Physical Stability of Salmon Calcitonin Spray-Dried Powders for Inhalation

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ABSTRACT: The effects of excipient crystallinity and water content on the physical stability of salmon calcitonin (sCT) in a spray-dried powder for inhalation have been investigated. sCT was dissolved in water with and without mannitol and then spray dried using a Büchi 190 spray dryer. The spray dried powders were stored for 5 days at 0, 29, 51, 58, 69, and 84% relative humidity at ambient temperature. The crystalline content, water content, secondary structure, and aggregation rates were determined for each powder immediately following spray drying and after storage at various relative humidities. In addition, the water sorption isotherms and reactivity to water vapor were determined using DVS and isothermal calorimetry, respectively. No sCT aggregation occurred during the spray drying process. Crystallinity depended on the amount of mannitol in the formulation. Powders containing up to 50% mannitol were fully amorphous, and those containing 70 and 90% mannitol contained some crystalline polyol. The powders remained aggregate free for over 2 years when stored below the critical RH (e.g., <20% for the powder containing 30% mannitol). Above this RH, sCT aggregation increased as a function of time. The amount of aggregate observed correlates with the amount of intermolecular β-sheet formed, determined by FTIR. The sCT aggregation rate in powders containing 70% mannitol was significantly lower than that in powders containing 30% mannitol at all RH tested, presumably because of a higher ratio of amorphous mannitol to sCT, which inhibits the formation of β-sheet structure. Moisture-induced crystallization of mannitol was observed in all powders stored at RH >50%. The moisture induced thermal activity trace (MITAT) offers a useful description on the physical stability of the spray dried powders. In conclusion, spray drying sCT and sCT/mannitol mixtures yields dry powders that contain physically intact peptide. In addition, sCT aggregation and mannitol crystallization in spray dried powders can be prevented during long-term storage if stored in low humidity environments, which can be easily assessed by MITAT.
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INTRODUCTION

The successful pulmonary delivery of dry powders containing therapeutic proteins or peptides requires the preparation of a stable formulation. It has been demonstrated that room-temperature stability of dry powders containing proteins or peptides can be achieved using glass stabilization

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technology.[1] Spray drying is most suitable for the production of dry powders that have controlled particle size distribution.[2] In contrast to common perception, the temperature experienced by formulations during this processing is sufficiently low to avoid many degradation reactions.[3,4] Spray drying also leads to the formation of a significant air–liquid interface due to atomization, a stress that can denature proteins. This challenge has been overcome by the use of surface-active excipients.[5] In addition, controlling the final physical state of the solid formed by conventional spray drying is difficult. Depending on the excipients used, as stabilizers or bulking agents, spray drying may produce solids that are amorphous or partially crystalline.[6] Manufacturing dry powders for use as inhaled therapeutics with consistent aerosol performance requires that the powders have consistent amounts of amorphous and crystalline phases.[7] In addition, conditions used for storage (storage temperature and relative humidity) must maintain the physical state and moisture content of the powder.[8–10] The latter maintains the glass transition temperature of the powder.

The stability of the pharmacologically active protein is also of critical importance. Stabilizing excipients can form hydrogen bonds with the protein when the two are in an *amorphous* dry state. This helps preserve the native conformation and chemical stability of the protein.[9] Thus, when excipient crystallization occurs, protein-excipient hydrogen bonding is disrupted, and the protective effect of the excipient is diminished. Other processes that adversely affect protein stability include: (1) water-induced plasticization of the powder (i.e., depressing the glass transition temperature, T_g), and, (2) reactions in which water is a reactant.[1]

Thus, understanding the factors that contribute to powder and protein degradation in spray-dried powders is complex. Although there are many studies on the solid-state stability of proteins, similar work on peptides is relatively scarce.[11–19] The present study investigates the impact of moisture content, crystallinity, and the presence of an excipient on the aggregation stability of a model peptide and the physical stability of the spray dried particles.

The model chosen for this study is salmon calcitonin (sCT) because of its well-known propensity to undergo aggregation.[20,21] This pharmacologically active peptide contains 32 amino acids, has an isoelectric point of 9.3, and a single disulfide bond (Cys1—Cys7) at the NH_2-terminus.

MATERIALS AND METHODS

Sample Preparation

Solutions of sCT were spray-dried as binary mixtures with D-mannitol (hereafter referred to as mannitol). For this study, seven powders containing 0:100, 10:90, 30:70, 50:50, 70:30, 90:10 and 100:0 (in wt %) of mannitol:sCT were obtained. All solutions were spray dried using a modified Büchi 190 spray dryer under the following conditions: liquid feed rate of 5 mL/min, atomising pressure of 60 psi, and the (controlled) inlet and (measured) outlet air temperatures were 125 and 70°C, respectively. Powder collected in the cyclone was recovered and used without further treatment. The solutions were prepared by dissolving known amounts of sCT (Peninsula Labs, San Carlos, CA) and mannitol (USP grade, Mallinckrodt, St. Louis, MO) to a total solid concentration of 10 mg/mL in deionized water. The pH of each solution was adjusted to pH 7.0 using concentrated NaOH.

Storage Stability at Controlled Relative Humidity

Approximately 2–5 mg of each of three selected powders (0, 30, and 70% mannitol) was aliquoted into a small glass vial. These open vials were stored for 5 days over saturated salt solutions in desiccators, which were placed in a temperature-controlled incubator at 25°C. Each of the following salt solutions was used to provide an environment of a given relative humidity at 25°C: $CaCl_2$ (20% RH), $Ca(NO_3)_2$ (51% RH), NaBr (57.6% RH), KI (68.9% RH), and KCl (84.3% RH).

