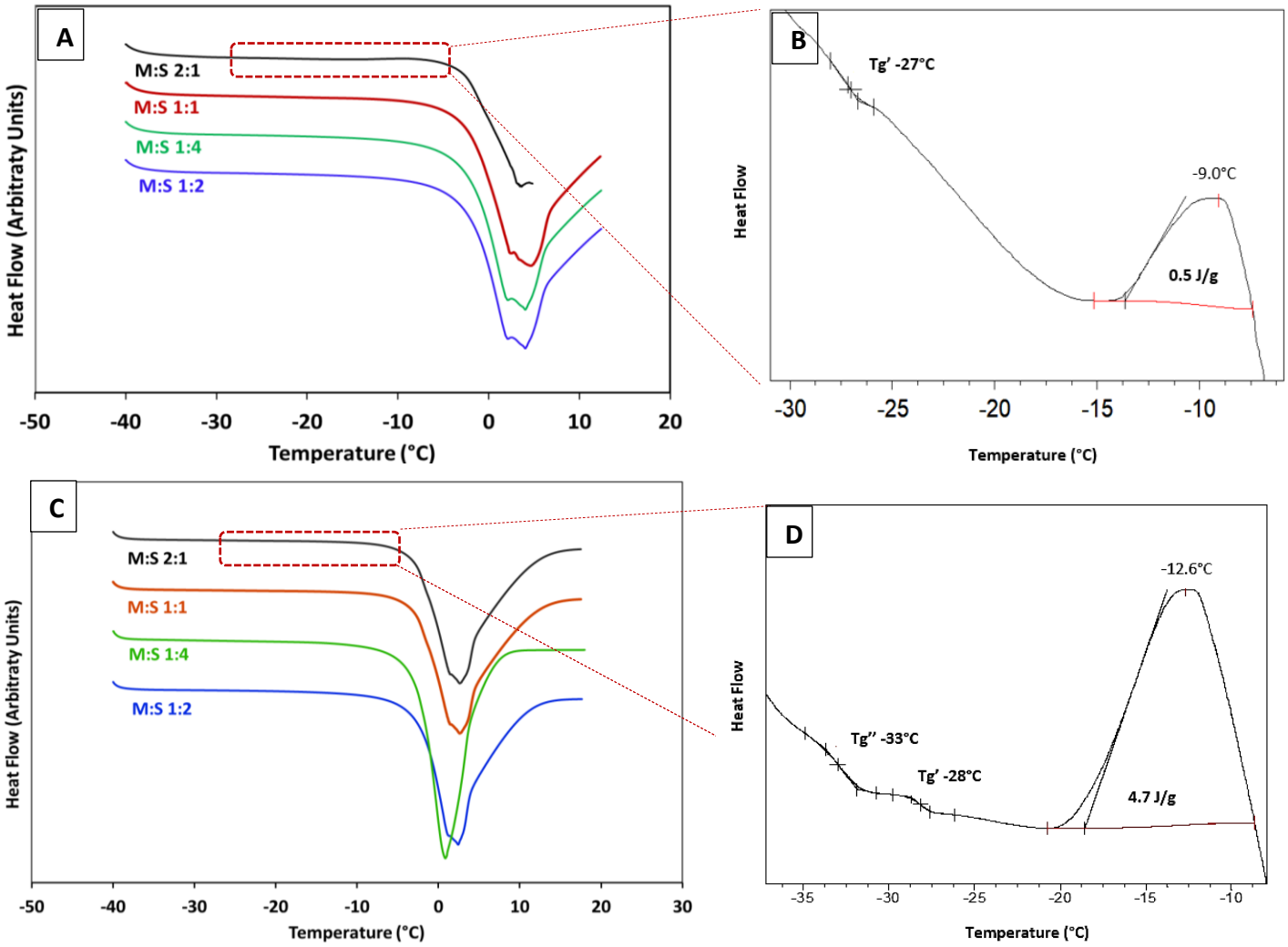


Figure 1. *Overlaid DSC heating curves for four different mannitol-sucrose ratios (1:4, 1:2, 1:1 and 2:1) with a solute concentration of 5% w/v. Panel A. The solutions were initially cooled from room temperature to -40°C at 10°C/minute held for 5 minutes and heated to 15°C at 10°C/minute. Only the heating curves are shown. Panel B. Shows a magnified region with Tg' and crystallization exotherm for mannitol-sucrose 2:1 solution from panel A. Panel C. The solutions were initially cooled from room temperature to -40°C at 5°C/minute held for 5 minutes and heated to 15°C at 5°C/minute. Only the heating curves are shown. Panel D. Shows a magnified region with Tg' and crystallization exotherm for mannitol-sucrose 2:1 solution from panel C.*

