# Protein Aggregation in Frozen Trehalose Formulations: Effects of Composition, Cooling Rate, and Storage Temperature

**BRIAN D. CONNOLLY,[1] LAN LE,[1] THOMAS W. PATAPOFF,[1] MARY E. M. CROMWELL,[2] JAMIE M. R. MOORE,[3] PHILIPPE LAM[4]**

[1]Department of Early Stage Pharmaceutical Research and Development, Genentech, Inc., South San, Francisco, California 94080
[2]Department of Manufacturing Sciences and Technology, Genentech, Inc., South San, Francisco, California 94080
[3]Department of Late Stage Pharmaceutical Development, Genentech, Inc., South San, Francisco, California 94080
[4]Department of Pharmaceutical Processing and Technology Development, Genentech, Inc., South San, Francisco, California 94080

*Received 15 January 2015; revised 4 August 2015; accepted 26 August 2015*

*Published online 23 September 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24646*

**ABSTRACT:** This study was designed to assess the effects of cooling rate, storage temperature, and formulation composition on trehalose phase distribution and protein stability in frozen solutions. The data demonstrate that faster cooling rates (>100°C/min) result in trehalose crystallization and protein aggregation as determined by Fourier Transform Near-Infrared (FT-NIR) spectroscopy and size-exclusion chromatography, respectively. Conversely, at slower cooling rates (≤1°C/min), trehalose remains predominantly amorphous and there is no effect on protein stability. Evaluation of storage temperatures demonstrates that aggregation increases more rapidly at −14°C compared with higher (−8°C) and lower (−20°C) storage temperatures; however, a relatively higher amount of cumulative aggregation was observed at lower (−20°C) temperature compared with higher storage temperatures (−14°C and −8°C). Further evaluation of the effects of formulation composition suggests that the phase distribution of amorphous and crystallized trehalose dihydrate in frozen solutions depends on the ratio of trehalose to mAb. The results identify an optimal range of trehalose–mAb (w/w) ratio, 0.2–2.4, capable of physically stabilizing mAb formulations during long-term frozen storage—even for fast cooled (>100°C/min) formulations.  J Pharm Sci 104:4170–4184, 2015

**Keywords:** crystallization; formulation; stability; stabilization; degradation products; protein aggregation; FTIR; nucleation; precipitation; protein formulation

## INTRODUCTION

Frozen storage can be an effective method for long-term stabilization of therapeutic proteins. Lower temperatures and freeze concentration of solutes increase the viscosity of the frozen solution, which limits molecular mobility. In addition, frozen storage temperatures typically decrease the rates of most chemical reactions (e.g., oxidation, deamidation, hydrolysis, etc.) and some physical reactions (e.g., aggregation) that affect proteins.[1] For these reasons, liquid formulations are generally frozen and stored at low temperatures (≤−20°C) to preserve protein stability prior to final fill finish operations.

However, the freezing process introduces morphological and physicochemical changes that can stress proteins. The formation of ice crystals, freeze concentration of solutes (including the protein), and phase separation can result in denaturation, conformational changes, and/or aggregation of proteins.[2] Irreversible protein aggregation has numerous consequences: in some cases, it has been shown to decrease potency (lowering treatment efficacy), whereas in other cases, it increases the potency of the drug (causing potential safety issues).[3] Additionally, it has been suggested that aggregates may elicit immune responses.[3]

For these reasons, protective excipients (e.g., carbohydrates, amino acids, polyols, etc.) are commonly included to stabilize proteins during freezing, thawing, and during frozen storage. Presumably, amorphous carbohydrates and amino acids stabilize proteins by forming a glassy matrix capable of hydrogen bonding to proteins. The protein–excipient interactions provide proteins with preferential interactions and excipient–excipient interactions provide a kinetic barrier to protein aggregation by decreasing the rate of molecular diffusion.[1,4,5]

It has been reported that some excipients (e.g., mannitol, sorbitol, etc.) that are effective cryoprotectants in the amorphous phase can crystallize during frozen storage and result in protein aggregation.[6] Although trehalose has been established as an effective cryoprotectant for protein formulations,[1,5,7–10] there have been reports of spontaneous trehalose crystallization during freezing, frozen storage, and lyophilization in recent years.[11–14] Similarly, Singh et al.[14] describe trehalose crystallization-induced protein aggregation during long-term frozen storage of mAb formulations. These studies did not comprehensively explore ranges of processing conditions and formulation compositions that could either lead to or prevent crystallization of trehalose. Controlling or preventing trehalose crystallization would have a significant positive impact on protein stability during freezing and frozen storage. Thus, it is of interest to further evaluate the effects of processing conditions and formulation compositions on trehalose crystallization.

Furthermore, it has been shown that cooling rate and storage temperatures are two conditions that can be critical for controlling the morphological and physicochemical properties of the frozen solution—including the extent of ice crystal formation, freeze concentration, and phase separation of solution components.[1,2,15–23] To study trehalose crystallization and

*Correspondence to*: Brian D. Connolly (Telephone: +650-467-4813; Fax: +650-225-3613, E-mail: connolly.brian@gene.com)
Brian D. Connolly and Lan Le contributed equally to this work.

Journal of Pharmaceutical Sciences, Vol. 104, 4170–4184 (2015)

**Table 1.** mAb2 Sugar/Polyl Formulations

| mAb2 (mg/mL) | mAb2 (%, w/v) | Sugar/Polyol (%, w/v) | Sugar/Polyol | Sugar (or Polyol) to mAb Ratio |
|---|---|---|---|---|
| 25 | 2.5 | 6 | Sucrose | 2.40 |
| 25 | 2.5 | 6 | Trehalose | 2.40 |
| 25 | 2.5 | 6 | Mannitol | 2.40 |

**Table 2.** mAb Sample Composition

| Protein | mAb (mg/mL) | mAb (%, w/v) | Trehalose (%, w/v) | Trehalose to mAb Ratio |
|---|---|---|---|---|
| mAb1 | 25 | 2.5 | 2.1 | 0.84 |
| mAb2 | 25 | 2.5 | 5.4 | 2.16 |
| mAb3 | 20 | 2.0 | 8.2 | 4.10 |

protein aggregation that result from different freeze processes and storage temperatures, three mAbs in different formulations were frozen at three cooling rates: fast (>100°C/min), intermediate (<1°C/min), and slow (<0.10°C/min) and subsequently stored at −20°C, −14°C, and −8°C for 12 months. These storage temperatures were selected based on their relevancy to large-scale manufacturing processes.

In addition, it has been reported that the stabilizer to protein ratio can mediate excipient crystallization (e.g., mannitol) and protein stability during freeze-drying.[24] To evaluate the ability of the stabilizer to protein ratio to mediate trehalose crystallization during frozen storage, we performed further studies to systematically investigate trehalose phase distribution and evaluate effects on the physical stability of mAbs in frozen solutions across a broad range of trehalose to mAb ratios using previously established FT-NIR and size-exclusion chromatography (SEC) methods, respectively.[25]

The results of this study have numerous practical implications for large-scale manufacturing and storage of biopharmaceuticals in addition to elucidating the low temperature phase behavior and composition of trehalose-containing solutions and of trehalose crystallization in frozen solutions. Presumably, the effectiveness of trehalose as a stabilizer of proteins depends on the phase distribution of trehalose in the frozen solution.[14,26] Thus, the results from this comprehensive study can provide guidance for the development of robust formulations, freezing processes, and frozen storage temperatures that sufficiently control the phase distribution of trehalose in the solid state.

## MATERIALS AND METHODS

### Materials and Sample Preparation

#### *mAbs and Excipients*

Three IgG1 full-length monoclonal antibodies (mAb1, mAb2, and mAb3) with an approximate molecular weight of 145 kDa were cloned, expressed in Chinese hamster ovary cell lines, and purified at Genentech, Inc. (South San Francisco, California).

#### *Excipient Solubility Study*

For the excipient solubility study, mAb2 is formulated at 25 mg/mL in 51 mM sodium phosphate, at pH 6.2 with 0.04% (w/v) polysorbate 20, water for injection, USP (control sample) with 6.0% (w/v) of sucrose, trehalose, or mannitol (Table 1).

### Excipient Crystallization Study

Additional protein-free solutions were prepared with 0.0%, 2.0%, 4.0%, and 8.0% (w/v) trehalose in 20 mM histidine acetate, at pH 5.5, and water for injection. Fifty microliters of pHydrion (pH range: 0–7) pH-indicator dye (Micro Essential Laboratory, Brooklyn, New York) was dispensed into a 10cc glass vial and allowed to evaporate. Approximately 4 mL of the various trehalose formulations were added to the vial and the dye was allowed to dissolve in the solution. Samples were frozen at −20°C for 24 h and then seeded with trehalose dihydrate crystals to promote nucleation. Photographs of frozen trehalose solutions were obtained using an Olympus Stylus 770SW digital camera (Olympus America Inc., Center Valley, Pennsylvania) in supermacro mode.

#### *Cooling Rate and Storage Temperature*

For the storage temperature and cooling rate studies, clinical formulations were obtained from Genentech, Inc. in their corresponding formulations. mAb1 is formulated at 25 mg/mL in 2.1% (w/v) trehalose, 5 mM histidine hydrochloride, at pH 6.0 with 0.01% (w/v) polysorbate 20, and water for injection, USP; mAb2 is formulated at 25 mg/mL in 5.4% (w/v) trehalose, 51 mM sodium phosphate, at pH 6.2 with 0.04% (w/v) polysorbate 20, and water for injection, USP; mAb3 is formulated at 20 mg/mL in 8.2% (w/v) trehalose, 20 mM histidine acetate, at pH 6.2 with 0.02% (w/v) polysorbate 20, and water for injection, USP (Table 2).

To prepare the samples for slow and intermediate cooling rate, 2 mL sample aliquots were dispensed into autoclaved 5cc glass vials and sealed with 20 mm Lyo-Stoppers using aseptic technique in a ventilated biosafety hood with laminar air flow.