Size-Exclusion HPLC

Size-exclusion chromatography was employed to determine the monomer content of sCT samples at time zero and upon storage at elevated relative humidity. Samples (20 μL) of sCT were injected onto a silica-based Tosohaas TSK-GEL $G2000SW_{XL}$ column maintained at 25°C. Two injections were made per sample, and samples were prepared in duplicate. The mobile phase, 0.25 M Na_2SO_4, was run at 0.7 mL/min. The column eluent was monitored at 210 nm.

The peak area of native monomer sCT was normalized to the total peak area of the sample.

FTIR Spectroscopy

Secondary structural analysis of the peptide in the mannitol-sCT powders was conducted using a ThermoNicolet Magna 760 IR spectrometer (Nicolet, Madison, WI) equipped with a dTGS detector and KBr beamsplitter. IR spectra (256 scans, 4 cm^{-1} resolution) were recorded at 25°C. Spray-dried samples (approx. 0.2–0.4 mg protein) were ground with 300 mg KBr and pressed into pellets, following a procedure that has been shown not to alter the structure of proteins in the dried state.[9] The spectra were corrected with the background spectrum and the second derivative amide I spectra were calculated with the ThermoNicolet Omnic software. The final second derivative spectra were smoothed with a seven point smoothing function via the Omnic software and area normalized with Grams/386 software, as outlined previously.[22,23]

Dynamic Water Vapor Sorption

The water sorption isotherms at 25°C of mannitol–sCT powders were measured using a dynamic vapor sorption (DVS) microbalance (Surface Measurement Systems, London, UK). This instrument measures gravimetric uptake and loss of water vapor of the powder by means of an electronic microbalance with a resolution of $\pm$ 0.1 μg. The RH of the vapor flowing around the sample was controlled by mixing saturated and dry carrier gases using mass-flow controllers. The whole system was kept isothermal by enclosure in a temperature-controlled incubator. In the first step of an experimental run, the sample was dried at 25°C and 0% RH for at least 600 min to bring the sample to near zero wt % H_2O. Following the drying step, the instrument was programmed to increase the RH to target values in a stepwise fashion. The *target* RH was the RH setpoint targeted by the mass-flow controllers; the *sample* RH was the actual RH measured by a fixed probe adjacent to the sample. For the experiment, the equilibrium water content was measured at *target* RH values from 0% RH to 90% RH in increments of 5% RH, and from 90% to 0% RH in increments of 10% RH. A weight change of less than 0.005% per min was chosen as the criterion for reaching equilibrium at each RH step before proceeding to the next RH step.

Modulated Temperature Differential Scanning Calorimetry (MTDSC)

Measurement of the glass transition temperature, T_g, was conducted using a differential scanning calorimeter (TA Instruments DSC-2920) with a TA refrigerated cooling system (New Castle, DE). Helium gas was used with a flow rate of 120 cm^3/min, and the cell flow rate was set at about 40 cm^3/min. Hermetic aluminum pans were filled with about 2–6 mg of powder and sealed. For open pan analysis, pans were punctured just prior to the run. The scans were performed at 2°C/min modulated (with a 1°C amplitude and a 60 s period) with an equilibration temperature of −30°C for 15–30 min, followed by heating to 200°C. Reported T_g values have been measured at the onset of the step-wise heat capacity change.

Raman Spectroscopy

Raman spectroscopy was employed to investigate the crystallinity of sCT powders for the storage stability study. The Raman instrument used in this study was custom designed at Nektar Therapeutics. It is a dispersive system with excitation in the red spectral region and detection by a cryogenically cooled CCD sensor. The laser (Process Instruments # PI-ECL-670-150-FS) was operated at a wavelength of 669.85 nm, and a power of 100 mW at the sample. Samples were placed on a holder in an environmentally controlled sample chamber (22°C, 0% RH). The radiant flux density at the sample was approximately 10^7 W/m^2. The Raman signal was collected at an angle of 90° with an exposure time of typically 20 min per spectrum. The spectrograph is a modified single stage Czerny-Turner spectrograph (Acton Research Corporation, 500i). The resolution of the spectrograph was set to 3 cm^{-1}. The positional accuracy was better than 1 cm^{-1}. The signal was detected by a digital CCD spectroscopy system (Princeton Instruments, LN/CCD-400EHR-G1) outfitted with a front-illuminated, deep depletion CCD array (EEV type 36).

Isothermal Calorimetry (TAM)

The interaction of the spray-dried formulations with water vapor was studied using a heat-conduction isothermal microcalorimetric system, TAM (Thermal Activity Monitor, Model 2277 Thermometric AB, Sweden). At a given temperature, this technique measures the total,

nonspecific heat flux, P (typically measured in μW), of interaction between the solid sample and water vapor.[24] During the experiment, the sample was placed into an ampoule connected to a gas pressure controlled device (Model 2255-120 Thermometric AB, Sweden) that accurately controls the RH of the incoming gas stream (0 to 90 ± 0.1% RH) as a step function or a linear ramp. An empty, closed ampoule was used as a reference cell. Dry N_2 was used as carrier gas at a constant flow rate (1.48 standard cm^3/min). The total flow of the carrier gas was split into two streams to control the relative humidity. One stream (the "dry stream") was fed directly to the sample ampoule, while the so-called "wet stream" was fed to two successive humidification chambers. A stream of a given RH was then produced by controlling the flow rate ratio of the dry to wet streams. The method used here required a mathematical correction to the RH to account for the change in mass flow rate as the nitrogen stream passed through the humidification chambers. This correction was used throughout this work even though it was at most 3% of the nominal RH value (at 25°C).

