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Physicochemical Characterization of the Freezing Behavior of Mannitol–Human Serum Albumin Formulations

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ABSTRACT

The goal of the study was to analyze the impact of human serum albumin (HSA) quality (stabilized or nonstabilized HSA), the addition of NaCl, and the HSA stabilizers Na-octanoate and Na-N-acetyltryptophanate on the freezing behavior of mannitol-HSA formulations. The focus was on crystallization, Tg' (glass transition temperature of the maximally freeze-concentrated phase), and Tc (collapse temperature). Differential scanning calorimetry (DSC), cryomicroscopy, and low-temperature x-ray powder diffraction (LTXRD) were used to study the frozen state. In mannitol-HSA formulations, mannitol crystallization was inhibited and Tg' lowered to a greater extent by stabilized HSA (containing Na-octanoate, Na-N-acetyltryptophanate, and NaCl) than by unstabilized HSA. Detailed DSC and LTXRD studies showed that in the concentrations used for stabilizing HSA, NaCl led to changes in the freezing behavior, an effect that was less pronounced for the other stabilizers. NaCl further lowered the Tc, which was determined by cryomicroscopy. As the freezing behavior governs the lyophilization process, the changes have to be taken into consideration for the development of a lyophilization cycle, to avoid collapse and instabilities.

KEYWORDS: Mannitol, HSA, DSC, LTXRD, freezing.

INTRODUCTION

Lyophilization is the technique most frequently used for producing dry and stable protein formulations. Within the lyophilization process, the applied freezing protocol and a potential annealing step have a major effect on the subsequent drying procedure—in particular, on drying rate and drying time.[1-3] The freezing process also governs the structure and morphology of the lyophilized products.[4] The physicochemical properties of the selected excipients are influenced by the presence of salts that are added as buffer components or stabilizers.[5,6] Salts can also be introduced into the formulation within the bulk purification process or by pH adjustment. A slight increase in salt concentration can lead to significant changes in the physicochemical properties of the excipients during freezing and drying.[7-10] Therefore, it is important to investigate and understand how the freezing step, the low-temperature behavior, and the subsequent drying process of a formulation are influenced by the salt concentration.

As the model system, a formulation with mannitol as the crystallizing bulking agent and human serum albumin (HSA) as the amorphous stabilizer was used. A combination of the excipients HSA and mannitol for protein stabilization has been described in the literature and patents, especially for hydrophobic proteins like interleukins and interferons.[11] Mannitol is a standard excipient for lyophilization that has excellent cake-forming qualities. The nonreducing sugar alcohol can be dried under relatively harsh conditions, because of its high eutectic temperature of –1.5°C. The crystallization of mannitol during freezing and drying depends on various factors. Besides the employed mannitol concentration,[4] the lyophilization process, especially the freezing rate, has a significant impact on the crystallization and the morphology of mannitol. The presence of other excipients like lyoprotectants, buffer salts, or proteins can also promote and inhibit mannitol crystallization.[12-15] Thus, mannitol is an adequate model excipient for the studies. HSA is still widely used as an excipient in both liquid and lyophilized protein formulations. The loss of protein in low-dose formulations because of adsorption onto the inside walls of the container can be overcome by the addition of HSA,[16] usually in concentrations between 0.05% and 0.1%.[17] For lyophilization HSA is generally used in higher concentrations, as lyoprotector and cryoprotector. According to the US Food and Drug Administration, HSA used in protein formulations or as a drug has to be pasteurized for 10 hours at temperatures of 60°C.[18] Consequently, stabilization against the heat-induced stress is required. The sodium salt of caprylic acid[19] and the amino acid derivative Na-N-acetyltryptophanate[20] protect HSA during pasteurization.[21] HSA is further stabilized by NaCl.[20,22] Thus the ionic strength in protein formulations stabilized with HSA inevitably increases, and the physicochemical properties can be affected because of the presence of NaCl and HSA. Earlier

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studies had shown that the physicochemical properties of the mannitol-HSA system after lyophilization were affected by NaCl.[23] The goal of the present study was to investigate the impact of NaCl and the other HSA stabilizers on the physicochemical properties of mannitol-HSA formulations at low temperatures.

Material and Methods

Materials

Different types of HSA were used for the experiments, namely nonstabilized HSA from Sigma Chemicals (Steinheim, Germany) and stabilized HSA from Grifols (Langen, Germany). Grifols HSA was used as 20% solution and contained 16mmol Na-octanoate, 16mmol Na-N-acetyltryptophanate, and NaCl. For the experiments the solution was diluted to the desired concentration. Sigma HSA was provided as a solid powder; it contained 97% HSA according to the specifications and was used without further purification. Mannitol in the quality of Ph Eur was purchased from Caelo (Hilden, Germany); NaCl, Na-octanoate, and N-acetyl-DL-tryptophanate were purchased from Sigma. The pH of the solutions was adjusted to 7.0 ± 0.1 using HCl and NaOH. The declaration of the used concentrations is given in % (wt/vol) if not stated otherwise.

