Overcoming Long Reconstitution Times of High Concentration Lyophilized Protein Formulations

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Highly concentrated therapeutic protein products stabilized by lyophilization often have undesirably long and variable reconstitution times. The overall objective of this work was to identify factors governing the reconstitution times of these formulations. Fundamental understanding of these factors will reduce the barrier to efficient formulation and process design presented by poorly reconstituting drug products.

Properties of the lyophilized “cake” that have been previously implicated in influencing reconstitution are cake wettability, liquid penetration into cake, cake disintegration, and cake porous structure. Methods to quantify these properties of intact cakes in vials were refined and further developed. The effects of formulation and processing on each of these properties were investigated to understand their role in mediating reconstitution times. Additionally, the role of viscosity near the surface of the dissolving cake (“concentrated formulation viscosity”) in influencing reconstitution time was also examined.

While “concentrated formulation viscosity” was the key factor governing reconstitution of amorphous cakes, crystallinity and larger pores governed reconstitution of partially crystalline cakes. Cake hydration results in a viscous region at the dissolving cake surface in contact with the reconstitution fluid. This viscous layer provides resistance to the diffusion of the reconstitution fluid through the layer into the remaining undissolved cake, delaying wetting and hydration of the cake core and prolonging the reconstitution time. Dissolution of amorphous cakes proceeded via hydration followed by gradual surface erosion. Partial crystallinity and larger pores tremendously improved wettability and liquid penetration in partially crystalline cakes, ultimately resulting in small, well dispersed cake pieces. Thus, cake dissolution involved wetting, disintegration followed by hydration. The “concentrated formulation viscosity” is expected to
influence reconstitution of both amorphous and partially crystalline cakes. However, in the latter case, there is an increase in surface area of the dispersed solids, lowering the barrier provided by the viscous layer.

Based on these findings, both formulation and processing conditions can be tailored to achieve faster reconstitution. Including a crystallizable excipient and incorporating an annealing step to facilitate its crystallization and to promote larger pores were beneficial. A viscosity lowering excipient may be advantageous but this approach needs to be explored further.
Overcoming Long Reconstitution Times of High Concentration Lyophilized Protein Formulations

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Overcoming Long Reconstitution Times of High Concentration Lyophilized Protein Formulations

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Dedication

To Aai and Baba for their unconditional love and unwavering support
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Chapter 1

Introduction, Aims and Organization of the Dissertation
**Introduction**

Recombinant DNA technology has enabled the commercialization of many protein therapeutics over the past three decades. Protein therapeutics are used for treating a variety of diseases including cancers and chronic conditions such as arthritic diseases, asthma and psoriasis. Subcutaneous delivery is a common administration route for biological products and is often preferred over conventional intravenous delivery owing to convenient patient self-administration and reduced treatment costs [1, 2]. The typical injection volume for sub-cutaneous administration is limited to 1-1.5 ml. In order to accommodate a high drug dose (particularly for monoclonal antibodies) in a low injection volume, these therapeutics are formulated at protein concentrations typically > 50 mg/ml and as high as 200 mg/ml. Since high concentration liquid protein products often have storage stability challenges [2-4], lyophilization (freeze-drying) is commonly used to overcome these stability related issues [2, 5, 6]. The lyophilization process transforms aqueous protein solutions into a solid, porous cylinder known as a “cake”. Prior to patient administration, the lyophilized cake is reconstituted into a solution by adding an appropriate fluid (usually Sterile Water for Injection). The endpoint of reconstitution as described by the United States Pharmacopeia is the complete dissolution of solids after addition of the reconstitution fluid [7].

Rapid reconstitution is an important product quality attribute for lyophilized formulations. However, lyophilized products containing highly concentrated proteins often have long and variable reconstitution times [8-13]. Reconstitution times of some FDA-approved high concentration lyophilized protein products range from less than 5 minutes (Raptiva®[14], Nucala® [15]) to as long as 15-40 minutes (Ilaris® [16], Xolair® [11], Cosentyx® [12], and Cimzia® [10]). Long reconstitution times can be problematic, particularly for self-administered drug products. For
these products, long reconstitution times are likely associated with the risk of immunogenicity due to undissolved proteinaceous small particles if the patient tries to self-administer the drug prior to complete reconstitution. Administration of the partially reconstituted product can also lead to incomplete dosing. In addition to being problematic from a patient perspective, long reconstitution times are also frustrating for the health care provider such as doctors, nurses or pharmacists in today’s face-paced clinical settings. As biosimilars and additional competing protein products come to market, reduced reconstitution time is expected to be a factor in product differentiation.

Research efforts have recently been directed towards understanding the causes of long reconstitution time and identifying ways to reduce it [9, 13, 17-19]. Properties of the lyophilized cake that have been previously implicated in influencing reconstitution are the presence of partial crystallinity in the final cake [8, 9, 20], cake wettability [8, 18], liquid penetration into the cake [8], cake dispersibility/disintegration [8, 18], cake solids rehydration rate [18], cake specific surface area (SSA) and cake porous structure [8, 13]. These cake properties are influenced by both formulation composition and processing conditions. A detailed understanding of the effects of each of these properties on reconstitution time as mediated by formulation and processing will enable pharmaceutical scientists to overcome the challenge of long reconstitution times.

Effect of partial crystallinity on reconstitution time

While evaluating multiple approaches to improve the reconstitution of an Fc-fusion protein formulated with sucrose and mannitol, Cao et al. found that partially crystalline lyophilized cakes reconstituted in 3 minutes when compared to fully amorphous cakes which took as long as 13
The authors attributed the significant reduction in reconstitution time to crystalline mannitol (identified using X-ray powder diffraction; XRPD). They observed that during reconstitution, partially crystalline cakes “absorbed” the reconstitution fluid much faster than their amorphous counterparts. The authors surmised that partially crystalline cakes were more wettable, making the cake core more accessible to the reconstitution fluid. They also noted that sometimes the partially crystalline cakes disintegrated on exposure to the reconstitution fluid resulting in an increased surface area for faster dissolution. Although insightful, the inferences regarding the mechanisms by which crystalline mannitol lowered reconstitution time were not supported by quantitative experimental data. Thus, underlying reasons for the improvement offered by crystalline mannitol are yet to be fully elucidated. Nonetheless, the work by Cao et al. pointed to a potential relationship between mannitol crystallinity and cake properties such as cake wettability, penetration of reconstitution fluid into cakes and cake disintegration.

The same authors also explored annealing for its ability to improve reconstitution. Annealing involves holding the freeze concentrate at a specific temperature above the glass transition temperature of the freeze concentrate ($T_g'$) for a certain period of time to facilitate the crystallization of excipients and to promote larger ice crystals. In that study, although the annealed samples showed traces of crystalline mannitol, no improvements were noted in reconstitution time. The crystallinity in the annealed samples was visible under polarized light microscope but undetectable using XRPD. Based on these observations the author speculated that the degree of crystallinity in freeze-dried cakes had to be in substantial amounts to impact reconstitution.
Effect of cake wettability, disintegration and rehydration on reconstitution time

Sane et al. attempted to elucidate the role of cake wettability, disintegration and hydration during reconstitution of amorphous cakes of a monoclonal antibody formulated at increasing protein concentrations with sucrose as the lyoprotectant [18]. The authors introduced methods to quantify the wettability and disintegration rates of lyophilized cakes [18]. Increasing the protein concentration from 0-85 mg/ml resulted in an increase in the reconstitution time. At protein concentrations ranging between 1-30 mg/ml, the wettability of cake solids (assessed by measuring the contact angle of an aqueous drop on compacted cake solids) increased with protein concentration. However, in the protein concentration range associated with long reconstitution times (> 50 mg/ml), the contact angle remained constant. The rates of cake disintegration and hydration were found to decrease with increasing protein concentration. However, in this work, as in most others [13, 17], the increase in protein concentration was accompanied by a concomitant increase in the % total solids, which can also affect reconstitution time [3, 9]. Protein-to-sugar ratio was suggested as a potentially more important factor than % total solids, but the conclusion was not strong. Hence, longer reconstitution times associated with higher protein concentration could be due to increased total solids, higher protein-to-sugar ratio, increased protein concentration, or a combination. Isolating the individual contribution of each of these factors would lead to better strategies for reducing reconstitution times. Furthermore, the methods for characterizing cake properties developed by Sane et al. relied on either formulations lyophilized in glass tubes (rather than in vials) or on samples that were crushed and removed from the vial for analyses. In doing so, the measured cake properties are not representative of the cake in the vial. Characterization methods that reduce sample handling by making measurements on intact cakes without disrupting the cake structure during measurement are expected to be more meaningful to actual products.
**Effect of specific surface area (SSA) on reconstitution time**

A lower surface area of the lyophilized cake was suggested to contribute to poor rehydration behavior and decreased dissolution rate, thereby prolonging the reconstitution time [21]. Hence, investigation of the effect of cake SSA on reconstitution time has been the subject of many studies. In the study by Cao et al. [9], both fast reconstituting partially crystalline cakes and slow reconstituting amorphous cakes had SSA of 1 m²/g. Moreover, there was no evident correlation between protein concentration and SSA, indicating that SSA did not influence the reconstitution time. The lack of correlation between SSA and reconstitution time was also confirmed in other studies for amorphous cakes [13, 17, 18]. Thus, there is enough prior evidence establishing that reconstitution time is independent of the cake SSA.

