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Research Paper

Sorbitol Crystallization Can Lead to Protein Aggregation in Frozen Protein Formulations

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Purpose. This work examines the cause of aggregation of an Fc-fusion protein formulated in sorbitol upon frozen storage for extended periods of time at −30°C.
Materials and Methods. We designed sub-ambient differential scanning calorimetry (DSC) experiments to capture the effects of long-term frozen storage. The physical stability of formulation samples was monitored by size exclusion high performance liquid chromatography (SE-HPLC).
Results. DSC analysis of non-frozen samples shows the expected glass transitions (T_g') at −45°C for samples in sorbitol and at −32°C in sucrose. In time course studies where sorbitol formulations were stored at −30°C and analyzed by DSC without thawing, two endothermic transitions were observed: a melting endotherm at −20°C dissipated over time, and a second endotherm at −8°C was seen after approximately 2 weeks and persisted in all later time points. Protein aggregation was only seen in the samples formulated in sorbitol and stored at −30°C, correlating aggregation with the aforementioned melts.
Conclusions. The observed melts are characteristic of crystalline substances and suggest that the sorbitol crystallizes over time. During freezing, the excipient must remain in the same phase as the protein to ensure protein stability. By crystallizing, the sorbitol is phase-separated from the protein, which leads to protein aggregation.

KEY WORDS: crystallization; formulation; frozen state; protein aggregation; sorbitol.

INTRODUCTION

The process of freezing can subject a protein to several potentially destabilizing stresses: adsorption of the protein to the ice surface, high concentrations of solutes in the freeze-concentrate, large changes in pH of the freeze-concentrate due to the crystallization of buffer salts, cold denaturation, and phase separation of the protein and its cryoprotectant (1–3). These varied freezing stresses can cause protein denaturation, which typically manifests as aggregation (4). Excipients are often used to mitigate the destabilizing effects of freezing on proteins (5).

When assessing the suitability of an excipient to improve the frozen stability of a protein, changes to the physical state of the excipient that can occur during freezing should be considered. As the temperature of a solution is lowered, the crystallization of ice results in the concentration of the protein and excipient (termed freeze-concentrate), which are confined to the space between the ice crystals. As the freeze-concentrate becomes supersaturated, the amorphous non-crystallizing solutes undergo a viscoelastic transition (from a rubbery to a highly viscous glassy state) characterized by the glass transition temperature or T_g' (5–8). The nature and concentration of solutes left in the freeze-concentrate determines its physical characteristics. For instance, non-crystallizing excipients such as sucrose remain in the amorphous phase during freezing, whereas excipients such as mannitol and glycine crystallize readily and therefore phase separate. The "glass dynamics hypothesis" proposes that the mechanism of protein stabilization is due primarily to the decreased molecular mobility of the protein in the glass matrix (7,9). Moreover, specific protein-excipient interactions that favor the native state of the protein (7,10) necessitate that the excipient must be in the same phase as the protein. Conversely, the phase separation of a stabilizing excipient from the protein should result in the destabilization of the protein (11,12).

Phase separation of other formulation components can also subject the protein to potentially destabilizing concentrations of solutes. For example, the concentration of an isotonic solution of sodium chloride was reported to increase more than 20-fold when the temperature was decreased to −21°C (1,13). This extreme concentration of protein and

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ABBREVIATIONS: 10 mM sodium acetate, pH 4.0 with 300 mM sorbitol, (A4S); 10 mM sodium acetate, pH 4.0 with 320 mM sucrose, (A4Su); 10 mM sodium acetate, pH 5.0 with 300 mM sorbitol, (A5S); differential scanning calorimetry, (DSC); fragment crystallizes easily, (Fc); glass transition temperature, (T_g'); size exclusion high performance liquid chromatography, (SE-HPLC).

solute that occurs in the non-ice phase can increase the concentration of solutes beyond their solubility. It is well known that sodium phosphate buffers undergo a large change in pH upon freezing when the dibasic salt reaches its eutectic temperature and crystallizes out of solution, which hampers the buffering capacity. This can subject the protein to a drastic, destabilizing change in pH (14).

A protein may also encounter freezing-induced stresses due to ice. Upon freezing, ice formation can provide a surface onto which the protein adsorbs. Strambini and Gabellieri (15,16) demonstrated protein destabilization due to adsorption at the ice surface. Such interfacial phenomenon can be explored by using different freezing rates to control the size of ice crystals, thereby altering the surface area of the ice.

Excipients are used to counteract the destabilizing stresses caused by freezing. Surfactants (such as polysorbate 20) offer protection against freezing-induced protein denaturation, presumably by protecting the protein at the ice interface (17,18). Carpenter and Crowe (5) reported that diverse excipients (such as sugars, polyols, amino acids, methylamines, and inorganic salts) stabilize proteins against freezing-induced stresses through the same preferential exclusion mechanism Lee and Timasheff (19) proposed for aqueous solutions.

Sorbitol (D-glucitol) is a polyol commonly used as an excipient in liquid parenteral biologic formulations, as a tablet or capsule diluent in small molecule formulations, and even as a food sweetening agent (20). Sorbitol provides effective protein stabilization in the liquid state, and several marketed biologics are formulated in sorbitol, including Neulasta® (pegfilgrastim) and Neupogen® (filgrastim). In the frozen state, sorbitol is traditionally known to be an amorphous, non-crystallizing solute, making it amenable to lyophilization, but its low T_g' (reported as −44°C) and low collapse temperature make it non-ideal as a lyophilization excipient (21,22). Most of the relevant literature on the stability of formulated proteins in the frozen state is written in the context of lyophilization development, and the unsuitability of sorbitol as a lyophilization excipient means that scant literature exists regarding the frozen behavior of sorbitol-containing formulations (7,23).

During formulation development, we identified optimal stability for a 60,116 Da N-terminal Fc-fusion protein (2 mg/ml) in the liquid state in 10 mM sodium acetate at pH 5.0 with sorbitol (274 mM or 5% w/v). The fusion protein is composed of a 35-residue peptide attached through a glycine linker to each of the IgG1 Fc heavy chain N-terminal residues. The stability of the Fc-fusion protein in the frozen state was monitored to assess degradation in case of accidental freezing upon transport as well as to address the potential for frozen storage of the bulk drug substance. It is preferable to store frozen formulations in the glassy state (for sorbitol, below its literature T_g' of −44°C), but practical considerations dictated that warmer temperatures be evaluated for storage of process intermediates (e.g., −20 and −30°C). Upon monitoring the stability of the protein, we noticed an increase in aggregation by size exclusion high performance liquid chromatography (SE-HPLC) during long-term storage of the protein at −30°C, but not at −20°C or −70°C. The aggregation in the frozen state at −30°C was also pH dependent and was more pronounced when the protein was formulated at pH 4.0 (in 10 mM sodium acetate with 300 mM sorbitol) than when formulated in the same buffer at pH 5.0 (data not shown).

