Sorbitol Crystallization-Induced Aggregation in Frozen mAb Formulations

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ABSTRACT: Sorbitol crystallization-induced aggregation of mAbs in the frozen state was evaluated. The effect of protein aggregation resulting from sorbitol crystallization was measured as a function of formulation variables such as protein concentration and pH. Long-term studies were performed on both IgG1 and IgG2 mAbs over the protein concentration range of 0.1–120 mg/mL. Protein aggregation was measured by size-exclusion HPLC (SE-HPLC) and further characterized by capillary-electrophoresis SDS. Sorbitol crystallization was monitored and characterized by subambient differential scanning calorimetry and X-ray diffraction. Aggregation due to sorbitol crystallization is inversely proportional to both protein concentration and formulation pH. At high protein concentrations, sorbitol crystallization was suppressed, and minimal aggregation by SE-HPLC resulted, presumably because of self-stabilization of the mAbs. The glass transition temperature (T_g') and fragility index measurements were made to assess the influence of molecular mobility on the crystallization of sorbitol. T_g' increased with increasing protein concentration for both mAbs. The fragility index decreased with increasing protein concentration, suggesting that it is increasingly difficult for sorbitol to crystallize at high protein concentrations. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:686–697, 2015

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INTRODUCTION

A strategy commonly used in the biopharmaceutical industry to extend the shelf life of mAb formulations is to freeze process intermediates such as drug substance. Although the frozen state can afford improved stability, it is well documented that freezing can subject proteins to several potentially destabilizing stresses: protein exposure to the ice surface, high concentrations of solutes in the freeze concentrate, changes in the pH of the freeze concentrate (due to the crystallization of buffer salts that become concentrated upon freezing, thereby exceeding their solubility), and cold denaturation.[1–4] Presumably, colder temperatures (ca. −80°C) afford improved long-term stability because of reduced molecular mobility in the frozen state. Because limited molecular mobility can translate into decreased rates of chemical and physical degradation, ideally samples should be stored in the glassy state (below the T_g'). However, practical requirements often necessitate that process intermediates are stored in warmer, walk-in freezers.

Excipients are used to stabilize a protein in the frozen state by mitigating the aforementioned stresses. However, for an excipient to stabilize a protein during freezing and throughout frozen storage, the excipient must remain in the same amorphous phase as the protein.[5,6] It is important to understand the phase transitions that an excipient may undergo during frozen storage in order to ensure adequate protein stability. Moreover, monitoring for excipient phase transitions during freeze drying is important to ensure protein stability, as was reported by Sundaramurthi et al.[7] who discovered the crystallization of trehalose during the annealing step of lyophilization. Parameters critical to effectively stabilize proteins in the frozen state include protein concentration and recommended storage temperature, which can be influenced by other factors such as dosing regimen and large-scale freezer availability, respectively.

The excipient sorbitol affords long-term stability at 2°C–8°C and is commonly used to stabilize liquid parenteral formulations, such as Neupogen® (filgrastim)[8] and Neulasta® (pegfilgrastim).[9] Although sorbitol was considered an amorphous excipient in the frozen state,[10,11] we previously reported that a sorbitol-containing formulation (2 mg/mL N-terminal Fc (fragment, crystallizes easily) fusion protein) crystallized upon long-term storage at −30°C. Upon crystallization, sorbitol undergoes phase separation from the protein, which ultimately induces protein aggregation.[12]

Building upon our previous work, here we assessed sorbitol crystallization-induced aggregation in mAbs primarily as a function of protein concentration (from 0.1 to 120 mg/mL). We also monitored the effect of pH on sorbitol crystallization and consequent protein aggregation. The covalent character of the resulting aggregate was also assessed. The role of molecular

Abbreviations used: SE-HPLC, size-exclusion HPLC; CE-SDS, capillary-electrophoresis SDS; DSC, differential scanning calorimetry; T_g', glass transition temperature; XRD, X-ray diffraction.

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mobility in the propensity of sorbitol formulations to crystallize was explored by measuring the glass transition temperatures of sorbitol-containing formulations over a 1000-fold range of protein concentration. In addition to routine T_g' measurements, measuring fragility index by differential scanning calorimetry (DSC) has been used to understand dynamics in the supercooled liquid.[13–15] Approaching the glass transition from the liquid side, it has been observed that supercooled liquids differ in the rate of change of viscosity with temperature. This difference in rate of change of viscosity among supercooled liquids as a function of temperature is related to differences in dynamic heterogeneity. This observation has led to the classification of supercooled fluids in terms of their fragility as "kinetic fragility."[16] When approaching the glass transition, fragile liquids show a faster change in viscosity with temperature than strong liquids. Correlations between fragility and other glass properties have been sought and tested over the years to better understand the glassy state.[17–20] In this paper, we report fragility index measurements of sorbitol-containing formulations over a broad concentration range. Such measurements can improve our understanding of the dynamic heterogeneity of systems with different protein concentrations and can also shed light on the potential for identifying formulation conditions where sorbitol crystallization may occur. The goal of this work was to understand sorbitol crystallization-induced aggregation, which may then provide the flexibility to freeze process intermediates of sorbitol-containing formulations.