#### *Formulation Composition*

For the formulation studies, mAb2 was evaluated at three mAb concentrations (0, 25, and 100 mg/mL) in 20 mM histidine hydrochloride at pH 6.0 with varying amounts of trehalose (Tables 3 and 4). Two milliliters of each of the 64 different formulations as well as 32 vehicle blanks containing 0 mg/mL of mAb2 were prepared and then dispensed into various

**Table 3.** 25 mg/mL mAb2 Formulations

| mAb2 (mg/mL) | mAb2 (%, w/v) | Trehalose (%, w/v) | Trehalose to mAb Ratio |
|---|---|---|---|
| 25 | 2.5 | 0.0 | 0.00 |
| 25 | 2.5 | 1.7 | 0.68 |
| 25 | 2.5 | 3.4 | 1.36 |
| 25 | 2.5 | 5.1 | 2.04 |
| 25 | 2.5 | 6.8 | 2.72 |
| 25 | 2.5 | 8.6 | 3.44 |
| 25 | 2.5 | 10.3 | 4.12 |
| 25 | 2.5 | 12.0 | 4.80 |
| 25 | 2.5 | 13.7 | 5.48 |
| 25 | 2.5 | 15.4 | 6.16 |
| 25 | 2.5 | 17.1 | 6.84 |
| 25 | 2.5 | 20.5 | 8.20 |
| 25 | 2.5 | 24 | 9.60 |
| 25 | 2.5 | 27.4 | 10.96 |
| 25 | 2.5 | 30.8 | 12.32 |
| 25 | 2.5 | 34.2 | 13.68 |

**Table 4.** 100 mg/mL mAb2 Formulations

| mAb2 (mg/mL) | mAb2 (%, w/v) | Trehalose (%, w/v) | Trehalose to mAb Ratio |
|---|---|---|---|
| 100 | 10 | 0.0 | 0.00 |
| 100 | 10 | 1.7 | 0.17 |
| 100 | 10 | 3.4 | 0.34 |
| 100 | 10 | 5.1 | 0.51 |
| 100 | 10 | 6.8 | 0.68 |
| 100 | 10 | 8.6 | 0.86 |
| 100 | 10 | 10.3 | 1.03 |
| 100 | 10 | 12.0 | 1.20 |
| 100 | 10 | 13.7 | 1.37 |
| 100 | 10 | 15.4 | 1.54 |
| 100 | 10 | 17.1 | 1.71 |
| 100 | 10 | 20.5 | 2.05 |
| 100 | 10 | 24 | 2.40 |
| 100 | 10 | 27.4 | 2.74 |
| 100 | 10 | 30.8 | 3.08 |
| 100 | 10 | 34.2 | 3.42 |

96-well Greiner microplates and 96-well glass plates (Zinsser, Germany). For each formulation, 200 μL sample volumes were aliquoted into each sample well. Multiple independent sample replicates (≥8) were prepared for each set of sample composition, timepoint, and temperature conditions and used to calculate the statistical values (i.e., average, SD, and range) for the FT-NIR and HP(high-performance)-SEC data.

Formulation study samples were prepared by exhaustively dialyzing the mAb into formulation buffers using Pierce Slide-A-Lyzer dialysis cassettes or Millipore (Billerica, Massachusetts) Amicon Ultra centrifugation tubes with10 kDa Molecular Weight Cutoff (MWCO). The solution pH was verified postdialysis. The mAb was further concentrated by ultrafiltration using Amicon Ultra centrifugal filtration devices (10 kDa MWCO).

All formulation buffers were prepared with compendial grade material, including L-histidine free base (USP, Ph. Eur.), glacial acetic acid (USP, FCC, ACS), L-histidine hydrochloride monohydrate (Ph. Eur.), sodium phosphate monobasic monohydrate (USP, ACS), sodium phosphate dibasic anhydrous (USP, FCC, ACS), α,α-trehalose dihydrate (USP-F, Ph. Eur.), sucrose (NF, EP, JP High Purity), D-mannitol (USP, EP), and polysorbate 20 (USP-NF, Ph. Eur.). In-house deionized water was further purified using an Elga PURELAB Ultra (Celle, Germany) water purification system prior to use.

### Controlled Ice Nucleation

To limit the extent of supercooling, ice nucleation was initiated prior to controlled cooling (i.e., freezing) processes. Sample vials were first placed on a laboratory-scale lyophilizer shelf and equilibrated to −1°C to −3°C (slightly below the solution's normal freezing point of ~−0.5°C). Here, the lyophilizer was used merely as a convenient way of controlling the temperature of the samples. Ice nucleation was then initiated by placing the bottom edge of each vial in contact with a small piece of frozen $CO_2$ (i.e., dry ice). Upon visual evidence of ice formation, the dry ice was removed and the vial was immediately returned to the cooled lyophilizer shelf. During this process, the majority of the solution remained liquid and the bulk solution temperature did not change outside the target temperature range (−1°C to −3°C) as confirmed by thermocouple probes inside a few selected samples.

### Controlled Cooling Rates

It is difficult to accurately control and measure the actual freezing rate (rate of ice formation) of a sample. However, the freezing rate is directly proportional to the temperature difference between the liquid-ice front and the surface providing the cooling (driving force). In our studies, we actively control the cooling rate of the samples in order to obtain a corresponding freezing rate, although the later quantity is not specifically measured, only inferred.

Following ice nucleation, samples to be subjected to the slow cooling regiment were left in the lyophilizer. A slow cooling rate of less than 0.1°C/min (and therefore, a slow cooling rate) was obtained by programming the lyophilizer to ramp the shelf temperature at a constant rate from −1°C to −40°C over 144 h.

Samples intended for the intermediate cooling rate regiment were removed from the lyophilizer and immediately placed in a −20°C freezer. Resulting cooling rates of about 1°C/min could be achieved in this manner.

The fast cooling rate (>100°C/min) was accomplished by dripping 50 μL sample aliquots of solution directly into a wide mouth cryogenic dewar filled with liquid nitrogen (quench cooling) and equipped with a stainless steel mesh basket. A number of the frozen spherical pellets, equivalent to a total of 1 mL of solution, were collected and swiftly transferred to prechilled 6cc glass vials, sealed with lyophilization stoppers, and stored on frozen $CO_2$. The vials were subsequently placed at the appropriate study storage temperature.

As the formulation composition screen involved a large number of conditions, samples were fast cooled in 96-well microplates using liquid nitrogen. Immediately after freezing, the ice surface was scratched using standard 24 gauge hypodermic needles (BD, Franklin Lakes, New Jersey) in order to promote crystallization of trehalose dihydrate. The plates were then placed at the target storage temperature for long-term storage.

### Controlled Trehalose Dihydrate Nucleation

Two experimental methods were employed to promote nucleation of trehalose dihydrate in frozen solutions. For excipient solubility studies, samples were stored at −20°C for at least 24 h to ensure that the samples were completely frozen. Seeding was accomplished by sprinkling small amounts (<1 mg) of compendial grade trehalose dihydrate crystals on to the surface of the frozen samples. For the formulation composition study, frozen samples were scratched in the 96-well microplate using a 24-gauge needle.

### Isothermal Hold

Samples frozen at the three cooling rates (slow, intermediate, and fast) by the methods described above were transferred to three freezer units with set points of −20°C, −14°°C, and −8°C for frozen storage.

Sample vials were pulled after 0, 1, 2, 3, 6, 9, and 12 months of isothermal, frozen storage. Samples intended for SEC analysis were placed on the laboratory counter bench under ambient conditions (~22°C) and allowed to thaw prior to analysis.

Samples intended for FT-NIR analysis were transferred on dry ice to a precooled shelf in a lyophilizer to prevent thawing prior to lyophilization.

In all cases, storage freezers were constantly monitored by a Yokogawa recording system (Yokogawa Meters and Instruments Corporation, Tokyo, Japan) to ensure that the temperature remained within ±2°C of set point.

Sample microplates for SEC analysis were stored isothermally for up to 12 months. Following frozen storage, microplates were thawed on the laboratory counter bench under ambient conditions (~22°C) prior to analysis. Control (0 day) samples were thawed immediately following completion of the freezing process. Sample glass microplates for Fourier transform infrared analysis were pulled after 12 months of isothermal, frozen storage and lyophilized for trehalose crystallization analysis. Control (0 month) samples intended for Fourier transform infrared analysis were lyophilized immediately following completion of the freezing process.

### Lyophilization

Samples intended for trehalose crystallization determination were first freeze-dried using a LyoStar II lyophilizer unit controlled by LyoManager II software (FTS Systems, Stone Ridge, New York). The frozen samples were placed on a precooled shelf and held at −35°C for 7 h. The chamber was then evacuated to a pressure of 150 μm Hg and primary drying was conducted by ramping shelf temperature to 20°C at a constant rate of 0.2°C/min followed by a 40-h hold at 20°C. Secondary drying was performed by linearly ramping shelf temperature to 30°C at 0.2°C/min followed by an 8-h hold at 30°C. Thermocouples were placed in various sample vials to monitor temperature during freeze-drying.

Upon completion of drying, samples in vials were stoppered at 150 μm Hg and the chamber vented to atmospheric pressure with nitrogen. Formulation screen samples lyophilized in 96-well glass plates under the same conditions were immediately sealed with aluminum foil upon removal from the lyophilizer to prevent samples from hydrating prior to analysis.

### Size-Exclusion Chromatography

To measure the molecular size distribution of the three drug substances, HP-SEC analysis was implemented using a TosohHaas TSKgel G3000 SWxl (7.8 mm × 30 cm, 250 Å pore size, 5 μm particle size) column on the Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, California) or equivalent. The mobile phase solution was 0.2 M potassium phosphate at pH 6.2 with 0.25 M potassium chloride. The flow rate was 0.5 mL/min and the run time was 30 min. Sample chamber temperature was 5°C and injection mass was 100 μg. The column outlet signal was monitored with a Diode Array Detector measuring absorbance at 280 nm using 360 nm as a reference signal. Data analysis and UV peak integration were then performed using Chromeleon Software (Dionex, Sunnyvale, California) to quantify the percent of molecular aggregate, monomer, and fragment present in each sample. Low volume, formulation screen samples were analyzed in 96-well polypropylene microplates (Greiner Bio-One, Kremsmünster, Austria) using the SEC method described with a flow rate of 1.0 mL/min and a run time of 15 min. Prior to SEC analysis, the mAb2 samples were diluted in 20 mM histidine hydrochloride pH 6.0 and held at 30°C for 24 h to resolve dissociable aggregates prior to SEC analysis. Sample chamber temperature was 30°C and injection mass was 100 μg. This SEC method is capable of detecting small increases (≥0.2) in percent aggregate.

### Concentration Measurement

The UV absorbance of each sample was measured by recording the absorbance at 279 and 320 nm in a quartz cuvette with 1-cm path length on an Agilent 8453 spectrophotometer using Chemstation software (Agilent Technologies). The UV concentration determination was calculated by using the experimentally determined absorptivities for each mAb. The measurements were blanked against the appropriate buffers.

### Turbidity Analysis

The turbidity of the samples was measured by recording the average absorbance from 340 to 360 nm in a quartz cuvette with 1-cm path length[27] on an HP8453 spectrophotometer using Chemstation software (Agilent Technologies). Sterile water for injection was used to blank the instrument prior to analyses. Turbidity analysis of formulation screen samples was performed on a UV transparent, Corning half area, 96-well microplate using Spectramax M2 instrument (Molecular Devices, Sunnyvale, California). The turbidity was measured against a Sterile Water for Injection blank and the turbidity value measured by recording the average of the absorbance from 340 to 360 nm.

### FT-NIR Spectroscopy

The percent trehalose crystallization was determined for lyophilized samples using near-infrared diffuse reflectance spectroscopy. Data collection, calibration, and analysis methods used in this study were adapted from previous work focused on characterizing the spectral differences between amorphous trehalose and crystalline trehalose dihydrate.[25] Using these methods, sample NIR spectra were recorded from 10,000 to 4000 $cm^{-1}$ using 32 averaged scans at 4 $cm^{-1}$ resolution on a Nicolet Antaris FT-Near IR Analyzer equipped with an integrating sphere module (Thermo Fisher Scientific, Waltham, Massachusetts). Relative percentage of crystallized trehalose, defined as the ratio of (%, w/v, crystalline trehalose dihydrate)/(%, w/v, crystalline trehalose + %, w/v, amorphous trehalose), was calculated using the ratio of the normalized peak intensities at 4306 and 4291 $cm^{-1}$, respectively.[25] The absolute concentration of crystalline trehalose is calculated as a product of (%, w/v trehalose prior to freezing) × (relative percent crystallized trehalose dihydrate as determined by FT-NIR). Data analysis was conducted using linear regression with MATLAB (R2007a; The MathWorks, Natick, Massachusetts). Previous work demonstrates that linear regression model using the ratio of normalized peak intensities is capable of detecting small changes (RMSEP: 1.56%) in percent trehalose dihydrate.[25] Low-volume (200 μL) formulation screen samples were lyophilized and then analyzed for percent trehalose crystallization using the same method.