RESULTS

Glass Transition Temperature Determination

The thermal properties of spray-dried sCT powders, both neat and formulated with mannitol, were analyzed by MTDSC. The glass transition temperature, T_g, of neat spray-dried sCT was determined to be 147°C. However, determination of the T_g of the different formulations was not straightforward, because many thermal events (i.e., water evaporation, crystallization, and melting of crystalline mannitol) complicate interpretation. Because knowing the T_g is paramount to understanding the stability of amorphous solids, a simple form of the Fox equation was used to estimate the T_g values of the dry formulations shown in Table 1. The Fox equation is the simplest model to predict the T_g of amorphous mixtures.[25] It is an inverse linear relationship with a general form for two components and is given by:

$$\frac{1}{T_g} = \frac{w_1}{T_{g(1)}} + \frac{w_2}{T_{g(2)}},$$

where w_i and $T_{g(i)}$ are the mass fraction and the T_g of component i, respectively. The estimated values shown in Table 1 correspond to events that can be interpreted as a T_g in the actual MTDSC thermograms (data not shown).

To use the Fox equation, the weight fraction of amorphous mannitol must be known. The amount of amorphous mannitol was determined using Raman spectroscopy, as described below. It is assumed that solid sCT is always amorphous. This assumption was corroborated by X-ray powder diffraction, which demonstrated that neat, spray-dried sCT is fully amorphous (data not shown). Estimated intrinsic T_g values (i.e., dry T_g) for the different spray dried sCT formulations are graphically depicted in Figure 8.

Crystallinity Measurements

The initial crystallinity and changes in the crystallinity of each powder upon storage and exposure to different humidity conditions of the various formulations was measured by Raman spectroscopy. Mannitol α-, γ-, and δ-polymorphs

Table 1. T_g Estimates for Mannitol/sCT Formulations Corrected for Crystalline Mannitol

Total Mannitol %	sCT %	Crystallinity by Raman %	Amorphous Mannitol (AmM) %	sCT/ AmM	AmM/ sCT	Wt Fraction Amorphous Mannitol	Wt Fraction Amorphous sCT	Estimated T_g^a (°C)
0	100	0	0	—	0	0	1	147[b]
10	90	0	10	9.0	0.11	0.1	0.9	128
30	70	0	30	2.3	0.44	0.3	0.7	94
50	50	0	50	1.0	1	0.5	0.5	66
70	30	50	20	1.5	0.67	0.4	0.6	79
90	10	86	4	2.5	0.4	0.29	0.71	96
100	0	100	0	—	—	0	0	11[c]

[a]Estimated using the Fox equation (see text for details). The intrinsic T_g values of mannitol and sCT are $T_g = 11$ and 147°C, respectively.
[b]Our own measurement for spray-dried sCT.
[c]From ref. 33.

were prepared by crystallization from solution, identified by XRPD and used as reference standards. Reference Raman spectra of the three crystalline polymorphs were found to be in agreement with previously published spectra.[26] All mannitol forms are easily distinguishable by characteristic peaks in their Raman spectra, and unambiguous assignment was achieved in all cases; this spectrum showed good agreement with a spectrum of an aqueous mannitol solution. The reference spectrum of amorphous mannitol was derived from the formulation containing 30% mannitol, which was demonstrated to be amorphous by XRPD. The Raman spectrum of amorphous mannitol is expected to be similar to the spectrum of mannitol in aqueous solution.[27] Neat spray-dried sCT was used to obtain a reference for sCT. The crystallinity of freshly made formulations was determined by subtracting the contributions of sCT and the various mannitol polymorphs in the formulation to the Raman spectra; results are reported in Table 1. Upon storage, the 30% mannitol formulation crystallized, with the amount of δ-mannitol increasing during exposure at 51% RH. Transformation to the α-form occurred at 69% RH (Fig. 1A). The 70% mannitol formulation initially contained amorphous, α- and δ-forms; upon storage at 69% RH the δ-form was transformed into the α-form (Fig. 1B). This finding is significant because the δ-form is stable when stored at low humidity for over 3 years in our laboratory. The results suggest a solution-mediated transformation, which will require that the deliquescence point of the δ-form be below 69% RH. The deliquescence point of the α-form is approximately 85% RH.

Moisture Uptake and Reactivity to Moisture

The moisture uptake properties of neat and formulated sCT spray-dried powders were characterized by their moisture sorption isotherms measured at 25°C (see Fig. 2). Neat spray-dried sCT (totally amorphous) and neat spray-dried mannitol (substantially crystalline) offer the most contrasting moisture isotherms. The amount of water absorbed below 50% RH increases proportionally to the amount of sCT in the formulation. The amount of water absorbed at RH >50% is defined not only by the initial formulation composition but also by the contribution of the moisture-induced crystallization (see Table 2). Even though formulations that contain significant amounts of amorphous mannitol, for example formulations containing less than 70% total mannitol (Table 1), experience a noticeable