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) was used to study Tg' (glass transition of the maximally freeze-concentrated solution) and crystallization behavior at low temperatures. Approximately 20 mg of the solution was analyzed in a crimped Al crucible. The samples were frozen from 20°C to –70°C and reheated to 20°C with a standard scanning rate of 10°C/min in a Netzsch DSC 204 Phoenix (Selb, Germany) calibrated with indium. The measuring cell was purged with gaseous nitrogen during the measurement. Tg' (onset and point of inflection) and crystallization (onset, peak, enthalpy) of the excipients were determined during the heating scan.

Cryomicroscopy

The collapse temperature, Tc, which is closely related to Tg', was determined by cryomicroscopy. To determine the collapse temperature, 5-µL samples of the solutions were frozen on a microscopic slide with an average cooling rate of 5°C/min to –50°C on the cooling stage (Linkman THM 600 S, Surrey, UK) under a microscope (Olympus BX 50, Hamburg, Germany). After a vacuum was applied to the system, a moving drying front could be observed, because of the sublimation of ice. Subsequently, the temperature was increased stepwise until collapse occurred. When the region of collapse was identified, the sample was recooled and smaller steps of 0.5°C with a lower heating rate were applied to bracket the collapse temperature. Tc was determined as the temperature at which the coherent and compact structure of the drying front became fragile with pores.

Low-Temperature X-ray Powder Diffraction

Crystallization was studied with low-temperature x-ray powder diffraction (LTXRD) using Cu-Kα_1-radiation (λ = 154.06 pm) on the powder diffractometer Stadi P from STOE (Darmstadt, Germany) with parafocused transmission geometry. Germanium was used as the primary monochromator, and the scattered x-rays were detected with a linear position sensing detector (PSD). The solutions were frozen in the rotating capillary (diameter 0.5 mm) in the cooling stage (Oxford Cryosystem) of the x-ray diffractometer. The diffraction patterns were analyzed with the program WinXPOW from STOE. For the LTXRD experiment a temperature profile similar to the conditions during lyophilization was chosen. The samples were frozen to –40°C with a cooling rate of 0.5°C/min. At –40°C the first measurement under isothermal conditions was performed. The temperature was subsequently increased to –20°C at 1°C/min, and several measurements were performed at designated time intervals. The temperature of –20°C was chosen because it represents the annealing temperature applied during lyophilization.

Results and Discussion

DSC Studies of Stabilized HSA and Mannitol (2.5% Total Solids)

During the first set of experiments, the total solid content of the solutions was kept constant at 2.5% and the ratio of mannitol to stabilized HSA was varied. DSC analysis of the freezing process for a 2.5% mannitol solution indicated partial crystallization of mannitol during cooling with 10°C/min. Generally, the nucleation of mannitol crystals prevails at low temperatures.[24] Because of the remaining amorphous mannitol fraction, 2 glass transition points, Tg'_1 = –29.9°C and Tg'_2= –26.1°C (point of inflection), followed by a crystallization with an onset of –25°C, where the nuclei grew to mature crystals,[24] were detected during the rewarming of the solution (Figure 1).

This was in agreement with the literature, which has found glass transition points at –32°C (Tg'_1) and –25°C (Tg'_2) for mannitol solutions.[25-27] However, it is often difficult to detect Tg'_2 because it overlaps with the subsequent crystallization of mannitol. Furthermore, the literature states that at higher cooling rates (20°C/min), crystallization of mannitol in solutions with 5% to 15% (wt/vol) cannot be observed during freezing, and reduced cooling rates (5°C-10°C/min) lead to partial crystallization. Even at the slow cooling rates

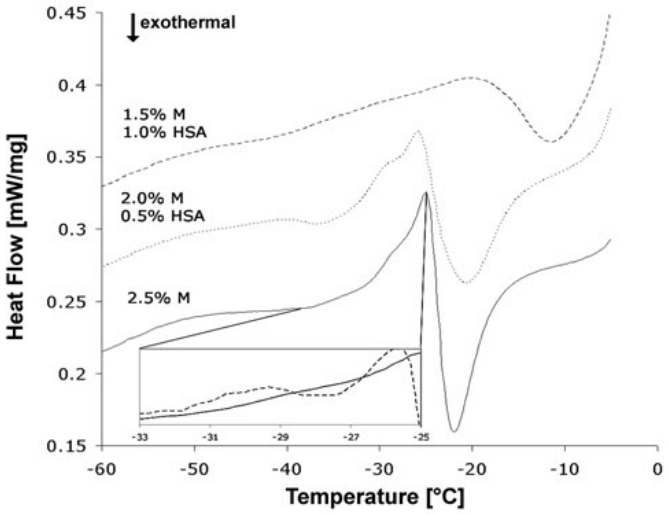

Figure 1. DSC heating scans of solutions with variable ratios of M to stabilized HSA at a total solid content of 2.5% at 10°C/min. The inset shows Tg'_1 and Tg'_2 of 2.5% M with the DSC signal (solid line) and the derivative (dotted line). M indicates mannitol; and HSA, human serum albumin.