## RESULTS AND DISCUSSION

### mAb Stability in Frozen Trehalose Formulations

Three IgG1 monoclonal antibodies (mAb1, mAb2, and mAb3) were evaluated as model proteins to investigate frozen storage

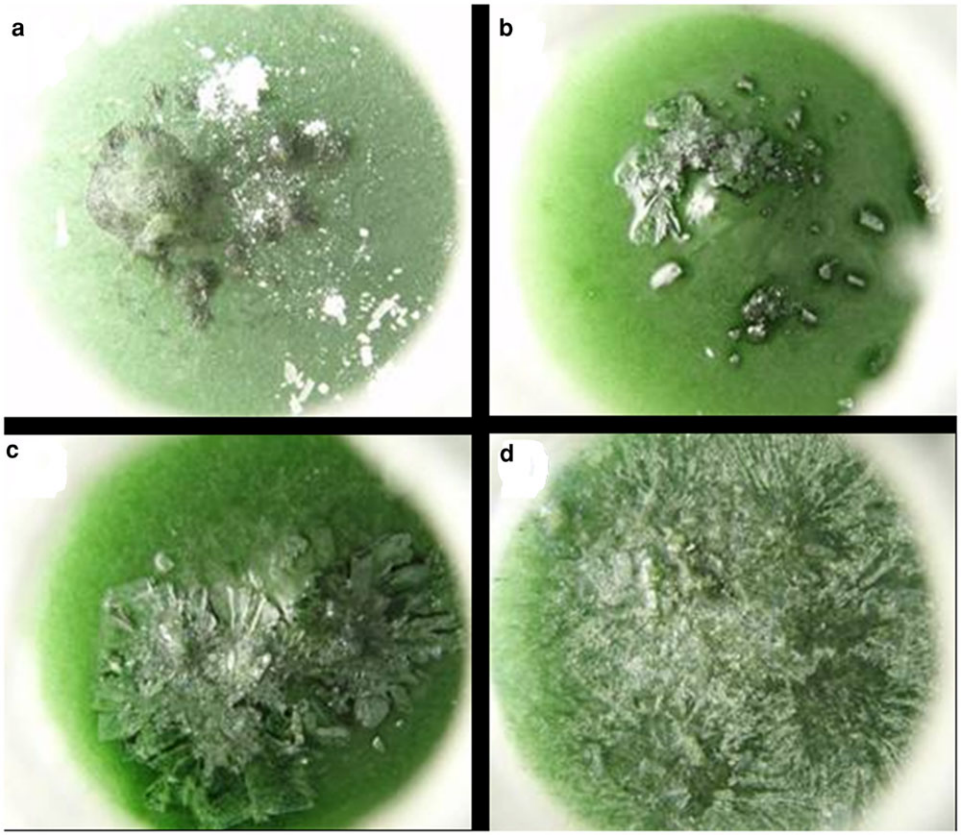


**Figure 1.** Images display visual trehalose crystallization after induced nucleation on the surface of frozen samples with pharmaceutically relevant concentrations of trehalose of (a) 0.0% (w/v) trehalose, (b) 2.0% (w/v) trehalose, (c) 4.0% (w/v) trehalose, and (d) 8.0% (w/v) trehalose.

stability in the presence of trehalose: mAb1 was selected as a negative control because it has not been observed to aggregate during frozen storage, whereas mAb2 and mAb3 were selected because they have been observed to aggregate during frozen storage at −20°C.

### *Effects of Excipient Solubility*

To evaluate the impact of nominal trehalose concentration on excipient crystallization, protein-free solutions were prepared with 0.0%, 2.0%, 4.0%, and 8.0% (w/v) trehalose dihydrate. A small amount of a pH-indicating dye was added to each sample to better visualize crystallization. The samples were frozen using the intermediate cooling rate, seeded with trehalose dihydrate crystals from the same raw material used to prepare the solutions, and stored at −20°C for 6 days. Following induced nucleation, trehalose crystallization was visually observed in all frozen trehalose solutions (Fig. 1). As expected, there was no evidence of trehalose crystallization in the samples prepared without any trehalose (Fig. 1a). The amount of visible crystallization is apparently higher in solutions that contain higher trehalose concentration prior to freezing.

Thus, our experiments confirm previously reported findings that solutions containing pharmaceutically relevant trehalose concentrations (≤10%, w/v) have the potential to crystallize at temperatures below the freezing point at trehalose concentrations that are well below the solubility limit at ambient temperatures above the freezing point (Fig. 2a). Additionally, the results demonstrate that higher trehalose concentrations prior to freezing increase the susceptibility and extent of trehalose crystallization at temperatures below the freezing point.

### *Effects of Excipient Crystallization*

Crystallization of carbohydrates during freezing, freeze-drying, and frozen storage has been shown to impact the physical stability of protein drugs. For example, mannitol has been shown to crystallize during freeze-drying and result in conformational changes, aggregation, and loss of activity for various proteins.[24,26,30,31] Although at lower temperatures (<40°C) trehalose is more soluble than mannitol, it is significantly less soluble than sucrose, which is generally regarded as a noncrystallizing excipient (Fig. 2a).[28,29]

To evaluate the effects of excipient solubility at temperatures above the freezing point on protein stability in frozen solutions, mAb2 solutions were prepared with equivalent concentrations of sucrose, trehalose, and mannitol. Samples were frozen at the intermediate cooling rate, seeded with the corresponding excipient, and stored frozen at −20°C for 28 days. SEC data demonstrate that after frozen storage, no increase in aggregation was observed in the sucrose formulation, a small increase (~1%) in aggregation was observed in the trehalose formulation, and a large increase (~3%) in aggregation was observed in the mannitol formulation (Fig. 2b). These results establish a rank-order correlation between excipient solubility at temperatures above the freezing point and protein aggregation. As expected, supercooled sugars/polyols with lower solubility at temperatures above the freezing point result in larger increases in aggregation (Fig. 2b). Specifically of interest, the results

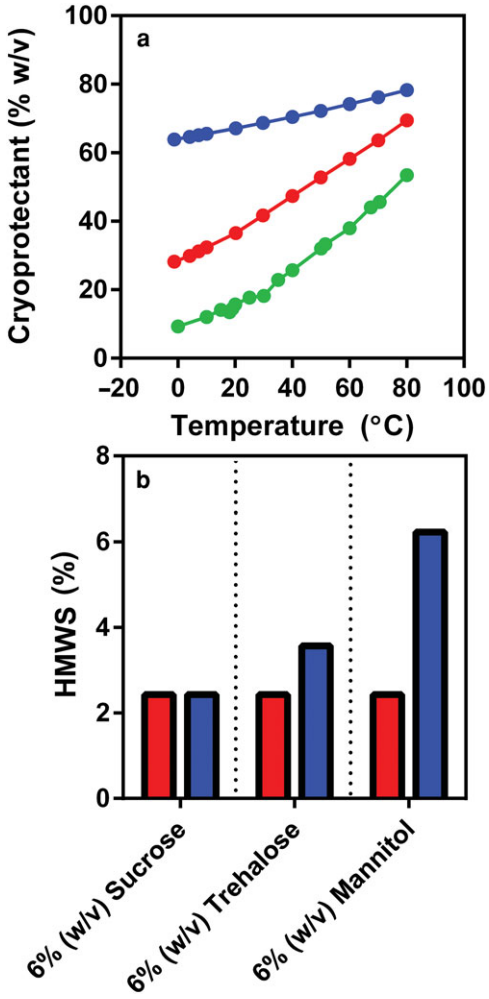


**Figure 2.** (a) Plot displays the solubility of sucrose (•), trehalose (•), and mannitol (•) as a function of temperature.[28,29] (b) Bar plot displays the percent high molecular weight species of mAb2 in various cryoprotectant formulations before (■) and after freezing and induced nucleation for 28 days at −20°C (■) as determined by HP-SEC.

suggest that trehalose at −20°C is sufficiently supersaturated that it may crystallize and result in protein aggregation at pharmaceutically relevant concentrations (2%–10%, w/v). These findings are consistent with a report that establishes that the aqueous solution solubility of carboxylic acids can be used to estimate the degree of supersaturation and predict the crystallization propensity of carboxylic acids during storage at temperatures below the freezing point.[32]

### *Effects of Cooling Rate*

The effects of cooling rate on ice crystal formation and protein stability have been studied extensively.[16,23,33–35] However, these studies do not systematically explore the combined effect of both cooling rate and storage temperature following freezing processes at different cooling rates. To study trehalose crystallization and protein aggregation that result from different freeze processes, three mAbs in different formulations were frozen at three cooling rates and each subsequently stored at three temperatures for 12 months.

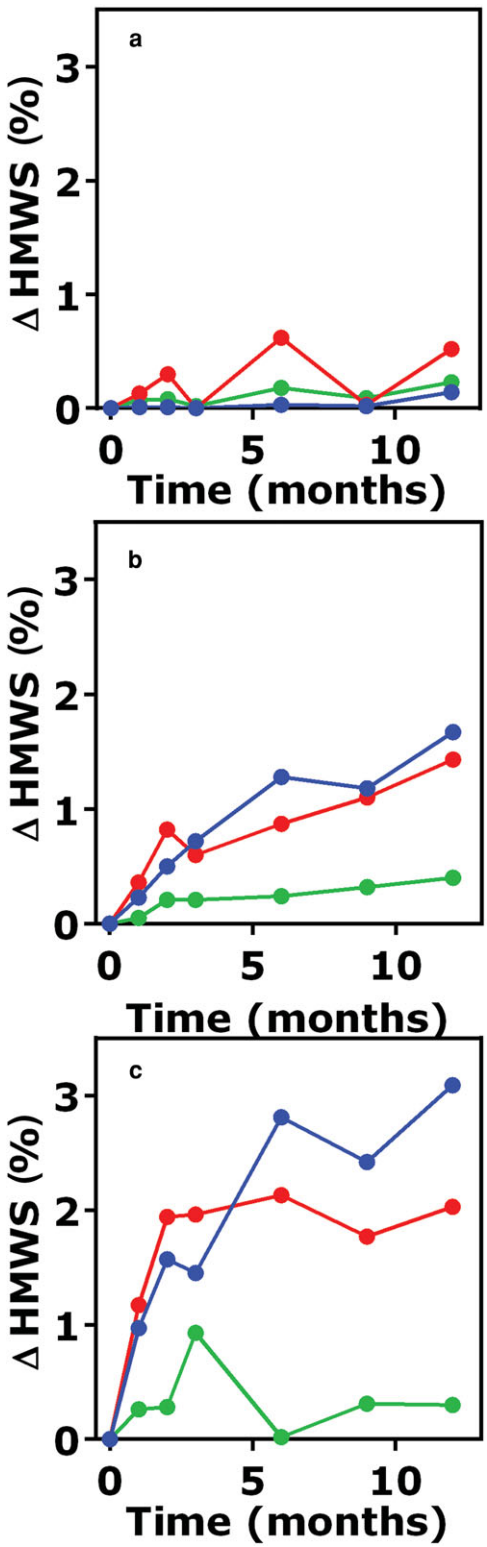


**Figure 3.** Plot represents the time dependent increase in aggregation of mAb1 (a), mAb2 (b), and mAb3 (c) fast-cooled samples stored frozen at −20°C •, −14°C, •and −8°C •.