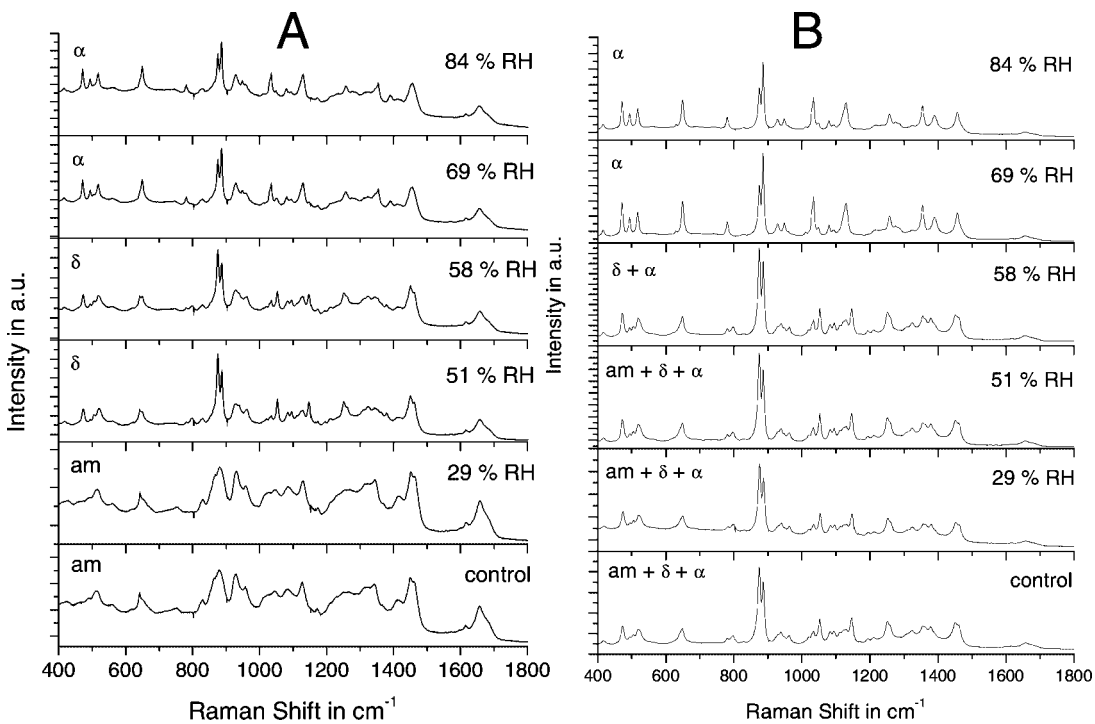

Figure 1. Raman spectra for formulations exposed to elevated RH. (A) 30% mannitol; (B) 70% mannitol.

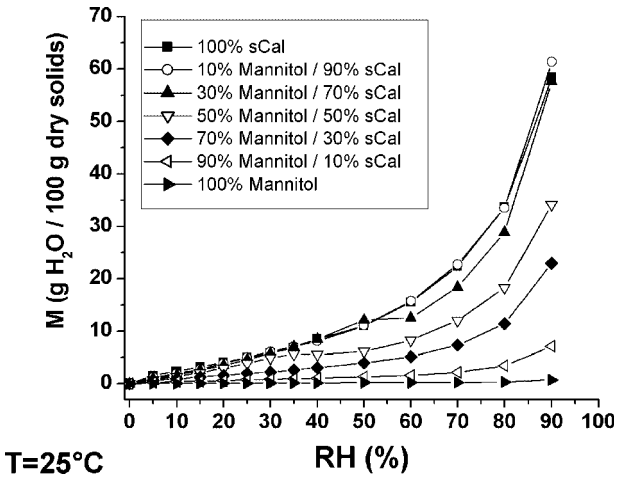

Figure 2. Moisture sorption isotherms (25°C) of spray-dried sCT powders.

weight loss upon exposure to RH >50% the total amount of water increases as shown in Figure 2. The observed weight loss is proportional to the amount of mannitol crystallization (Fig. 3). Over the time scale of these DVS experiments, moisture-induced crystallization implies that the glass transition temperature has been depressed well below the storage temperature (25°C in this case) as a result of the plasticizing effect of the absorbed water.[28] Data in Figure 3 can be used to identify excipient crystallization; however, its usefulness at predicting long-term stability is limited because crystallization of amorphous solids is known to occur even when stored at temperatures below their T_g.[29–31] The T_g of the formulations containing 30 and 70% mannitol is higher than the storage temperature (Fig. 8). Thus, to predict long-term stability, determination of the appropriate storage RH that prevents crystallization below the T_g is necessary.

The use of isothermal microcalorimetry to determine appropriate storage conditions to ensure long-term physical stability has been recently demonstrated.[24] Following this approach, the moisture induced thermal activity trace (MITAT) has been determined for sCT formulations (see Fig. 4). From analysis of the MITAT, a critical RH value for crystallization, RH_x, and the critical RH indicative of long-term stability, RH_p, can be assigned. It is clear that for the 30%-mannitol formulation, RH_x in the MITAT corresponds to 60% RH. Above this level, crystallization is observed in the kinetic DVS plot (Fig. 3). To achieve long-term stability at 25°C, this powder needs to be stored at <20% RH. Similarly, for the 70% mannitol formulation, a storage RH of <12% is determined from the MITAT at 25°C. RH_x is found to be 50% RH, similar to the crystallization RH observed in the dynamic DVS data.

The Effect of Spray Drying on sCT Stability

Spray-dried sCT powders, neat and formulated with mannitol, were reconstituted into dilute aqueous solutions and analyzed. The monomer content for freshly spray dried powders and after storage at different conditions is summarized in Table 3. In the case of mannitol-containing formulations, solutions of reconstituted powders were clear on visual inspection.

Native human calcitonin has been shown to contain a significant amount of α-helix and little or

Table 2. Effects of RH (25°C) on Moisture Content, Crystallinity, and sCT to Amorphous Mannitol Ratio of 30 and 70% Mannitol Powders after Storage for 5 Days

Formulation	Storage RH (%)	Moisture Content (%)[a]	Crystallinity (%)[a,b]	Moisture Content of the Amorphous Phase (%)	Amorphous Mannitol (AmM) (%)	sCT/AmM
30% mannitol	29	5.5	0	5.5	30	2.3
	51	12.1	30	17.3	0	—
	58	12.4	30	17.8	0	—
	69	17.8	30	25.4	0	—
	84	40.4	30	57.7	0	—
70% mannitol	29	2.2	63	5.9	7	10
	51	4.1	68	12.8	2	35
	58	4.9	70	16.3	0	—
	69	7.1	70	23.7	0	—
	84	16.0	70	53.3	0	—

[a]From moisture isotherm at 25°C (Fig. 2).
[b]Crystallinity was determined by Raman spectroscopy.

