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Research paper

Impact of freezing procedure and annealing on the physico-chemical properties and the formation of mannitol hydrate in mannitol–sucrose–NaCl formulations

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Abstract

The goal was to investigate the impact of NaCl on the physico-chemical properties of mannitol–sucrose formulations during freezing and drying, with special focus on mannitol hydrate formation. Differential scanning calorimetry (DSC) and low-temperature X-ray powder diffraction (LTXRD) were used to study the frozen solutions. After lyophilization the products were analyzed with DSC, temperature-modulated DSC (TMDSC), X-ray powder diffraction (XRD) and Karl-Fischer titration. DSC showed an inhibition of mannitol crystallization by sucrose and NaCl during freezing. The glass transitions of the maximally freeze-concentrated solutions (Tg') were lowered by both mannitol and NaCl. By the application of an annealing step during lyophilization mannitol crystallinity could be increased. However, lyophilization with an annealing step promoted the formation of mannitol hydrate, which is known to undergo conversion into the anhydrous polymorphs of mannitol upon storage. LTXRD revealed that mannitol hydrate was formed at temperatures below −30 °C, but not at −27 °C. The tendency that mannitol hydrate is predominantly formed at lower temperature was confirmed by XRD of lyophilized products, produced at different annealing temperatures. For the development of lyophilization cycles the lowered Tg', as well as the tendency to mannitol hydrate formation predominantly at lower temperature needs to be considered.

Keywords: Freezing; Lyophilization; Annealing; NaCl; Mannitol hydrate

1. Introduction

Lyophilization is frequently used for the stabilization of biopharmaceuticals. To achieve stable products it is essential to add excipients that protect the active ingredient, e.g., a protein against degradation and damage during freezing and drying. One common way to achieve elegant lyophilized products is combining a crystalline bulking agent, e.g., mannitol or glycine with a second excipient that remains amorphous, e.g., sucrose, trehalose, human serum albumin [1–3]. Combinations of glycine with sucrose, respectively, mannitol with sucrose are often employed. Johnson et al., as well as Passot et al. used a combination of 4.0% mannitol and 1.0% sucrose to successfully stabilize different proteins [4,5]. Liao et al. have studied the impact of an incorporated protein on the physical state of mannitol in formulations with mannitol and sucrose [6]. During lyophilization mannitol can crystallize in the α-, β- or δ-modification or as mannitol hydrate depending on the applied freezing protocol, a potential annealing step and the process conditions during primary and secondary drying [7–9]. The presence of other excipients like buffer components, lyoprotectants or proteins can both inhibit and promote mannitol crystallization [10–12]. Especially salts, that are added as buffer components, isotonicity agents or stabilizers can have a major impact on the physico-chemical properties of mannitol and other excipients [13,14]. A slight increase in salt

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concentration can lead to significant changes of the physico-chemical properties of the excipients during freezing and drying [15–18]. Besides the anhydrous modifications, mannitol can exist as a metastable crystalline hydrate after lyophilization [19]. The presence of mannitol hydrate can lead to stability problems during storage due to the release of hydrate water upon its conversion into the anhydrous crystal forms. Therefore, it is important to develop lyophilization cycles that result in products free of mannitol hydrate. Johnson et al. showed that mannitol hydrate content can be reduced by performing the secondary drying at temperatures above 40 °C [4]. Little is known about how an annealing procedure influences the formation of mannitol hydrate during lyophilization. Annealing is often applied for formulations with mannitol as bulking agent to maximize mannitol crystallization during the freezing step. On the other hand, several approaches are described to produce amorphous mannitol as lyoprotector, e.g., by adding NaCl, boric acid or sodium tetraborate [13,20,21]. Only in the amorphous state mannitol is able to adequately stabilize the active protein via molecular interactions [22,23]. However, amorphous mannitol tends to crystallize upon storage and thereby looses its ability to stabilize the protein [2]. Therefore, the employment of mannitol as crystalline bulking agent in combination with an amorphous lyoprotector is the more promising approach. Thereby, it is essential to ensure mannitol crystallization during lyophilization. Crystallization of excipients upon storage can reduce storage stability. This was described for *Humicola lanuginosa* Lipase formulated with sucrose by Kreilgaard et al., who attributed the reduced stability after crystallization to an increase in moisture content and a reduced glass transition (T_g) value of the remaining amorphous phase [24]. Different combinations of mannitol, sucrose and NaCl were used as model formulations for the studies. The impact of NaCl on the physico-chemical properties of the formulation during freezing, annealing and drying was studied. For the frozen state the glass transition of the maximally freeze-concentrated solution (Tg') of the formulations and the crystallization of mannitol were monitored. The lyophilized products were analyzed regarding mannitol modifications, as well as T_g and residual moisture. Special focus was set on the presence of mannitol hydrate in relation to the applied annealing steps.

2. Materials and methods

2.1. Materials

Mannitol was obtained from Caelo (Hilden, Germany), sucrose from Suedzucker (Mannheim, Germany) and NaCl from Sigma (Steinheim, Germany).

2.2. Low-temperature X-ray powder diffraction (LTXRD)

Crystallization was studied with LTXRD using Cu-Kα1 radiation ($\lambda = 154.06$ pm) on the powder diffractometer Stadi P from STOE (Darmstadt, Germany) with parafocussed transmission geometry. Germanium was used as primary monochromator and the scattered X-rays were detected with a linear PSD area detector. The sterile filtrated solutions were frozen in the rotating capillary (diameter 0.5 mm) in the cooling stage (Oxford Cryosystem) of the X-ray diffractometer. For the LTXRD experiment a temperature profile similar to the conditions during lyophilization was chosen. The samples were frozen to −50 °C with a cooling rate of 0.5 °C/min. At −50 °C the first measurement under isothermal conditions was performed. The temperature was subsequently increased to the different annealing temperatures with 1 °C/min and several measurements were performed at designated time intervals. The diffraction patterns were analyzed with the program WinXPOW from STOE (Darmstadt, Germany). Table 1 shows the XRD diffraction peaks used for the assignment of the different mannitol modifications.