(eg, 1°C/min) that are typically used during lyophilization, mannitol crystallizes only partially during cooling, leading to additional crystallization during warming.[14,25] The addition of stabilized HSA as noncrystallizing solute delayed and inhibited mannitol crystallization (Figure 1). Onset and peak maximum of the crystallization were shifted to higher temperatures when the experiments were performed at a constant scanning rate of 10°C/min. A similar effect was described for mannitol-BSA formulations in phosphate buffer.[28] The Tg' of the formulations was shifted to lower temperatures, with increasing amounts of stabilized HSA added to mannitol. The formulations with 2.5% mannitol and 2.0% mannitol–0.5% stabilized HSA showed nearly identical transition temperatures at –26°C and –30°C and crystallization with an onset of –25°C. At equal amounts of mannitol and stabilized HSA in the solution, mannitol crystallization was completely suppressed and only 1 Tg' at –35.2°C remained (curve not shown in Figure 1). The stabilized HSA employed for the experiments contained NaCl, Na-octanoate, and Na-N-acetyltryptophanate. It is well known that NaCl suppresses the crystallization of mannitol during freezing[5] and can lower the Tg' of amorphous excipients.[29]

Impact of HSA Quality on Freezing Behavior of Mannitol

To study the impact of HSA quality on the low-temperature behavior of mannitol, increasing amounts of unstabilized HSA and stabilized HSA were added to a constant mannitol concentration of 6.25%. With the constant mannitol concentration, the crystallization enthalpies (area of the crystallization peak using a linear baseline) of the different samples could be compared. In the DSC experiments, mannitol crystallization was delayed and finally completely suppressed with increasing amounts of stabilized HSA at a scanning rate of 10°C/min (Figure 2A and Table 1).

Stabilized HSA additionally influenced the Tg' of the formulations. Two Tg' values were detected up to a concentration of 3.125% stabilized HSA, whereas only 1 Tg' was left at higher concentrations. The Tg' dropped with increasing concentrations of stabilized-HSA. Both results were in agreement with the studies conducted with 2.5% total solid content. The same set of experiments was performed with unstabilized HSA. With the addition of 3.125% to 6.25% unstabilized has, the crystallization of mannitol moved to higher temperatures and the crystallization enthalpy decreased (Figure 2B). This showed that HSA itself delayed and inhibited mannitol crystallization. However, the glass transitions were only marginally affected by unstabilized HSA compared with how they were affected by stabilized HSA (Table 1).

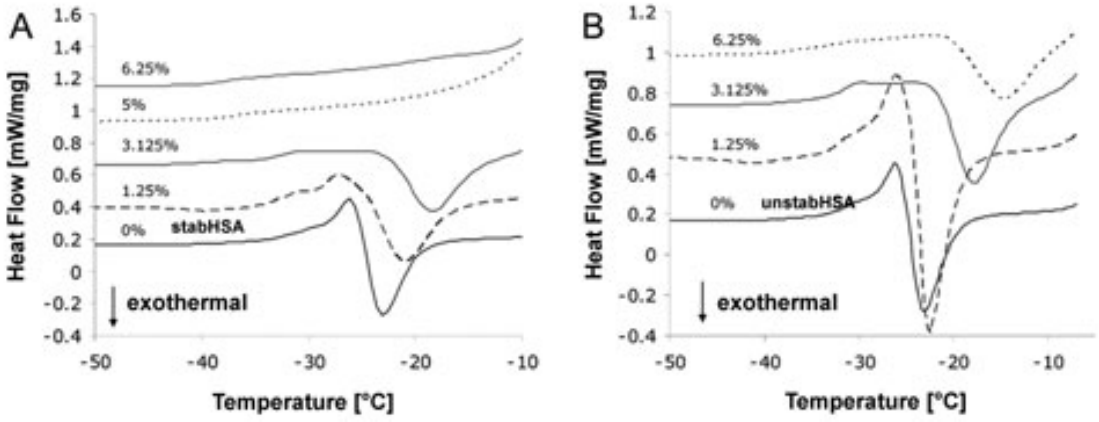

Figure 2. Differential scanning calorimetry heating scan of 6.25% mannitol with 0% to 6.25% stabilized HSA (A) and 0% to 6.25% unstabilized HSA (B) at 10°C/min. HSA indicates human serum albumin; stab, stabilized; and unstab, unstabilized.