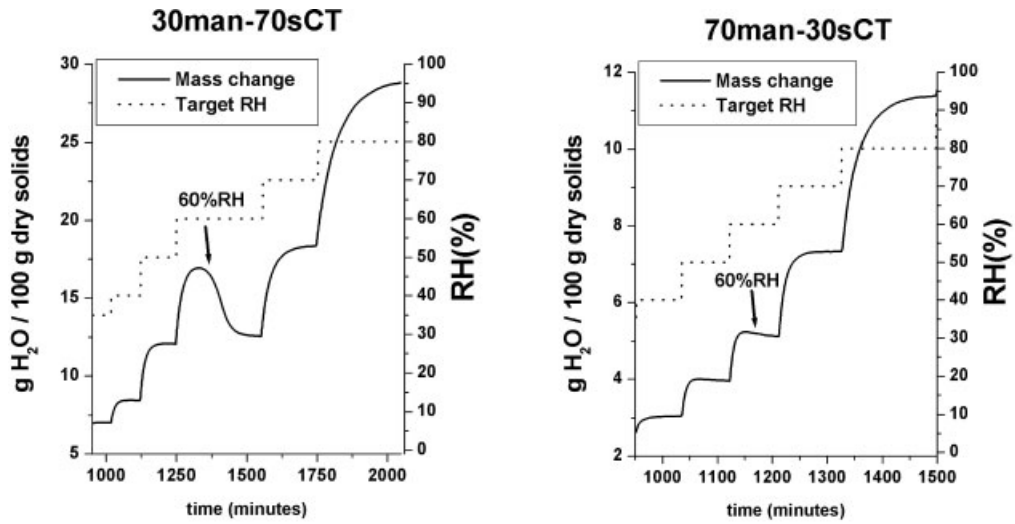

Figure 3. The moisture sorption kinetic profiles for the 30% mannitol (left) and the 70% mannitol (right) formulations. Moisture sorption was monitored using DVS at 25°C. The observed loss of mass corresponds to the crystallization of mannitol.

no β-sheet.[20] Native salmon calcitonin also appears to contain a significant amount of α-helix as indicated by the prominent band at 1656 cm^{-1} in the FTIR spectrum (see Fig. 5A). FTIR analyses of the spray-dried powders show that powders obtained from the 30% mannitol formulation retain maximum α-helicity (Fig. 5A). These data indicate that these powders are different, even though irreversible aggregation is not observable by SEC-HPLC.

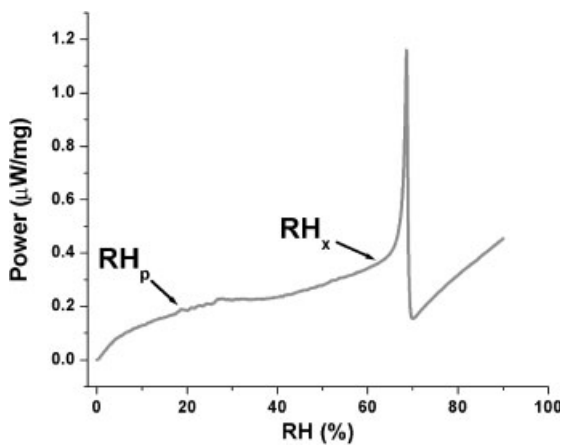

Figure 4. The moisture-induced thermal activity trace (MITAT) for the 30% mannitol.formulation. RH_p indicates the onset of water-induced thermal activity due to increased water mobility in the powder. RH_x indicates the onset of real-time crystallization.

The Effect of Relative Humidity on sCT Aggregation

Storage of sCT spray-dried powders at room temperature in the presence of moisture leads to aggregation (Table 3). For all powders, the aggregation rate peaks at 69% RH (Fig. 6). Powder stored at 84% RH forms less peptide aggregate than powders stored at 69% over a 5-day incubation period. At very high relative humidity, sufficient water is likely present to begin dilution of the reactant (sCT), which is expected to reduce the overall reaction rate.[32]

FTIR analyses were performed on samples stored at different RH conditions. We only show results for the 30% mannitol formulations due to space limitations (Fig. 5B). After storage at RH = 58% for 5 days, the amorphous powders (0 and 30% Mannitol) showed a large reduction in native α-helix structure (1656 cm^{-1}) by FTIR analysis (Fig. 5B). The loss of α-helicity is accompanied by an increase in intramolecular β-sheet formation (1632 cm^{-1}). In addition, sCT powders stored at RH = 58% show an increase in peak intensity at 1615 cm^{-1} and formation of a new band at 1690 cm^{-1}, indicating formation of intermolecular beta sheet aggregates. In contrast, sCT powders with significant amount of crystalline mannitol (i.e., 70% mannitol formulation), retained the native α-helix structure during storage except at the highest RH tested (84%). At this RH, a nearly complete loss of native α-helix structure occurred with a concomitant increase in

Table 3. Monomer Content Immediately after Spray Drying and after 5 Days of Storage at the Indicated Relative Humidity (25°C)

Formulation Mannitol:sCT (wt %)	Initial SD Powder	29% RH	51% RH	58% RH	69% RH	84% RH
70:30	97.3	94.5	89.1	67.6	58.6	73.8
30:70	97.8	95.0	94	79.8	73.5	78.5
0:100	95.7	93.4	91.3	66.4	56.1	67.8

the intramolecular β-sheet structure (1632 cm^{-1}) and formation of intermolecular β-sheet aggregates (1615 and 1690 cm^{-1}).