**Effect of cake porous structure on reconstitution time**

It is generally acknowledged that more porous cakes (i.e., greater porosity) with larger sized pores reconstitute faster [3, 20], based largely on scanning electron microscopic images. Hence, subsequent research efforts involved quantifying cake porous structure and evaluating the relationship between these quantitative measures of cake structure and reconstitution time. The size and morphology of ice crystals that form during the freezing portion of lyophilization largely determine the porous structure of the cake [22]. The pore size within the freeze-dried cake has been related to the specific surface area of the cake as shown in Eq. (1), where r is the pore radius, ρ₅ is the density of the solid and ε is the porosity. Using Eq. (1), specific surface area determined from Brunauer–Emmet–Teller (BET) gas adsorption has been used for many years as an indirect measure of porous structure.
In one study, Geidobler et al. controlled the ice crystal size by employing different freezing protocols, namely, ramp freezing following either spontaneous ice nucleation or controlled ice nucleation at -5°C and precooled shelf method [17]. No meaningful relationship was found between specific surface area and reconstitution time. In fact, contrary to the expectation, cakes with lower SSA obtained by controlled nucleation reconstituted faster. Since controlled nucleation, which results in larger pores, led to a significant reduction in the reconstitution time, it suggests that cake porous structure influences reconstitution time in some way that SSA does not capture. Using high pressure mercury intrusion porosimetry, Beech et al. studied the influence of cooling profile on the reconstitution time of amorphous cakes containing 50 and 200 mg/ml bovine serum albumin (BSA) as mediated by the SSA and pore size distribution. For a given formulation (50 or 200 mg/ml BSA), no significant differences were observed either in the reconstitution time or in the SSA as a function of the cooling protocol. While the cooling profiles produced very different pore size distributions, there was no effect on reconstitution time. However, formulation did influence reconstitution time, with the cakes containing 200 mg/ml BSA reconstituting in about 40 minutes compared to 2 minutes for cakes containing 50 mg/ml BSA, regardless of the cooling protocol. Although the SSAs for the two formulations were not significantly different, the pores in the 200 mg/ml BSA formulation were smaller than in the 50 mg/ml BSA formulation. The authors concluded that larger sized pores with an open, well connected structure facilitated faster reconstitution of cakes containing 50 mg/ml BSA. In this case, cake porous structure did have an impact on reconstitution. However, there are other variables such as % total solids that were not ruled out as the cause for the reconstitution time difference.
The above examples establish that SSA is not an adequate measure of cake porous structure, at least as far as reconstitution is concerned. This is expected because BET measurements yield a single value for SSA which gives an average value for the pore size as per Eq. (1). However, there is a distribution of different sized pores in the cake and this pore size distribution is possibly a better indicator of reconstitution behavior than the average pore diameter as suggested from the data by Beech et al. Furthermore, both BET and MIP analyses were performed on crushed cakes which are not representative of the intact cake structure. This further highlights the need for a fast and simple technique capable of in-situ analysis of intact cakes. The technique should also be capable of quantifying a variety of measures of porous structure such as pore size distribution, average pore diameter, total pore volume, porosity and bulk density for a complete understanding of the detailed effects of that structure on reconstitution time. The results also suggest that cake porous structure is not the sole parameter influencing reconstitution time. Hence, there is a scope to better define the relationship between reconstitution time and cake porous structure and to identify which measure of cake structure, if any, best correlates with reconstitution.

**Objective and Aims**

The overall objective of this work is to identify factors governing the reconstitution times of highly concentrated lyophilized protein formulations. Fundamental understanding of these factors will provide information critical to efficient formulation and process design with the end goal of overcoming long reconstitution times.

*The specific aims are:*
1. Develop novel methods to characterize cake properties of intact cakes in vials.

2. Examine the role of formulation and processing in influencing reconstitution time as mediated by the cake properties.

3. Identify the key factors governing reconstitution time of highly concentrated lyophilized protein formulations.

**Organization of the Dissertation**

**Chapter 2** aims to understand the mechanisms by which partial crystallinity of lyophilized cakes lowers reconstitution time. Novel methods were developed for quantifying the effect of crystalline mannitol on cake properties influencing reconstitution, specifically, wettability of the cake solids, liquid penetration into the cake and cake disintegration. Amorphous and partially crystalline cakes of the same composition were obtained by varying the freeze-drying conditions, particularly, the freezing rate (slow vs. fast), annealing (annealed vs. unannealed), and primary drying (aggressive vs. conservative). Mannitol crystallinity was quantified using X-ray powder diffractometry.

Phase separation of crystalline mannitol from the amorphous, protein rich matrix improved wettability of the cake solids and promoted penetration of the reconstitution fluid into the cake interior. The partially crystalline cakes offered less resistance to crushing in the dry state than the amorphous cakes. Crystalline mannitol provided “weak points” in the freeze-dried cakes, potentially enabling easier cake disintegration upon addition of the reconstitution fluid. There was no evident correlation between the degree of crystallinity and reconstitution time. While crystalline
mannitol generally decreased reconstitution time by favorably affecting the cake properties influencing reconstitution, it did not always reduce reconstitution time. Cake porous structure and foaming during reconstitution also seemed to be major contributing factors.

Chapter 3 presents a detailed investigation of the role of formulations and processing in influencing cake porous structure. Low pressure mercury intrusion porosimetry (LP-MIP) was explored as a characterization technique to provide quantitative measures of the porous structure of intact cakes. The T_g’ and viscosity of the freeze concentrate were found to be formulation dependent factors influencing mobility and hence ice crystal growth. A longer time to reach the T_g’ (i.e., a longer residence time in the more “mobile” state above T_g’) and lower viscosity of the freeze-concentrate promoted ice crystal growth producing cakes with larger pores. Both these mobility governing factors were not just dependent on the protein concentration but also on the specific protein in the formulation. The Williams-Landel-Ferry (WLF) dependence of freeze concentrate viscosity on temperature below 0°C had implications on the freezing, annealing and primary drying stages of the lyophilization cycle. For formulations containing highly concentrated proteins, controlled ice nucleation at a high temperature (-5°C) produced cakes with largest pores. Annealing at -16°C for 2 hours produced smaller pores in formulations yielding a highly viscous freeze concentrate as compared to formulations with a low freeze-concentrate viscosity. However, formulations were a high freeze-concentrate viscosity were more resistant to cake collapse during aggressive drying. The difference between product temperature (T_p) and T_g’ was also identified as a critical factor influencing micro-collapse during aggressive drying.
Chapter 4 presents a detailed understanding of the role of formulation in mediating the reconstitution time. A variety of formulation variables such as % total solids, protein concentration, protein-to-sugar ratio, different proteins and inclusion of a crystallizable excipient were investigated for their effect on cake properties influencing reconstitution namely, cake wettability, penetration of reconstitution fluid into the cake, cake disintegration and cake porous structure. Additionally, several measures of viscosity were also evaluated for their effect on reconstitution time.