Table 1. Tg'_1, Tg'_2 (Inflection Points), and Crystallization (Onset, Peak Maximum, and Enthalpy) of 6.25% Mannitol With Unstabilized HSA and Stabilized HSA Measured With DSC at 10°C/Min, 5°C/Min, and 1°C/Min (n = 3)*

HSA (Ratio Mannitol: HSA)	Unstabilized HSA (10°C/min)		Stabilized HSA (10°C/min)		Stabilized HSA (5°C/min)		Stabilized HSA (1°C/min)	
	Tg'_1 Tg'_2	Crystallization	Tg'_1 Tg'_2	Crystallization	Tg'_1 Tg'_2	Crystallization	Tg'_1 Tg'_2	Crystallization
0% (5:0)	–31.2°C –27.1°C	–25.4°C† –23.1°C‡ –17.1 J/g§	–31.2°C –27.1°C	–25.4°C† –23.1°C‡ –17.1 J/g	–34.0°C –29.4°C	–27.2°C† –25.3°C‡ –10.4 J/g	–34.2°C –31.6°C	–28.9°C† –27.9°C‡ –5.0 J/g
1.25% (5:1)	–32.0°C –27.2°C	–24.6°C† –22.6°C‡ –26.8 J/g§	–32.9°C –28.1°C	–25.1°C† –21.0°C‡ –17.0 J/g	–34.1°C –29.4°C	–27.9°C† –26.5°C‡ –12.9 J/g	–35.1°C –31.8°C	–30.0°C† –28.7°C‡ –5.3 J/g
3.125% (5:3)	–31.4°C –25.0°C	–20.2°C† –14.6°C‡ –12.5 J/g§	–39.5°C –32.7°C	–22.5°C† –18.4°C‡ –11.4 J/g	–34.6°C	–26.9°C† –24.0°C‡ –8.8 J/g	–36.1°C	–31.4°C† –29.6°C‡ –6.3 J/g
6.25% (5:5)	–31.4°C –25.0°C	–20.2°C‡ –14.6°C –12.5 J/g§	–37.7°C	No crystallization	–39.0°C	–14.6°C† –11.3°C‡ –1.1 J/g	–40.4°C	–29.0† –25.0‡ –6.6 J/g

*HSA indicates human serum albumin.

†Onset.

‡Peak maximum.

§Enthalpy of crystallization.

Both stabilized and unstabilized HSA had a significant impact on the crystallization behavior of mannitol during the heating scan of the DSC. At a concentration of 1.25% HSA, the onset of mannitol crystallization (–24.5°C) was unaffected by the addition of both unstabilized and stabilized HSA (Figure 2). The peak maximum of the crystallization was –23.1°C for pure mannitol and shifted to –22.6°C with the addition of 1.25% unstabilized HSA; it shifted to –21.0°C for stabilized HSA. However, a higher crystallization enthalpy in the heating scan was measured for 1.25% unstabilized HSA containing mannitol solutions (–26.8 J/g) compared with 6.25% pure mannitol (17.1 J/g) or 6.25% mannitol with 1.25% stabilized HSA (–17.0 J/g). The inhibitory effect of unstabilized HSA on mannitol crystallization was less pronounced than that of stabilized HSA, resulting in the increased crystallization enthalpy during the rewarming for unstabilized HSA. The lower crystallization enthalpy for mannitol as a single component could be explained by the fact that mannitol had the chance to crystallize during the cooling scan, seen by an exothermal peak at –40°C (data not shown). Complete inhibition of mannitol crystallization was achieved by adding 6.25% stabilized HSA, but not by adding 6.25% unstabilized HSA.

The DSC data suggested that the 2 types of HSA both have the ability to inhibit mannitol crystallization, with a stronger inhibitory effect for stabilized HSA. Stabilized HSA contained further additives, such as NaCl, which is known to inhibit mannitol crystallization. The role of the stabilizers Na-octanoate and Na-N-acetyltryptophanate on the low-temperature behavior was subsequently elucidated.

Influence of Applied Scanning Rate on Thermal Behavior of Mannitol-HSA Formulations

To reflect conditions predominating during lyophilization and to demonstrate how the scanning rate influences Tg' and the crystallization behavior of mannitol, the DSC was operated at 1°C/min, 5°C/min, and 10°C/min for the heating and cooling scans. At the scanning rates 1°C/min and 5°C/min 6.25% mannitol in a solution with 6.25% stabilized HSA had the chance to crystallize during the cooling scan. As a consequence, less material crystallized during heating, resulting in a lowered crystallization enthalpy of –5.0 J/g with 1°C/min and –10.4 J/g with 5°C/min compared with –17.1 J/g with 10°C/min. It was shown that mannitol crystallization is completely suppressed by the addition of more than 3.125% stabilized HSA to 6.25% mannitol at a scanning rate of 10°C/min. At scanning rates of 1°C/min and 5°C/min, mannitol crystallization was detected up to a concentration of 6.25% stabilized HSA.