The goal of this study was to understand the factors governing the aggregation of the Fc-fusion protein formulated in sorbitol and sodium acetate buffer. We chose to use the pH 4.0 formulation as a model because the instability was more apparent (meaning easier to monitor by SE-HPLC) in this formulation condition. We monitored the aggregation as a function of several variables including freezing rate (to study the effect of ice surface area) and increasing excipient concentrations in an attempt to correlate the noted instability to changes in the physical state of one of the excipients over time. SE-HPLC and sub-ambient differential scanning calorimetry (DSC) techniques were used to monitor protein aggregation and the physical state of the excipients, respectively. A novel DSC method was developed to capture the effects of long-term frozen storage. Here we present evidence for the first report of sorbitol crystallization in the frozen state.

MATERIALS AND METHODS

Sample Preparation and Storage

An N-terminal Fc-fusion protein with a molecular weight of 60,116 Da was provided by Amgen Inc. (Thousand Oaks, CA). The molecule was expressed in *Escherichia coli* and is therefore not glycosylated. The protein was dialyzed against 10 mM sodium acetate buffer (J.T. Baker, Phillipsburg, NJ) at pH 4.0 with isotonic concentrations of the excipients sorbitol (Roquette, Keokuk, IA) or sucrose (Pfanstiehl, Cleveland, OH). The formulated protein (1 ml at 2 mg/ml) was stored in 3 cc glass vials for analysis by SE-HPLC and 40 μl aliquots were stored in 50 μl aluminum DSC pans (Perkin-Elmer, Shelton, CT) for the thermal analyses.

Size Exclusion High Performance Liquid Chromatography (SE-HPLC)

Physical degradation of the protein such as the presence of soluble aggregates or clipped species was determined by SE-HPLC on an Agilent 1100 liquid chromatography system. SE-HPLC separations were performed at 25°C on a 30 cm Shodex KW-803 column with a 5 μm particle size and 8 mm inner diameter. The mobile phase consisted of 20 mM sodium phosphate, 305 mM sodium perchlorate with 3.5% ethanol and 0.3% isopropanol at pH 7.0. A 16 μg protein load was determined to provide optimal resolution. A flow rate of 0.5 ml/minute was used. The protein was monitored using UV detection at 215 and 280 nm for a total run time of 30 min. Three peaks were separated in the chromatograms. The peaks at 17.0 and 18.8 min were identified by light-scattering experiments to be the dimeric and monomeric species of the protein, respectively (data not shown). The peak at 16.0 min could not be identified due to its low intensity but is presumed to represent species that are larger than the dimer. Data was analyzed with ChemStation software, and the 215 nm signal was integrated to determine the area percent of monomer, dimer, and aggregate. Error bars (+2σ) for the main peak are calculated from several injections of the standard (Fc-fusion protein at 2 mg/ml formulated in 10 mM sodium acetate, pH

5.0 with 300 mM sorbitol and stored at −80°C). The standard is injected with every time point and the standard from each successive time point is added to the calculation of the standard error used for the error bars. Thus, the error bars reflect the variability of the chromatography of the standard over several different SE-HPLC runs.

Differential Scanning Calorimetry (DSC)

Sub-ambient thermal analysis was carried out on a Pyris 1 DSC (Perkin-Elmer Corp., Norwalk, CT) equipped with liquid nitrogen to cool to −100°C. Temperature calibration was performed (at a heating rate of 5°C/min) using the melting points of hexane (−95°C) and cyclohexane (6.5°C) standards. Protein and buffer samples (40 μl) were analyzed in 50 μl semi-hermetically sealed DSC pans. An empty sealed DSC pan was used as a reference. Two methods were used for analysis. The first method is a standard procedure; liquid samples were loaded into the DSC pans at room temperature and transferred to the DSC cell, also at room temperature. These samples were cooled to −70°C at a rate of 60°C/min, then held at −70°C for one minute before heating to +15°C at a rate of 5°C/min. The second method was designed to characterize the physical state of the excipient-protein mixture after long-term frozen storage. The samples were stored directly in the DSC pans in either −20°C or −30°C freezers for extended periods of time. To preserve the state of the samples for DSC analysis, the DSC pans were transferred on dry ice from the freezer to a DSC cell pre-cooled to −70°C. This procedure prevents the samples from being thawed and maintains the effect of long-term storage. Once loaded, the samples were held at −70°C for 5 min, then heated at 5°C/min from −70°C to +15°C. The baseline slopes were optimized and the glass transition temperatures were determined using the Pyris thermal analysis software for Windows version 3.81. To overlay thermograms of multiple samples, their profiles were manually offset on the y–axis for viewing clarity. Figures show the heating cycle profiles only.

Stability Studies

Excipient concentration studies

Protein formulations were prepared at 2 mg/ml either without excipient or containing 100 mM (1.8% w/v), 300 mM (5.5% w/v), 600 mM (11% w/v) or 1,200 mM (22% w/v) mM sorbitol or 100 mM (3.4% w/v), 320 mM (11% w/v) or 640 mM (22% w/v) sucrose. SE-HPLC assays were run immediately after dialysis into the selected formulation buffer to obtain a profile prior to freezing. Samples (1 ml) were transferred to 3 cc glass vials and stored at 4°C, −20°C, −30°C or −70°C for a period of 2, 4, 16, 32, 68, and 104 weeks. Samples were thawed at room temperature prior to analysis by SE-HPLC.

Selected protein formulations (300, 600, and 1,200 mM sorbitol and 320 mM sucrose) were transferred to DSC pans, sealed and stored at −20°C, −30°C and −70°C. The 300, 600 and 1,200 mM sorbitol buffers without protein were also stored in this manner for comparison. After different intervals of time, frozen samples were analyzed by the second DSC method. This allowed characterization of the samples as a function of frozen storage time, without thawing.

Rapid freezing

Samples (1 ml) were frozen rapidly by submerging the 3 cc glass vials in liquid nitrogen until the samples appeared frozen. The samples were then placed at the appropriate storage temperature (e.g., −30°C) for various periods of time. Samples were thawed, analyzed by SE-HPLC and compared to samples that were allowed to slowly equilibrate to the freezer temperature.