DISCUSSION

Phase Analysis of sCT and Mannitol in Spray-Dried Powders

In our experience, spray dried peptides like sCT are entirely amorphous. Therefore, the crystallinity of these sCT/mannitol mixtures is determined by the mannitol content in the formulation (see Table 1). Because amorphous sCT has a relatively high intrinsic glass transition temperature ($T_g^0 = 147$°C), elevated moisture content is required to depress the T_g to below room temperature. Even when this occurs, no spontaneous crystallization is expected. Instead, sCT is likely to form a gel phase as has been previously observed in concentrated solutions.[20] In contrast, the formation of amorphous or crystalline material of a small molecule excipient, such as mannitol, during spray drying is not easily predicted. For example, the crystallization propensity of common excipients used in the preparation of spray-dried powders for inhalation (i.e., trehalose, lactose, sucrose, mannitol) has been reported.[6] Mannitol stands out for its strong tendency to form a crystalline phase due, in part, to its low T_g^0 (11°C).[33] However, molecular symmetry also appears to be important since sorbitol, a diastereomer of mannitol, does not crystallize on spray drying even though its T_g^0 is −3°C.[33] Mannitol, when used in high concentrations (i.e., >20%), also tends to crystallize during freeze drying, which makes it a preferred bulking agent for the lyophilization of protein formulations.

In the case of the spray-dried binary mixtures studied here, the propensity of mannitol to crystallize is modulated by the amount of sCT in the formulation. Mannitol remains amorphous at concentrations up to 50% w/w and a significant amount of crystalline mannitol is observed in the formulations containing 70 and 90% mannitol, as shown in Table 1. The propensity of mannitol to crystallize in the binary mixtures is driven by:

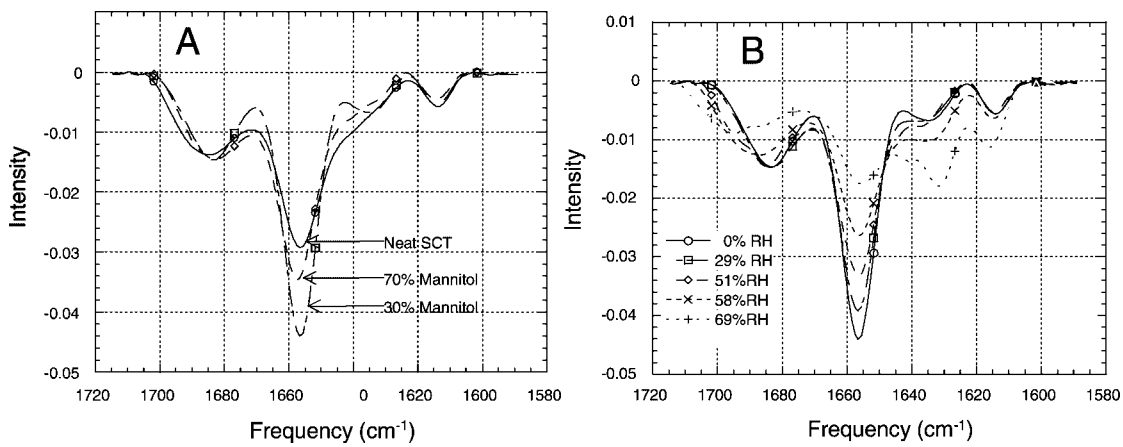

Figure 5. (A) FTIR spectra of neat sCT and formulations containing 30%, and 70% mannitol stored at room temperature in a dry box at RH <10%. (B) FTIR spectra of sCT formulations containing 30% mannitol after room temperature storage at the indicated relative humidity conditions. The spectrum of native sCT is similar to that of the neat spray dried sCT and is not included for simplicity.

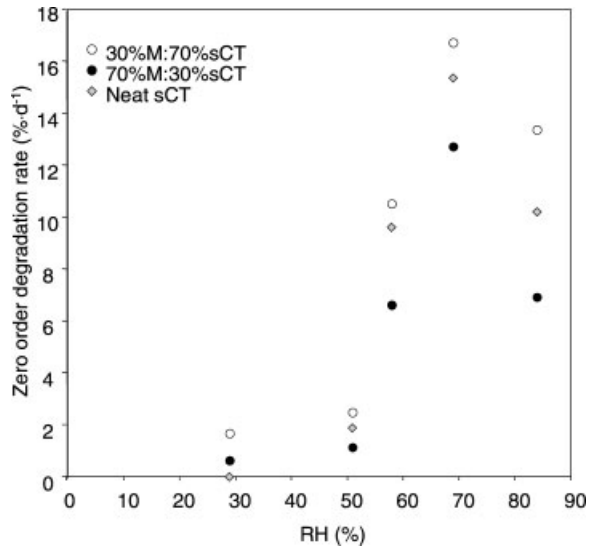

Figure 6. Zero-order rate of sCT aggregation during 25°C storage at various relative humidities.

(1) initial concentration of the mannitol, (2) extent of mixing with sCT, and (3) processing conditions. Spray drying is a rapid process with characteristic droplet lifetimes on the order of milliseconds. Therefore, nucleation and crystal growth kinetics need to be considered when predicting crystallinity in spray dried powders. While critical supersaturation—the concentration at which crystal nuclei start to grow—will always be reached at some point in the evaporation process, it will be reached late in the process for initially dilute solutions. Consequently, the crystal growth kinetics may be slow relative to the time it takes for the remaining moisture to evaporate. Conditions for crystallization are more favorable at higher initial concentrations where critical supersaturation is reached early in the evaporation process, when there is still sufficient time to grow crystals. Thus, it can be expected that at a fixed total solids content in the feed solution, the propensity of mannitol to crystallize will increase with the amount of mannitol present in the binary mixture.