Table 1
Assignment of X-ray diffraction peaks to the different mannitol modifications

Modification	Main peaks [° 2-Θ]	Intensity (%)	Peaks used for identification [° 2-Θ]	References
α-Mannitol	9.4	10	13.6	JCPDS-database
	13.6	20	17.2	
	17.2	45		
	18.7	100		
β-Mannitol	10.5	18	14.6	JCPDS-database
	14.6	65	16.8	
	16.8	85	23.4	
	18.8	100		
	23.4	90		
δ-Mannitol	9.7°	100	9.7	JCPDS-database
	20.4	50	No peak at 17.9	
Mannitol hydrate	9.6	80	9.6	[19]
	17.9	100	17.9	[19]

2.3. Differential scanning calorimetry (DSC) of the frozen solutions

The glass transition of the maximally freeze-concentrated solution (Tg') and the crystallization behavior of formulations with a total solid content of 5.0% [w/v] at low temperatures was studied by DSC. Thereby, the ratio of mannitol to sucrose was varied and 0.05–0.2% NaCl added to the formulations. Approximately 20 mg of the sterile filtrated solutions was analyzed in crimped Al-crucibles. The samples were frozen from 20 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. Tg' (onset and point of inflection) and crystallization (onset, peak, enthalpy) of the excipients were determined during the heating scan.

2.4. Lyophilization

One thousand microliters of the sterile filtrated formulations was dried in the Epsilon 2-12 D freeze-drier from Christ (Osterrode, Germany) using 2R-vials from Schott (Mainz, Germany), which were not further treated prior to lyophilization. The samples were frozen to −50 °C with a standard cooling rate of 0.45 °C/min. Different annealing steps were used for the production of the samples (Table 2). Primary drying was conducted at a shelf temperature of −15 °C and a pressure of 0.045 mbar. For secondary drying the shelf temperature was increased to 20 °C, respectively, 40 °C at a pressure of 0.01 mbar.

2.5. Differential scanning calorimetry (DSC) of lyophilized products

The lyophilized samples were analyzed with the Netzsch DSC 204 Phoenix® (Selb, Germany) from 0 to 150 °C using scanning rates of 10 °C/min. Approximately 10 mg of the lyophilized samples was analyzed in sealed Al-crucibles. The thermal events were analyzed in the heating scan of the DSC.

2.6. Temperature-modulated DSC (TMDSC) of lyophilized products

The lyophilized samples were further analyzed using the Mettler Toledo DSC 822e (Giessen, Germany) in the modulating mode with 40 μl Al-crucibles with Pin (ME 27331). The samples were scanned from 0 to 80 °C with an average heating rate of 1.0 °C/min at an amplitude of 0.5 °C and a period of 0.8 min. The glass transitions were detected in the reversing heat curve.

Table 2
Lyophilization cycles used for the production of the samples

Process	Annealing procedure	Secondary drying shelf temperature and duration
I	–	20 °C/10 h
II	−20 °C/2 h	40 °C/8 h
III	−25 °C/2 h	40 °C/8 h
IIIa	−25 °C/2 h	20 °C/10 h
IV	−30 °C/2 h	40 °C/8 h

2.7. X-ray powder diffraction (XRD)

The morphology of the lyophilized products was analyzed by X-ray powder diffraction (XRD) from 5° to 40° 2-Θ, with steps of 0.05° 2-Θ and a duration of 2 s per step on the X-ray diffractometer XRD 3000 TT (Seifert, Ahrenburg, Germany), equipped with a copper anode (40 kV, 30 mA, wavelength 154.17 pm).

2.8. Karl-Fischer titration

The residual moisture of the samples was determined by coulometric Karl-Fischer titration using the Aqua 40.00 titrator with a headspace module (Analytik Jena AG, Halle, Germany). The headspace method was validated against conventional Karl-Fischer titration, where anhydrous methanol was used to extract water from the lyophilized products. For the measurement at least 10 mg of the lyophilized sample was heated to 80 °C for 10 min. The evaporated water was transferred into the titration solution and the amount of H_2O was determined. As reference material Apura Water Standard Oven 1% (Merck, Darmstadt Germany) was used and the recovery was considered for the calculation of the residual moisture of the samples.

To determine the amount of mannitol hydrate Karl-Fischer titration was performed in a dynamic mode. The sample was heated with a heating rate of 5 °C/min from 30 to 90 °C and the water content was simultaneously titrated.

3. Results and discussion

3.1. Impact of NaCl on the physico-chemical properties of mannitol–sucrose formulations

3.1.1. DSC of frozen mannitol–sucrose–NaCl formulations

DSC indicated partial crystallization of mannitol during cooling for a 5.0% mannitol solution at scanning rates of 10 °C/min. During the cooling scan the nucleation of mannitol crystals prevailed [25]. Because of the remaining amorphous mannitol fraction, two glass transitions $Tg'_1 = -30.0$ °C and $Tg'_2 = -25.5$ °C were measured. This is in agreement with literature, where glass transitions at −32 °C (Tg'_1) and −25 °C (Tg'_2) are described for solutions with 10% mannitol [26,27]. The glass transition events were followed by mannitol crystallization with a peak maximum at −19.3 °C during the rewarming of the sample. Here the nuclei, formed at low temperatures grow to mature crystals [25]. The addition of sucrose inhibited mannitol crystallization in the heating scan, indicated by a decreased enthalpy of crystallization and a shift of the peak maximum to high-