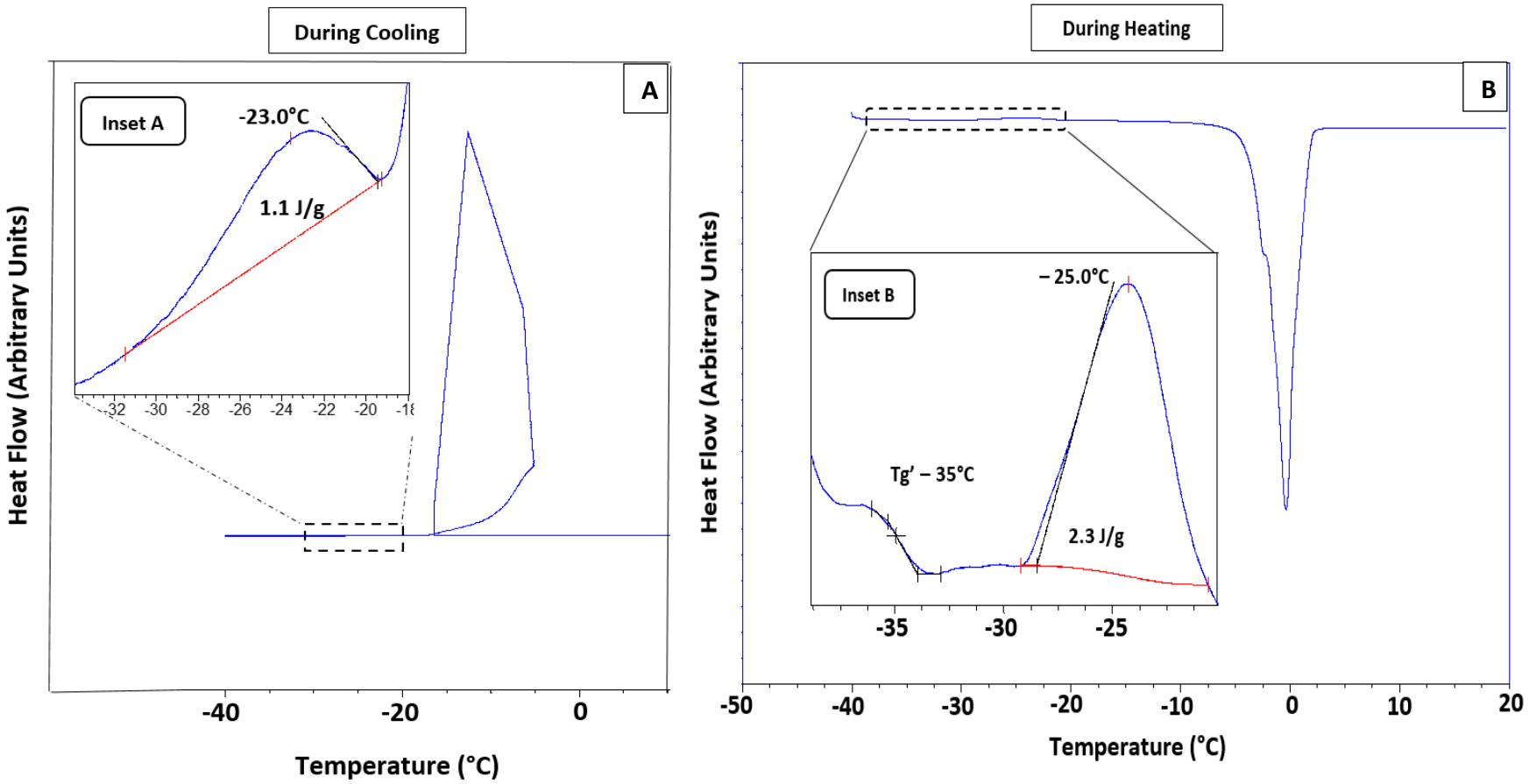


Figure 2. *DSC heating curves for mannitol-sucrose 2:1 ratio with a solute concentration of 5% w/v. Panel A. DSC cooling curve from room temperature to -40°C. The cooling rate was 0.5°C/minute and sample was held at -40°C for 5 minutes. The cooling curve shows an initial exotherm for ice crystallization followed by an additional exotherm. The exotherm at -23°C was attributed to partial mannitol crystallization (inset A). Panel B. DSC heating curve from -40°C to 25°C. The heating rate was 0.5°C. A glass transition was observed at -35°C followed by a crystallization exotherm at -25.0°C (inset B).*