Reconstitution time was primarily influenced by a measure of the formulation viscosity with negligible contributions from % total solids and protein concentration. Partial crystallinity in the final cake also expedited reconstitution. Wettability (assessed using contact angle), ability of aqueous liquids to penetrate into the cake (assessed using drop penetration time), cake disintegration tendency (assessed from the resistance offered by cakes to crushing in the dry state) and cake porous structure were found to be invariant for amorphous cakes and did not correlate with reconstitution time. However, these properties were sensitive to the presence of crystallinity and resulted in faster reconstitution at least of the partially crystalline cakes. “Concentrated formulation viscosity” strongly correlated with reconstitution times of amorphous cakes, providing insights on the steps involved in the reconstitution of these amorphous formulations.

Reconstitution time is dependent on a number on factors in a complex manner. Furthermore, factors governing the reconstitution of partially crystalline cakes are reportedly different from amorphous cakes. The goal in Chapter 5 was to identify the key factors governing reconstitution
and to understand the mechanisms involved in reconstitution of both amorphous and partially crystalline cakes. Partial crystallinity in the final cake, larger pores and low “concentrated formulation viscosity” (i.e., viscosity near the surface of the dissolving cake) were identified as desirable characteristics for expediting reconstitution. Crystallinity and larger pores dramatically improved wettability and liquid penetration in partially crystalline cakes, ultimately resulting in well dispersed small pieces of partially dissolved cake. The smaller disintegrated cake pieces dissolved faster because of the increased surface area. The amorphous cakes exhibited poorer wettability than partially crystalline cakes. Moreover, the ability of the reconstitution fluid to penetrate the pores, and the resulting cake disintegration was much lower than that observed for partially crystalline cakes. In fact, for some of the amorphous cakes, the reconstitution fluid did not penetrate the cake at all. As a result, the undissolved intact cake or a large cake chunk floated on the reconstitution fluid amidst foam or bubbles generated during reconstitution. Dissolution of the floating cake appeared to proceed via gradual surface erosion where reconstitution time was found to be highly correlated with the “concentrated formulation viscosity”. Based on the above findings, both formulation and processing conditions can be tailored to achieve faster reconstitution. Including a crystallizable excipient proved to be beneficial. Incorporating an annealing step to facilitate crystallization of the crystallizable excipient and to promote larger pores was also found to be advantageous. A viscosity lowering excipient in the formulation could potentially be helpful but this approach needs to be explored further to provide stronger evidence.

The overall results and significance of the research are summarized in Chapter 6.
References


Chapter 2

Mechanisms by Which Crystalline Mannitol Improves the Reconstitution Time of High Concentration Lyophilized Protein Formulations
Abstract

Lyophilized high concentration protein formulations often have long and variable reconstitution times. The aim is to understand the role of crystalline mannitol in lowering the reconstitution time of these formulations. Novel methods were developed for quantifying the effect of crystalline mannitol on cake attributes influencing reconstitution, specifically, cake wettability, liquid penetration into the cake and cake disintegration. Amorphous and partially crystalline cakes were obtained by varying the freeze-drying conditions, particularly, the freezing rate (slow vs. fast), annealing (annealed vs. unannealed), and primary drying (aggressive vs. conservative). Mannitol crystallinity was quantified using X-ray powder diffractometry. Phase separation of crystalline mannitol from the amorphous, protein rich matrix improved wettability of the cake solids and promoted penetration of the reconstitution fluid into the cake interior. The partially crystalline cakes offered less resistance to crushing in the dry state than the amorphous cakes. Crystalline mannitol provided “weak points” in the freeze-dried cakes, potentially enabling easier cake disintegration upon addition of the reconstitution fluid. There was no evident correlation between the degree of crystallinity and reconstitution time. While crystalline mannitol generally decreased reconstitution time by favorably affecting the cake attributes influencing reconstitution, it did not always reduce reconstitution time.

Keywords: Reconstitution, high-concentration proteins, lyophilization, crystalline mannitol, wettability, liquid penetration, disintegration, dissolution
Introduction

Over the past decade, there has been a considerable rise in the number of biologics approved by the FDA [1]. Subcutaneous delivery is a common administration route for biological products and is often preferred over conventional intravenous delivery owing to convenient patient self-administration and reduced treatment costs [2, 3]. The requirement for high doses, particularly for monoclonal antibodies (mAbs), coupled with the desire for subcutaneous delivery (injection volume limit < 1.5 ml) necessitates the development of certain biologics at protein concentrations higher than 100 mg/ml. Since the development of high concentration liquid protein products poses both in-process and storage stability challenges [3-5], lyophilization (freeze-drying) is commonly used to overcome these stability related issues [3, 6, 7]. Prior to patient administration, a lyophilized drug product is reconstituted into a solution by adding an appropriate fluid (usually Sterile Water for Injection). The endpoint of reconstitution as described by USP is the complete dissolution of solids after addition of the reconstitution fluid [8]. Rapid reconstitution is an important product quality attribute for lyophilized formulations. However, in the case of highly concentrated lyophilized proteins, reconstitution time is often long and variable. Reconstitution times of some FDA-approved high concentration lyophilized protein products range from less than 5 minutes (Raptiva® [9], Nucala® [10]) to as long as 15-40 minutes (Ilaris® [11], Xolair® [12], Cosentyx® [13], and Cimzia® [14]). Long reconstitution times are undesirable in fast-paced clinical settings. Moreover, long and variable reconstitution of self-administered products can be dangerous, particularly if the patient attempts self-administration prior to complete reconstitution. As biosimilars and additional competing protein products come to market, reduced reconstitution time is expected to be a differentiating factor.
Research efforts have recently been directed towards understanding the causes of long reconstitution time and identifying ways to reduce it [15-19]. While evaluating multiple approaches to improve the reconstitution of an Fc-fusion protein formulated with sucrose and mannitol, Cao et al. found that partially crystalline lyophilized cakes reconstituted in 3 minutes when compared to fully amorphous cakes which took as long as 13 minutes [15]. The authors attributed the significant reduction in reconstitution time to crystalline mannitol. They observed that during reconstitution, partially crystalline cakes “absorbed” the reconstitution fluid much faster than their amorphous counterparts. The authors surmised that partially crystalline cakes were more wettable, making the cake core more accessible to the reconstitution fluid. They also noted that sometimes the partially crystalline cakes disintegrated on exposure to the reconstitution fluid resulting in an increased surface area for faster dissolution. Although insightful, the inferences regarding the mechanisms by which crystalline mannitol lowered reconstitution time [15] were not supported by quantitative experimental data. Thus, underlying reasons for the improvement offered by crystalline mannitol are yet to be fully elucidated. Nonetheless, the work by Cao et al. pointed to a potential relationship between mannitol crystallinity and cake attributes such as cake wettability, penetration of reconstitution fluid into cakes and cake disintegration.

In the food industry, the important steps for reconstitution of milk, cocoa and whey protein powders as well as infant formulas have been identified as (a) powder wetting, (b) liquid penetration into the porous powder by capillary action leading to sinking of the powder into the reconstituting liquid, (c) disintegration of powder agglomerates into primary particles, and (d) dissolution of the primary particles [20-22]. Analogously, reconstitution of high concentration lyophilized protein formulations can be considered to depend on cake attributes such as (a) cake
wettability, (b) liquid penetration to the cake interior, (c) cake dispersibility or disintegration upon fluid addition, and (d) dissolution of the disintegrated particles.

The primary goal of the present work was to better understand the role of crystalline mannitol in decreasing reconstitution time by comparing the cake attributes (e.g., wettability, penetration of reconstitution fluid into the cake, and disintegration) of both amorphous and partially crystalline lyophilized formulations. A second goal was to develop methods to quantitatively assess wettability of cake solids, liquid penetration through the cake and cake dispersibility or disintegration. Sane et al. introduced methods to quantify the wettability and disintegration rates of lyophilized cakes [18]. However, their methods relied on either formulations lyophilized in glass tubes (rather than in vials) or on samples that were crushed and removed from the vial for analyses. Hence, the objective behind developing newer methods was to reduce sample handling by making measurements on intact cakes without disrupting the cake structure during measurement. In doing so, the measured cake attributes would be more representative of the cake in the vial. This is especially relevant while studying reconstitution because cake structure has been pointed out as an important factor influencing reconstitution of high concentration proteins in amorphous cakes [16, 17].