Comparing the results obtained at the different scanning rates, it was obvious that the thermal events occurred at lower temperatures at 1°C/min and 5°C/min (Table 1). Tg' is a kinetic parameter and therefore depends on the applied scanning rate,[24] with a shift of Tg' to lower temperatures at

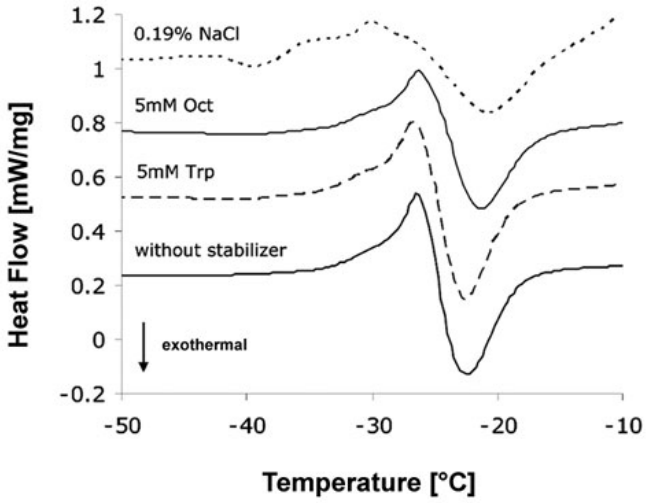

Figure 3. Differential scanning calorimetry heating scan of 6.25% mannitol without the addition of stabilizers and with 5 mM Na-N-acetyltryptophanate (Trp), 5 mM Na-octanoate (Oct), and 0.19% NaCl at 10°C/min.

reduced scanning rates.[30] This has to be considered when developing a lyophilization cycle, because of the lower cooling rates that are frequently used.

Influence of Na-Octanoate, Na-N-Acetyltryptophanate, and NaCl on Freezing Behavior of Mannitol

A more pronounced inhibitory effect on crystallization and the lowering of Tg' was noticeable for stabilized HSA compared with unstabilized HSA. A solution of 6.25% stabilized HSA used for the DSC experiments contained 5 mM (0.08%) Na-octanoate, 5 mM (0.13%) Na-N-acetyltryptophanate, and 31.5 mM (0.19%) NaCl. In the concentrations actually used for stabilizing HSA, Na-octanoate showed no inhibition and Na-N-acetyltryptophanate showed a slight inhibition of mannitol crystallization (Figure 3).

Furthermore, 5 mM Na-octanoate had no impact on Tg'. In the presence of 5 mM Na-N-acetyltryptophanate, Tg'_1 was lowered by 1.0°C and Tg'_2 by 1.5°C. NaCl in a concentration of 0.19% showed the most pronounced suppression of mannitol crystallization and further suppressed the Tg'_1 by 3.9°C and Tg'_2 by 2.8°C. Thus, among the HSA stabilizers, NaCl was primarily responsible for the differences in the low-temperature behavior in formulations with stabilized and unstabilized HSA.

The impact of 0.05% to 1.0% NaCl on the low-temperature behavior of 6.25% mannitol solution is shown in Figure 4A. The addition of NaCl inhibited mannitol crystallization and moved it to higher temperatures. At the same time, Tg' was significantly lowered, and this reduction was linear (Figure 4B).

The shift of Tg' in formulations with NaCl was due to the very low Tg' of NaCl itself, which lies below –60°C.[31] NaCl acts as a plasticizer by increasing the amount of unfrozen water in the amorphous phase, leading to a lowering of the Tg' of amorphous excipients. As lyophilization has to be conducted at product temperatures below Tg',[32,33] the samples with the lowered Tg' have to be dried at lower product temperatures, resulting in longer, less efficient drying processes.

Influence of Na-Octanoate, Na-N-Acetyltryptophanate, and NaCl on Freezing Behavior of Unstabilized HSA and Mannitol

The impact of the stabilizers was further investigated using the DSC heating scan at 10°C/min for the more complex formulations composed of 6.25% mannitol–6.25% unstabilized

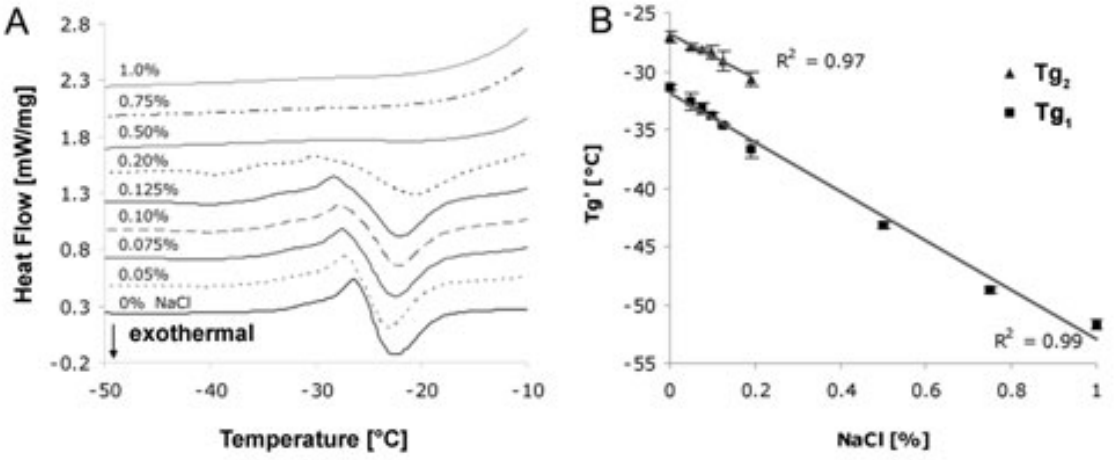

Figure 4. Differential scanning calorimetry heating scan at 10°C/min of 6.25% mannitol with 0% to 1.0% NaCl (A) and Tg'_1 and Tg'_2 (inflection points, n = 3) plotted for 0% to 1.0% NaCl (B).