The SEC data for the three mAb formulations demonstrates that cooling rate does impact protein stability (Fig. 3). In general, increases in mAb aggregation were observed following freezing at the fast rate (>100°C/min) and no increases in

**Table 5.** Summary of Trehalose Phases After 12-Month Frozen Storage

| Protein | Total Trehalose (%, w/v) | Cooling Rate | Δ Aggregation (%) | | | Crystallized Trehalose (%, w/v, Relative Percentage) | | | Amorphous Trehalose (%, w/v, Relative Percentage) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | −20°C | −14°C | −8°C | −20°C | −14°C | −8°C | −20°C | −14°C | −8°C |
| mAb1 | 2.1 | Fast | 0.1 | 0.6 | 0.2 | 0.2 (10%) | 0.1 (5%) | 0.2 (10%) | 1.8 (90%) | 1.9 (95%) | 1.9 (90%) |
| | | Intermediate | 0.0 | 0.0 | 0.1 | 0.0 (0%) | 0.1 (5%) | 0.1 (5%) | 2.0 (100%) | 2.0 (95%) | 2.1 (95%) |
| | | Slow | 0.0 | 0.0 | 0.1 | 0.0 (0%) | 0.0 (0%) | 0.0 (0%) | 2.0 (100%) | 2.0 (100%) | 2.1 (100%) |
| mAb2 | 5.4 | Fast | 1.7 | 1.4 | 0.4 | 2.5 (45%) | 2.5 (46%) | 1.6 (30%) | 3.0 (55%) | 2.9 (54%) | 3.8 (70%) |
| | | Intermediate | 0.1 | 0.0 | 0.1 | 0.3 (6%) | 0.3 (6%) | 0.3 (5%) | 5.1 (94%) | 5.1 (94%) | 5.2 (95%) |
| | | Slow | 0.2 | 0.2 | 0.1 | 0.3 (6%) | 0.3 (5%) | 0.2 (4%) | 5.1 (94%) | 5.2 (95%) | 5.2 (96%) |
| mAb3 | 8.2 | Fast | 3.1 | 2.1 | 0.9 | 5.1 (61%) | 5.7 (70%) | 5.5 (67%) | 3.2 (39%) | 2.5 (30%) | 2.7 (33%) |
| | | Intermediate | 0.0 | 0.0 | 0.1 | 0.1 (1%) | 0.5 (6%) | 0.2 (2%) | 8.1 (99%) | 7.7 (94%) | 8.0 (98%) |
| | | Slow | 0.0 | 0.0 | 0.1 | 0.3 (4%) | 0.2 (2%) | 0.2 (2%) | 7.9 (96%) | 8.1 (98%) | 8.0 (98%) |

Table 5 displays the change (Δ) in percentage aggregation, and the absolute and relative percentage of crystallized trehalose, and amorphous trehalose data for mAb1, mAb2, and mAb3 at three different cooling rates (fast, intermediate, and slow) after 12 months of storage at −20°C, −14°C, and −8°C.

aggregation were observed at the slower cooling rates (<1°C/min), irrespective of the long-term storage temperature. The mAb2 and mAb3 samples frozen at the fast cooling rate showed significant increases in aggregation over time (Table 5). Even fast-cooled mAb1, included as a negative control, showed minor increases in aggregation during the course of the study (Table 5). For all fast-cooled samples, the majority of aggregation observed during storage appears to occur within the first 6 months (Fig. 3). Subsequently, the rate of aggregation decreases significantly between 6 and 12 months—changes are sufficiently small during this period so that measured aggregation could potentially be attributable to sample and/or assay variability.

The SEC chromatogram overlay of mAb3 samples frozen at the three cooling rates and stored at −20°C for 12 months demonstrates that there is an observable increase in aggregated species (dimer and high molecular weight) in the fast-cooled mAb3 sample (Fig. 4). Conversely, the mAb3 samples frozen at the slow and intermediate cooling rates and stored at −20°C for 12 months show no significant increases in percent aggregate and the chromatograms for these samples closely match to the study control (Fig. 4). The fast-cooled mAb2 samples show increases in soluble aggregate (Table 5) and the formation of precipitates as determined by turbidity analysis and visual inspection (data not shown). These precipitates have previously been characterized as protein related, and have concomitant decreases in concentration and turbidity (340–360 nm) by UV absorbance following removal by filtration using a 0.2-μ PVDF filter (data not shown).

The FT-NIR spectra of pure samples of the two relevant trehalose phases illustrate the key spectral differences between amorphous and crystalline dihydrate (Fig. 5). Analysis of the 12-month samples by FT-NIR spectroscopy demonstrates that there was measurable amount of trehalose crystallization in the fast-cooled samples for mAb1 (Fig. 6a3), mAb2 (Fig. 6b3), and mAb3 (Fig. 6c3) at all storage temperatures (Table 5). There was no measurable amount of crystalline trehalose detected in the slow- and intermediate-cooled samples for any mAb formulations (Figs. 6a1, 6a2, 6b1, 6b2, 6c1, and 6c2). In contrast, fast-cooled sample spectra obtained following 12 months of frozen storage at the various storage temperatures display sharp peak shifts in key spectral regions associated with trehalose dihydrate (Figs. 6b3 and 6c3) and contain between 30 and 70 relative percent (1.6%–5.7%, w/v) crystallized trehalose

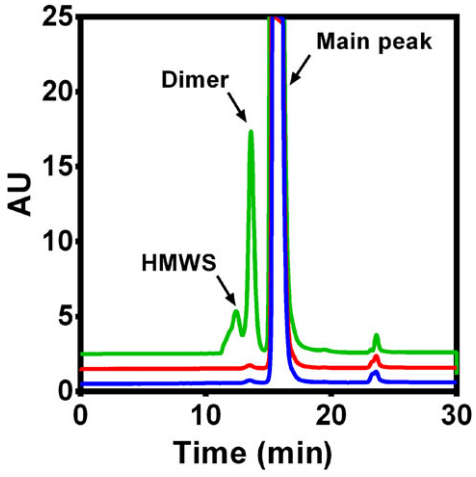


**Figure 4.** Representative SEC chromatograms of mAb3 samples frozen at the slow (•), intermediate (•), and fast-cooled (•) rates, and stored at −20°C for 12 months. Study control (•) (stored at −70°C) shown for comparison.

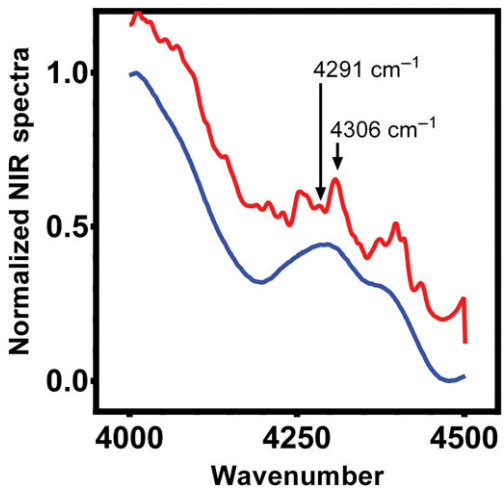


**Figure 5.** Normalized NIR spectra of amorphous trehalose (•), and trehalose dihydrate (crystalline) (•).


DOI 10.1002/jps.24646

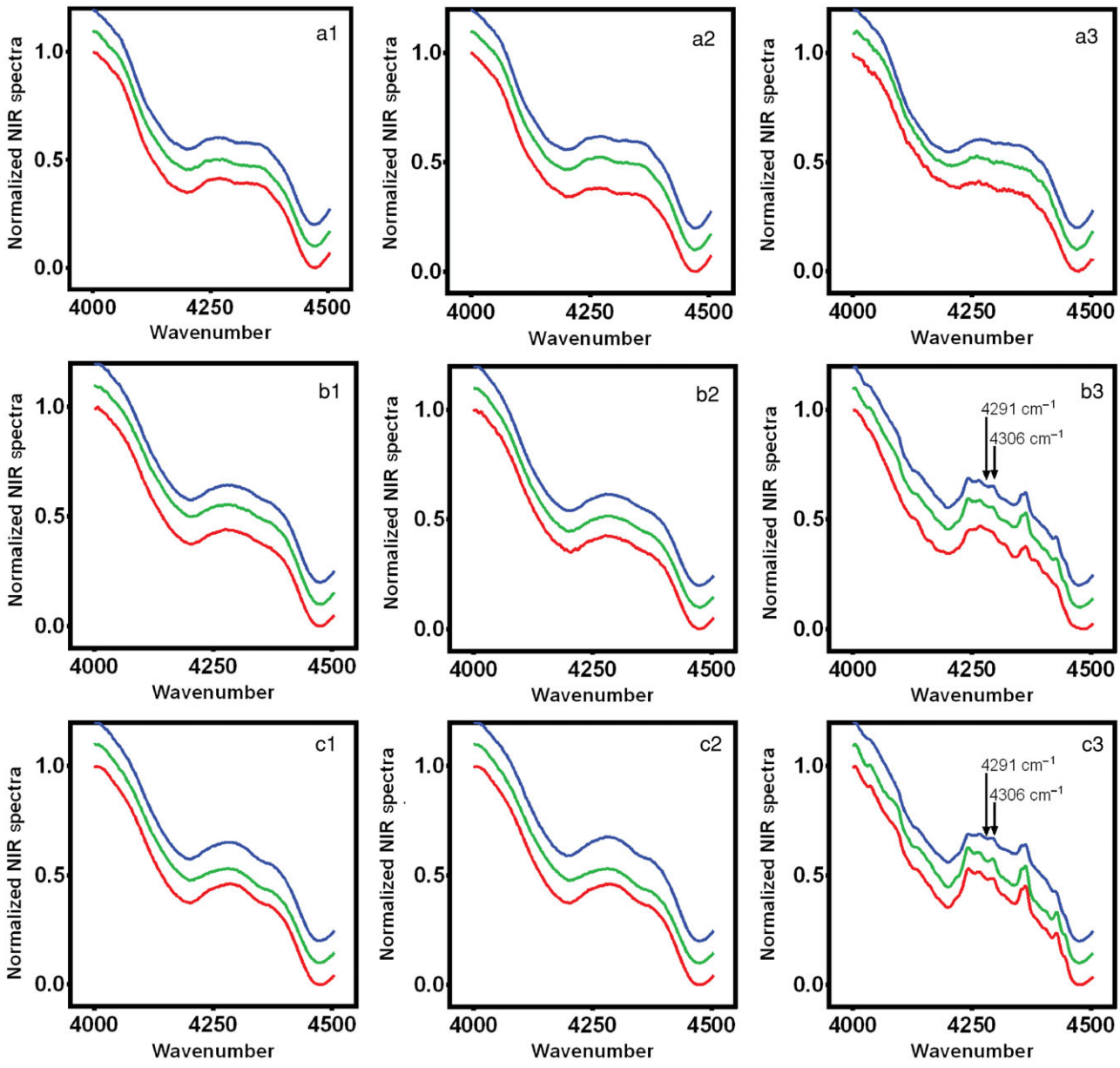


**Figure 6.** Normalized NIR spectra of the (a) mAb1, (b) mAb2, and (c) mAb3 samples frozen using (1) slow cooling, (2) intermediate cooling, and (3) fast-cooling rates following 12-month storage at −20°C (•), −14°C (•), and −8°C (•).

dihydrate compared with absolute trehalose concentration (%, w/v).