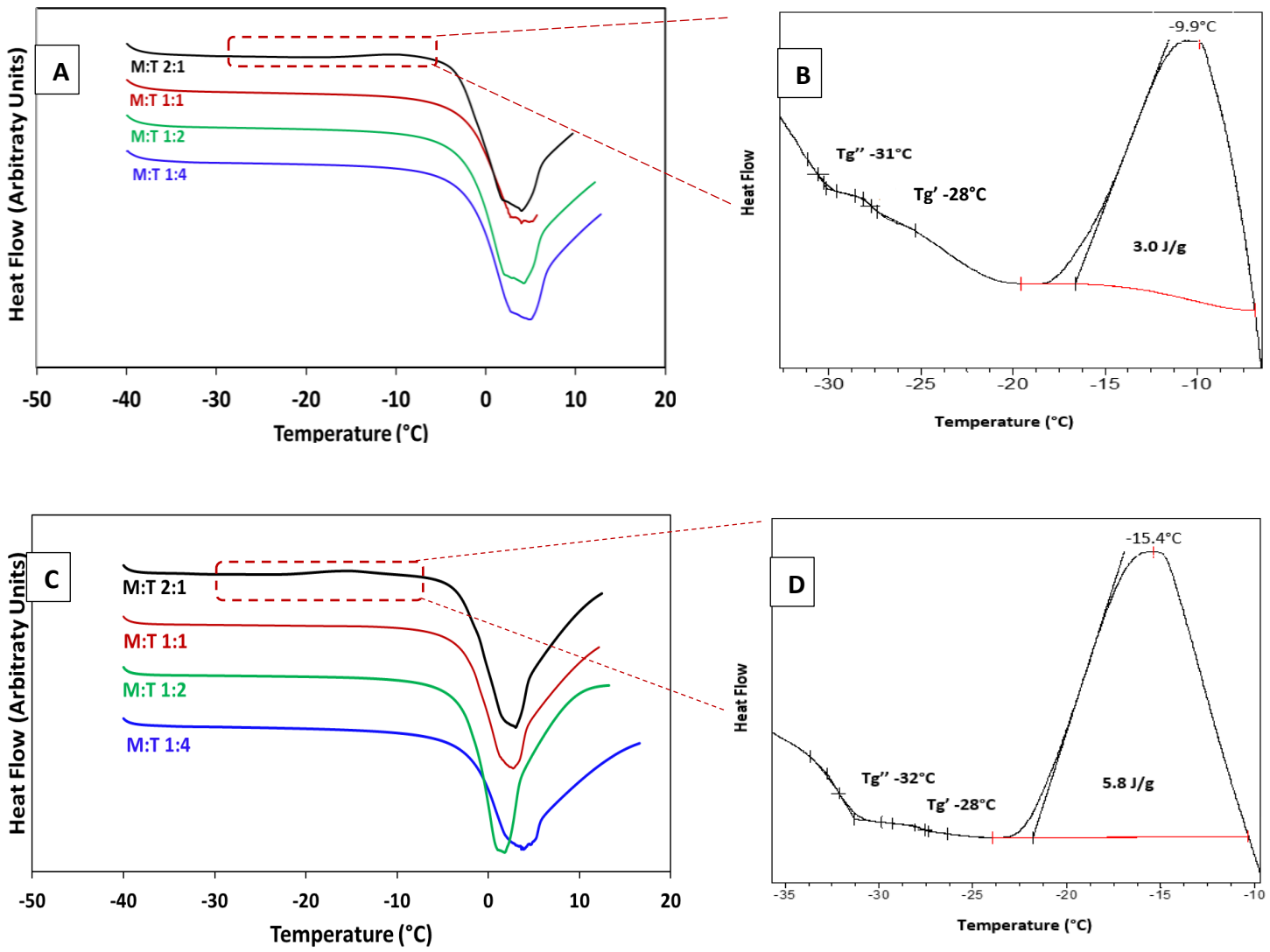


Figure 3. *Overlaid DSC heating curves for four different mannitol-trehalose ratios (1:4, 1:2, 1:1 and 2:1) with a solute concentration of 5% w/v. Panel A. The solutions were initially cooled from room temperature to -40°C at 10°C/minute held for 5 minutes and heated to 15°C at 10°C/minute. Only the heating curves are shown. Panel B. Shows a magnified region with Tg' and crystallization exotherm for mannitol-trehalose 2:1 solution from panel A. Panel C. The solutions were initially cooled from room temperature to -40°C at 5°C/minute held for 5 minutes and heated to 15°C at 5°C/minute. Only the heating curves are shown. Panel D. Shows a magnified region with Tg' and crystallization exotherm for mannitol-trehalose 2:1 solution from panel C.*

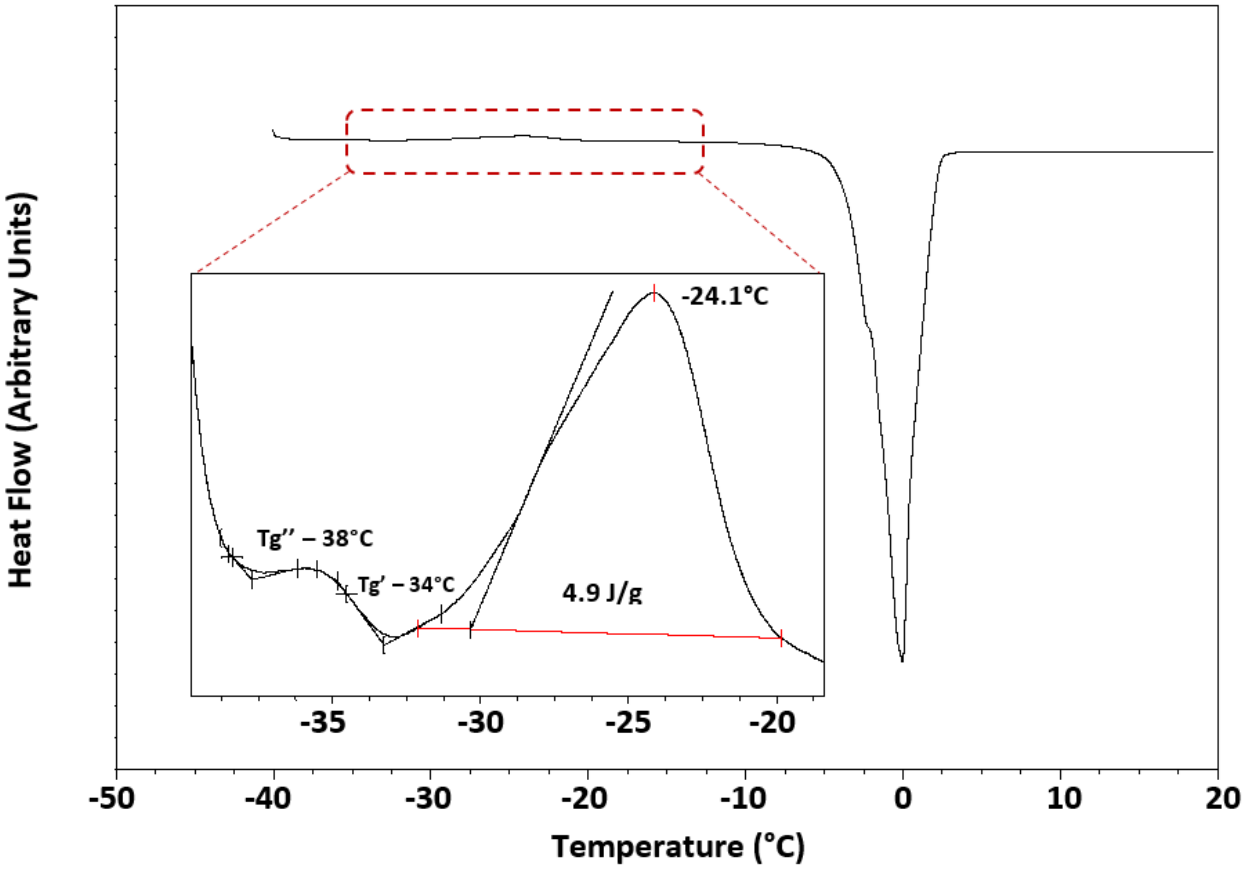


Figure 4. *Mannitol-trehalose 2:1 composition. DSC heating curve from -40°C to 25°C at 0.5°C/min. The inset shows glass transition and crystallization exotherm from -40 to -20°C. The sample was cooled from room temperature to -40°C at 0.5°C/min (cooling curve not shown).*