er temperatures (Figs. 1a and b). When the sucrose fraction in the formulation exceeded 50%, mannitol crystallization was completely suppressed in the heating scan. In formulations with 4.0% mannitol–1.0% sucrose three glass transition events Tg'_1 at −41.5 °C, Tg'_2 at −32.0 °C and Tg'_3 at −28.0 °C could be detected. For a similar formulation with 4.0% mannitol and 1.0% sucrose in 10 mM Tris as buffer Passot et al. described two glass transitions Tg'_1 at −41 °C and Tg'_2 at −31 °C, while Tg'_3 was not measured [5]. However, it is often difficult to detect the Tg' which is close to the onset of mannitol crystallization even in solutions with mannitol as single component, as it can overlap with the subsequent mannitol crystallization [26]. The origin of multiple glass transition processes was discussed by Liao et al. who focused on the lowest Tg' for their studies [6]. With increasing amounts of sucrose, the two glass transitions at higher temperatures were no longer present. The glass transition at lower temperatures was raised from −41.8 °C for 4.0% mannitol–1.0% sucrose to −33 °C for 5.0% sucrose (Fig. 1c). Liao et al. showed a constant Tg' of −43 °C for mannitol to sucrose ratios between 1.5 and 3 and increasing Tg' values for mannitol to sucrose ratios below 1.5 for formulations with a total solid content of 7.0% using cooling rates of 20 °C/min and heating rates of 5 °C/min for the DSC measurements [6].

Upon the addition of NaCl to mannitol–sucrose formulations the Tg' of the formulations was lowered for all studied mannitol–sucrose combinations (Table 3). The shift of

Table 3
Tg' in °C for mannitol–sucrose formulations with increasing NaCl concentrations ($n = 3$)

	Tg' [°C] 0% NaCl	Tg' [°C] 0.05% NaCl	Tg' [°C] 0.1% NaCl	Tg' [°C] 0.2% NaCl
4.0% M 1.0% S	−42.0	−42.0	−42.5	−43.5
3.0% M 2.0% S	−41.0	−41.0	−42.0	−43.0
2.5% M 2.5% S	−40.0	−40.5	−41.0	−42.0
2.0% M 3.0% S	−39.0	−39.5	−41.0	−41.0
1.0% M 4.0% S	−36.5	−37.5	−38.0	−39.0
0.0 %M 5.0% S	−34.0	−34.0	−36.0	−37.5

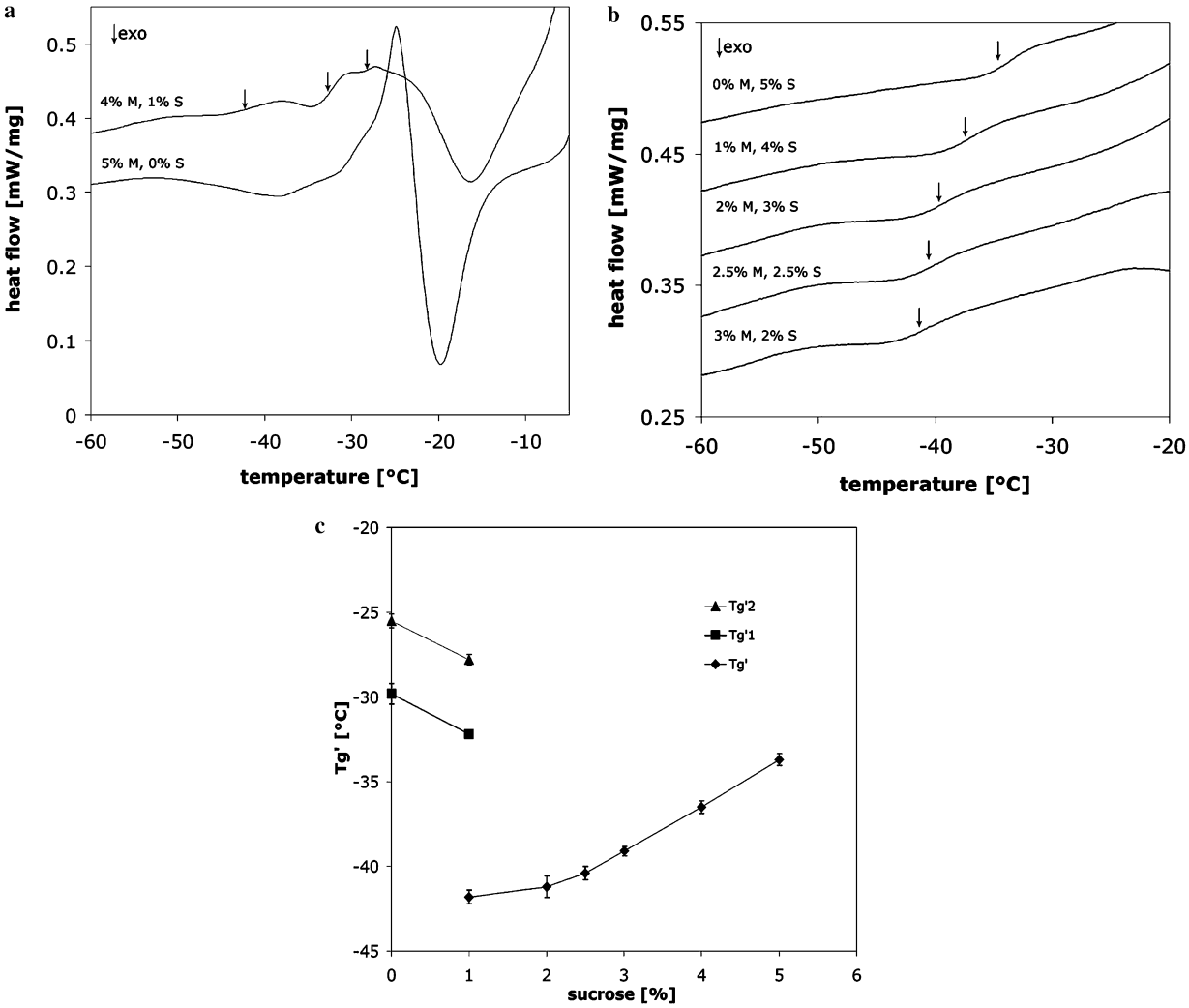

Fig. 1. DSC heating curve (10 °C/min) of mannitol (M)–sucrose (S) formulations with total solid content of 5.0% and different ratios of mannitol to sucrose (a and b). The different Tg' determined with DSC ($n = 3$) for the formulations (total solid content 5.0%) of the formulations is shown in (c).