Interestingly, observed increases in protein aggregation are correlated with increases in trehalose crystallization. For example, fast-cooled mAb1 samples stored at −20°C for 12 months were found to have less than 10 relative percent (<0.2%, w/v) crystalline trehalose dihydrate and no significant increase (<0.2%) in aggregation, whereas the fast-cooled mAb2 and the mAb3 samples stored at −20°C for 12 months were found to have 45 and 61 relative percent (2.5% and 5.1%, w/v) crystallized trehalose dihydrate and 1.7% and 3.1% increase in aggregation, respectively (Table 5).

As a control, samples were fast-cooled and either immediately analyzed by SEC or lyophilized for FT-NIR analysis to evaluate the immediate impact of rapid cooling and freeze-drying on crystal formation and protein aggregation. No change in protein aggregation was observed (data not shown). Additionally, FT-NIR spectra obtained for the fast-cooled $T_0$ sample set reveal that the sugar is predominantly amorphous in character, containing less than 5% crystallized trehalose dihydrate. These results demonstrate that fast cooling does not immediately impact protein aggregation or amorphous/crystalline trehalose phase distribution. Thus, measured increases in trehalose crystallization and protein aggregation during this study represent changes that occur in a time-dependent fashion during storage.

The results from the cooling rate studies provide insight into the effects of cooling rate on both trehalose crystallization and mAb aggregation. As discussed previously, the cooling rate affects the size, shape, and directionality of the ice crystals formed during freezing. It has been established that rapid cooling rates result in the formation of numerous, small ice crystals

that creates a larger ice-aqueous interfacial surface area.[2,33] It is possible that the larger interfacial surface areas formed in rapidly cooled samples increases the probability that a surface-induced nucleation event will occur. It is also possible that the freezing process induces phase separation and the formation of microenvironments with higher local trehalose concentration, resulting in higher probability for nucleation. These findings are consistent with a previous report of colocalized deposits of trehalose and lysozyme in the freeze concentrate following rapid cooling.[34]

Although fast cooling increases the probability of a nucleation event, it does not guarantee consistent nucleation. It is likely that the high variability of the aggregation results can be attributable to the chaotic nature of nucleation events following fast cooling (Fig. 3). Conversely, the larger ice crystals formed in the samples frozen at the slow and intermediate cooling rates have a lower surface area, which decreases the probability of a nucleation event. This would be consistent with the higher percent of trehalose crystallization and higher increases in mAb aggregation observed in samples frozen at the fast-cooling rates compared with samples frozen at slow and intermediate cooling rates.

It is important to note that the observed increases in protein aggregation are not proportional to the percent of amorphous trehalose remaining in solution (Table 5). Although more amorphous trehalose remains in mAb3 than in mAb1, greater increases in aggregation are observed for mAb3 samples The total amount of amorphous trehalose should be sufficient to protect mAb3 from interactions with ice crystals; however, the molecules in these solutions are not conferred the same protection by the remaining cryoprotectant. These results demonstrate that the observed increases in aggregation cannot be explained solely by the total amount of amorphous trehalose.

Instead, the changes in protein aggregation seem to be better correlated with the total amount of crystallized trehalose. A strong correlation ($R^2 = 0.99$) of percent trehalose dihydrate and mAb aggregation suggests that the concentration of crystallized trehalose is impacting protein stability. However, numerous confounding factors (e.g., protein concentration, buffer type, buffer concentration, trehalose concentration, etc.) prevent direct comparison of the effects of formulation composition between mAb formulations. Additional studies designed to further evaluate the correlation between trehalose crystallization and protein aggregation are described in the section "*Effects of Formulation Composition*."

Presumably, the formation of trehalose dihydrate crystals introduces a large, potentially denaturing, interfacial surface area into the freeze concentrate. It is possible that trehalose crystals interact with protein molecules in the frozen solution and denature protein in a manner similar to ice-induced denaturation.[2,16,22,23] If this is indeed the case, increasing amounts of crystallized trehalose would also lead to larger increases in protein aggregation.

### *Effect of Storage Temperature*

It is well established that crystallization of excipients and protein aggregation decreases at temperatures at or below the glass transition temperature. However, because of the very large volumes involved in large-scale commercial pharmaceutical production, frozen storage of drug substance at temperatures below the glass transition temperature (which, for many products is around −40°C or below) is not common because of significantly higher capital and operating costs. Typically, bulk drug substance is stored in large containers (up to 300 L) at higher temperatures (e.g., −20 ± 4°C).

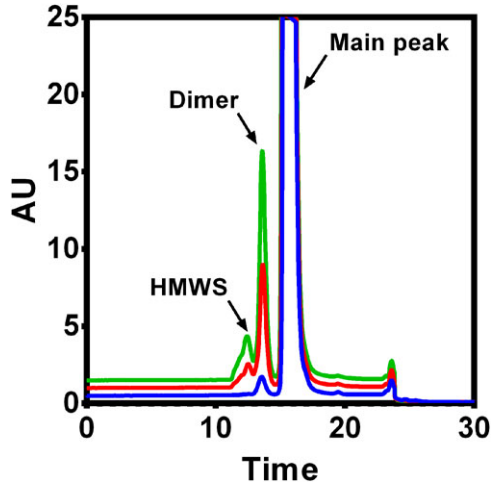


**Figure 7.** Representative SEC chromatograms of mAb3 samples frozen at the fast cooling rate and stored at −20°C (•), −14°C (•), and −8°C (•) for 12 months. Study control (•) (stored at −70°C) is shown for comparison.

Thus, it is of interest to understand the stability of these solutions within this pharmaceutically relevant temperature range. Also, it is of interest to understand the impact of higher temperature exposures as there may be short duration temperature excursions during long-term storage and transportation operations.

Analysis of cooling rate study samples following long-term storage by SEC and FT-NIR demonstrates that storage temperature does impact protein stability (Fig. 3). In general, mAb2 and mAb3 samples fast cooled and stored 12 months at −20°C aggregated to the greatest extent (Fig. 3c). However, the initial rate of aggregation of mAb2 and mAb3 was more rapid in fast-cooled samples stored at −14°C with the rate of aggregation decreasing earlier than those stored at −20°C (Fig. 3). Samples fast cooled and stored at −8°C, the highest storage temperature, generally showed both the lowest rate and extent of aggregation throughout the course of the study (Fig. 3). The chromatogram overlay in Figure 7 shows the observed differences in SEC profiles for fast-cooled mAb3 samples stored 12 months at the three respective storage temperatures.

Interestingly, fast-cooled samples stored at higher temperatures (e.g., −8°C, and −14°C) have smaller increases in aggregation; and conversely, samples stored at lower temperatures (e.g., −20°C) have larger increases in aggregation for mAb2 and mAb3. For example, the increases in aggregation for mAb3 was higher at −20°C (3.1%) relative to −14°C (2.1%) and −8°C (0.9%), even though all temperatures had similar amounts of crystallized trehalose (61–67 relative percent). This trend demonstrates that larger increases in protein aggregation occur at lower temperatures (−20°C) than at higher temperatures (−14°C and −8°C, respectively), even though the samples have comparable amount of trehalose crystallization. This finding is surprising because the influence of frozen storage temperature on protein aggregation is typically interpreted in the context

of molecular mobility. It has been shown that an increase in the viscosity of the freeze concentrate leads to a decrease in molecular mobility. Thus, the degradation rates for reactions that are coupled with viscosity (i.e., molecular mobility) decrease at higher viscosity, leading to a reduction in degradation at lower temperatures. Tang and Pikal[36] established that protein unfolding rates are highly coupled with system viscosity at low temperatures in highly viscous systems. For example, the protein unfolding rate for phosphoglycerate kinase and β-lactoglobulin decreases significantly at lower frozen temperatures above the glass transition temperature.[36] Although these studies were performed in the context of freeze-drying, the findings provide insights into protein unfolding rates in frozen systems.

In this study, the range of storage temperatures surveyed (−20°C, −14°C, and −8°C) is above the glass transition temperatures (midpoint $T_g'$ range: −22°C to −32°C, data not shown) for these solutions. The $T_g'$ would be expected to be relatively more negative at the higher trehalose–mAb ratios (trehalose:mAb ratio range: 0.84–4.10; Table 2). A broad range of molecular mobility is expected for each formulation across the range of temperature and trehalose–mAb ratios evaluated.

It has been well established that the temperature dependence of viscosity, η(T), for small molecule solutions at temperatures above the glass transition temperature can be estimated relative to the viscosity at the glass transition temperature, $\eta(T_g)$, using the Williams–Landel–Ferry (WLF) equation[37] with two previously established universal constants.[37,38]

$$\log\left(\frac{\eta}{\eta_g}\right) = \frac{-17.44\,(T - T_g)}{51.6 + (T - T_g)} \quad (1)$$

The WLF equation may be used to estimate the relative change in viscosity (i.e., molecular mobility) of solutions across the various storage temperatures evaluated in this study to inform the contribution of molecular mobility to the observed changes in trehalose crystallization and protein aggregation. The system viscosity is considerably higher at lower temperatures and the temperature dependence of viscosity is more significant at lower temperatures. For example, the estimated increase in viscosity for mAb2 formulations is approximately 20-fold with a decrease in temperature from −8°C to −14°C. For comparison, the estimated increase in viscosity is approximately 1100-fold with a decrease in temperature from −8°C to −20°C. At temperatures below the glass transition temperature (e.g., −40°C), molecular mobility is negligible.

The estimates provided by the WLF equation indicate that there is a significant increase in molecular mobility at −8°C relative to −20°C. However, as discussed previously, more protein aggregation was observed following storage at low temperature (−20°C) relative to high temperature (−8°C). If molecular mobility (i.e., viscosity) was the primary factor governing protein aggregation rates, then the opposite temperature dependence would be expected. Thus, the results from this study indicate that molecular mobility is not the only factor that is important for determining protein aggregation rates during frozen storage.

The storage temperature dependence of protein aggregation in this study provides additional insight into the mechanism of protein aggregation. Presumably, the higher initial rate of aggregation post storage at −14°C relative to the higher and lower storage temperatures (of −8°C and −20°C, respectively) observed can be attributed to the competing temperature-dependent effects of molecular mobility and freeze concentration, which in turn influence the rate of molecular interactions. For example, at the lower storage temperatures, the extent of freeze concentration is higher and the molecular mobility in the freeze concentrate is lower. Conversely, at the higher storage temperatures, the extent of freeze concentration is lower, whereas molecular mobility is higher.

These two competing factors influence the overall rate and extent of trehalose crystallization and protein aggregation. This could be a possible explanation for an intermediate storage temperature (i.e., −14°C) resulting in the highest initial aggregation rate that exists between the freezing point and the glass transition temperature in the temperature range (range: −20°C to −8°C) evaluated. However, it is also possible that observed temperature dependence of the initial aggregation rate between −14°C and −20°C could be attributed to the high variability associated with trehalose crystallization in frozen samples.