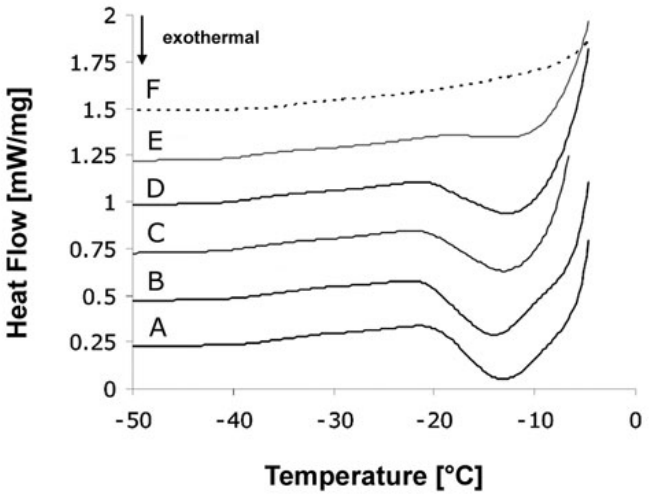

Figure 5. Differential scanning calorimetry heating scan at 10°C/min of 6.25% unstabilized HSA + 6.25% mannitol, without additives (A), with 5 mM Na-octanoate (B), with 5 mM Na-N-acetyltryptophanate (C), with 0.19% NaCl (D), and with a combination of all stabilizers (E), compared with 6.25% stabilized HSA + 6.25% mannitol (F). HSA indicates human serum albumin.

HSA. Again, mannitol crystallization was less affected by 5 mM Na-octanoate or 5 mM Na-N-acetyltryptophanate than by the addition of 0.19% NaCl (Figure 5).

When a combination of the stabilizers (5 mM Na-N-acetyltryptophanate, 5 mM Na-octanoate, 0.19% NaCl) was added to 6.25% mannitol–6.25% unstabilized HSA, the low-temperature behavior of the formulation resembled that of a formulation with stabilized HSA. The crystallization of mannitol was inhibited and the Tg' shifted to lower temperatures. These data demonstrated that the HSA stabilizers, especially NaCl, are responsible for the change in freezing behavior of the mannitol-HSA formulation using stabilized or unstabilized HSA.

Influence of NaCl on Freezing Behavior of Mannitol With Stabilized HSA

As it is crucial to consider the lowered Tg' when developing a lyophilization cycle to avoid collapse, the impact of NaCl was further investigated. Therefore, the addition of NaCl to mixtures of mannitol–stabilized HSA at a ratio of 1:1 (at a total solid content of 2.5% or 12.5% of mannitol and stabilized HSA, respectively) was analyzed by DSC and LTXRD. This ratio was chosen for the more detailed studies, as it is often found in protein formulations and commercial lyophilized products.[12] The addition of 0.5% NaCl lowered the Tg' of 1.25% mannitol–1.25% stabilized HSA in a linear fashion from –37°C to –47°C. The results could be confirmed by solutions with a 5-fold total solid content. With the addition of 0.5% to 2.5% NaCl to a 6.25% stabilized HSA–6.25% mannitol solution, a comparable depression of Tg' was detected (Figure 6A). With a Tg' near –50°C measured for the highest NaCl concentrations, the development of a feasible and economical lyophilization process is hardly possible. The results showed that it is not the total amount of NaCl present in a formulation but the ratio of NaCl to other excipients that determines the lowering of the Tg' (Figure 6B). This is due to the fact that the Tg' is concentration-independent for diluted systems.[27,34]

Determination of Tc With Cryomicroscopy

Cryomicroscopy was used to determine the Tc of the formulations. Tc is the temperature at which the interstitial water in the frozen matrix becomes significantly mobile.[35] To