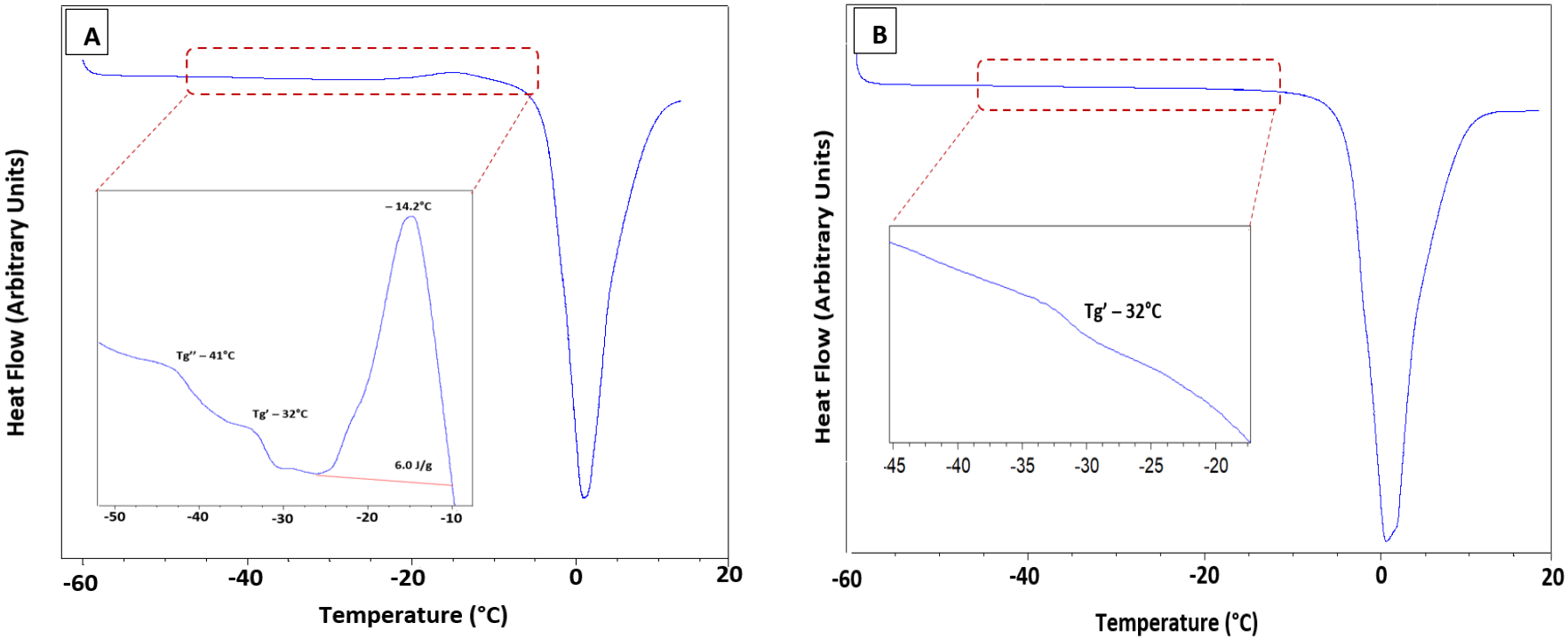


Figure 5. *DSC heating curves for mannitol-trehalose 3:1 (A) sample was cooled to -20°C at 0.5°C/min held for 2 minutes and further cooled to -60°C at 5°C/min. The frozen sample was heated to 20°C at 5°C/min. Only the heating curve is shown in the figure. (B) sample was cooled to -20°C at 0.5°C/min held for 2 hours and further cooled to -60°C at 5°C/min. The frozen sample was heated to 20°C at 5°C/min. The heating curve in the figure shows the presence of only glass transition event.*

Table 1. *Mannitol-trehalose (3:1) – DSC data for isothermal hold (-20°C) experiments*

Formulation	Annealing time at -20°C during cooling (hours)	Tg"		Tg'	Tc		ΔH (J/g) at Tm (Ice and mannitol-ice eutectic melting)
		Temperature (°C)	ΔCp (J/g°C)	Temperature (°C)	Temperature (°C)	ΔH (J/g)	
Mannitol:trehalose 3:1	No annealing	-41.5 ± 0.1	0.19 ± 0.0	-32 ± 0.1	-21	6.28 ± 0.2	323.0 ± 2.0
	2			-31 ± 0.2			336.7 ± 2.0
	4			-32 ± 0.0			339 ± 2.0
	8			-32 ± 0.0			336.1 ± 3.2
	16			-31 ± 0.0			333.0 ± 0.4

Variability in the results are reported in terms of standard deviation (n=3)

Table 2. *Mannitol-sucrose (3:1) – DSC data for isothermal hold (-20°C) experiments*

Formulation	Annealing time at -20°C during cooling (hours)	Tg"		Tg'	Tc		ΔH (J/g) at Tm (Ice and mannitol-ice eutectic melting)
		Temperature (°C)	ΔCp (J/g°C)	Temperature (°C)	Temperature (°C)	ΔH (J/g)	
Mannitol:sucrose 3:1	No annealing	-42 ± 0.5	0.1 ± 0.0	-33 ± 0.0	-15 ± 0.2	7.1 ± 0.3	328.1 ± 2.6
	2			-34.5 ± 0.7			332.5 ± 0.7
	8			-33.7 ± 1.1			334.5 ± 0.8
	16			-34.5 ± 0.7			332.8 ± 0.6

Variability in the results are reported in terms of standard deviation (n=3)