Tg' in formulations with NaCl could be due to the very low Tg' of NaCl below −60 °C [28]. NaCl could act as plasticizer by increasing the amount of unfrozen water in the amorphous phase, leading to a depression of Tg'. The lowered Tg' in the formulations could further be indicative for the amorphous state of NaCl in the frozen state in the presence of mannitol and sucrose. When mannitol was present in the formulations the depression of Tg' by NaCl was less pronounced. While Tg' was lowered by 1.5–2.5 °C in mannitol containing formulations, a decrease of 3.5 °C is measured for 5.0% sucrose in the presence of 0.2% NaCl. In the amorphous state, mannitol itself can act as plasticizer and increase the amount of unfrozen water in the amorphous phase [12]. Even at the highest concentration of 4.0% mannitol in combination with 1.0% sucrose, amorphous mannitol was existent, evident by the crystallization in the DSC heating scan with an onset of −25 °C (Fig. 1a). At higher sucrose concentrations mannitol crystallization was further inhibited and the Tg' of the amorphous phase was lower compared to the Tg' of mannitol and sucrose as single components. This was ascribed to a higher amount of unfrozen water within the freeze-concentrate with increasing mannitol to sucrose ratio by Lueckel et al. [12]. A lowering of Tg' has to be considered for the development of the lyophilization cycles to avoid the higher mobility of the amorphous phase above Tg' and potential collapse during lyophilization. To avoid collapse the product temperature has to be kept below T_c, which ranges about 1–3 °C higher than Tg' [29]. Besides the depression of Tg' NaCl inhibited mannitol crystallization in the DSC heating scan, exemplarily shown for 4.0% mannitol–1.0% sucrose in Fig. 2.

3.1.2. DSC and XRD of lyophilized mannitol–sucrose formulations

For the stabilization of proteins the presence of an amorphous fraction in the lyophilized product is beneficial. Many attempts have been made to achieve amorphous mannitol to stabilize proteins, e.g., by using fast freezing rates or adding excipients that inhibit crystallization [13,20,21]. However, mannitol frequently crystallizes upon storage which can lead to stability problems for the protein. Crystalline mannitol looses its molecular interaction with proteins and with it the ability to stabilize the protein [2]. This indicates that the approach to produce amorphous mannitol as protein stabilizing component in a lyophilized formulation often fails. Therefore, a high degree of mannitol crystallization and the addition of a second amorphous stabilizer, e.g., sucrose or trehalose is the favored route. To ensure sufficient mannitol crystallization during the lyophilization process the formulation compositions, as well as the lyophilization processes have to be optimized. The addition of amorphous excipients can inhibit mannitol crystallization, leading to partially crystalline system. This was shown for formulations with a total solid content of 5.0% and varying ratios of sucrose to mannitol. After lyophilization without annealing the products were largely amorphous when more than 2.5% sucrose was present (Fig. 3a). In the DSC no crystallization was detected for 2.5% sucrose–2.5% mannitol during freezing and rewarming. However, the lyophilized products were partially crystalline, indicating that crystallization occurred to a great extent during the drying process. This observation is in agreement with Pyne et al. [30]. The LTXRD experiments further supported this finding, as no peaks for crystalline mannitol appeared after freezing the samples to −50 °C (Fig. 8).

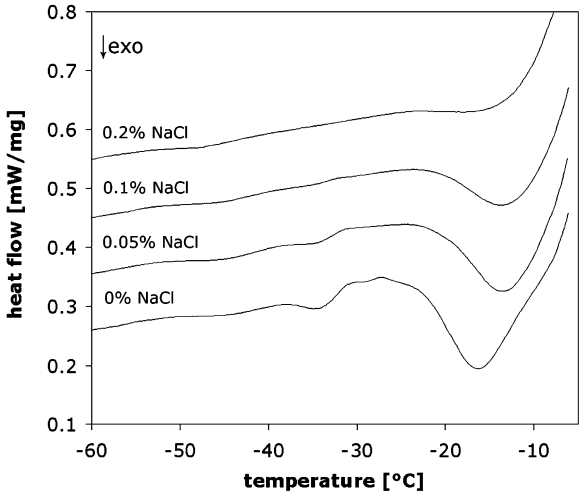

Fig. 2. DSC heating scan of 4.0% mannitol–1.0% sucrose with 0–0.2% NaCl.