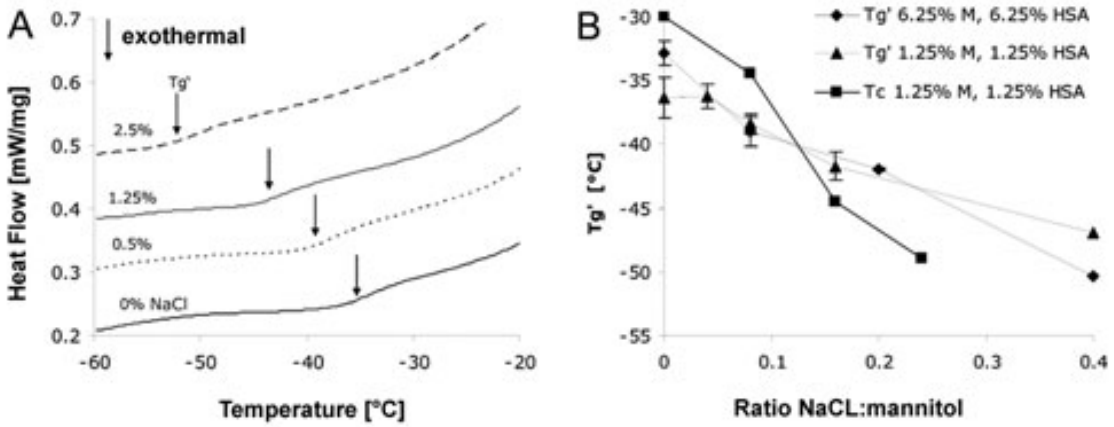

Figure 6. Differential scanning calorimetry heating scan of 6.25% mannitol, 6.25% stabilized HSA, 0% to 2.5% NaCl (A). Tg' (inflection point, n = 3) determined by DSC at 10°C/min and Tc determined by cryomicroscopy of 6.25% mannitol, 6.25% HSA plotted against the ratio of NaCl:mannitol are compared with Tg' of 1.25% mannitol and 1.25% HSA (B). HSA indicates human serum albumin; and M, mannitol.

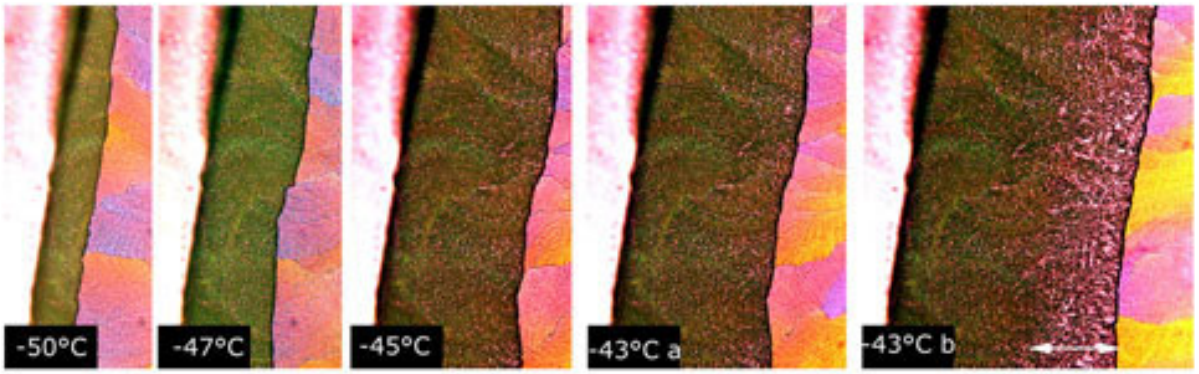

Figure 7. Cryomicroscopy of 1.25% mannitol, 1.25% stabilized human serum albumin, and 0.2% NaCl from –50°C to –43°C; –43°Ca is the starting point at –43°C, and –43°Cb is the image after 5 minutes. The arrow marks the collapsed area.

avoid collapse during lyophilization, the product temperature has to be kept below the Tc. The collapse temperature approximately coincides with the Tg' measured by DSC[32,33] and is usually ~2°C higher than the Tg'.[36] In Figure 7 the behavior of 1.25% mannitol–1.25% stabilized HSA with 0.2% NaCl is illustrated as an example.

The dark section represents the moving drying front. The bright section derives from the frozen solution, with colors due to the use of polarization filters to achieve a better contrast between the drying front and the frozen solution. At temperatures between –50°C and –45°C the drying front was compact and did not show structural changes. At –43°C the drying front lost its compact structure. Holes and cracks appeared, through which the colored background became visible. For the formulation with 0.2% NaCl, the collapse temperature was determined to be between –45°C and –43°C. When smaller temperature increments were used, Tc was determined to be –44.5°C.

Tc and Tg' both were lowered by the addition of NaCl (Figure 6B). The results from the cryomicroscopic experiments suggested that there were significant differences between Tg' and Tc values and that NaCl had a more substantial effect on Tc. This could be explained by the different experimental setups and time-temperature profiles used in DSC and cryomicroscopy. While DSC involves working with a constant dynamic heating scan of 10°C/min, cryomicroscopy involves working with isothermal and dynamic segments and lower scanning rates. The lower scanning rates could explain the steeper decline of Tc and the shift to lower temperatures in the cryomicroscopy experiments compared with the DSC experiments. Correspondingly, in the DSC experiments, thermal events were shifted to lower temperatures when the scanning rate was reduced. Knopp et al[37] found lower values for the Tc than for the Tg' of sucrose solutions using cryomicroscopy. A Tc of –37.7°C was measured for 5% and 10% sucrose, while the Tg' lay at –32.0°C. However, sucrose solutions at higher concentrations (>40%) can exhibit 2 transitions. The transition at –40°C is usually assigned to the real glass transition, while the transition at –32°C is due to the onset of melting of ice crystals.[29] The Tc below –45°C for mannitol–stabilized HSA formulations with 0.2% and 0.3% NaCl can become critical during lyophilization.