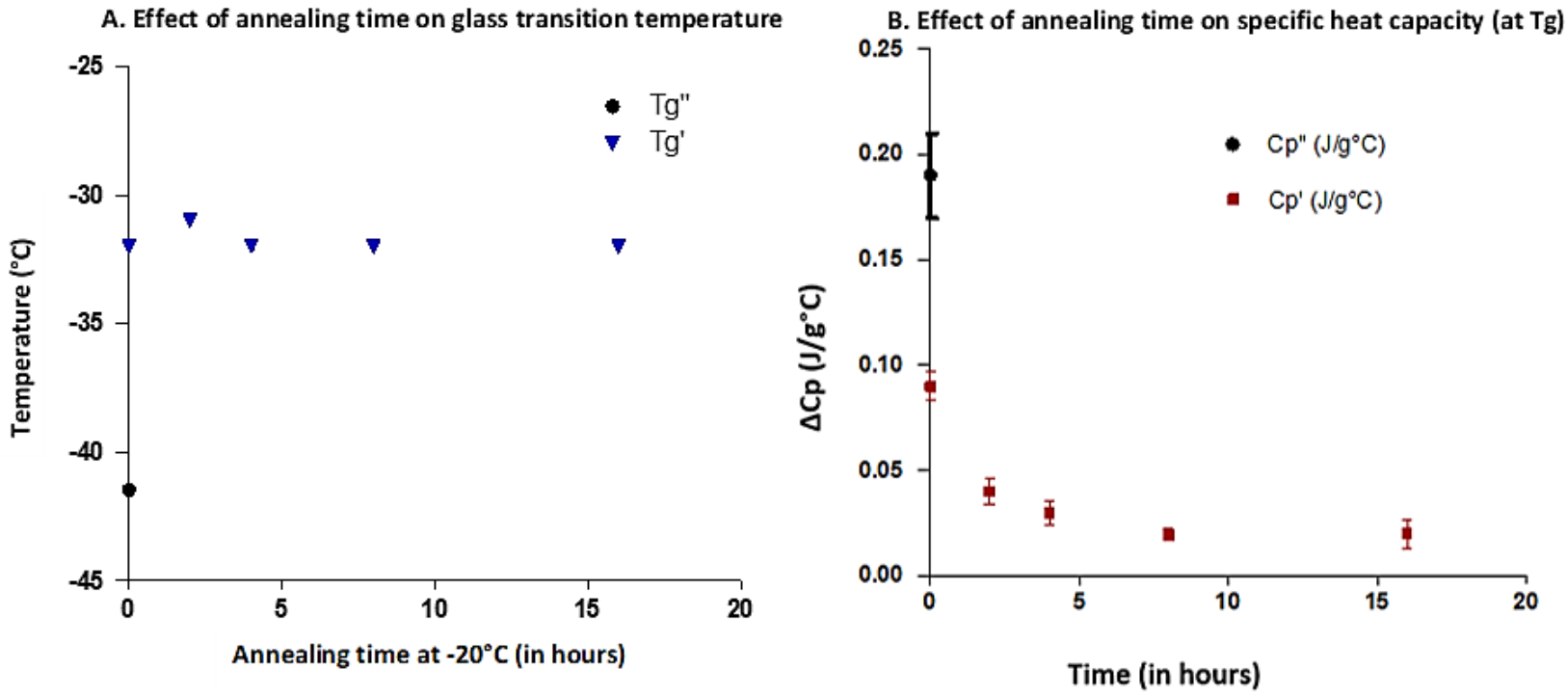


Figure 6. *Graphical representation for mannitol:trehalose 3:1 (A) Change in glass transition temperature as a function of annealing time at -20°C (B) Change in heat capacity associated with glass transition temperature as a function of annealing time at -20°C.*

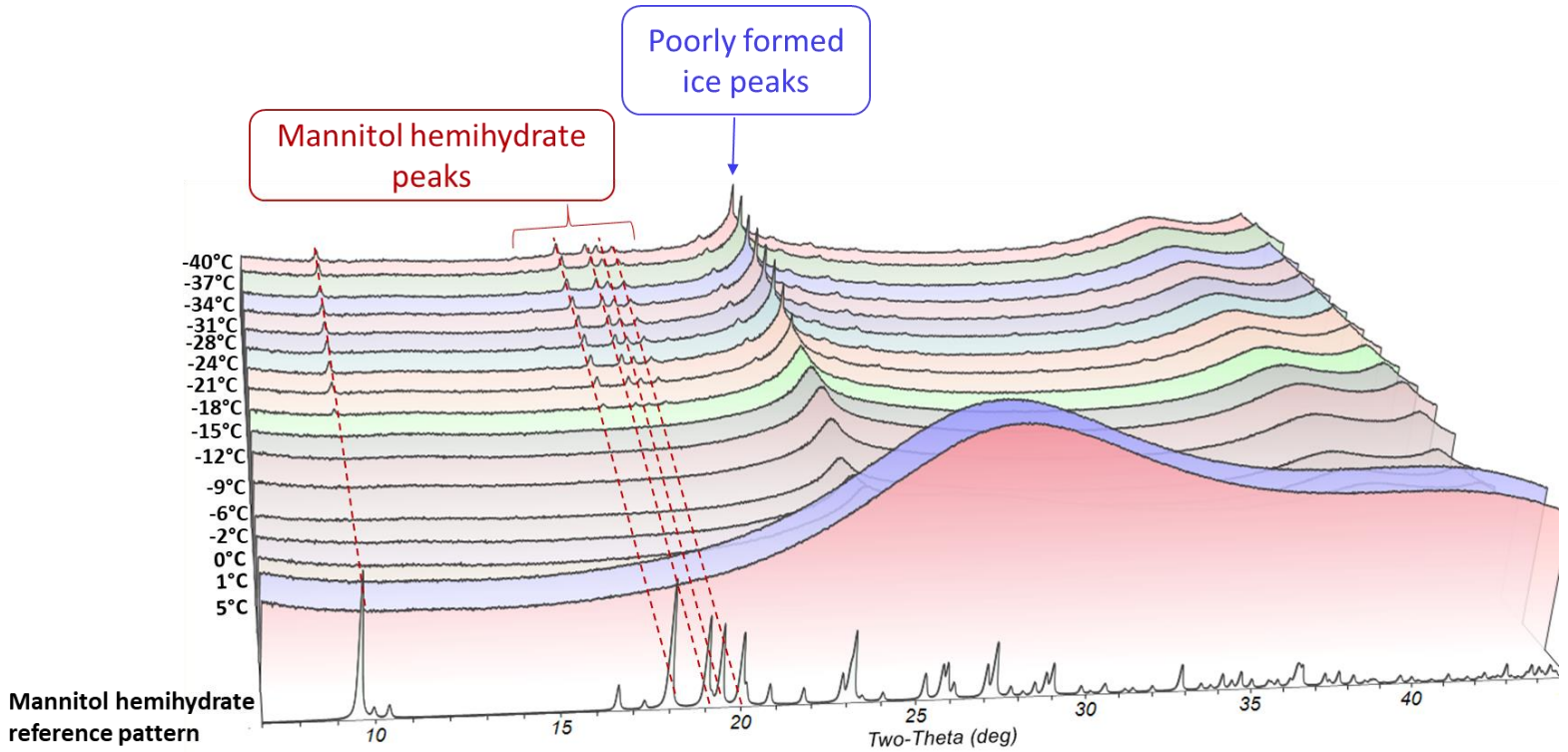


Figure 7A. *In situ synchrotron XRD patterns for mannitol-trehalose (3:1, 5% w/v) solution. Panel A shows overlays of XRD patterns during cooling from 5°C to -40°C; the solution was frozen from room temperature to -45°C at 1°C/min and held at -45°C for 10 minutes followed by heating the samples back to room temperature at 1°C/min. Mannitol hemihydrate reference pattern is shown at the bottom of the overlays. The data were collected using synchrotron radiation (0.45Å). They were converted and plotted for Cu Kα radiation (1.54 Å), so as to enable direct comparison with the reference patterns.*