Freeze–thaw study

Five cycles of freeze–thaw were performed on 1 ml protein formulations (2 mg/ml in 10 mM sodium acetate, pH 4.0) containing 300 mM sorbitol. At each cycle, the samples were removed from the initial storage temperature (−20°C, −30°C, or −70°C) and allowed to thaw unperturbed at room temperature. The samples were mixed to insure homogeneity and returned to the respective storage temperature, where the samples were frozen for 24 h before the next thaw cycle. SE-HPLC was conducted after cycles 1, 3 and 5.

RESULTS

The stability of an Fc-fusion protein in the frozen state was monitored in formulations containing 300 mM sorbitol or 320 mM sucrose excipients after storage at −20°C, −30°C and −70°C. Figure 1A compares the SE-HPLC overlays of a 2 mg/ml protein formulation containing 300 mM sorbitol and 10 mM sodium acetate buffer at pH 4.0, which was stored in glass vials at −20°C, −30°C and −70°C for 68 weeks. Increased dimer and aggregate formation (2.4%) is observed in the sample held at −30°C (Fig. 1A, dotted line), whereas the samples held at −70°C or −20°C show no increase in either species. The stability at −70°C (well below the T_g' of sorbitol) can be explained by the "glass dynamics hypothesis" (7,9), but the stability at −20°C (above the T_g' of sorbitol) is intriguing, and suggests a complex temperature dependence. Interestingly, when the excipient is changed from sorbitol to 320 mM sucrose and stored at −30°C for the same 68-week duration, the instability is not seen as illustrated in Fig. 1B. These data suggest that the aggregation is specific to sorbitol-containing formulations stored at −30°C.

The effect of excipient concentration on the stability of the 2 mg/ml protein in 10 mM sodium acetate at pH 4.0 was also investigated. Different excipient concentrations were studied: 100, 300 or 600 mM sorbitol, and 100, 320, or 640 mM sucrose as well as an excipient-free formulation. Figure 2 shows the trend in percent main peak area by SE-HPLC for all formulations stored at −20°C (top panel) and −30°C (bottom panel) over a total of 104 weeks. When stored at −20°C, only the excipient-free formulation shows a 2% decrease in main peak after 104 weeks; all other formulations are stable, regardless of the excipient concentration. At −30°C, the excipient-free formulation and all sorbitol formulations show decreased main peak areas, while the sucrose formulations remain stable. The decrease in main peak area is due to an increase in the dimer and aggregate species. The instability seen in the sorbitol formulations at −30°C appears to be independent of the

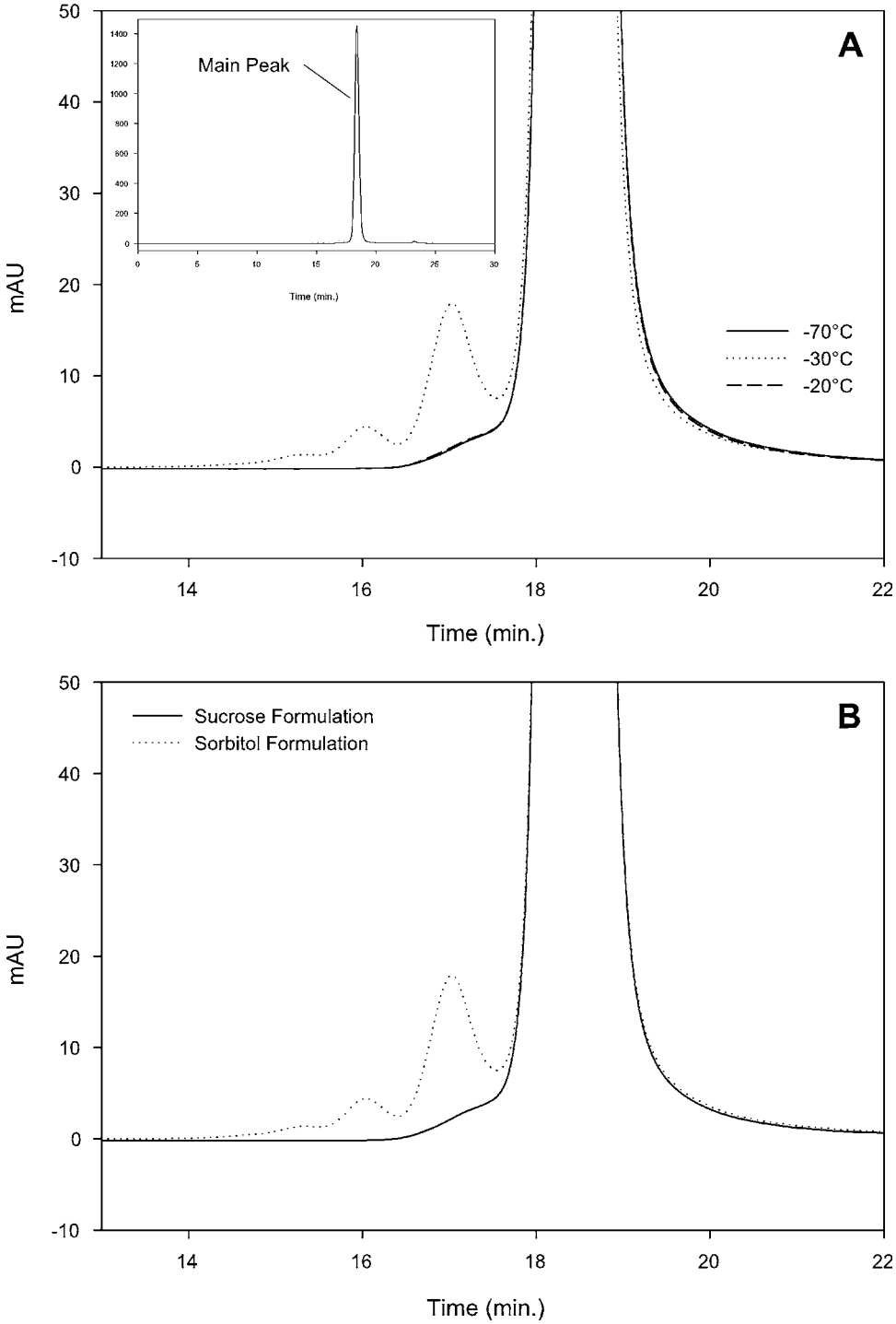

Fig. 1. (A) SE-HPLC overlays obtained from 2 mg/ml Fc-fusion protein samples (formulated in 300 mM sorbitol, 10 mM sodium acetate buffer at pH 4.0) and stored at −70°C, −30°C or −20°C for 68 weeks. The sorbitol formulation stored at −30°C is shown as a *dotted line*. The 215 nm trace is shown from 13–22 min, and the *inset* shows the overlaid chromatograms for the entire 30 min run duration, and highlights the main peak. (B) SE-HPLC overlays obtained from 2 mg/ml Fc-fusion protein samples stored at −30°C for 68 weeks formulated in 10 mM sodium acetate buffer at pH 4.0 and either 300 mM sorbitol (*dotted line*) or 320 mM sucrose (*solid line*).

sorbitol concentration and roughly comparable to the formulation containing no excipient. None of the formulations stored at −70°C show a decrease in the main peak area (data not shown).