In formulations with a 1:1 mannitol to sucrose ratio, peaks of δ-mannitol (9.7° 2-Θ) and β-mannitol (23.4° 2-Θ) were detected. Furthermore, mannitol hydrate indicated by the peak at 17.9° 2-Θ was present. In the first DSC heating scan of 2.5% mannitol–2.5% sucrose, lyophilized with program I two glass transitions (T_g) at 12 and 36 °C followed by mannitol crystallization with an onset of 43.3 °C and a peak maximum of 55.5 °C were detected (Fig. 4). The first glass transition at 12 °C could be attributed to the amorphous mannitol fraction. For amorphous mannitol a T_g of 13 °C is described by Kim et al. [11]. In the second scan mannitol was present in the crystalline form, as confirmed by XRD (data not shown). Here, only the higher T_g at 42 °C, which corresponds to sucrose remained. Increasing the mannitol content to 3.0% and 4.0% led to a higher intensity of the mannitol peaks in the XRD. However, formulations with 3.0% mannitol–2.0% sucrose, dried with program I also showed a crystallization event in the first scan pointing at the presence of amorphous mannitol. For 4.0% mannitol–1.0% sucrose no crystallization was detected in the DSC heating scan. This could be ascribed to a high degree of mannitol crystallinity, which could be confirmed by the high sucrose T_g of 58 °C. For lyophilized sucrose formulations with a residual moisture of 0.7–2.0% a T_g between 59 and 63 °C is described by te Booy et al. [31].

After lyophilization with annealing (program IIIa) a higher degree of crystallinity was achieved (Fig. 3b). There-

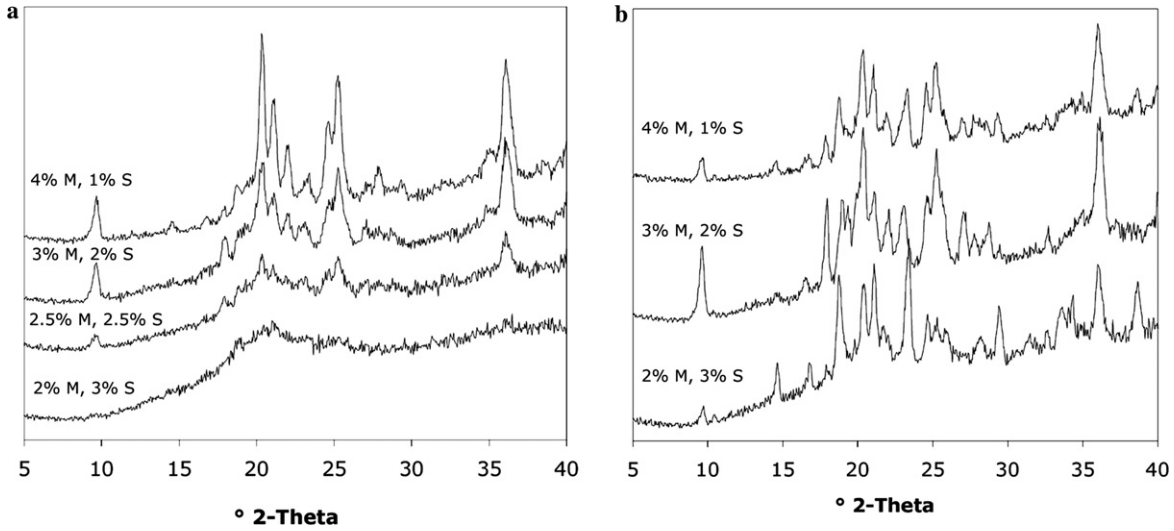

Fig. 3. XRD of mannitol (M)–sucrose (S) formulations after lyophilization with process I (a) and with process IIIa which includes an annealing step at −25 °C for 2 h (b).

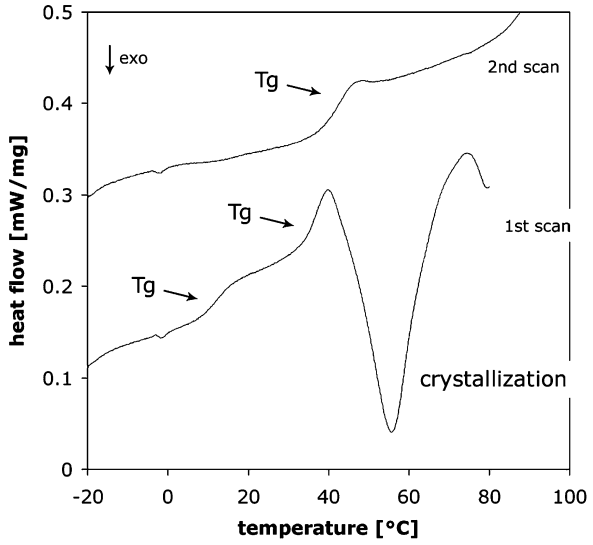

Fig. 4. DSC heating scans of 2.5% mannitol–2.5% sucrose lyophilized with program I.

by, mannitol crystallized mainly in the β-modification in formulations consisting of 2.0% mannitol–3.0% sucrose, which were amorphous after lyophilization without annealing. Formulations with more than 2.0% mannitol were composed of β- and δ-mannitol, comparable to lyophilization without annealing. However, a larger fraction of mannitol hydrate (peak at 17.9° 2-Θ) was present after drying the samples with annealing. This could be due to the fact that mannitol hydrate is a modification which emerges mainly at low temperatures during lyophilization. The impact of annealing on the formation of mannitol hydrate was studied in the further progress of the study. Therefore, the focus was set on formulations consisting of 4.0% mannitol–1.0% sucrose, as a high degree of mannitol crystallization can be achieved here.

3.1.3. Impact of NaCl on lyophilized mannitol–sucrose formulations

NaCl inhibited the crystallization of mannitol during freezing, which was shown by DSC for formulations with 4.0% mannitol–1.0% sucrose (Fig. 2). After lyophilization of 4.0% mannitol–1.0% sucrose mannitol crystallization was observed for all studied NaCl concentrations and lyophilization cycles. Fig. 5 shows the XRD diffraction patterns after lyophilization with program III. For 0–0.8% NaCl mannitol crystallized as a mixture of the β- and δ-modification and mannitol hydrate (17.9° 2-Θ), with a slight decline of the mannitol hydrate fraction at 0.8% NaCl. No crystallization events were measured in the DSC of the lyophilized products as well, indicating that mannitol and NaCl have crystallized during lyophilization.