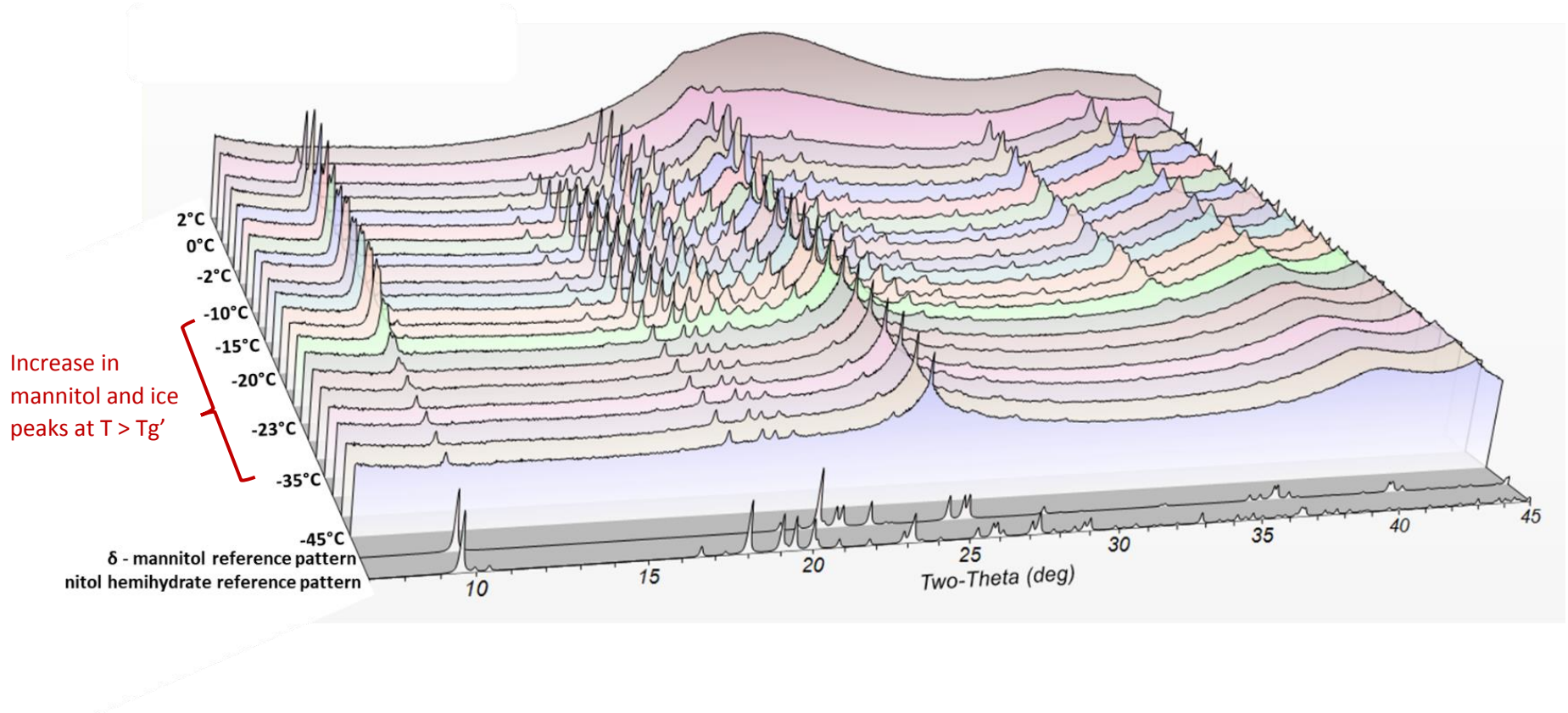


***Figure 7B**. In situ synchrotron XRD patterns for mannitol-trehalose (3:1, 5% w/v) solution. Panel B shows overlays of XRD patterns during heating from -45°C to 2°C at 1°C/min; mannitol hemihydrate and δ-mannitol reference patterns are shown at the bottom of the overlays. The data were collected using synchrotron radiation (0.45Å). They were converted and plotted for Cu Kα radiation (1.54 Å), so as to enable direct comparison with the reference patterns.*