Mannitol crystallization in multicomponent systems comprising of a protein, lyoprotectant and mannitol is affected by both formulation composition [15, 23, 24] and processing conditions [23, 25, 26]. Hence, a range of formulations and freeze-drying cycles were used to obtain amorphous and partially crystalline cakes with the end goal of understanding the effect of crystalline mannitol
on cake attributes influencing reconstitution. The percent solids in the cake has been proposed as a major factor governing reconstitution time [4, 15, 19, 27]. In general, cakes with higher density have longer reconstitution times [4, 27]. To eliminate the influence of cake density on reconstitution time, formulations were designed with the same total solids content (21% w/v) but with different compositions.

Materials and Methods

Materials

β-D-Mannitol (catalog # M4125; Sigma-Aldrich, St. Louis, MO, USA) was used to prepare a 20% w/v solution, which was filtered through a 0.22 µm syringe filter (Millex®, MilliporeSigma, Burlington, MA, USA). The pre-lyophilization (prelyo) solution was provided by GlaxoSmithKline and consisted of 140 mg/ml recombinant protein in 40 mg/ml mannitol, 28 mg/ml trehalose and 10 mM sodium phosphate buffer (pH 7.0) with total solid content of 20.9% w/v. Portions of the 140 mg/ml prelyo solution were diluted with the filtered 20% w/v mannitol to obtain protein concentrations at 127, 122, 117, 100 and 70 mg/ml. The resulting prelyo solution compositions are listed in Table 1. All the solutions had approximately the same total solids at 21% w/v. The prelyo solution provided by GSK also contained 0.01% w/v polysorbate 80. Upon dilution with 20% w/w mannitol solution, the surfactant concentration was < 0.01%. Polysorbate 80, at concentrations up to 1%, has been shown to have no observable effect on reconstitution time [15]. Hence, the surfactant concentration in the formulations was considered negligible from a reconstitution point of view and has not been reported in Table 1.
Table 1: Prelyo solution compositions and solution characteristics

<table>
<thead>
<tr>
<th></th>
<th>Protein (mg/ml)</th>
<th>140^a</th>
<th>127^b</th>
<th>122^b</th>
<th>117^b</th>
<th>100^b</th>
<th>70^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trehalose (mg/ml)</td>
<td></td>
<td>40</td>
<td>36</td>
<td>35</td>
<td>33</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>Mannitol (mg/ml)</td>
<td></td>
<td>28</td>
<td>44</td>
<td>50</td>
<td>57</td>
<td>77</td>
<td>106</td>
</tr>
<tr>
<td>Sodium phosphate buffer (mg/ml)</td>
<td></td>
<td>1.3</td>
<td>1.2</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Mannitol fraction (w/w relative to total solids)</td>
<td></td>
<td>0.13</td>
<td>0.21</td>
<td>0.24</td>
<td>0.27</td>
<td>0.37</td>
<td>0.51</td>
</tr>
<tr>
<td>Weight ratio (w:w) of mannitol to trehalose</td>
<td></td>
<td>0.7</td>
<td>1.2</td>
<td>1.4</td>
<td>1.7</td>
<td>2.7</td>
<td>3.5</td>
</tr>
<tr>
<td>Weight ratio (w:w) of protein to sugars</td>
<td></td>
<td>2.1</td>
<td>1.6</td>
<td>1.4</td>
<td>1.3</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Total solids (% w/v)</td>
<td></td>
<td>20.9</td>
<td>20.8</td>
<td>20.8</td>
<td>20.8</td>
<td>20.7</td>
<td>20.7</td>
</tr>
<tr>
<td>Osmolality^c (mOsmol/kg)</td>
<td></td>
<td>369 ± 1</td>
<td>414 ± 2</td>
<td>446 ± 0</td>
<td>524 ± 2</td>
<td>606 ± 1</td>
<td>979 ± 4</td>
</tr>
</tbody>
</table>

^a As received from GSK.
^b Prelyo solutions prepared by diluting the original 140 mg/ml solution as received from GSK with 20% w/v mannitol solution.
^c Osmolality values were measured using a vapor pressure osmometer (Vapro®, Model 5520, Wescor Inc., Logan, UT, USA) and are reported as mean ± standard deviation (n = 3).

Methods

Pre-lyophilization solution characterization

**Determination of glass transition temperature of the freeze concentrates (T_g’)**

Aliquots of each solution (10-15 mg) were hermetically sealed in aluminum pans. Each solution was cooled to -50°C at 1°C/min, held for 30 minutes and then scanned at 10°C/min to room temperature in a differential scanning calorimeter (DSC; Model Q1000, TA Instruments, New Castle, DE, USA). To study the effect of annealing on T_g’, the solutions were cooled to -50°C at 1°C/min, heated to -20°C at 1°C/min, held at -20°C for 12 hours, then cooled back to -50°C before scanning at 10°C/min to room temperature. The midpoint of the glass transition was reported as T_g’ (Table 2). The T_g’ values were utilized to design primary drying protocols that were either
“aggressive” (i.e., product temperatures maintained above $T_g'$) or “conservative” (i.e., product temperatures maintained below $T_g'$).

Table 2: Glass transition temperatures of the freeze concentrates ($T_g'$) and average product temperatures during primary drying of the six prelyo solutions.

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>$T_{g'}$ (°C)$^a$</th>
<th>Average Product Temperature (°C)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unannealed</td>
<td>Annealed</td>
</tr>
<tr>
<td>140</td>
<td>-29.6</td>
<td>-29.9</td>
</tr>
<tr>
<td>127</td>
<td>-31.9</td>
<td>-24.3</td>
</tr>
<tr>
<td>122</td>
<td>-32.5</td>
<td>-25.4</td>
</tr>
<tr>
<td>117</td>
<td>-33.5</td>
<td>-25.6</td>
</tr>
<tr>
<td>100</td>
<td>-35.4</td>
<td>-25.9</td>
</tr>
<tr>
<td>70</td>
<td>-37.5 ($T_{g1'}$)$^b$</td>
<td>-29.7</td>
</tr>
<tr>
<td></td>
<td>-30.7 ($T_{g2'}$)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $T_{g'}$ values are midpoints.
$^b$ For 70 mg/ml formulation, $T_{g1'}$ value was used in designing the primary drying protocol.
$^c$ Product temperatures during primary drying of the six prelyo solutions processed using Cycles 1 and 2 and of the prelyo solution containing 100 mg/ml protein processed using Cycles 1-5 were monitored using thermocouples placed at the bottom of the product. Product temperature values are averaged over the entire primary drying time. When there was vial-to-vial variation in product temperature for the same formulation, the values are represented as mean ± standard deviation (n=3).
$^d$ Not tested
Lyophilization
Solutions (0.5 ml) were filled in 3 ml clear tubing glass vials (Wheaton Type 1, Fischer Scientific, Waltham, MA, USA) and partially stoppered (V50 lyo 4432/50 Gray 13 mm lyo stoppers, West Pharmaceutical Services, Exton, PA, USA). Vials containing the prelyo solutions were placed in the center of a hexagonal array of surrogate-containing vials. This was done to minimize the effect of radiative heat transfer from the door and walls, the so-called “edge vial effect”, thereby ensuring vial-to-vial uniformity of the lyophilized cakes [28]. To prepare the surrogate, the GSK protein was replaced with bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA), and trehalose (catalog # T-104-4; α,α trehalose dihydrate, high purity, low endotoxin, USP grade, Pfanstiehl, Waukegan, IL, USA) and mannitol were added to produce a composition equivalent to the 140 mg/ml prelyo solution. For each freeze-drying cycle, a total of 308 vials were arranged in a hexagonal array within a 32×25 cm stainless steel band and were loaded in the center on the middle shelf of a lab freeze dryer (Revo® , Millrock Technology, Kingston, NY, USA) at room temperature. Amorphous and partially crystalline cakes were obtained by using a range of freezing-drying cycles described below. Since mannitol crystallization from multicomponent systems is known to be affected by freezing rate [29, 30], annealing (time and temperature) [23, 31], and primary drying conditions [25, 32], the lyophilization cycles were designed to study the effects of each of these processing parameters on mannitol crystallinity, reconstitution time and cake attributes influencing reconstitution. Cycles were designed to study (a) the effect of annealing and (b) the combined effects of freezing rate and primary drying temperature.