DSC thermograms showing the low temperature thermal events of 300 mM sorbitol and 320 mM sucrose formulations containing 10 mM sodium acetate buffer, pH 4.0 and 2 mg/ml protein are shown in Fig. 3. Both DSC measurements were performed at 'time zero' before the samples were stored frozen, meaning liquid samples were loaded onto the DSC cell. A T_g' is observed at a temperature of ca. −45°C for the sorbitol sample and at ca −32°C for the sucrose sample, consistent with published T_g' values of −44°C for sorbitol (22) and −33°C for sucrose (24).

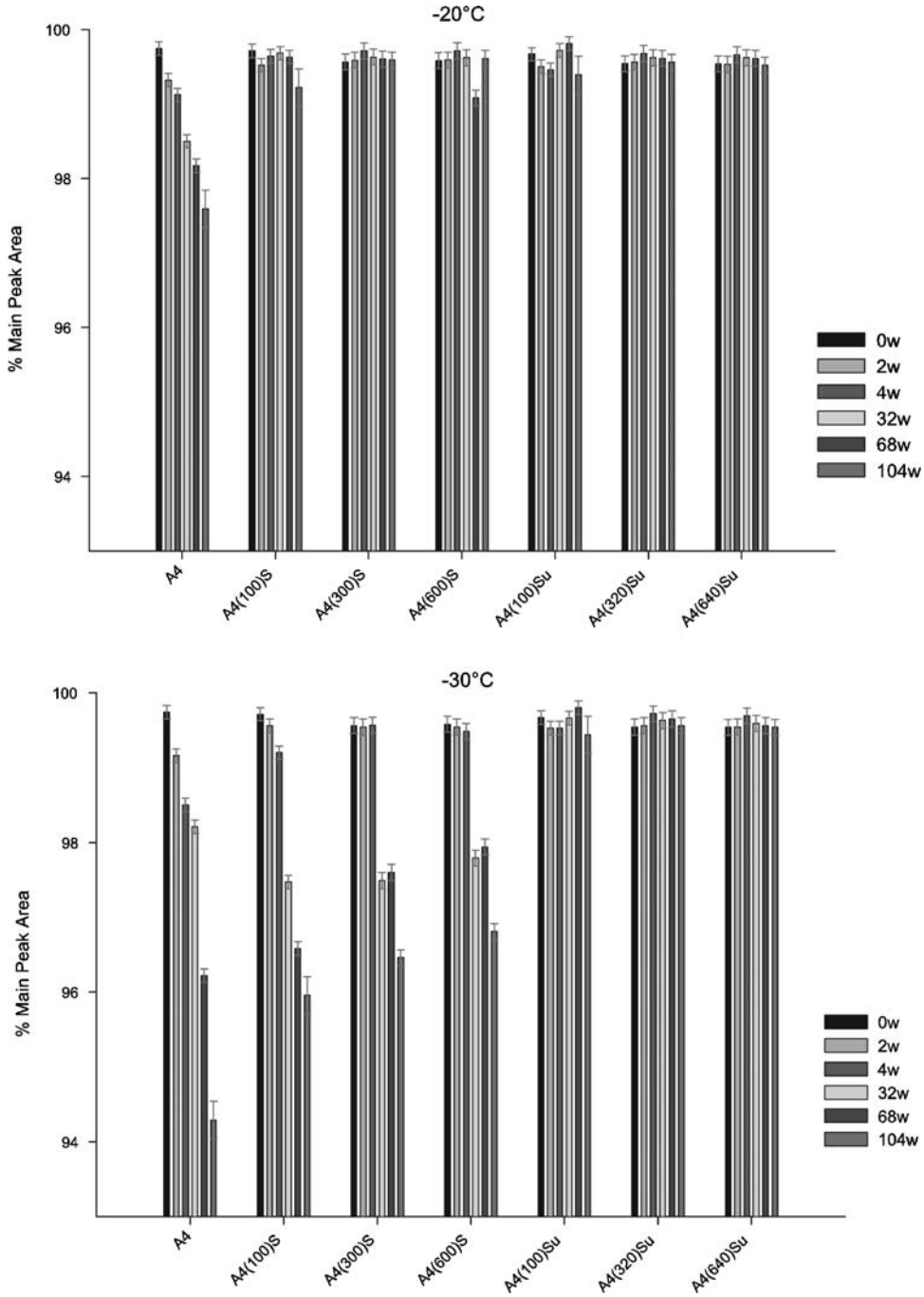

Fig. 2. Percent main peak area (by SE-HPLC) is shown for 2 mg/ml Fc-fusion protein samples stored in glass vials at −20°C (*top panel*) and −30°C (*bottom panel*) in 10 mM sodium acetate at pH 4.0 for 0, 2, 4, 32, 68, and 104 weeks in increasing sorbitol (0, 100, 300, or 600 mM S) or sucrose (100, 320, or 640 mM Su) concentrations. The excipient concentration is indicated in parentheses [e.g., A4(100)S denotes 10 mM sodium acetate, pH 4.0 with 100 mM sorbitol]. The stability of the Fc-fusion protein in 10 mM sodium acetate, pH 4.0 in the absence of excipient (e.g., A4) was also monitored, and is the first formulation shown in each graph.