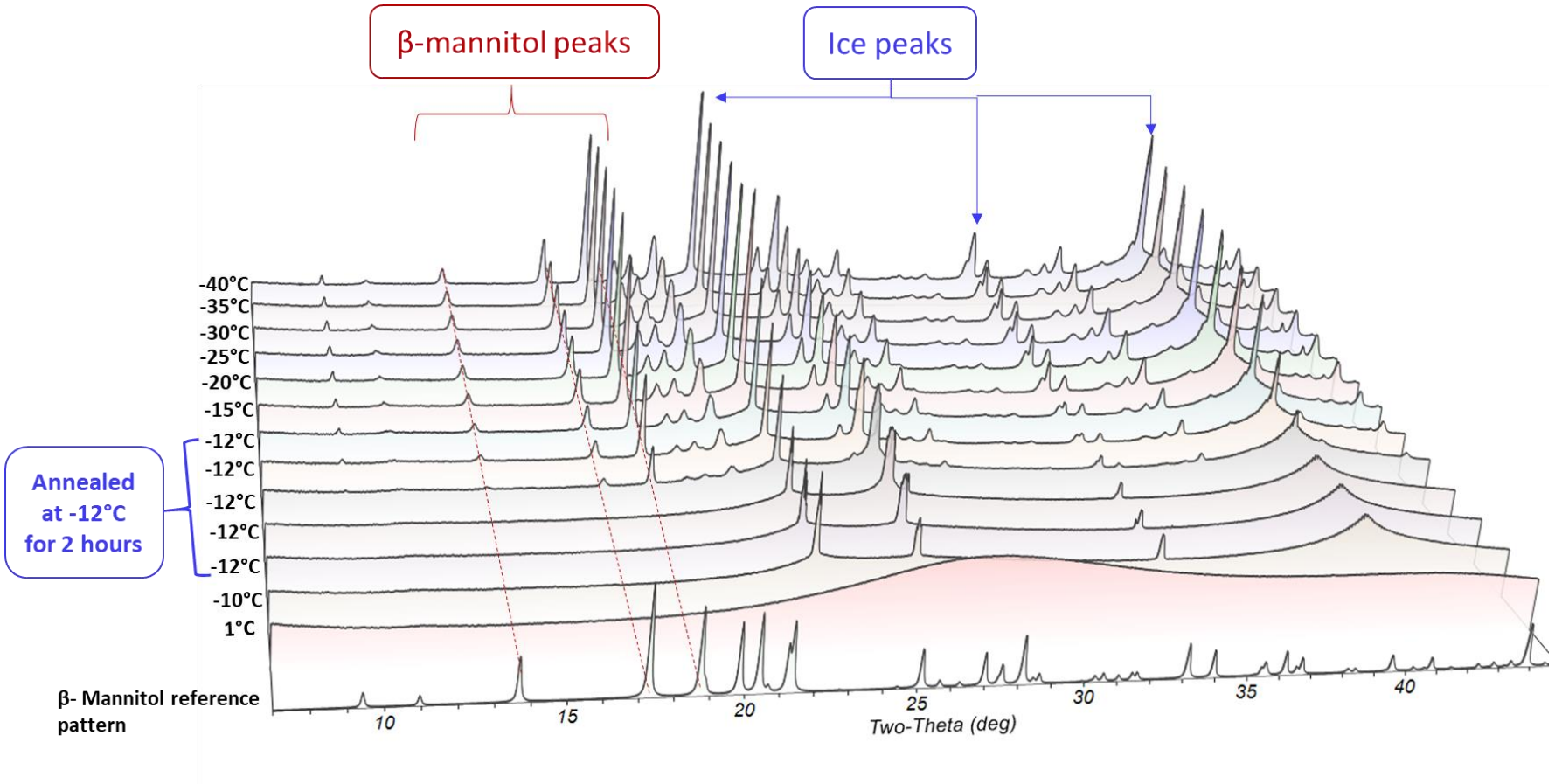


***Figure 8A**. In situ synchrotron XRD patterns for mannitol-trehalose (3:1, 5% w/v) solution during cooling. Panel A shows overlays of XRD patterns during cooling from 5°C to -40°C; mannitol hemihydrate reference pattern is shown at the bottom of the overlays. The solution was frozen from room temperature to -12°C, held for 2 hours further cooled to -45°C at 1°C/min and held at -45°C for 10 minutes. The data were collected using synchrotron radiation (0.45Å). They were converted and plotted for Cu Kα radiation (1.54 Å), so as to enable direct comparison with the reference patterns.*

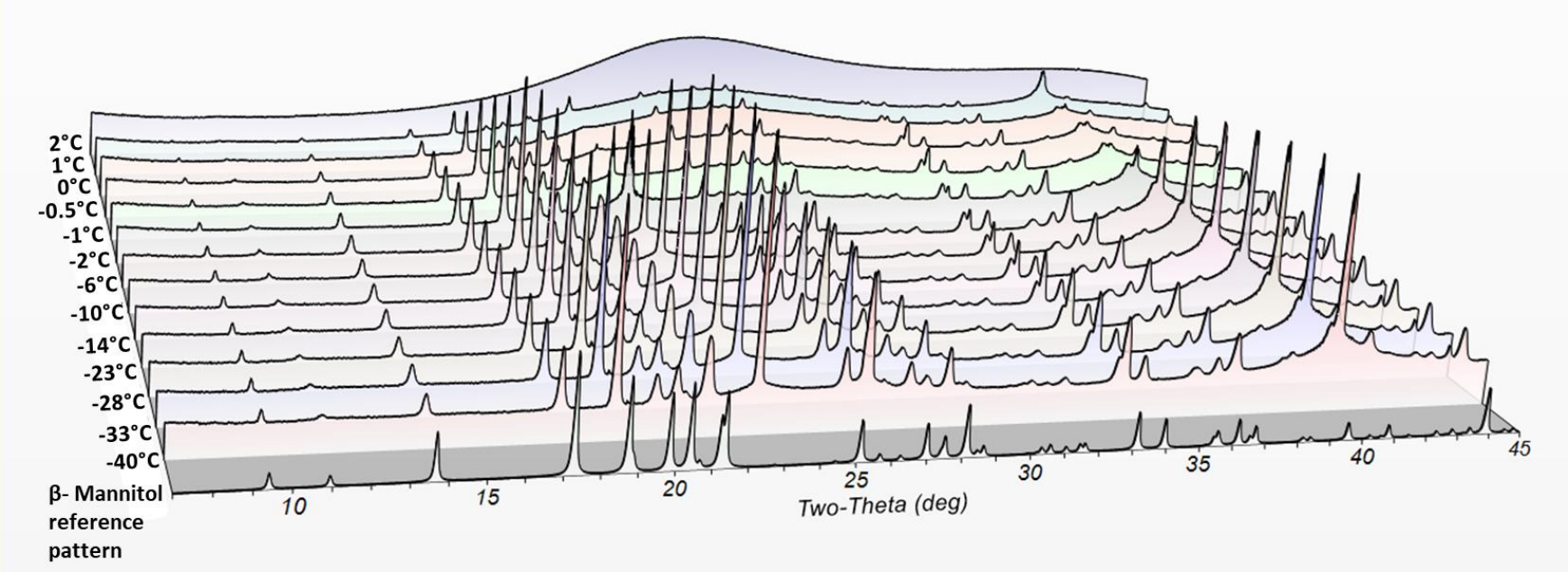


***Figure 8B**. Insitu synchrotron XRD patterns for mannitol-trehalose (3:1, 5% w/v) solution during heating. Panel B shows overlays of XRD patterns during heating from -45°C to 2°C. The frozen solution was heated from -45°C back to room temperature at 1°C/min. Mannitol hemihydrate and δ-mannitol reference patterns are shown at the bottom of the overlays. The data were collected using synchrotron radiation (0.45Å). They were converted and plotted for Cu Kα radiation (1.54 Å), so as to enable direct comparison with the reference patterns.*