Although these two competing factors can adequately describe the observed temperature dependence of the initial kinetics of protein aggregation, there are consequences of the phase separation of trehalose that add additional complexity to the observed time dependence of protein aggregation. For example, the rate of aggregation decreases with time for all storage temperatures (Fig. 3). As the solution components (i.e., ice, trehalose) redistribute during frozen storage, proteins can be stabilized by re-establishing interactions with colocalized amorphous trehalose molecules. It is also possible that the ice and trehalose crystals grow by Ostwald ripening, thereby decreasing the surface area of potentially denaturing surfaces and reducing the rate of aggregation. Both these possible explanations would be consistent with the observed decrease in aggregation rate as a function of time (Table 5; Fig. 3). These phenomena may also explain why the lowest rate and extent of aggregation was observed for molecules at the highest temperature (e.g., −8°C) where solutes diffuse and redistribute most rapidly.

It is also important to consider the role of annealing in phase separation. It has been shown that annealing can lead to phase separation in the frozen state.[39] In this study, freezing and subsequent isothermal storage is performed at temperatures above the $T_g'$. Although each sample had equivalent amounts of trehalose crystallization following 12-month frozen storage, it is possible that storage (i.e., annealing) at these different temperatures after freezing may be contributing to the kinetics of phase separation and solute redistribution in the frozen solutions. Additional studies demonstrate that the onset of trehalose crystallization occurs within days and reaches a plateau within 2 weeks of frozen storage (data not shown). If the crystallization and aggregation rates are both influenced by molecular mobility in the frozen state, then it is possible that the more rapid onset of trehalose crystallization relative to protein aggregation could be attributed to the size difference between trehalose (~342 Da) and the IgG1 (~145,000 Da). This hypothesis is consistent with the shorter duration of the trehalose crystallization process that occurs within (≤2 weeks) relative to the much longer duration of protein aggregation (≤12 months).

Overall, the results from this study provide additional insights into the crystallization of cryoprotectants in frozen, aqueous solutions in the context of cooling rate, solution

composition, and storage temperature. However, further studies designed to characterize the kinetics of phase separation, solute redistribution, and crystal size distribution that are beyond the scope of this work are required to fully elucidate the mechanism of crystallization-induced protein aggregation.

### *Effects of Formulation Composition*

To determine the effects of formulation composition on trehalose crystallization and mAb aggregation during frozen storage, mAb2 was evaluated at a range of trehalose concentration [(trehalose) range: 0%–34.2% (w/v)] and mAb2 concentrations [(mAb2 range: 0, 25, and 100 mg/mL)]. Samples were fast cooled and stored for 12 months at both −20°C and −40°C and analyzed for trehalose crystallization and protein aggregation using FT-NIR and SEC, respectively.

The results from this study demonstrate that following fast cooling, trehalose crystallizes in frozen solutions stored at −20°C ($>T_g'$) for 12 months (Fig. 8). Conversely, no increases in trehalose crystallization or mAb aggregation were observed in frozen solutions stored at −40°C ($<T_g'$) for 12 months (data not shown). Although no changes are expected to occur at temperatures below the glass transition temperature, mannitol has been shown to crystallize at temperatures below $T_g'$ during freeze-drying.[40]

Immediately following fast cooling and scratching, the relative amount of crystallized trehalose is less than 10% (data not shown). However, following 12-month frozen storage at −20°C, trehalose crystallization increases significantly (Figs. 8a–8c). In general, the results demonstrate that increasing the trehalose concentration results in higher percentages of crystallized trehalose. For example, the relative percent crystallized trehalose is directly proportional to the trehalose concentration prior to freezing (Fig. 8). Similarly, mAb2 aggregation also generally increases after 12-month frozen storage at −20°C (Figs. 8d and 8e). However, the results show that there is an optimal range of trehalose that stabilizes mAb2 solutions during long-term frozen storage. Alternatively, trehalose concentrations above the optimal range result in significant increases in both trehalose crystallization and protein aggregation (Figs. 8d and 8e). At trehalose concentrations below the

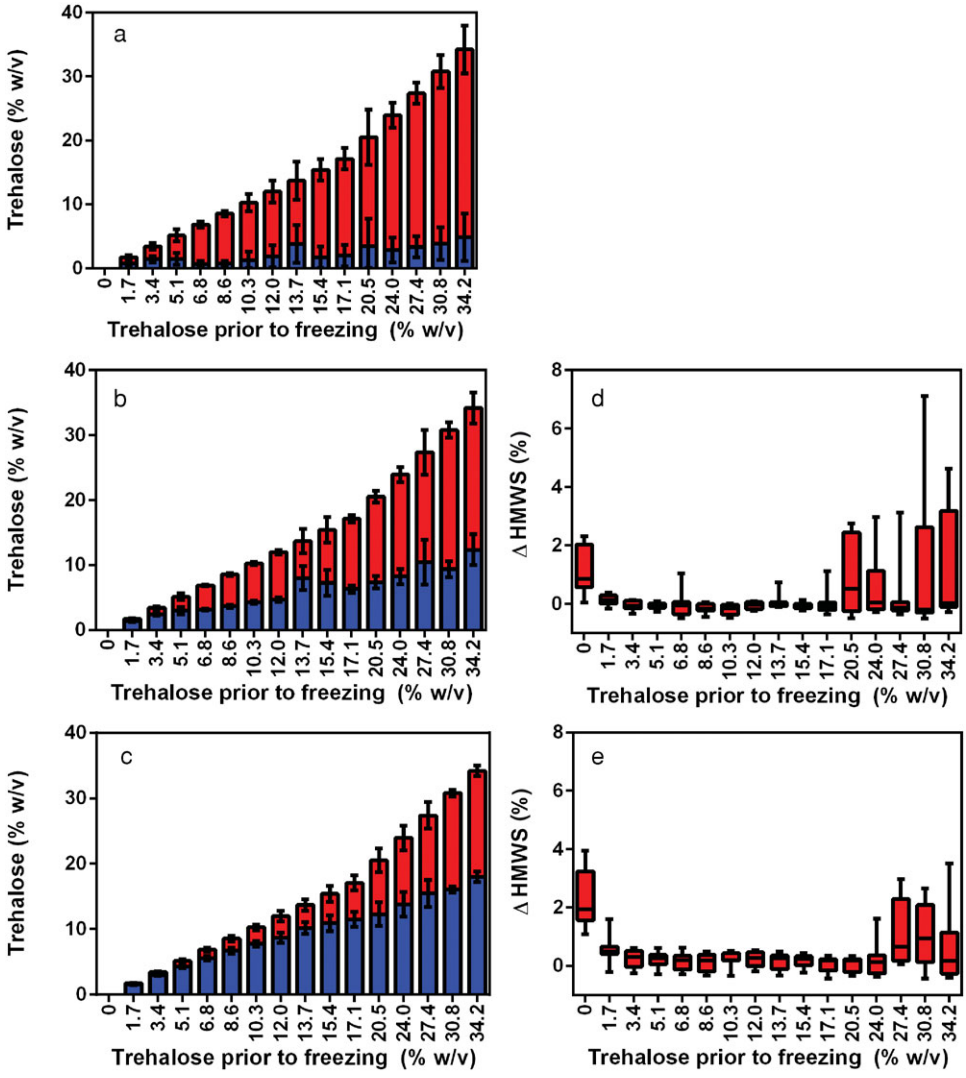


**Figure 8.** Bar plots display the concentrations of amorphous (■) and crystallized (■) trehalose in formulations with (a) 0 mg/mL mAb2, (b) 25 mg/mL mAb2, and (c) 100 mg/mL mAb2 following 12-month storage at −20°C. Box plots display the change in percent high molecular weight species for trehalose formulations with (d) 25 mg/mL mAb2, and (e) 100 mg/mL mAb2 following 12-month storage at −20°C.

 DOI 10.1002/jps.24646

optimal range, there is an increase in protein aggregation but no increase in trehalose crystallization—presumably because of another mechanism (e.g., insufficient cryoprotectant).

The data indicate that the percent of crystallized trehalose formed depends on the concentrations of both protein and trehalose. Higher mAb2 concentration results in lower amounts of trehalose crystallization. For example, at fixed nominal trehalose concentration (1.7%, w/v), increasing the mAb2 concentration from 0 to 100 mg/mL decreases the relative percent of crystallized trehalose from 53% to 9%. Table 6 summarizes the trehalose crystallization and protein aggregation data for each trehalose to mAb ratio (w/w) evaluated in the formulation composition study. These results can be interpreted in the context of the trehalose to mAb ratio (w/w) on trehalose crystallization (Fig. 9a) and changes in percent mAb2 aggregation (Fig. 9b). Analysis of mAb2 physical stability demonstrates that sufficient trehalose–mAb (≥0.2:1, w/w) is required to stabilize the mAb2 solutions during long-term frozen storage. However, excessive trehalose–mAb (≥2.4:1, w/w) results in higher proportions of crystallized trehalose dihydrate and significant increases in percent mAb2 aggregation. Therefore, there appears to be optimal range of trehalose to mAb ratio between 0.2:1 and 2.4:1 (w/w) for mAb2.

Interestingly, polysorbate did not impact the protein stability or phase distribution of trehalose in any of the frozen mAb2 formulations. For samples with equivalent compositions, the addition of polysorbate (0.04%, w/v) did not result in any measurable changes in percent trehalose crystallization or protein aggregation (data not shown). The results from the formulation composition study indicate that increasing protein concentration decreases the occurrence and extent of trehalose crystallization in frozen samples.

It has been shown that cosolutes can reduce the tendency for excipient crystallization (i.e., the poisoning effect). The importance of the excipient to protein ratio in determining solute crystallization for sucrose, trehalose, and mannitol during lyophilization has been established previously.[24,41–44] The proteins may function as crystallization inhibitors that provide a physical barrier to crystal propagation. Similarly, it has been proposed that increasing the protein concentration effectively retards the rate and extent of crystal formation. For example, Singh et al.[14] suggest that at increased concentrations, proteins can provide steric hindrance to the crystallization of trehalose. It is also possible that protein molecules have specific interaction with trehalose, reducing the activity (effective concentration) of trehalose in the frozen solution and thereby lowering its supersaturation level. Another possibility is that the trehalose is simply diluted by the protein that also results in lower supersaturation level.

Although the glass transition temperature for each formulation would be expected to play a role, it does not appear to be the primary factor that governs the effect of composition on trehalose crystallization at −20°C that is well above the glass transition temperature of all formulations evaluated. In this study, we have shown that the amount of crystalline trehalose increases at higher trehalose concentration (Figs. 8a–8c). Thus, the results demonstrate that under the conditions evaluated the relative amounts of trehalose and protein play a more important role in governing trehalose phase distribution. However, the results demonstrate that the glass transition temperature is important in governing the crystal formation at temperatures at or below the glass transition temperature. For example, no crystal formation was observed following 12-month storage at −40°C.