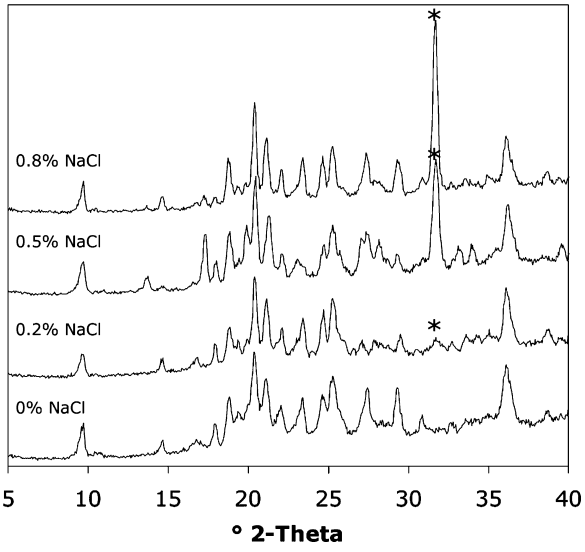

Fig. 5. XRD of 4% mannitol–1% sucrose with 0–0.8% NaCl (∗ marks characteristic peak of NaCl) lyophilized with program III.

However, the T_g of the formulations was affected by the addition of NaCl. Both DSC and TMDSC showed that the T_g of the formulations was decreased by about 25 °C when 0.4% NaCl was added to the formulations (Fig. 6). The residual moisture increased from 0.8% without NaCl to 1.7% with 0.5% NaCl. This could explain the lowered glass transitions as water can act as plasticizer for the amorphous phase.

Although mannitol crystallization was not affected by increasing NaCl concentrations, the lowered glass transition temperatures of the lyophilized products need to be considered for the storage stability of a protein. A higher T_g is often beneficial for protein stability upon storage which was for example shown by Prestrelski et al. for interleukin-2 [32]. However, Chang et al. demonstrated that storage of a lyophilized recombinant human interleukin-1 receptor antagonist formulations below T_g was necessary, but not always sufficient to ensure long-term stability, e.g., when the protein was already damaged during lyophilization process [33]. On the other hand, Davidson and Sun demonstrated for sucrose–raffinose formulations that an increase in T_g of the lyophilized samples was not related to a higher recovery of glucose-6-phosphate dehydrogenase activity after reconstitution when stored above T_g [34].

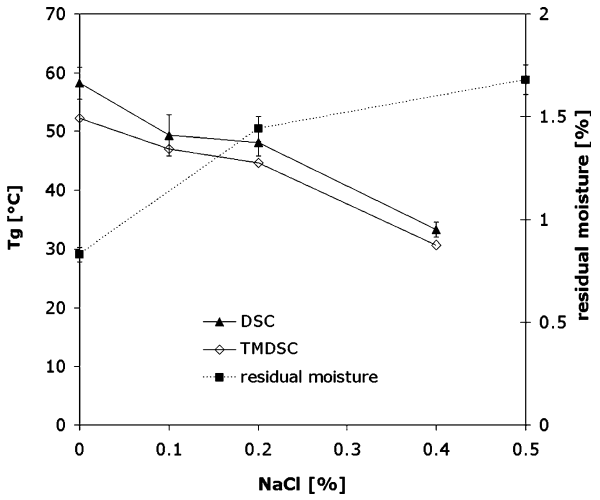

Fig. 6. T_g determined with TMDSC and DSC (second scan) and residual moisture of 4.0% mannitol–1.0% sucrose with 0–0.5% NaCl after lyophilization with program III.

3.2. Impact of annealing on the formation of mannitol hydrate

To study the impact of annealing on the formation of mannitol hydrate, formulations of 4.0% mannitol–1.0% sucrose were lyophilized with different lyophilization cycles. Furthermore, LTXRD was used to monitor crystallization of mannitol during an annealing step at low temperatures. For the LTXRD experiments solutions with higher concentrations (8.0% mannitol–2.0% sucrose) were used, due to the detection limit of the method.

3.2.1. LTXRD of mannitol–sucrose formulations at different annealing conditions

DSC offers information on crystalline and amorphous phases in the frozen state. However, with DSC it is not possible to identify and characterize the composition and modifications of the crystalline phase [35], which is on the other hand possible with LTXRD [36,37]. After freezing a formulation of 8.0% mannitol–2.0% sucrose to −50 °C with a cooling rate of 0.5 °C/min no peaks of crystalline mannitol were detected (Fig. 7). The cooling rate of 0.5 °C/min was chosen as it is comparable to the cooling rate used during lyophilization. After an isothermal phase of 1 hour at −50 °C the temperature was increased with 1 °C/min to the annealing temperatures of −23, −27 and −32 °C, where three measurements were performed under isothermal conditions. After heating the samples to the annealing temperatures peaks of crystalline mannitol were detected. At −23 and −27 °C mannitol crystallized in the δ-modification, seen by peaks at 9.7° 2-Θ and 20.4° 2-Θ (Fig. 7a). Annealing at −32 °C on the other hands led to a crystallization of