MATERIALS AND METHODS

Sample Preparation and Storage

The IgG1 and IgG2 molecules were expressed in mammalian cells and purified at Amgen Inc. (Thousand Oaks, California). In studies performed with the IgG1 molecule, a 70 mg/mL protein solution was diluted with formulation buffer (10 mM acetate at pH 5.2 with 274 mM sorbitol) to the desired protein concentrations. Protein concentrations were verified by UV–vis spectroscopy at 280 nm using the appropriate extinction coefficient. A 1% stock solution of polysorbate 20 was added to each formulation to a final polysorbate 20 concentration of 0.004% (w/v). For the IgG2, protein concentrations greater than 30 mg/mL were achieved by concentration in an Amicon Centricon Centrifugal Filter Device (Millipore, Billerica, Massachusetts) with a molecular weight cutoff of 10 K. For frozen stability studies and subsequent analysis by size-exclusion HPLC (SE-HPLC) and capillary-electrophoresis SDS (CE-SDS), volumes of the formulated protein ranging from 100 μL to 1.25 mL (depending on the study) were stored in 3 cc glass vials. Subambient DSC was performed on 40 μL aliquots in 50-μL aluminum DSC pans (PerkinElmer, Waltham, Massachusetts). Time course studies were performed in both glass vials (for SE-HPLC analysis) and DSC pans (for subambient DSC measurements). Samples were stored at –30°C. We originally intended to use the same primary container for both the DSC and SE-HPLC analyses, but saw high levels of clipping over time in the DSC pans (particularly in the 1 mg/mL samples at both –30°C and the –20°C controls), presumably because of the metal composition of the pans. It is for this reason that stability samples were stored separately in glass vials.

Stability samples formulated in sorbitol were also stored at –20°C as a negative control for sorbitol crystallization, as our previous work showed that sorbitol crystallizes only upon storage at –30°C.[12] Some samples formulated in sucrose (in place of sorbitol) were included at limited time points, stored at both –20°C and –30°C, to assess aggregation levels in the presence of a stabilizing excipient that remains in the amorphous phase.

Table 1. All Formulations are 10 mM Acetate and 0.004% (w/v) Polysorbate 20

Excipient	pH	Protein Concentration (mg/mL)	Isotype
274 mM sorbitol	5.2	0.1, 1, 10, 30, 70	IgG2
274 mM sorbitol	5.2	40, 50, 60, 80, 100, 120	IgG2
274 mM sorbitol	5.2	0.1, 1, 10, 30, 70	IgG1
None	4.5	1	IgG1
300 mM sorbitol	4.5	1	IgG1
None	5.0	1	IgG1
300 mM sorbitol	5.0	1	IgG1
300 mM mannitol	5.0	1	IgG1
None	5.5	1	IgG1
300 mM sorbitol	5.5	1	IgG1

The sorbitol crystallization and protein aggregation of an IgG1 and IgG2 were monitored over a concentration range of 0.1–70 mg/mL. Six additional protein concentrations were analyzed for the IgG2 samples with the 70 mg/mL formulation chosen as a midpoint. In total, nine formulations were studied, including both IgG1 and IgG2 formulations ranging in protein concentration from 0.1 to 120 mg/mL and pH from 4.5 to 5.5. Proteins were formulated with 10 mM acetate at the protein concentration and pH presented in Table 1.

Calorimetry

The subambient DSC method used to detect crystalline sorbitol melts has been described previously.[12] In brief, 40 μL samples were semihermetically sealed in DSC pans and stored at –30°C until analysis, when they were transferred on dry ice to a DSC cell (Pyris 1 DSC instrument, PerkinElmer, Massachusetts) that was precooled to –70°C. In contrast to traditional subambient DSC methods where liquid samples are loaded and frozen by rapidly cooling to –70°C, this method preserves the frozen state of the samples, which is necessary to capture the crystallization of sorbitol. The melting endotherms indicating sorbitol crystallization were integrated for total area. Sorbitol formulations stored at –20°C and sucrose formulations at both –20°C and –30°C were included and analyzed as negative controls for sorbitol crystallization. A standard method was used to determine glass transition temperatures: a sample was aliquoted into a DSC pan, which was then placed in the DSC cell, where both the sample and DSC cell were at room temperature. Samples were cooled to –70°C at a rate of 60°C/min, held at –70°C for 1 min before heating to +15°C at a rate of 5°C/min. Baseline slopes of the heating scans were optimized, and glass transition temperatures were calculated using the Pyris thermal analysis software for Windows, version 3.81 (PerkinElmer). The reported T_g' is the temperature at half of the change in heat capacity (C_P = J/g °C) over the temperature range of the baseline shift.

Chromatographic Separations

Size-exclusion HPLC was used as the primary stability indicating assay because physical instability (e.g. aggregation) is often