FTIR analysis and moisture sorption measurements suggest that there is an interaction between the peptide and mannitol in the solid state. This is discussed in greater detail in the following section. However, analysis of the ratio of amorphous mannitol to sCT (Table 3) shows no trends to explain the observed levels of mannitol crystallinity.

Interactions between sCT and Mannitol in Spray-Dried Powders

To investigate possible interactions between amorphous mannitol and sCT, FTIR spectroscopic analysis was employed to determine the secondary structure of sCT at various storage relative humidities. The secondary structure of sCT is estimated by analyzing IR bands in the conformationally sensitive amide I region (1620–1700 cm^{-1}), which is primarily due to C=O stretching vibrations. C–N stretching, and C–C–N bending vibrations also contribute this region. The frequency of the amide I vibration is determined by the geometry of the peptide backbone and by hydrogen bonding, and is therefore sensitive to changes in secondary structure (α-helices, β-sheets, turns, and disordered structures). Second derivative spectral band

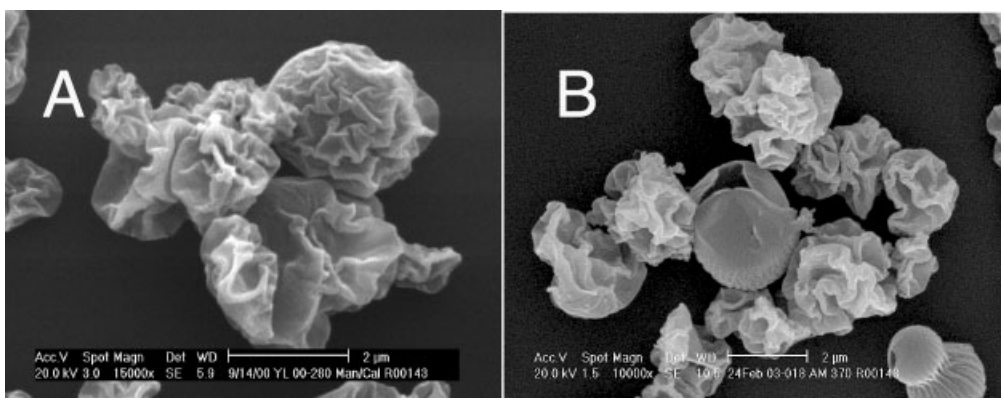

Figure 7. SEM of the 30% mannitol/70% sCT formulation. (A) freshly made and (B) after 2 years of room temperature storage in a dry box kept at RH <10%. Morphology variations have not occurred during storage, they are observed in both freshly made and stored sample. These particular SEM's were selected because they both had the same magnification.

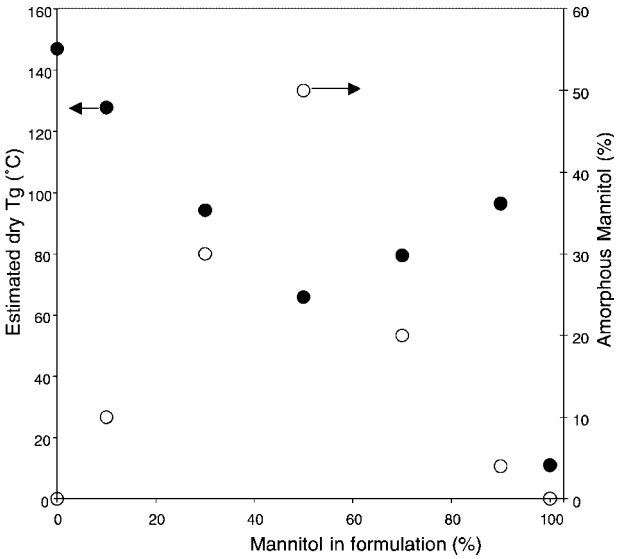

Figure 8. Estimated T_g and amorphous mannitol as a function of amorphous mannitol in the spray-dried powder as measured by Raman spectroscopy.

assignments for different secondary structures have been assigned: α-helix = 1656 cm^{-1}, β-strand/sheet = 1620–1642 cm^{-1}, 1690–1698 cm^{-1}; disordered = 1648 cm^{-1}; turn = 1670–80 cm^{-1}, other helices = 1663 cm^{-1}.[22,34] FTIR observation of the α-helical content indicates that the 30% mannitol sample experiences a greater degree of hydrogen bonding compared to the 70% mannitol sample. Because only mannitol in its amorphous state can hydrogen bond to the sCT, we speculate that the increased hydrogen bonding is due to the increased interaction between the sCT and mannitol. However, controversy exists in the literature[35,36] on whether hydrogen bonding between protein and excipient can be distinguished from hydrogen bonding due to water present at the protein surface.

Additional indirect evidence that supports the existence of a non-specific interaction between amorphous mannitol and sCT is given by the analysis of the water sorption isotherms.[37] At any given RH, the water content (g H_2O/100 g dry solids) of a physical mixture of sCT, amorphous mannitol and crystalline mannitol can be expressed by:

$$M_{\text{total}} = w_{\text{sCal}} M_{\text{sCal}} + w_{\text{mannitol, amorphous}} M_{\text{mannitol, amorphous}} + w_{\text{mannitol, crystalline}} M_{\text{mannitol, crystalline}}$$

where w_i and M_i are the mass fraction and the water content of the component in g H_2O/100 g dry solid, respectively. To make such a calculation, the isotherms of pure sCT, 100% crystalline mannitol, and 100% amorphous mannitol must be known. Because it is difficult to produce 100% amorphous mannitol, the isotherm of 100% amorphous mannitol was estimated by a rearrangement of the above equation for the sCT formulation containing 10% mannitol:

$$M_{\text{mannitol, amorphous}} = (M_{\text{Totals}} - 0.9 M_{\text{sCal}})/0.10$$