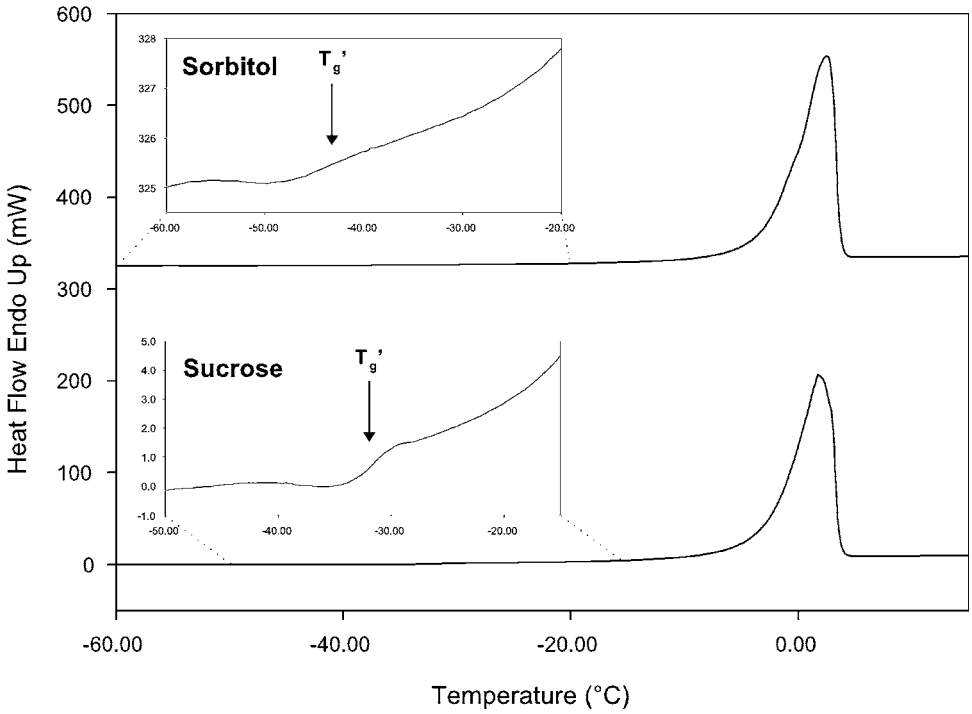

Fig. 3. DSC thermogram obtained from a 300 mM sorbitol formulation containing 10 mM sodium acetate buffer, pH 4.0 and 2 mg/ml Fc-fusion protein. The T_g' of sorbitol at ca −45°C is indicated in the *inset* (*top*). DSC thermogram obtained from a 320 mM sucrose formulation containing 10 mM sodium acetate buffer, pH 4.0 and 2 mg/ml Fc-fusion protein. The T_g' of sucrose at ca −32°C is indicated in the *inset (bottom)*. These samples were not frozen prior to DSC analysis, but were loaded into the DSC pans at room temperature and transferred to the DSC cell also at room temperature.

Samples stored frozen in DSC pans at −30°C were analyzed by sub-ambient DSC after 1 day, 5 days and 2, 3, 4, 6, and 20 weeks. Thermograms obtained by heating the samples at 5°C/min are shown in Fig. 4A. After one day, the sample exhibits a profile similar to that obtained before freezing. However, after 5 days, the sorbitol-containing samples show a melting endotherm with an onset temperature of −20°C in addition to the ice melt. This endotherm is seen for all samples that have been stored at −30°C in 300 mM sorbitol for up to 4 weeks. However, the endotherm is no longer detectable in samples stored longer than 4 weeks. A second melting event occurs at an onset temperature of −8°C and is evident after 2 weeks. The area under this peak increases until it reaches a maximum at 6 weeks and persists until the last measured time point at 20 weeks. Samples stored at −20°C do not show either of these endothermic transitions, as seen in Fig. 4B.

A similar time-course experiment was performed in 10 mM sodium acetate buffer at pH 4.0 with 300 mM sorbitol in the absence of protein, and the thermograms obtained are shown in Fig. 5. Qualitatively, the results are identical to those obtained with 2 mg/ml protein; i.e., an endotherm with an onset temperature of −20°C is observed after 5 days, while a second endotherm that increases in intensity at −8°C is seen in the later time points. In the absence of protein, the −20°C endotherm is no longer evident after 4 weeks of storage at −30°C (Fig. 5), whereas in the presence of protein, this endothermic peak persists out to 4 weeks (Fig. 4A). These experiments were repeated several times, and in all experiments we saw endothermic peaks at ca −20°C and ca −8°C. It is important to note that the T_g' for sorbitol [−44°C, (22)] is only detected in the thermograms obtained from samples prior to freezing (meaning buffer was loaded into the DSC cell) and after one day of frozen storage at −30°C. A T_g' is not evident in any sorbitol samples stored longer than two days at −30°C.

The physical stability of the protein after frozen storage in the DSC pans was also assessed. Following the thermal analysis, samples were recovered from the pans and analyzed by SE-HPLC. Once again, after 16 weeks of storage at −30°C, increased dimer and aggregate formation was seen only in the sorbitol formulation, but not in the sucrose formulation, as shown in Fig. 6. None of the sorbitol formulations stored at −20 or −70°C showed increased dimer or aggregate (by SE-HPLC) over the time course studied (not shown).

Experiments using increasing concentrations of sorbitol were designed to investigate the effect of higher sorbitol to protein ratios on aggregation. Figure 7 shows thermograms of formulations with increasing sorbitol concentrations (300, 600, and 1,200 mM) after 16 weeks of storage at −30°C. Melting endotherms with an onset temperature of −8°C are seen in all the samples. Thermograms obtained from samples stored at −70 or −20°C for the same period of time do not have these melting endotherms but instead show a T_g'. The sub-ambient DSC profiles (not shown) are similar to those seen in Fig. 3. We see a difference in the area of the endothermic melts in Fig. 7 that corresponds with the increase in sorbitol concentration. It was also observed that the ice melting endotherms were shifted to lower temper-