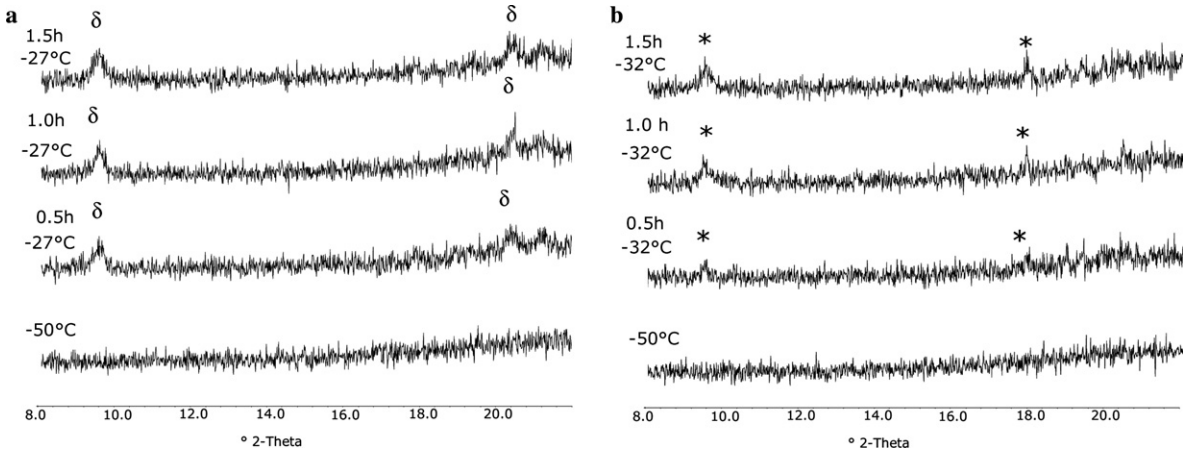

Fig. 7. LTXRD of 8.0% mannitol–2% sucrose after freezing to −50 °C and during an isothermal annealing step at −27 °C, with crystallization of δ-mannitol (a) and −32 °C (b), with crystallization of mannitol hydrate (marked with *).

mannitol hydrate which was evident by the peak at 17.9° 2-Θ (Fig. 7b). In the DSC heating scan mannitol crystallized with an onset of −25 °C. At −32 °C the crystallization process was decelerated as indicated by the lower intensity of the peaks in the first scan at −32 °C.

Thus, according to LTXRD annealing at a temperature of −32 °C is not suitable due to the formation of mannitol hydrate. To investigate whether the findings of the LTXRD could be transferred to formation of mannitol hydrate in lyophilized products, lyophilization was conducted at different annealing temperatures and the samples were analyzed by XRD.

3.2.2. XRD of lyophilized samples produced with different lyophilization cycles

The impact of annealing and secondary drying on the formation of mannitol hydrate during lyophilization was investigated for 4.0% mannitol–1.0% sucrose formulations with 0 and 0.2% NaCl. Annealing during lyophilization was conducted for 2 h at a shelf temperature of −20, −25 or −30 °C (Table 2). All processes resulted in crystalline formulations which was confirmed by DSC (data not shown). XRD revealed the presence of β- and δ-mannitol, as well as different amounts of mannitol hydrate (Fig. 8a). The characteristic peak of mannitol hydrate at 17.9° 2-Θ is enlarged in Fig. 8b. To compare the amount of mannitol hydrate formed after the different processes, the peak area and the relative intensity of the mannitol hydrate peak at 17.9° 2-Θ were considered (Fig. 9). The peak area was calculated by integrating the area under the peak from 17.4° 2-Θ to 18.4° Θ in the diffraction pattern. For the relative intensity the height of the mannitol hydrate peak was compared to the highest peak of the diffraction pattern, which was set as 100%. In formulations dried without annealing the lowest fraction of mannitol hydrate was formed, although secondary drying was performed at 20 °C. Com-

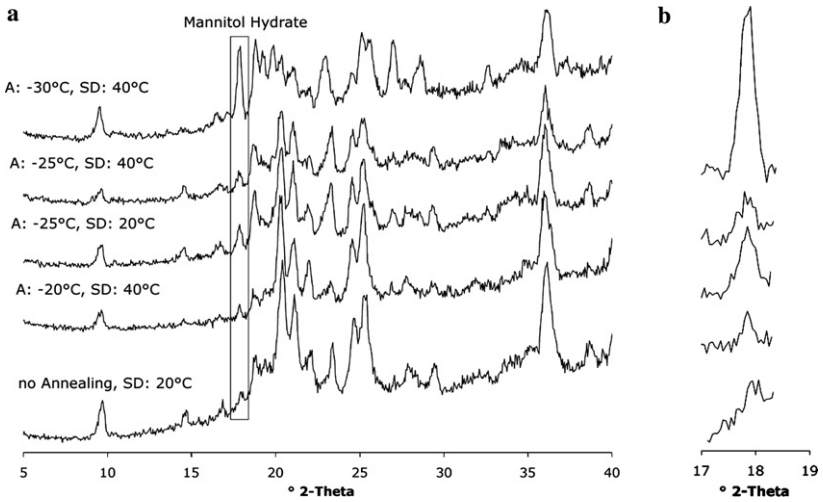

Fig. 8. XRD of lyophilized 4.0% mannitol–1.0% sucrose after different annealing (A) and secondary drying (SD) parameters (a) and mannitol hydrate peak at 17.9° 2-Θ (b).