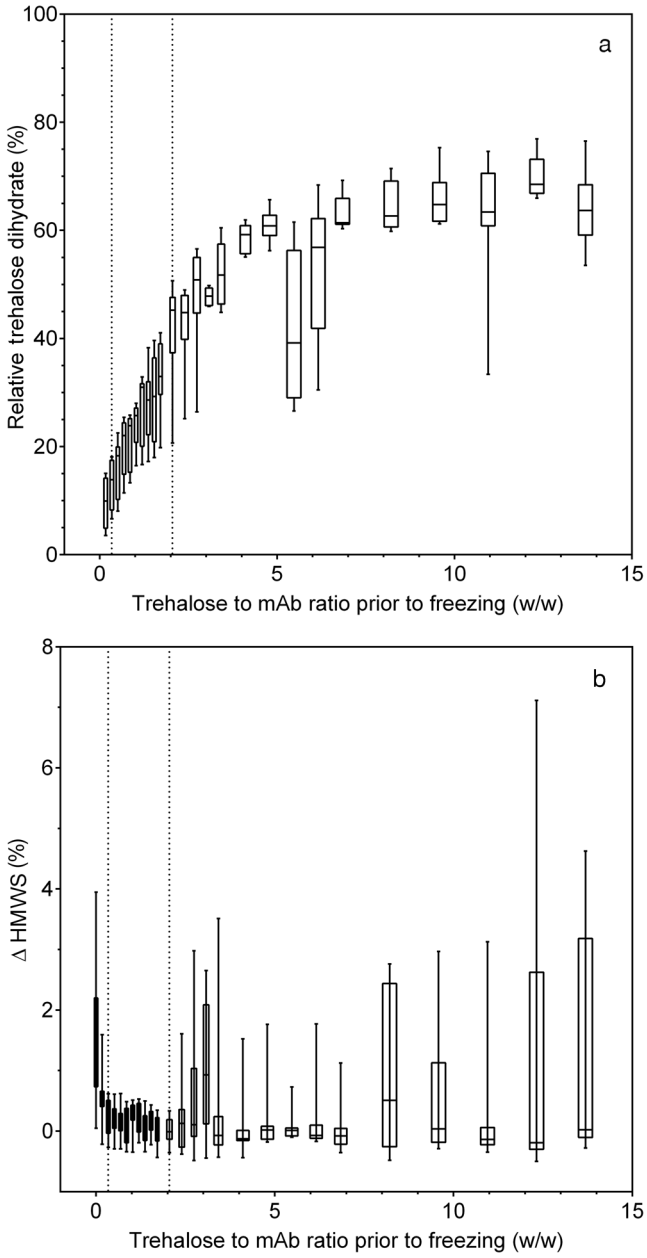


**Figure 9.** Box plots display the (a) percent of crystallized trehalose dihydrate, and (b) change in percent high molecular weight species as a function of total trehalose–mAb ratio (w/w). High molecular weight species was measured using HP-SEC and trehalose dihydrate concentration was determined using FT-NIR.

Interestingly, the mAb2 samples in the cooling rate study (Table 5) and formulation composition study (Figs. 8 and 9) have similar trehalose–mAb ratios; however, they do not have equivalent changes in aggregation and trehalose crystallization. This suggests that trehalose–mAb ratio is not the only factor that affects mAb stability. This difference most likely results from other components of formulation composition such as the buffer, buffer concentration, and pH. The mAb2 samples assessed in the cooling rate study contain 51 mM sodium phosphate at pH 6.2, and the comparable sample assessed

**Table 6.** Impact of mAb2–Trehalose Ratio on Protein Aggregation and Trehalose Crystallization

| Trehalose–mAb Ratio | FT-NIR Trehalose Dihydrate (Relative Percentage) | | HP-SEC Aggregate (Relative Percentage) | |
|---|---|---|---|---|
| % (w/v): % (w/v) | Average ± SD | Range (Minimum to Maximum) | Average ± SD | Range (Minimum to Maximum) |
| 0.00 | 0.00 ± 0.00 | 0.00–0.00 | 1.67 ± 1.06 | 0.05–3.95 |
| 0.17 | 9.70 ± 4.54 | 3.54–15.06 | 0.56 ± 0.50 | −0.22–1.59 |
| 0.34 | 13.20 ± 4.54 | 6.65–18.10 | 0.24 ± 0.30 | −0.27–0.62 |
| 0.51 | 16.20 ± 5.35 | 8.04–22.51 | 0.21 ± 0.27 | −0.29–0.61 |
| 0.68 | 20.40 ± 5.00 | 11.41–25.41 | 0.14 ± 0.24 | −0.29–0.62 |
| 0.86 | 21.20 ± 5.14 | 13.30–25.82 | 0.12 ± 0.30 | −0.34–0.49 |
| 1.03 | 24.30 ± 4.12 | 16.45–28.02 | 0.21 ± 0.26 | −0.35–0.51 |
| 1.20 | 27.50 ± 6.67 | 16.66–32.88 | 0.23 ± 0.26 | −0.19–0.53 |
| 1.37 | 27.30 ± 6.26 | 17.25–38.28 | 0.05 ± 0.25 | −0.34–0.50 |
| 1.54 | 28.90 ± 7.87 | 17.95–39.62 | 0.18 ± 0.21 | −0.23–0.43 |
| 1.71 | 32.80 ± 6.74 | 19.78–41.03 | 0.06 ± 0.26 | −0.43–0.35 |
| 2.05 | 41.10 ± 9.59 | 20.65–50.65 | −0.01 ± 0.20 | −0.35–0.33 |
| 2.40 | 42.40 ± 7.79 | 25.17–48.98 | 0.22 ± 0.62 | −0.38–1.61 |
| 2.74 | 49.00 ± 7.69 | 26.42–56.54 | 0.58 ± 1.04 | −0.48–2.98 |
| 3.08 | 47.80 ± 1.57 | 45.93–49.77 | 1.06 ± 1.09 | −0.45–2.65 |
| 3.42 | 52.30 ± 5.63 | 44.84–60.45 | 0.22 ± 0.98 | −0.43–3.51 |
| 4.11 | 58.70 ± 2.66 | 55.06–61.93 | 0.07 ± 0.60 | −0.44–1.53 |
| 4.79 | 60.90 ± 2.84 | 56.25–65.65 | 0.19 ± 0.64 | −0.18–1.77 |
| 5.48 | 41.70 ± 13.59 | 26.57–61.53 | 0.08 ± 0.27 | −0.10–0.73 |
| 6.16 | 53.00 ± 12.90 | 30.48–68.40 | 0.17 ± 0.65 | −0.17–1.77 |
| 6.85 | 63.10 ± 3.23 | 60.33–69.23 | 0.03 ± 0.46 | −0.35–1.12 |
| 8.22 | 64.20 ± 4.52 | 59.82–71.43 | 0.93 ± 1.36 | −0.48–2.76 |
| 9.58 | 65.70 ± 4.85 | 61.19–75.32 | 0.57 ± 1.12 | −0.29–2.97 |
| 10.95 | 61.80 ± 12.56 | 33.38–74.58 | 0.27 ± 1.16 | −0.35–3.13 |
| 12.32 | 69.70 ± 3.92 | 65.96–76.92 | 1.15 ± 2.75 | −0.50–7.12 |
| 13.68 | 64.00 ± 6.96 | 53.51–76.51 | 1.06 ± 2.09 | −0.28–4.63 |

Table 6 displays the change (Δ) in percent aggregation and relative percent of trehalose dihydrate for mAb2 samples after 12 months of storage at −20°C. Multiple (≥8) independent sample replicates were used to calculate statistical values (i.e., average, SD, minimum, and maximum).

in the formulation composition study contains 20 mM histidine hydrochloride at pH 6.0. It has been shown that sodium phosphate has a propensity to crystallize and stress proteins during frozen storage.[2,45] Presumably, crystallization of the sodium phosphate buffer is contributing to the observed differences in trehalose crystallization and mAb2 aggregation between the cooling rate and formulation composition studies. This could be a possible explanation for the instability observed in the cooling rate study for mAb2 samples that are within the range of stabilizing trehalose–mAb ratios (>0.2:1 and <2.4:1, w/w) established for mAb2 in the absence of a crystallizing buffer.

### *Practical Implications for Cooling Rate, Storage Temperature, and Formulation Composition*

This study investigated three parameters that can be controlled to optimize frozen stability of a protein: formulation composition, cooling rate, and frozen storage temperature. The tested ranges of the selected parameters are all pharmaceutically relevant and attainable. However, selection of the formulation for a specific product must also take into account stability of the liquid in the vial, processing equipment, transportation, storage, and other considerations. The resulting formulation may then place constraints upon the cooling rate and frozen storage temperature. The final cooling rate and storage temperature will be a balance of all three parameters. The results of this study shed light upon how these parameters can impact frozen storage stability.

### *Cooling Rate*

The effects of cooling rate on the development of robust freezing processes are of interest in the biopharmaceutical industry.[1,2,33,35] The results from this study demonstrate that high cooling rate (e.g., >100°C/min) leads to nucleation of supersaturated trehalose. Conversely, no changes were observed following freezing at the slow or intermediate cooling rates at any of the storage temperatures evaluated. Thus, freezing processes for trehalose formulations that implement lower cooling rates (≤1°C/min) are preferred. In developing a freezing process, it is also important to consider the maximum cooling rate small volumes may be exposed to within the bulk solution, as opposed to the average bulk cooling rate. The results from this study suggest that following nucleation, trehalose crystallization will propagate throughout the freeze concentrate. Thus, the maximum cooling rate is likely a more important factor than bulk cooling rate.

### *Storage Temperature*

The results from the storage temperatures study demonstrate that following fast cooling, less protein aggregation is observed at higher temperature (i.e., −8°C) relative to lower temperatures (−14°C and −20°C, respectively) for each of the three mAb formulations evaluated. Interestingly, this suggests that it is possible to decrease the rate and amount of protein aggregation that result from trehalose crystallization by increasing the storage temperature. The ability to support a

temperature excursion for a frozen storage unit that contains numerous bulk containers of drug substance can result in a tremendous cost savings for a company and ensure a constant supply of medicine for patients. Thus, evaluating stability in this temperature range has tremendous practical relevance for biopharmaceutical applications. However, it will be necessary to also monitor other critical product quality attributes, as chemical degradation rates typically increase at greater temperature.

Additionally, the results demonstrate that storage at a lower temperature (i.e., −40°C) below the glass transition temperature prevented trehalose crystallization and protein aggregation, regardless of cooling rate or formulation composition. Although storage at this temperature can be costly, it is an effective alternative to optimizing cooling rate or formulation composition and can enable faster development timelines for problematic molecules in early development.

### *Formulation Composition*

The results from the formulation composition study demonstrate that there is an optimal range of trehalose–mAb ratios (range: 0.2–2.4, w/w) that minimize trehalose crystallization and protein aggregation for the set of formulations and conditions evaluated. In similar systems, it is possible that the specific ratio range identified may be limited in application to the specific scale, freeze processes, storage temperatures, formulations, and molecule types evaluated. Thus, determination of optimal ratio ranges across different conditions may necessitate independent evaluations. However, the general finding that decreasing the trehalose–mAb ratios will generally decrease the amount of trehalose crystallization can be used to inform the development of formulations and processes that significantly differ from those described in this work. It is possible that protein aggregation may also occur if the trehalose concentration is too low, thus it is also important to ensure the there is sufficient trehalose to prevent protein aggregation that is independent of trehalose crystallization.

The results from the excipient screen study also suggest that noncrystallizing stabilizers (e.g., sucrose) should be considered as an alternative to trehalose if optimal cooling and storage temperature conditions cannot be achieved. However, the limitations of the alternative excipient should be considered as well. For example, sucrose is a reducing sugar and can lead to glycation at lower pH.[46,47] Thus, it is important to select pH and buffer excipients that control for pH in liquid and frozen formulations. For example, sodium phosphate should be avoided as it has been shown to crystallize and result in acidic pH during frozen storage.[2,45]

## CONCLUSIONS

This study was designed to investigate the impact of cooling rate, storage temperature, and formulation composition on protein stability and trehalose crystallization. The results demonstrate that cooling rate and storage temperature have an effect on protein stability in trehalose mAb formulations. Samples frozen at the fast cooling rate (>100°C/min) exhibited increases in protein aggregation, whereas samples frozen at the slow and intermediate cooling rate remained unaffected. Additionally, samples stored at lower temperatures (i.e., −20°C) demonstrated a greater extent of aggregation over the course of the 12-month study.