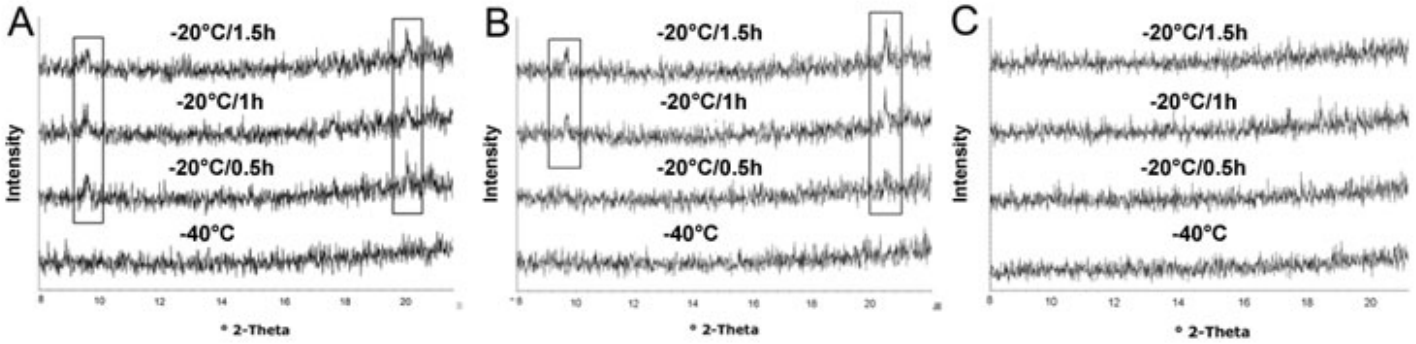

Figure 8. Low-temperature x-ray powder diffraction of 6.25% mannitol, 6.25% stabilized human serum albumin with 0% NaCl (A), 1.25% NaCl (B), and 2.5% NaCl (C) after cooling to –40°C and during the 3 scans of the annealing phase at –20°C.

Analysis of Mannitol Freezing Behavior With LTXRD

DSC offers information on crystalline and amorphous phases in the frozen state. However, only LTXRD, not DSC, can identify and characterize the crystalline phases according to their composition and modifications.[37] Mannitol can crystallize in the α, β, and δ modification. Furthermore, a crystallization of mannitol in a metastable hydrate form during freezing and lyophilization is reported.[38,39]

After a solution with 6.25% mannitol–6.25% stabilized HSA was frozen to –40°C at 0.5°C/min in LTXRD, no peaks of crystalline material could be detected other than the peaks of ice, which were present at angles larger than 22° 2-θ. After the temperature was increased to –20°C, corresponding to an annealing step, mannitol crystallized in the δ modification, which was apparent by the peak at 9.7° 2-θ and 20.4° 2-θ and the absence of a signal at 17.9° 2-θ (Figure 8A). Unlike in LTXRD, no crystallization was measured in the heating scan of the DSC. The kinetic mechanism of crystallization is influenced by temperature.[40] During the dynamic heating scan of the DSC at 10°C/min, mannitol did not have a chance to crystallize, whereas the isothermal steps in LTXRD provided enough time for crystallization. The addition of 1.25% NaCl delayed the crystallization of mannitol. Here, not until the second scan, after about 30 minutes at –20°C, could the peaks at 9.7° and 20.4° 2-θ be detected at full intensity (Figure 8B). Increasing the NaCl concentration to 2.5% led to a complete inhibition of crystallization and no crystalline material being detected with LTXRD (Figure 8C).

LTXRD provides insight into the crystallization behavior of mannitol in the frozen formulation. The thermal conditions during LTXRD are comparable to the freezing and annealing step during the lyophilization process. Therefore, LTXRD is a valuable tool for the development of lyophilized formulations.

CONCLUSIONS

DSC showed that unstabilized HSA, stabilized HSA, and NaCl can delay and inhibit the crystallization of mannitol, which was confirmed by LTXRD. Stabilized HSA exhibited different behavior than unstabilized HSA because of the presence of NaCl, Na-octanoate, and Na-N-acetyltryptophanate. In the concentration used for the stabilization of HSA, NaCl had the most distinct impact on the low-temperature behavior of mannitol. The addition of small quantities of NaCl shifted the Tg' and Tc of mannitol formulations to lower temperatures, which could become critical for a freeze-drying process with respect to collapse of the lyophilized products. The study demonstrated that the freezing step had a significant effect on the physicochemical properties of mannitol-HSA formulations, which could affect the subsequent drying process and the physicochemical properties of the lyophilized products.

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