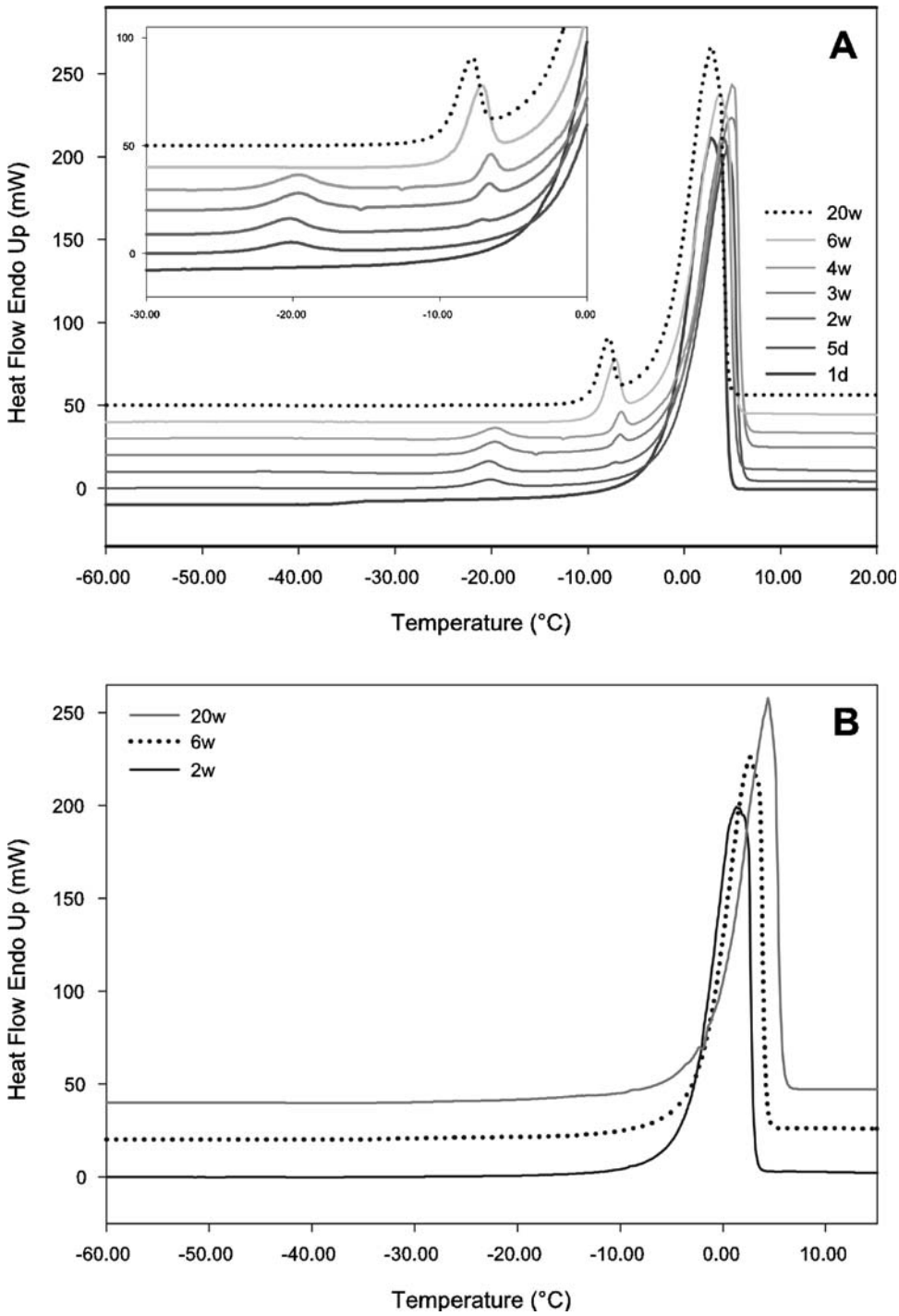

Fig. 4. (A) DSC thermograms obtained from 300 mM sorbitol formulations containing 10 mM sodium acetate buffer, pH 4.0 and 2 mg/ml Fc-fusion protein stored in sealed DSC pans for 1 day, 5 days, 2, 3, 4, 6, and 20 weeks at −30°C. The *inset* shows a more detailed view of the two melting endotherms at ca −20°C and ca −8°C. (B) DSC thermograms of 300 mM sorbitol formulations containing 10 mM sodium acetate buffer, pH 4.0 and 2 mg/ml Fc-fusion protein obtained after storage at −20°C in sealed DSC pans for 2, 6 and 20 weeks.

atures as the sorbitol concentration was increased, likely due to freezing point depression. The 600 mM sorbitol sample has an area (1,257 mJ) nearly twice that of the 300 mM sorbitol endotherm (654 mJ), and the area of the endotherm from the 1,200 mM sorbitol sample (2,617 mJ) is four times greater that of the 300 mM sorbitol sample. We did not see a significant increase in protein aggregation of the Fc-fusion protein in the presence of increasing concentrations of sorbitol (Fig. 2).

Experiments were conducted to study the effect of freezing rate on the observed aggregation. Presumably, rapid freezing can create a larger ice surface area than slow

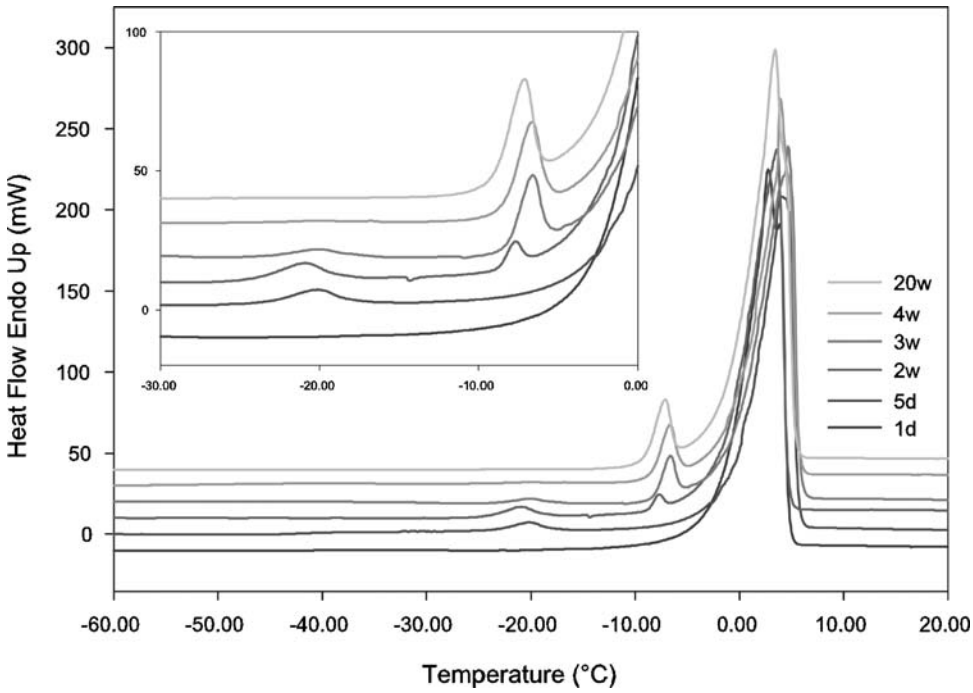

Fig. 5. Sub-ambient DSC thermograms obtained from formulations without protein, containing 300 mM sorbitol in 10 mM acetate buffer, pH 4.0 and stored in aluminum pans for 1 day, 5 days, 2, 3, 4, and 20 weeks at −30°C. The samples were transported frozen and loaded onto the DSC cell that was pre-cooled to −70°C. The inset shows a more detailed view of the two melting endotherms at −20°C and ca −8°C.