(a) Effect of annealing

Two protocols were used to study the effect of annealing:

Cycle 1: slow freezing, no annealing, conservative drying
Cycle 2: slow freezing, annealing, conservative drying

Each of the six solutions was lyophilized using a freeze drying cycle that included annealing and one that did not include an annealing step between freezing and primary drying. The shelf temperature was ramped down to -5°C at 1°C/min followed by a 1 hour hold at -5°C. Ice nucleation was induced at this temperature using an ice fog (FreezeBooster® nucleation station, Millrock Technology, Kingston, NY, USA). The shelf was then cooled to -40°C at 0.2°C/min (slow freezing) and held for 90 minutes. For the annealing step, the shelf temperature was raised to -20°C at 1°C/min and held for 12 hours. This atypically long annealing time was employed to maximize any mannitol crystallization. Following annealing, the shelf temperature was decreased to -40°C at 1°C/min and held for 90 minutes. The chamber pressure was then reduced to 50 mTorr for Cycle 1 and 100 mTorr for Cycle 2 and the shelf temperature was increased at 1°C/min to the primary drying shelf temperature, -35°C for Cycle 1 and -10°C for Cycle 2. The primary drying shelf temperatures and chamber pressures ensured that the product temperatures were maintained below their T_g’ (Table 2) throughout primary drying (i.e., conservative drying). The primary drying product temperatures during Cycle 1 (no annealing) were compared with the T_g’ of the unannealed prelyo solutions and temperatures during Cycle 2 (annealing) were compared with T_g’ values of the annealed prelyo solutions. When the Pirani gauge pressure was within 3 mTorr of the pressure measured by the capacitance manometer (i.e., the end of primary drying), the shelf temperature was raised to 40°C, at 0.2°C /min, and secondary drying was conducted for 3 hours. Product temperatures during freezing and drying were recorded by placing 36-gauge T-type thermocouples (Omega Engineering, Inc., Norwalk, CT, USA) in the bottom center of the vials. At the end of secondary drying, the shelf temperature was lowered to 20°C. The vials were stoppered at 700
Torr. Aluminum seals were crimped over the stoppers and the vials were stored at 5°C until further use.

(b) Effect of freezing rate and primary drying temperature

Freezing rate (slow vs. fast) and primary drying temperature (aggressive vs. conservative) are known to affect mannitol crystallization from multicomponent systems containing combinations of protein, mannitol, and lyoprotectant [25, 30, 32]. Four protocols were used to study the effect of freezing rate and primary drying temperature on reconstitution time and other cake attributes:

- Cycle 1: slow freezing, conservative drying (as described above)
- Cycle 3: slow freezing, aggressive drying
- Cycle 4: fast freezing, aggressive drying
- Cycle 5: fast freezing, conservative drying

High protein concentration and low mannitol to lyoprotectant ratio (< 2) are known to inhibit mannitol crystallization in multicomponent protein formulations [15, 23, 30]. Hence, only the moderately concentrated prelyo solution containing 100 mg/ml protein, with a mannitol to trehalose ratio of 2.7, was subjected to the additional Cycles 3, 4 and 5. The slow freezing (in Cycle 3) and conservative drying (in Cycle 5) conditions were the same as described for Cycle 1 previously. In contrast, fast freezing (Cycles 4 and 5) consisted of controlled ice nucleation at a shelf temperature of -10°C followed by cooling at 5°C/min to -40°C. For aggressive drying (Cycles 3 and 4), the product temperature was above the Tg’ throughout primary drying, using a shelf temperature of -10°C and chamber pressure of 100 mTorr in the absence of annealing. The same chamber pressure was maintained during secondary drying. The secondary drying temperature, as well as the ramp rates and hold times were the same as in Cycle 1. The primary drying product temperatures for the prelyo solution containing 100 mg/ml protein and processed using the
lyophilization Cycles 3-5 are listed in Table 2. The freeze-drying cycle parameters for Cycles 1-5 are summarized in Table 3.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Ice nucleation temperature (°C)</th>
<th>Freezing ramp rate (°C/min)</th>
<th>Annealing</th>
<th>Primary drying shelf temperature (°C) and chamber pressure (mTorr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 1</td>
<td>-5</td>
<td>0.2</td>
<td>-</td>
<td>-35 and 50</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>-5</td>
<td>0.2</td>
<td>Yes</td>
<td>-10 and 100</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>-5</td>
<td>0.2</td>
<td>-</td>
<td>-10 and 100</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>-10</td>
<td>5</td>
<td>-</td>
<td>-10 and 100</td>
</tr>
<tr>
<td>Cycle 5</td>
<td>-10</td>
<td>5</td>
<td>-</td>
<td>-35 and 50</td>
</tr>
</tbody>
</table>

**Characterization of freeze-dried cakes**

*Mannitol phase identification and quantification by X-ray powder diffractionmetry (XRPD)*

The contents of a vial were powdered and transferred to a sample holder. X-ray powder diffractograms were collected from 5° to 40° 2θ with a step size of 0.02° using Cu Kα radiation (40 kV × 44 mA) at room temperature (Ultima IV system, Rigaku Americas Corporation, Andover, MA, USA) with Bragg-Brentano geometry. Unique peaks of anhydrous mannitol α-form (13.6 and 17.3° 2θ), β-form (14.6, 16.8 and 23.4° 2θ), and δ-form (9.7, 20.4 and 24.6° 2θ) and mannitol hemihydrate (17.9° 2θ) were used to identify the different physical forms of mannitol [33]. Samples were analyzed in triplicate.

Based on the physical forms of mannitol in the lyophilized cakes (discussed in the Results and Discussion section), the integrated peak intensities of only δ-mannitol were determined after baseline subtraction (Spectragryph® optical spectroscopy software [34]). The degree of crystallinity in the freeze-dried cakes was quantified based on a standard curve, where the
“standard mixtures” with known degree of crystallinity were prepared by mixing appropriate amounts of pure crystalline δ-mannitol (catalog # 1.12635.1000; Emprove® exp Parteck® Delta Mannitol, MilliporeSigma, Bellerica, MA, USA) and crushed powder of the amorphous cakes of 140 mg/ml formulation. The standards were prepared in a glove bag maintained at a relative humidity (RH) < 2% at room temperature (20 ± 2°C). The amount of powder used, the sample preparation method, and XRPD instrument settings for the standards were the same as those used to analyze the freeze-dried cakes. For each of the standards, the sum of the areas of 9.7, 20.4 and 24.6° 20 peaks (attributed to δ-mannitol) was plotted as a function of the degree of crystallinity. Linear regression on the data yielded the line of best fit (r² = 0.989) where \( \text{sum of the peak areas} = 47.5 \times \text{degree of crystallinity} - 81.1 \). This regression equation was rearranged to calculate the degree of crystallinity in the freeze-dried cakes as shown in Eq. (1).

\[
\text{Degree of crystallinity (°C)} = \frac{\text{Sum of peak areas} + 81.1}{47.5}
\] (1)

The extent of mannitol crystallization was calculated from the degree of crystallinity (Eq. (1)) as shown in Eq. (2).

\[
\text{Extent of mannitol crystallization (°C)} = \frac{\text{Degree of crystallinity (°C) from Eq. (1)}}{\text{Mannitol fraction (Table 1)}}
\] (2)

Degree of crystallinity and extent of mannitol crystallization are represented as mean ± standard deviation (n = 3).

**Reconstitution time**

Freeze-dried cakes were equilibrated to room temperature before being reconstituted manually using Sterile Water for Injection USP (Hospira Inc., Lake Forest, IL, USA). A 1 ml syringe, attached to a 3/8 inch long 26 G needle, was filled with the appropriate volume of reconstitution
fluid depending on the measured loss of water during freeze-drying. The needle was pierced through the center of the vial stopper such that the needle was completely inside the vial (up to the needle hub). The reconstitution fluid was injected into the vial in about 4-5 seconds with the needle positioned over the center of the cake during injection. The start time for reconstitution was the end of fluid addition.