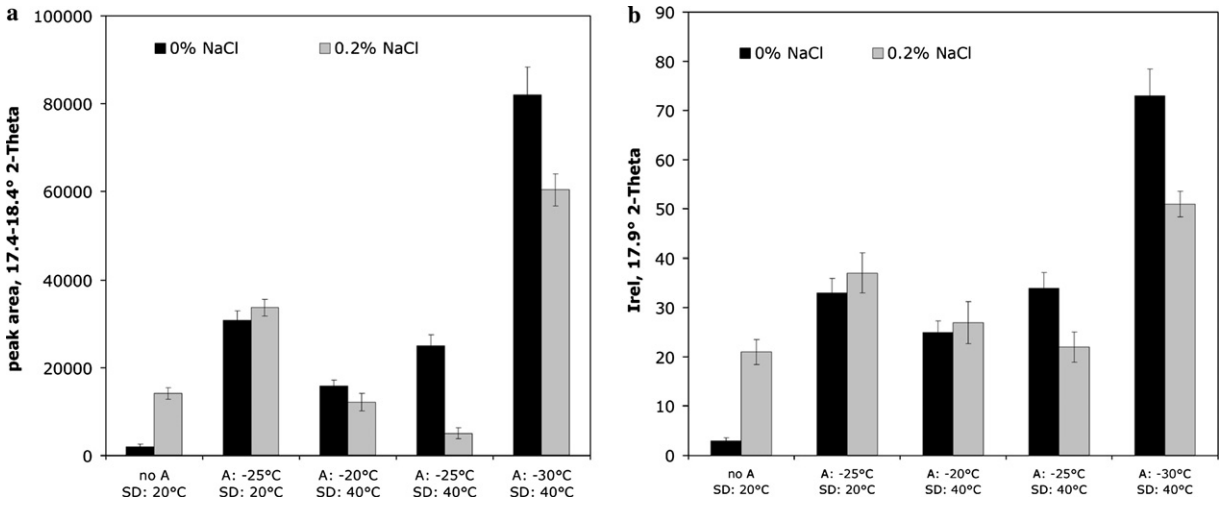

Fig. 9. Peak area (a) and relative intensity (b) of mannitol hydrate peak after lyophilization of 4.0% mannitol–1.0% sucrose without and with 0.2% NaCl using different annealing (A) and secondary drying (SD) conditions ($n = 3$).

paring the different annealing conditions the formation of mannitol hydrate was more distinct at lower annealing temperatures. This was in agreement with LTXRD, where mannitol hydrate was also formed at a lower temperature. The addition of 0.2% NaCl to the formulations slightly inhibited the formation of mannitol hydrate during lyophilization with annealing. However, NaCl tended to promote the formation of mannitol hydrate when annealing was waived. Comparing the different secondary drying temperatures less mannitol hydrate was formed when secondary drying was performed at 40 °C, which was in agreement with Johnson et al. [4]. However, the effect was less pronounced than the impact of the selected annealing temperature.

To estimate the amount of mannitol hydrate in the lyophilized formulation with 4.0% mannitol–1.0% sucrose dried with program IV dynamic Karl-Fischer titration was performed (Fig. 10). Water was released in two steps from the lyophilized product upon heating. The first step between 30 and 45 °C could be attributed to the release of sorbed water and the second step from about 55 to 70 °C mainly to the release of hydrate water from mannitol. In the second step about 0.6% water was released an in total the residual moisture content was 1.1%. The data showed that about 15–20% of mannitol were present as hydrate, when calculated as hemihydrate in the worst case of annealing at −30 °C.

LTXRD and XRD both showed that annealing can promote the formation of mannitol hydrate. Although there were differences in volumes and containers used during lyophilization and for the LTXRD experiment it was evident that mannitol hydrate is formed especially at lower temperatures. Therefore, it is beneficial to conduct lyophilization without annealing if possible. For the formulations with 4.0% mannitol–1.0% sucrose lyophilization without annealing was feasible, because mannitol crystallinity comparable to the processes with an extra annealing step was achieved. If annealing is required it is important to select a shelf temperature during annealing above −30 °C to keep the level of mannitol hydrate as low as possible.

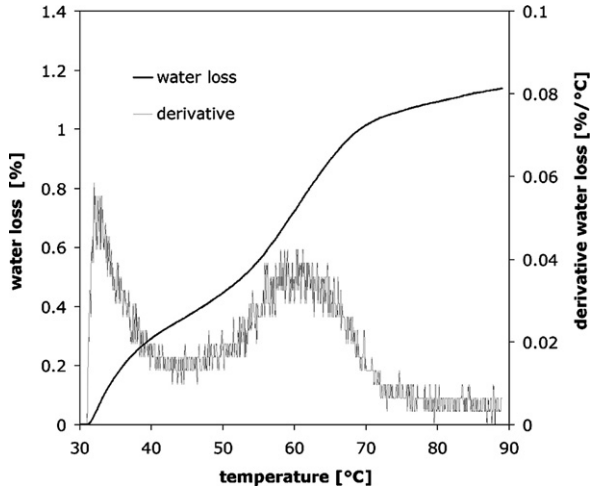

Fig. 10. Water loss from 30 to 90 °C determined with dynamic Karl-Fischer titration for 4.0% mannitol–1.0% sucrose dried with program IV.

4. Conclusions

DSC results of the system mannitol–sucrose at low temperatures indicated a lowering of Tg' with increasing amounts of mannitol due to the plasticizing effect of mannitol on the amorphous phase. The addition of NaCl further reduced the Tg' of the formulations, which was more pronounced for formulations with lower mannitol contents. Mannitol crystallization during freezing was inhibited by sucrose and NaCl, resulting in partially crystalline systems. However, the T_g of these formulations was depressed by NaCl. Lyophilization of 4.0% mannitol–1.0% sucrose led to extensive crystallization of mannitol, even without annealing. Annealing increased mannitol crystallinity, however, a higher mannitol hydrate content was often found after lyophilization of 4.0% mannitol–1.0% sucrose with annealing. LTXRD of 8.0% mannitol–2.0% sucrose formulations showed that mannitol hydrate was preferably formed at lower annealing temperatures, while annealing at higher temperatures led to the formation of δ-mannitol. The results of LTXRD were confirmed by XRD after lyophilization, which showed that the highest amount of mannitol hydrate was formed after annealing at −30 °C. NaCl enhanced the formation of mannitol hydrate when lyophilization was conducted without annealing. Compared to the impact of annealing on the formation of mannitol hydrate the secondary drying temperature only played a minor role, shown by a slight reduction of mannitol hydrate when secondary drying temperature was increased from 20 to 40 °C. Here higher temperatures and longer drying times would be required. If annealing during lyophilization is necessary, a sufficient high temperature needs to be selected to avoid the formation of mannitol hydrate.

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