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

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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

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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