The formulation composition studies demonstrate that excessively high trehalose–mAb ratios (≥2.4, w/w) result in trehalose crystallization and protein aggregation, but insufficient trehalose–mAb ratios (≤0.2, w/w) did not provide adequate cryoprotection. However, no increases in trehalose crystallization or protein aggregation were observed following 12-month storage at −40°C for any of the formulation compositions evaluated. These results suggest that trehalose crystallization and protein aggregation can also be prevented by storing formulations at or below the glass transition temperature.

A quantitative comparison of trehalose crystallization following long-term frozen storage across different samples shows that the amount of trehalose that remains in the amorphous form is adequate to protect concentrated proteins from forming unfavorable interactions with ice crystals along the interfacial ice aqueous surface. This leads us to believe that the crystallization of concentrated trehalose introduces additional, unique stresses that destabilize the frozen solution and contribute to protein aggregation. These stresses may include phase separation of the protein from the amorphous cryoprotectant and introduction of a potentially denaturing surface, that is, trehalose dihydrate crystals.

Future work will focus on investigating the microscopic phase separation of IgG1 monoclonal antibodies from cryoprotectants in frozen media and their rates of rearrangement (i.e., diffusion), and the effects of crystallized carbohydrates surfaces on protein stability to assess the impact of these unique physical changes that result from nonbuffer component crystallization in frozen aqueous solutions.

## ACKNOWLEDGMENTS

The authors would like to acknowledge Tim Kamerzell, Tom Bewley, Al Stern, Jun Liu, Steve Shire, Y. John Wang, Chung Hsu, Bruce Kabakoff, and Vikas K. Sharma for useful discussions and feedback. We would also like to thank Chris Heynes for providing lyophilization support.

## REFERENCES

**1.** Avis KE, Wagner CM, Eds. 1999. Cryopreservation—Applications in pharmaceuticals and biotechnology. Denver, Colorado: Interpharm Press.

**2.** Bhatnagar BS, Bogner RH, Pikal MJ. 2007. Protein stability during freezing: Separation of stresses and mechanisms of protein stabilization. Pharm Dev Technol. 12:505–523.

**3.** Rosenberg AS. 2006. Effects of protein aggregates: An immunologic perspective. AAPS J 8:E501–E507.

**4.** Carpenter JF, Crowe JH. 1989. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. Biochemistry 28:3916–3922.

**5.** Green JL, Angell CA. 1989. Phase relations and vitrification in saccharide-water solutions and the trehalose anomaly. J Phys Chem 93:2880–2882.

**6.** Piedmonte DM, Summers C, McAuley A, Karamujic L, Ratnaswamy G. 2007. Sorbitol crystallization can lead to protein aggregation in frozen protein formulations. Pharm Res 24:136–146.

**7.** Beattie GM, Crowe JH, Lopez AD, Cirulli V, Ricordi C, Hayek A. 1997. Trehalose: A cryoprotectant that enhances recovery and preserves function of human pancreatic islets after long-term storage. Diabetes 46:519–523.

**8.** Crowe LM, Reid DS, Crowe JH. 1996. Is trehalose special for preserving dry biomaterials? Biophys J 71:2087–2093.
**9.** Leslie SB, Israeli E, Lighthart B, Crowe JH, Crowe LM. 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl Environ Microbiol 61:3592–3597.
**10.** Willart JF, De Gusseme A, Hemon S, Descamps M, Leveiller F, Rameau A. 2002. Vitrification and polymorphism of trehalose induced by dehydration of trehalose dihydrate. J Phys Chem 106:3365–3370.
**11.** Aldous BJ, Auffret AD, Franks F. 1995. The crystallization of hydrates from amorphous carbohydrates. Cryo-Letters 16:181–186.
**12.** Sundaramurthi P, Patapoff TW, Suryanarayanan R. 2010. Crystallization of trehalose in frozen solutions and its phase behavior during drying. Pharm Res 27:2374–2383.
**13.** Sundaramurthi P, Suryanarayanan R. 2010. Influence of crystallizing and non-crystallizing cosolutes on trehalose crystallization during freeze-drying. Pharm Res 27:2384–2393.
**14.** Singh SK, Kolhe P, Mehta AP, Chico SC, Lary AL, Huang M. 2011. Frozen state storage instability of a monoclonal antibody: Aggregation as a consequence of trehalose crystallization and protein unfolding. Pharm Res 28:873–885.
**15.** Arakawa T, Prestrelski SJ, Kenney WC, Carpenter JF. 2001. Factors affecting short-term and long-term stabilities of proteins. Adv Drug Deliv Rev 46:307–326.
**16.** Bhatnagar BS, Pikal MJ, Bogner RH. 2008. Study of the individual contributions of ice formation and freeze-concentration on isothermal stability of lactate dehydrogenase during freezing. J Pharm Sci 97:798–814.
**17.** Franks F, Hatley RHM. 1991. Stability of proteins at subzero temperatures: Thermodynamics and some ecological consequences. Pure Appl Chem 63:1367–1380.
**18.** Franks F. 1995. Protein destabilization at low temperatures. Adv Protein Chem 46:105–139.
**19.** Franks F, Hatley RH, Friedman HL. 1988. The thermodynamics of protein stability. Cold destabilization as a general phenomenon. Biophys Chem 31:307–315.
**20.** Hsu CC, Nguyen HM, Yeung DA, Brooks DA, Koe GS, Bewley TA, Pearlman R. 1995. Surface denaturation at solid-void interface—A possible pathway by which opalescent particulates form during the storage of lyophilized tissue-type plasminogen activator at high temperatures. Pharm Res 12:69–77.
**21.** Lashmar U, Vanderburgh M, Little S. 2007. Bulk freeze-thawing of macromolecules: Effects of cryoconcentration on their formulation and stability. BioProcess Tech 5:44–54.
**22.** Strambini GB, Gabellieri E. 1996. Proteins in frozen solutions: Evidence of ice-induced partial unfolding. Biophys J 70:971–976.
**23.** Strambini GB, Gonnelli M. 2007. Protein stability in ice. Biophys J 92:2131–2138.
**24.** Sharma VK, Kalonia DS. 2004. Effect of vacuum drying on protein-mannitol interactions: The physical state of mannitol and protein structure in the dried state. AAPS PharmSciTech 5:E10.
**25.** Connolly B, Patapoff TW, Wang YJ, Moore JM, Kamerzell TJ. 2010. Vibrational spectroscopy and chemometrics to characterize and quantitate trehalose crystallization. Anal Biochem 399:48–57.
**26.** Izutsu K, Yoshioka S, Terao T. 1993. Decreased protein-stabilizing effects of cryoprotectants due to crystallization. Pharm Res 10:1232–1237.
**27.** Eckhardt BM, Oeswein JQ, Bewley TA. 1991. Effect of freezing on aggregation of human growth hormone. Pharm Res 8:1360–1364.
**28.** Miller DP, de Pablo JJ, Corti H. 1997. Thermophysical properties of trehalose and its concentrated aqueous solutions. Pharm Res 14:578–590.
**29.** Yalkowsky SH. 2003. Handbook of aqueous solubility data. Boca Raton, Florida: CRC Press.
**30.** Cavatur RK, Vemuri NM, Pyne A, Chrzan Z, Toledo-Velasquez D, Suryanarayanan R. 2002. Crystallization behavior of mannitol in frozen aqueous solutions. Pharm Res 19:894–900.
**31.** Izutsu K, Yoshioka S, Terao T. 1994. Effect of mannitol crystallinity on the stabilization of enzymes during freeze-drying. Chem Pharm Bull (Tokyo) 42:5–8.
**32.** Sundaramurthi P, Suryanarayanan R. 2011. Predicting the crystallization propensity of carboxylic acid buffers in frozen systems—Relevance to freeze-drying. J Pharm Sci 100:1288–1293.
**33.** Cao E, Chen Y, Cui Z, Foster PR. 2003. Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions. Biotechnol Bioeng 82:684–690.
**34.** Dong J, Hubel A, Bischof JC, Aksan A. 2009. Freezing-induced phase separation and spatial microheterogeneity in protein solutions. J Phys Chem B 113:10081–10087.
**35.** Miller MA, Rodrigues MA, Glass MA, Singh SK, Johnston KP, Maynard JA. 2013. Frozen-state storage stability of a monoclonal antibody: Aggregation is impacted by freezing rate and solute distribution. J Pharm Sci 102:1194–1208.
**36.** Tang XL, Pikal MJ. 2005. Measurement of the kinetics of protein unfolding in viscous systems and implications for protein stability in freeze-drying. Pharm Res 22:1176–1185.
**37.** Soesanto T, Williams MC. 1981. Volumetric interpretation of viscosity for concentrated and dilute sugar solutions. J Phys Chem 85:3338–3341.
**38.** Roos YH, Karel M. 1991. Amorphous state and delayed ice formation in sucrose solutions. Int J Food Sci Tech 26:553–566.
**39.** Sundaramurthi P, Suryanarayanan R. 2010. Trehalose crystallization during freeze-drying: Implications on lyoprotection. J Phys Chem Lett 1:510–514.
**40.** Pyne A, Surana R, Suryanarayanan R. 2002. Crystallization of mannitol below $T_g$' during freeze-drying in binary and ternary aqueous systems. Pharm Res 19:901–908.
**41.** Liao X, Krishnamurthy R, Suryanarayanan R. 2005. Influence of the active pharmaceutical ingredient concentration on the physical state of mannitol—Implications in freeze-drying. Pharm Res 22:1978–1985.
**42.** Cleland JL, Lam X, Kendrick B, Yang J, Yang TH, Overcashier D, Brooks D, Hsu C, Carpenter JF. 2001. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. J Pharm Sci 90:310–321.
**43.** Wang B, Tchessalov S, Warne NW, Pikal MJ. 2009. Impact of sucrose level on storage stability of proteins in freeze-dried solids: I. Correlation of protein-sugar interaction with native structure preservation. J Pharm Sci 98:3131–3144.
**44.** Wang B, Tchessalov S, Cicerone MT, Warne NW, Pikal MJ. 2009. Impact of sucrose level on storage stability of proteins in freeze-dried solids: II. Correlation of aggregation rate with protein structure and molecular mobility. J Pharm Sci 98:3145–3166.
**45.** Pikal-Cleland KA, Cleland JL, Anchordoquy TJ, Carpenter JF. 2002. Effect of glycine on pH changes and protein stability during freeze-thawing in phosphate buffer systems. J Pharm Sci 91:1969–1979.
**46.** Gadgil HS, Bondarenko PV, Pipes G, Rehder D, Mcauley A, Perico N, Dillon T, Ricci M, Treuheit M. 2007. The LC/MS analysis of glycation of IgG molecules in sucrose containing formulations. J Pharm Sci 96:2607–2621.
**47.** Fischer S, Hoernschemeyer J, Mahler HC. 2008. Glycation during storage and administration of monoclonal antibody formulations. Eur J Pharm Biopharm 70:42–50.