A standardized protocol was developed for monitoring reconstitution. The vial was manually swirled at 60-70 rpm in a circular motion. Swirling of the vial involved rotating around the circumference of a circle with diameter ~8.7 cm. Swirling was paused at 2 minutes for 15 seconds to make a thorough visual observation. During the pause, the contents of the vial were observed from below and from the sides while taking care to hold the vial steady and avoid tipping of the vial to eliminate any agitation during the observational pause. The vial was again swirled for 1 minute followed by another 15 second observational pause. The next swirling period was 1 minute (with 15 second pause), and subsequent periods were 2 minutes (each with 15 second pauses) until reconstitution was complete. The time at which there were no undissolved cake solids in the vial was noted as the endpoint of reconstitution. Total reconstitution time included the observational pause periods, because the cake was in contact with the fluid during that time. Reconstitution times of 8 vials of each formulation/freeze-drying cycle combination are reported as mean ± standard deviation.

**Water content**

The residual water (moisture) content in each vial was determined using Karl Fischer titration. Anhydrous methanol (2.0 ml) was injected into the sealed vials. The vials were vortexed for 20 minutes to disperse the cake and were then allowed to stand for at least an hour to ensure complete
extraction of water. The 20 minute duration was necessary, particularly for mannitol-containing cakes, which in our experience are difficult to disperse, at least relative to sucrose-containing cakes. Aliquots of 0.50 ml were injected into the titration vessel (831 KF Coulometer, Metrohm, Riverview, FL, USA). The measurements were made in duplicate for each vial with 2 vials for each formulation/freeze-drying cycle combination. Residual water content (average of 4 readings) was < 1.2 % w/w in all samples.

**Wettability of compacted cakes**

The method developed by Sane et al. [18] was refined to assess the wettability of compacted cakes. A nearly saturated solution of mannitol and trehalose (17% w/v mannitol and 78% w/v trehalose) was prepared. A portion (80-100 mg) of the powdered contents of a vial was transferred to a 12 mm die and compressed at 10,000 psi in a laboratory press (Model 3912, Carver Inc., Wabash, IN, USA). The press was enclosed in a glove bag maintained at < 2% RH and 20 ± 2°C. The contact angle of a sessile drop of the solution on the compacted disc of the lyophilized cake was measured using a goniometer (Model 100-00, Ramé-hart Inc., Succasunna NJ, USA). The drop was composed of a nearly saturated solution to reduce dissolution of the solids in the compact during measurement of the contact angle. The compact was placed on the sample stage and 10 µl of the freshly prepared drop solution was placed on its surface using a micropipette. The drop resting on the surface of the compact was observed through the eyepiece and the contact angle was measured using the goniometer in the eyepiece. Measurements were made in duplicate for each compact (left and right angles of the same drop) with 4 compacts analyzed for each sample. Contact angle values are reported as mean ± standard deviation (n = 8).
Liquid penetration into cakes

To measure the accessibility of the cake core to the reconstitution fluid, a drop penetration time test was modified from that used for studying liquid penetration into porous pharmaceutical powders [35]. An aqueous solution (with the same composition used for contact angle measurements) was tinted with a blue dye to aid visibility. A 10 µl drop of the freshly prepared solution was gently placed, using a micropipette, on the surface of the intact cake in a vial (Figure 1A). The time for the drop to disappear from the surface was recorded to the nearest second. The test was terminated at 15 minutes. The test was performed on 3 vials of each sample.

Cake resistance to crushing

While breaking up dried cakes in vials to remove them for XRPD measurements, it was noted that some cakes were easier to crush into powders than others. The resistance of the cake to crushing in the dry state was evaluated to potentially serve as a measure of the ease with which the cake disperses or disintegrates upon addition of the reconstitution fluid. A method was developed to quantitatively assess cake crushing resistance. Specifically, the force necessary for displacement of a flat faced, 3 mm diameter, stainless steel cylindrical probe into an intact cake at a constant rate of 0.5 mm/min was recorded (Figure 1B). The cylindrical probe was attached to a calibrated 1 kN load cell (Model 5866, Instron Corporation, Norwood, MA, USA). The crimped vials containing the freeze-dried cake were equilibrated at 21 ± 1°C before being tested. The vials were decrimped, unstoppered and placed on the sample stage. The test was terminated when the probe displacement was 1.4 mm into the cake which corresponded to approximately half the cake height. Displacement was stopped at half cake height because beyond this point, sample densification resists further compression [36]. The force versus displacement of 7 replicate samples was recorded in an environment of 21 ± 1°C and 40% relative humidity. The time from opening the
vial to completion of the measurement was about 4 minutes. The slope of the initial linear portion of the force vs. displacement curve was reported as cake crushing resistance.

**Statistical analysis**

For each of the cake attributes measured, a conclusion regarding statistical significance was based on a two-tailed t-test (MS Excel) with significance at $\alpha = 0.05$, $\alpha = 0.01$, or $\alpha = 0.001$ as specified in the text and figures.
Figure 1: Experimental set up for (A) drop penetration time test for quantifying the penetration of the reconstitution fluid into the cake, and (B) crushing resistance test for quantifying the resistance of the cake to crushing as a potential measure of cake dispersibility or disintegration upon addition of the reconstitution fluid.

Results and Discussion

Crystalline mannitol has been reported to influence the reconstitution time of high concentration lyophilized protein formulations [15, 37]. In an effort to control mannitol crystallinity, at a fixed pre-lyophilization solution composition, the freeze-drying protocols were modified. The cake attributes that influence reconstitution time were identified and quantified, for both amorphous and partially crystalline cakes obtained from the various lyophilization cycles. The effect of processing parameters, specifically, (a) the effect of annealing and (b) the combined effects of freezing rate and primary drying temperature on mannitol crystallinity, reconstitution time and cake attributes influencing reconstitution are discussed below so as to understand the mechanisms by which crystalline mannitol improves reconstitution time.
**Effect of annealing**

*Mannitol phase identification and quantification in freeze-dried cakes*

Mannitol was in the amorphous form in the unannealed (i.e., Cycle 1) formulations with protein concentrations of 100-140 mg/ml. This finding is consistent with the literature showing that protein, at a high concentration and in the presence of lyoprotectant, has the potential to inhibit mannitol crystallization [15, 23, 38]. Additionally, the low mannitol fraction (< 0.3 relative to the total solids) prevented mannitol from crystallizing in these formulations as has also been reported [24]. At the lowest protein concentration (70 mg/ml), mannitol crystallized even without annealing (Figure 2A). The low protein concentration (70 mg/ml) coupled with the high mannitol fraction (0.51) and the high mannitol to trehalose ratio (3.5) allowed mannitol crystallization during processing, as previously reported [23].

Annealing led to mannitol crystallization in all solutions processed using Cycle 2 except for the one with the highest protein concentration (140 mg/ml; Figure 2B). Annealing is known to promote crystallization of mannitol from formulations containing a protein at concentrations of 10 - 100 mg/ml [15, 23]. Moreover, in the absence of protein, while solutions with a mannitol to sucrose ratio of 1 or 2 have shown the need for annealing to promote mannitol crystallization, inhibition of mannitol crystallization has been noted in solutions with a ratio lower than 1 [23, 30]. In the present study, mannitol crystallization was inhibited in the 140 mg/ml sample even upon annealing for 12 hours at -20°C, owing to the high protein concentration and low mannitol to trehalose ratio of 0.5. Annealing was required for inducing mannitol crystallization in samples containing 100-127 mg/ml protein where the mannitol to trehalose ratio ranged from 1.2 to 2.7 and mannitol fraction ranged from 0.21-0.37.
The sharp peaks at 9.7, 20.4 and 24.6° 2θ indicate that mannitol predominantly crystallized as the δ- polymorph. Traces of α-mannitol were found in the annealed samples (Cycle 2) with higher protein concentration (117, 122 and 127 mg/ml) and in the unannealed 70 mg/ml sample (Cycle 1). The annealed 117 and 122 mg/ml samples from Cycle 2 also showed traces of mannitol hemihydrate. In freeze-dried formulations comprising of protein, lyoprotectant and mannitol, the polymorphic form of mannitol was previously shown to depend on both the formulation composition and freeze-drying conditions [25, 32, 39]. In those studies, mannitol crystallized predominantly as the δ- polymorph and as the hemihydrate upon annealing and a fraction of the mannitol remained amorphous. While α- and/or β- polymorphs were present, the relative percentages of these polymorphs were very low. The presence of predominantly δ-mannitol and traces of the hemihydrate and/or α-mannitol in the present study were consistent with the previous reports. Furthermore, the ratio of δ-mannitol to hemihydrate in the cake has been shown to depend on the protein concentration, the annealing time and temperature, and the drying conditions [25, 32, 38, 39]. At a given annealing temperature, higher protein concentrations favored the formation of mannitol hemihydrate over δ-mannitol [25]. In the present study, the presence of the hemihydrate peak in the annealed 117 and 122 mg/ml formulations, but not in the 70 and 100 mg/ml formulations, is in agreement with this previous finding. A higher protein concentration (127 mg/ml) decreased the overall crystallinity after lyophilization, reducing any hemihydrate, if present, to below the detection limit. In any case, since the peaks for the δ-form of mannitol clearly predominated in all the samples, the degree of crystallinity was calculated based on those peaks alone.
The degree of crystallinity in solutions lyophilized with an annealing step increased with decreasing protein concentration (Table 4). In all cases, the degree of crystallinity in the lyophilized formulations remained low (≤ 15 %) and the extent of mannitol crystallization in the formulations was < 35%. Low crystallinity values are expected because of the strong inhibitory effect of high protein concentrations (≥ 70 mg/ml) on mannitol crystallization.