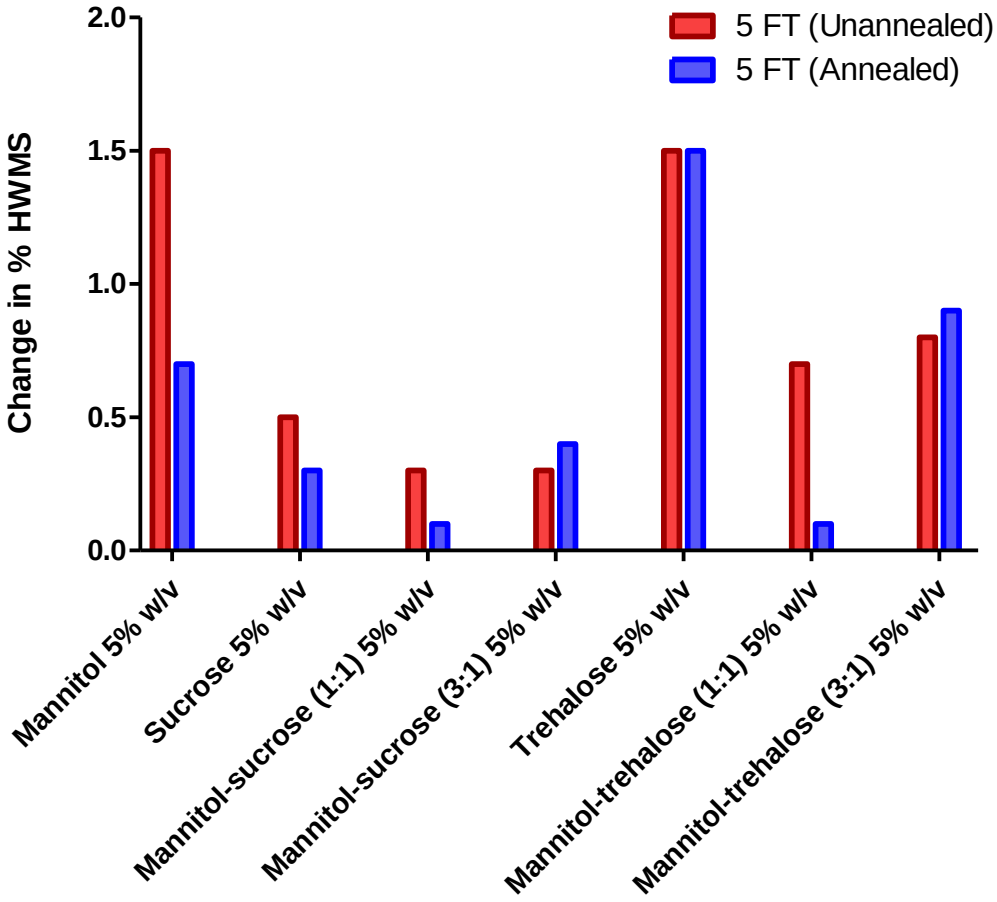
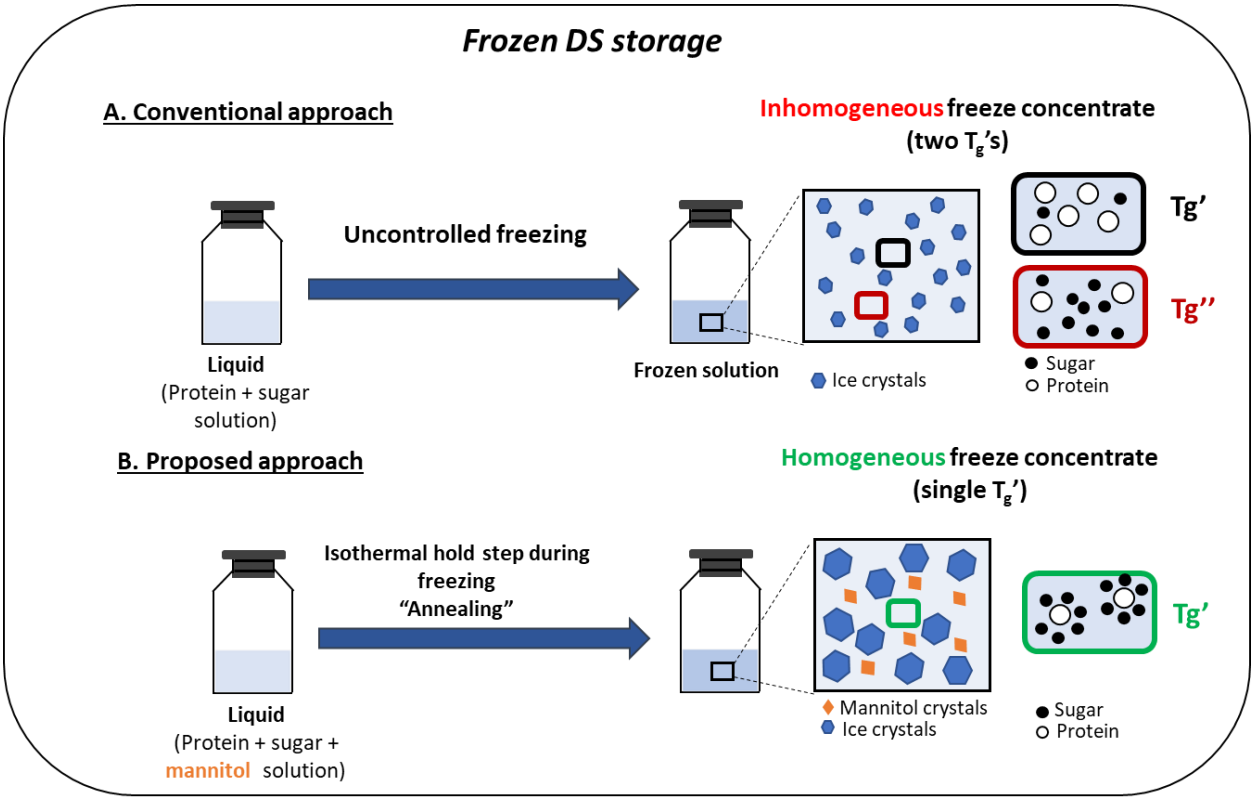


***Figure 9**. SE-HPLC results for 1 mg/mL HSA with (i) 5% w/w Mannitol (ii) 5% w/w Sucrose (iii) 5% w/w mannitol-sucrose 1:1 (iv) 5% w/w mannitol-sucrose 3:1 (v) 5% w/w Trehalose (vi) 5% w/w mannitol-trehalose 1:1 and (vii) 5% w/w mannitol-sucrose 3:1. The 'change in %HMWS' on the Y-axis refers to the %HMWS in the F/T samples minus the %HMWS in the respective control formulations that were not subjected to 5 F/T cycles. One set of formulations were cooled from room temperature to -45°C and held for 30 minutes at -45°C and reheated back to room temperature at 1°C/min, these formulations were labelled as 'unannealed'. Another set of samples were cooled to -20°C held for 2 hours and further cooled to -45°C and reheated back to room temperature at 1°C/min, these formulations were labelled as 'annealed'.*

683 Graphical Abstract



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