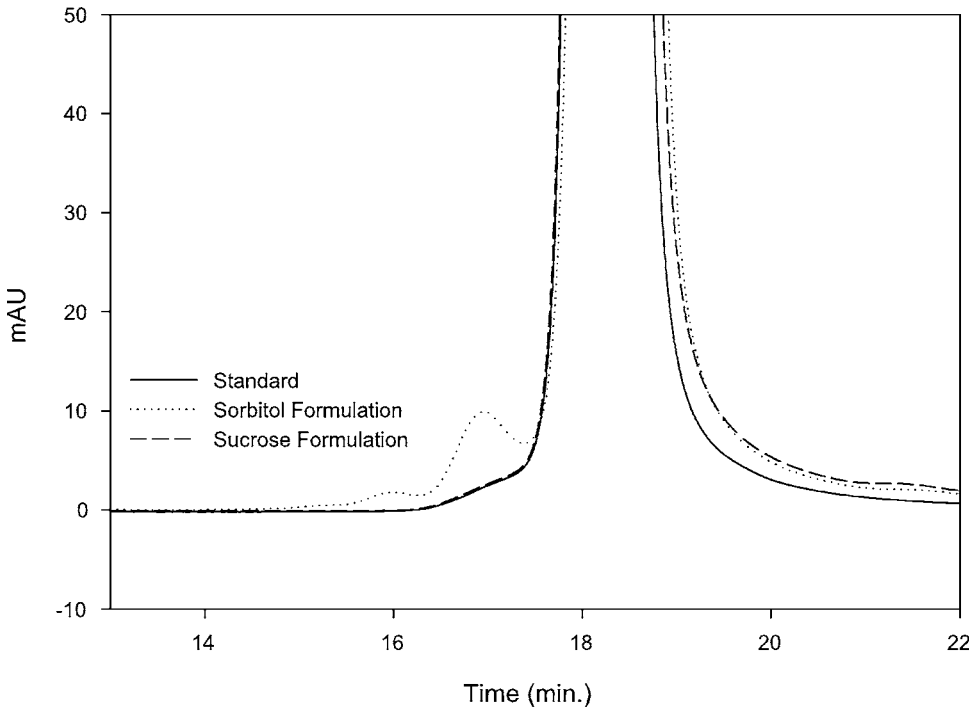

Fig. 6. SE-HPLC overlays obtained from the sorbitol and sucrose formulations containing 10 mM sodium acetate buffer at pH 4.0 and 2 mg/ml Fc-fusion protein stored in sealed DSC pans for 16 weeks at −30°C are shown. The percent main peak for both the standard and the sucrose formulation was 99.8%, compared to 98.8% main peak for the sorbitol formulation (shown as a *dotted line*). The standard was formulated in A5S (10 mM sodium acetate, pH 5.0 with 300 mM sorbitol) and stored at −80°C.

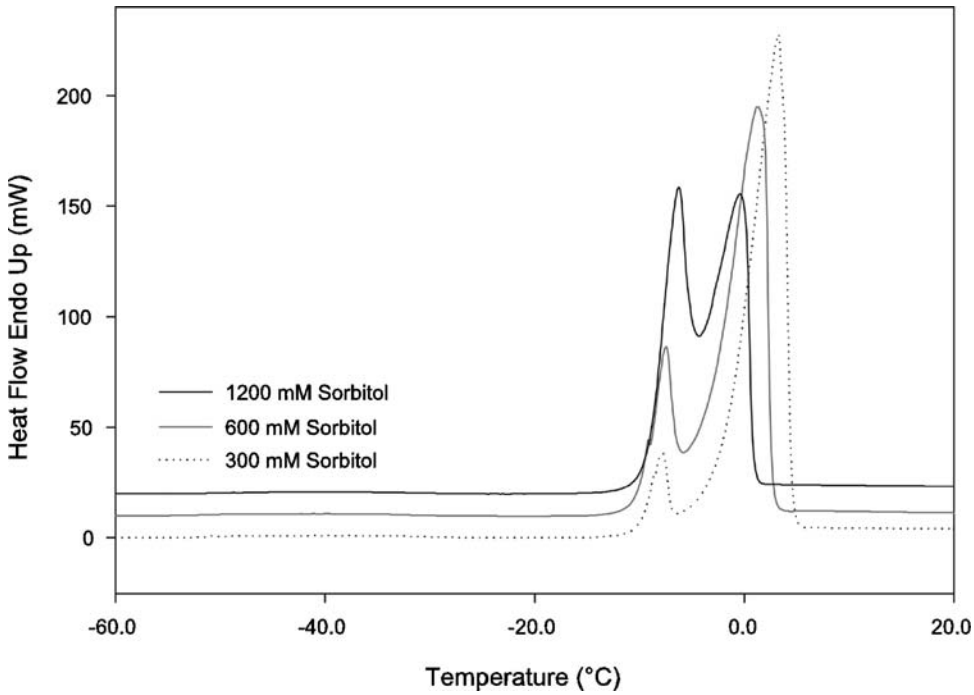

Fig. 7. Sub-ambient DSC thermograms obtained from formulations with 2 mg/ml Fc-fusion protein and increasing sorbitol concentrations (300, 600 and 1,200 mM) after storage for 16 weeks at −30°C. The 300 mM sorbitol formulation is shown as a *dotted line*.

freezing by generating a greater number of smaller ice crystals. However, we did not see a consistent pattern of higher aggregation in samples frozen rapidly in liquid nitrogen compared to samples that were frozen conventionally (data not shown).

We also monitored the stability of the molecule to five freeze thaw cycles (at −20°C, −30°C, and −70°C) and saw no increase in aggregation (by SE-HPLC) upon freeze–thaw (data not shown).

DISCUSSION

Our experiments were designed to investigate the relative importance of various freezing stresses in inducing aggregation of an Fc-fusion protein formulated in sorbitol and stored at −30°C. We studied aggregation as a function of temperature, excipient, time, freezing rate, sorbitol concentration, and successive freeze–thaw cycles. We also monitored the physical state of the excipients as a function of storage temperature.