Figure 2: XRPD patterns of the cakes obtained from (A) Cycle 1: unannealed and (B) Cycle 2: annealed. Mannitol physical forms, namely anhydrous α-, δ- and mannitol hemihydrate (MHH), were identified based on the characteristic peak positions.

Table 4: Degree of crystallinity and extent of mannitol crystallization in the partially crystalline lyophilized cakes determined using Eqs. (1) and (2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein Conc. (mg/ml)</th>
<th>Annealing</th>
<th>Cycle</th>
<th>Degree of crystallinity (%)</th>
<th>Extent of mannitol crystallization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>127</td>
<td>Yes</td>
<td>2</td>
<td></td>
<td>4 ± 0</td>
<td>20 ± 0</td>
</tr>
<tr>
<td>122</td>
<td>Yes</td>
<td>2</td>
<td></td>
<td>8 ± 1</td>
<td>34 ± 3</td>
</tr>
<tr>
<td>117</td>
<td>Yes</td>
<td>2</td>
<td></td>
<td>9 ± 1</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>100</td>
<td>Yes</td>
<td>2</td>
<td></td>
<td>12 ± 0</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>100</td>
<td>No</td>
<td>3</td>
<td></td>
<td>6 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>100</td>
<td>No</td>
<td>4</td>
<td></td>
<td>9 ± 0</td>
<td>25 ± 0</td>
</tr>
<tr>
<td>70</td>
<td>Yes</td>
<td>2</td>
<td></td>
<td>15a</td>
<td>29a</td>
</tr>
<tr>
<td>100</td>
<td>No</td>
<td>3</td>
<td></td>
<td>6 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>100</td>
<td>No</td>
<td>4</td>
<td></td>
<td>9 ± 0</td>
<td>25 ± 0</td>
</tr>
<tr>
<td>70</td>
<td>No</td>
<td>1</td>
<td></td>
<td>12a</td>
<td>23a</td>
</tr>
</tbody>
</table>

mean ± standard deviation (n = 3)

a n = 1
**Reconstitution time**

In formulations lyophilized without annealing (Cycle 1), as the protein concentration increased from 70 to 140 mg/ml, there was a progressive increase in reconstitution time from 6 to 16 minutes (Figure 3). All the cakes except the one with the lowest protein concentration (70 mg/ml in the prelyo solution) were fully amorphous. These results were consistent with previous studies where reconstitution time has been reported to increase with protein concentration in amorphous cakes [15-17]. Interestingly, reconstitution times of the annealed, partially crystalline cakes (Cycle 2) over the entire range of protein concentrations from 70 - 127 mg/ml fell within a relatively small range between 5 – 7 minutes (Figure 3). Thus, the effect of protein concentration on reconstitution time is very different for amorphous and partially crystalline formulations.

A comparison between reconstitution times of the annealed (Cycle 2) and the unannealed (Cycle 1) cakes of the same composition also showed an interesting trend. At a protein to sugar ratio of 2 (i.e., 140 mg/ml protein), there was no difference in the reconstitution time of the amorphous cakes, with or without annealing. For formulations with protein to sugar ratios between 0.9 and 1.6 (i.e., 100, 117, 122 and 127 mg/ml protein), annealing led to mannitol crystallization and the partially crystalline cakes had a significantly (Figures 3 and 4A) shorter reconstitution time when compared to the corresponding unannealed, amorphous cakes. Of particular note, in formulations containing 117, 122 and 127 mg/ml protein, mannitol crystallinity lowered reconstitution times dramatically (from 13 minutes to 5 minutes). However, at 100 mg/ml protein, although the difference was statistically significant (p < 0.01, Figure 4A), mannitol crystallinity offered only a slight reduction in reconstitution time when compared to unannealed, amorphous cakes at the same protein concentration (7 vs. 9 minutes). These results suggest that improvement in reconstitution time
offered by crystalline mannitol is more pronounced in formulations with relatively high protein to sugar ratio. This is expected because at low protein to sugar ratios and low protein concentrations, reconstitution time is short irrespective of the presence or absence of crystallinity in the cakes. At a protein to sugar ratio of 0.5 (i.e., 70 mg/ml protein), where both unannealed and annealed cakes were partially crystalline, there was no significant difference in the reconstitution time. Based on the effects of mannitol crystallinity on reconstitution described above, a more detailed analysis of the 122, 117 and 100 mg/ml formulations was undertaken with the goal of understanding the mechanisms by which crystalline mannitol decreased the reconstitution time.

Figure 3: Reconstitution times plotted against the protein to sugar (trehalose + mannitol) ratio of the respective formulation. Cakes obtained from Cycle 1 were unannealed and from Cycle 2 were annealed. Reconstitution times are indicated as mean ± standard deviation for n = 8 vials.
Figure 4: (A) Reconstitution time (n = 8), (B) contact angle (n = 8), (C) drop penetration time (n = 3), and (D) crushing resistance (n = 7) expressed as the force exerted on the freeze-dried cake per distance travelled by the probe into the cake for 122, 117 and 100 mg/ml formulations obtained from Cycle 1 (unannealed, amorphous cakes) and Cycle 2 (annealed, partially crystalline cakes). All values are represented as mean ± standard deviation. Statistically significant differences between the unannealed and annealed cakes are denoted by **(p < 0.01) and *** (p < 0.001).

**Wettability of compacted cakes**

Contact angle is a measure of the ability of a liquid to wet the solid, where lower values of contact angle correspond to better wetting. Strictly speaking, the relationship between contact angle and surface energies as described by the Young’s equation applies only to smooth, flat surfaces [40]. Lyophilized cakes are neither smooth nor flat. To focus on the solids, and minimize the contribution of the porous structure, lyophilized solids were compacted into discs for measurement of contact angle. The compaction process reduced, but could not eliminate all pores in the freeze-dried solids. Hence, the reported contact angles should be interpreted relative to each other rather than to any reference smooth, flat surface. Wenzel described the effect of surface roughness on contact angle using a roughness ratio [41]. Thus, before comparing contact angles of an aqueous
drop on the compacted solids, roughness ratios for all samples were measured and found to be within a narrow range of 1.05 ± 0.02 (Nexview profiler, Zygo Corporation, Middlefield, CT, USA).

The aqueous drop on compacts of the annealed, partially crystalline cakes had significantly lower contact angles than on the corresponding compacts from the unannealed, amorphous cakes, indicating better wetting of the former (Figure 4B). The difference between contact angles of partially crystalline and amorphous cakes was the first quantitative confirmation of the insightful speculation of Cao et al. that partially crystalline freeze-dried cakes were more wettable than amorphous cakes. Furthermore, for samples tested in the present study, the wettability of the cakes (i.e., contact angle) was less influenced by the formulation than by processing.