This is a reasonable assumption, because Raman data show that this formulation is completely amorphous (within resolution of the technique). Raman data are also needed to estimate the % crystallinity of mannitol in each of the formulations. Then, from the measured and calculated isotherms of the three pure species and the crystallinity data, all other isotherms can be estimated. The deviation between the measured and predicted isotherm can be used as a measure of molecular interactions in the solid state. Presumably, molecular interactions would satisfy some of the hydrogen bonding of the interacting species, and would result in nonadditive water absorption. The deviation of each isotherm is shown in Figure 9. Because the sCT formulation containing 10% mannitol was used to calculate the isotherm of amorphous mannitol, the deviation of the isotherm of this formulation is forced to be zero. The deviations are always negative, which means that the measured isotherm is less than that which would be predicted from linear superposition. This

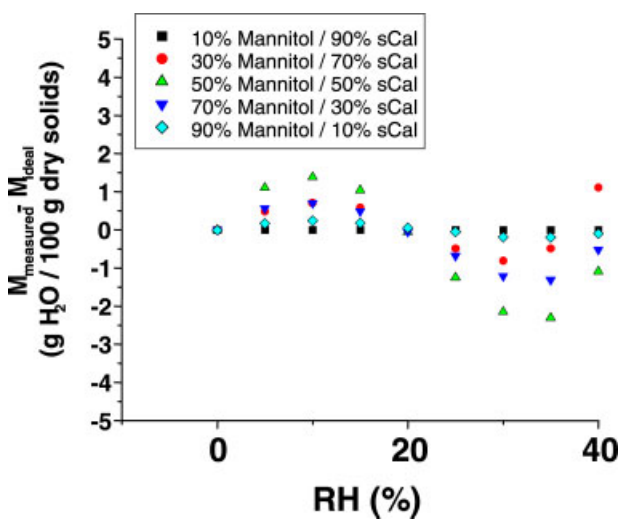

Figure 9. Deviation of each moisture sorption isotherm (25°C) from ideal isotherm given by simple superposition model.

suggests that less water is absorbed into the binary, sCT:mannitol, amorphous phase because some of the hydrogen bonding is satisfied by interactions between the components. The same conclusion is obtained when the data are treated according to a model developed by Chan et al.[38]

Physical Stability of sCT Spray-dried Powders

The physical stability of sCT spray-dried powders for inhalation is critical for maintaining good aerosol properties.[6,7,39–43] Physical stability of these powders is dependent on the presence of amorphous mannitol because crystallization of amorphous mannitol will cause changes in the particle morphology, which is likely to adversely affect aerosol performance.[39]

Two situations need to be considered regarding the impact of amorphous mannitol crystallization: (1) long-term stability to ensure shelf life requirements, and (2) stability during patient use in humid environments.[44] Mannitol crytallization during storage causes the formation of crystalline bridges between particles, increasing their aerodynamic and geometric diameters and decreasing powder dispersibility. To ensure long-term stability, storage conditions can be predicted using MITAT.[24,45] The validity of the prediction is supported by these data because powders stored below the critical RH for over 2 years look the same as those that are freshly prepared (see Fig. 7).

Short-term stability will have an impact on the patient-use scenario, which can be evaluated by kinetic analysis of the time course of moisture uptake[46] (see Fig. 3) or the MITAT[24] (Fig. 4), which can determine the RH required to induce spontaneous crystallization. These data suggest that the 70% mannitol formulation is less susceptible to environmental effects since it contains a lower fraction of amorphous mannitol.

Powder Crystallinity and sCT Aggregation Stability

Although formulations, that are primarily crystalline (e.g., 70% mannitol), have been shown to be physically stable, sCT may not have acceptable aggregation stability. This is a concern because amorphous mannitol likely contributes to the protection of this peptide.

The zero-order reaction rate of aggregate formation, under storage at 29% RH, measured for the 30% mannitol formulation is higher than the one obtained for the 70% mannitol formulation (Fig. 6). This trend is independent of the storage RH. Water absorption in turn promotes changes in the crystallinity through moisture-induced crystallization. The observation is therefore that the rate of aggregate formation in the originally largely crystalline system (70% mannitol) is *lower* than that observed in the completely amorphous system (30% mannitol).

At first glance, the degradation rate of sCT would appear to be unrelated to the crystallinity of the formulation because, in such a case, mannitol would be totally phase separated from sCT. However, the water content normalized to the amorphous content must be considered.[38,47] and the concentration of the protein in the amorphous phase (which depend on crystallinity changes). This is necessary because, as discussed above, most of the water is absorbed by the amorphous phase and protein will only be mixed on a molecular scale with amorphous mannitol.

Once these factors are considered (see Table 2), we find that water content of the amorphous phase is higher in the 30% mannitol formulation (where RH $>51\%$) than in 70% mannitol formulation. This is consistent with the data in Figure 6.

CONCLUSIONS

Spray-dried neat sCT is amorphous and its physical stability was unaffected by spray drying. All powders remained stable during the course of the study when stored at RH $<29\%$. Moisture-induced crystallization of mannitol was observed in all powders when stored at RH $>50\%$. The presence of mannitol forming partially crystalline powders does not affect sCT physical stability. Physical stability of particles is demonstrated for over 2 years if powders are kept below a critical RH determined by the MITAT. The amount of intermolecular β-sheet formed during storage, determined by FTIR, correlated to the observed aggregation rate. However, the observed spectral differences in the peptide-mannitol interactions do not correlate with the aggregation rate. The sCT aggregation rate in powders containing 70% mannitol was significantly lower than that in powders containing 30% mannitol at all RH tested. The physical stability was most affected by moisture content in the amorphous phase. The aggregation stability of sCT and the physical stability of the dry powders for inhalation are optimal if stored in a low humidity environment.

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