Under DSC experimental conditions where liquid samples were rapidly frozen (down to ca −70°C) then heated, sorbitol formulations exhibited a T_g' at ca −45°C, indicating sorbitol is an amorphous non-crystallizing solute. It was only upon modification of our DSC experiments to avoid thawing that we were able to capture the effects of long-term storage in the frozen state. The endothermic transitions that we observed at −20°C and −8°C (shown in Figs. 4A and 5) are characteristic of crystalline substances, suggesting that a component of the formulation crystallizes during frozen storage at −30°C. In samples showing endothermic melts, the T_g' (at −45°C) typically attributed to the amorphous sorbitol, is not seen. Samples without sorbitol (containing just protein and acetate buffer) did not show endothermic melts upon DSC analysis (not shown), indicating that sorbitol is the component of the formulation that crystallizes. Moreover, we see that the area of the observed endotherms increases proportionally as a function of sorbitol concentration (Fig. 7). Thermograms from the sorbitol formulation in the presence and absence of protein are similar; endotherms are detected in all samples (Figs. 4A and 5), suggesting the protein does not contribute to this transition. We noted some variation in the rate of crystallization (meaning the timing of the appearance of the two endothermic transitions), which we attributed to both the stochastic nature of nucleation and the fact that the rate of freezing was not controlled in our standard laboratory freezers.

We propose that storage of sorbitol-containing formulations at −30°C (well above the T_g' of −44°C) for a period of more than 24 h can lead to sorbitol crystallization. Presumably, storage above the T_g' of the formulation allows sufficient mobility within the matrix to induce crystallization of sorbitol. Crystallization is one type of phase separation that has been reported to occur upon freezing mixtures (e.g., glycine, mannitol and PEG; 25–29). Phase separation of the protein from its stabilizing excipient can result in protein instability.

The increased protein aggregation in sorbitol-containing formulations stored at −30°C can be explained by the crystallization of sorbitol. When the sorbitol crystallizes, it is depleted from the amorphous phase, and the stabilizing interactions between the sorbitol and the protein are removed. This destabilizes the protein and ultimately leads to protein aggregation. In fact, SE-HPLC profiles of the protein formulated without an excipient stored at either

−20°C or −30°C (Fig. 2) are roughly comparable to that of sorbitol formulations stored long-term at −30°C (Fig. 1). This further reinforces the idea that formulations in which sorbitol has crystallized behave similarly to protein formulated in the absence of excipient.

The two observed endotherms (with onset temperatures of ca −8°C and ca −20°C) likely correspond to eutectic melts of sorbitol polymorphs. Similar observations have been reported for glycine. Multiple glycine endotherms have been observed, some of which correspond to eutectic mixtures of different glycine polymorphs (26). The time-course DSC data we generated for sorbitol also suggests that the form that melts at −20°C is metastable because it decreases over time, while a presumably more stable form that melts at −8°C grows. This suggests that there is an inter-conversion from one form to the other. Conversion of a metastable to a more stable polymorph has been observed in solids [termed Ostwald's law of stages (30)]. Extending this theory to the frozen state can explain our observation.

In the freeze–thaw experiments where the samples were held at −30°C for a period of 24 h before thawing, aggregation was not detected by SE-HPLC (data not shown). In conjunction with DSC data obtained after storage for 1 day at −30°C (shown in Fig. 4), this suggests that crystallization of sorbitol in the presence of 2 mg/ml of protein does not occur within 24 h of freezing at −30°C.

The physical stability (as determined by SE-HPLC) of the Fc-fusion protein at −20°C, in contrast to its instability at −30°C, can be explained by the temperature dependence of sorbitol crystallization. If the sorbitol eutectic that melts at −20°C must form before the polymorph that melts at −8°C (that is presumably more stable), storage at −20°C likely prevents formation of the first polymorph. This reinforces the hypothesis that the formation of the two polymorphs is interdependent; our data suggest the polymorph that melts at −20°C must form before the polymorph that melts at −8°C.

Our data also show that sorbitol in the absence of protein (Fig. 5) appears to crystallize at a faster rate than in the presence of protein (Fig. 4), suggesting that the protein may suppress the rate of crystallization. The crystallization of mannitol during the freezing step in preparing lyophilized formulations is suppressed by high concentrations of protein (31). However, since the rates of freezing were not controlled in our experiments, differences in these rates could also contribute to the observed differences.

Chang and Randall (32) grouped salts commonly used in lyophilization into three categories based on their tendency to crystallize during freezing: those that crystallize readily, those that partially crystallize and glass-forming salts that remain amorphous during freezing. We propose that sorbitol be considered a slowly crystallizing excipient, and this should be taken into consideration during formulation development and the freezing of process intermediates.

Although the structural characterization of the sorbitol polymorphs is beyond the scope of this work, a brief discussion of sorbitol and its isomers is warranted. Mannitol and sorbitol are isomeric, unbranched hexitols, with different stereochemistry only at C2. Unlike sorbitol, mannitol is known to crystallize readily from the frozen state, making it a widely used bulking agent in lyophilized formulations. It has been reported that alditols exhibit different tendencies to crystallize from their melts, even between isomers whose stereochemistry differs at only one carbon (33). In fact, mannitol and galacticol crystallize readily whereas sorbitol and iditol crystallize extremely slowly (all are in the C6 series). After examining the conformations of several alditols in their crystalline form, Jeffrey and Kim (34) explained them as follows: "the carbon chain adopts the extended, planar zigzag when the configurations at alternate carbon centers are different and is bent and non-planar when they are the same." They then predicted that these differences in conformation would affect the rate of crystallization. Mannitol is observed in the extended form and sorbitol has been shown to crystallize in the bent form. This may explain the differences in the rates of crystallization between mannitol and sorbitol in the frozen state. Our future work will focus on characterizing the structures of the sorbitol polymorphs.

CONCLUSION

The stability of a protein in the frozen state is dependent upon both the preservation of the native conformation of the protein that occurs by specific interaction with the excipient as well as decreased mobility in the solid frozen state (7,10). We report here that by crystallizing, sorbitol is unavailable to stabilize the protein in the frozen state; this leads to aggregation of sorbitol-containing formulations stored long-term at −30°C. Future work will focus on the study of sorbitol crystallization as a function of protein concentration and other formulation variables. In addition, we will attempt to correlate the rate of sorbitol crystallization with subsequent protein aggregation.

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