The presence of mannitol crystals can introduce both surface roughness and chemical heterogeneity in the surface being analyzed (i.e., solid compacts of the lyophilized cakes). Since surface roughness of both amorphous and partially crystalline compacts was found to be the same, surface roughness does not account for the improved wetting of the partially crystalline compacts. Chemical heterogeneity arises from distinct patches of crystalline mannitol separated from the remaining amorphous mixture. Wetting of chemically heterogeneous surfaces was described by Cassie as shown in Eq. (3) [42].

$$\cos \theta_C = f_1 \cos \theta_1 + f_2 \cos \theta_2$$  \hspace{1cm} (3)

As per Eq. (3), the cosine of the measured contact angle for a chemically heterogeneous surface (\(\cos \theta_C\)) can be expressed as a weighted sum of the cosines of the contact angles of the pure
components at the surface \( (i.e. \cos \theta_1 \text{ and } \cos \theta_2) \) where \( f_1 \) and \( f_2 \) are fractions of the surface area occupied by the respective components. Annealed cakes are composed of two phases – pure crystalline \( \delta \)-mannitol and the amorphous mixture containing the protein, trehalose and remaining amorphous mannitol. To test the applicability of the Cassie equation to the annealed compacts, contact angles of the aqueous drop were measured on compacts made from physical mixtures of pure \( \delta \)-mannitol and the amorphous powder containing protein, trehalose and mannitol obtained by crushing the 122 mg/ml cakes from Cycle 1. The contact angle of this remaining amorphous phase can be approximated by the contact angle of the compacts made from the unannealed, amorphous cakes from Cycle 1 i.e., 72°. The average contact angle of the aqueous drop on a compact of pure \( \delta \)-mannitol was found to be 30°. The experimentally measured contact angles for these standards (Table 5) agreed favorably with those calculated using Eq. (3), where reduction in the contact angle with increasing \( \delta \)-mannitol amounts followed the expected trend.

Table 5: Comparison of measured and calculated contact angles of compacts of different compositions.

<table>
<thead>
<tr>
<th>Weight fraction of pure ( \delta )-mannitol ( (f_1) ) (^a)</th>
<th>Weight fraction of amorphous powder containing protein, trehalose and mannitol ( (f_2) ) (^a,b)</th>
<th>Measured contact angle(^c) ( (°) )</th>
<th>Calculated contact angle(^d) ( (°) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>0.00</td>
<td>30 ± 1</td>
<td>-</td>
</tr>
<tr>
<td>0.00</td>
<td>1.00</td>
<td>72 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>0.08</td>
<td>0.92</td>
<td>60 ± 3</td>
<td>69</td>
</tr>
<tr>
<td>0.50</td>
<td>0.50</td>
<td>51 ± 2</td>
<td>54</td>
</tr>
</tbody>
</table>

\(^a\)Surface fraction in Eq. (3) is assumed to be equal to weight fraction.  
\(^b\)Powder obtained by crushing cakes freeze-dried from 122 mg/ml protein solution using Cycle 1.  
\(^c\)Mean ± standard deviation \((n = 8)\)  
\(^d\)Contact angle values calculated using Eq. (3) with \( \theta_1 \) of 30° and \( \theta_2 \) of 72°.

In summary, as the hydrophilic mannitol crystallizes, it phase separates from the relatively hydrophobic, amorphous, protein-rich phase, leading to the formation of hydrophilic areas in the
cake which are wetted relatively easily. The improved wettability promotes faster reconstitution of these partially crystalline cakes. Notably, a degree of crystallinity as low as 8% in the 122 mg/ml formulation improved wettability of the cake solids, resulting in a dramatically shorter reconstitution time (from 13 minutes at 0% crystalline to 4 minutes at 8% crystalline).

**Liquid penetration into cakes**

For rapid reconstitution, the reconstitution fluid should be able to penetrate the cake in order to gain access to the interior of the cake, without requiring erosion from the surface. The porous lyophilized cake can be considered a network of capillaries. Penetration of the reconstitution fluid into the porous cake can be understood by applying the concepts of capillary penetration and by measuring the drop penetration time. Drop penetration time measurements have been applied to other similar pharmaceutical and food formulations [35, 43]. For a drop of a constant volume of a given liquid, the drop penetration time is inversely proportional to the pore size, porosity and cosine of the contact angle [35, 44]. Hence, a more porous cake structure with larger pores and good wettability of the cake solids will promote rapid drop penetration.

The drop penetration times for the annealed, partially crystalline cakes were dramatically lower than the unannealed, amorphous cakes (Figure 4C). Drops with penetration times > 15 minutes remained on the surface for at least 24 hours. In order to reduce dissolution of the cake and minimize the changes in the drop viscosity and surface tension during the measurement, drop penetration times were measured using a nearly saturated solution of mannitol and trehalose (17% w/v mannitol and 78% w/v trehalose). All six samples presented in Figure 4C had approximately
the same pore size and porosity as determined by mercury intrusion porosimetry (data not shown). Thus, in the absence of pore size and porosity differences, the drop penetration time was governed by wettablility of the cake solids. The annealed, partially crystalline cakes displayed lower contact angles and hence better wettablility, resulting in faster drop penetration times compared to unannealed, amorphous cakes. Faster drop penetration in the unannealed 100 mg/ml formulation (compared to unannealed 117 and 122 mg/ml) suggests a significantly lower contact angle and better wetting of the 100 mg/ml formulation. Since the protein concentration did not significantly affect the contact angles of amorphous cakes (Figure 4B), it is possible that there was some incipient mannitol crystallinity that remained undetected by XRPD.

The drop penetration results suggest that the reconstitution fluid can access the interior of the partially crystalline cake more rapidly than the amorphous cakes. Cao et al. had observed partially crystalline cakes to “immediately absorb” the dissolution medium during reconstitution [15]. They alluded to the cake core being more accessible to the reconstitution medium in the partially crystalline cakes. The drop penetration time protocol developed in the present study quantified that accessibility.

**Cake resistance to crushing**

As has been previously suggested, cake dispersion or disintegration prior to hydration is favorable for achieving rapid reconstitution [18, 20, 21]. Resistance of the cake to crushing in the dry state was evaluated to potentially serve as a measure of the difficulty with which the cake disperses or disintegrates upon addition of the reconstitution fluid. The force (or resistance to breakage) versus
displacement (of a probe pushing into the cake) curve for freeze-dried cakes have been characterized by three stages [36, 45, 46]: (i) an initial linear rise in force with small displacements\(^1\), (ii) collapse and fracture of the pore walls of the porous cake for intermediate displacements, and (iii) densification under large displacements, which was not of interest here.

The force vs. displacement curves for the unannealed and annealed cakes of 100, 117 and 122 mg/ml formulations are presented in Figure 5. The initial portion of the force-displacement profiles, for both amorphous and partially crystalline cakes, was linear until the transition point between stages (i) and (ii) (indicated by an arrow). The initial slopes of the amorphous cakes were steeper than the corresponding partially crystalline cakes indicating that more force was required to break the porous structure of the amorphous cakes. To quantify these differences, the resistance to crushing is reported as the slope of the initial linear portion of the force vs. displacement curve (Figure 4D). The crushing resistance\(^2\) for the annealed, partially crystalline cakes of all three formulations was found to be significantly lower than for the unannealed, amorphous cakes of the same chemical composition.

The second stage of the force vs. displacement curve for the unannealed, amorphous cakes (Cycle 1) is distinct from that of the annealed, partially crystalline cakes (Cycle 2) (Figure 5). The second stage is characterized by a jagged, saw-tooth pattern in the amorphous cakes, which is absent in the partially crystalline cakes. Differences between amorphous and partially crystalline cakes can

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\(^{1}\) Mechanical behavior of freeze-dried cakes during stage (i) has previously been described as elastic [36, 45]. However, in the present study, the cakes exhibited “partially elastic” behavior.

\(^{2}\) The units of N/mm used in the present study to describe crushing resistance are the same as those for stiffness, which is the resistance offered by an elastic body to deformation. However, freeze-dried cakes are not elastic, thus the slope of the force-displacement curve is better described as ‘crushing resistance’ instead of ‘stiffness’.

44
be explained based on the nature of compressive failure in porous freeze-dried cakes called fracture-controlled crushing [36, 45]. Fracture-controlled crushing is the result of rapid fracture events that involve breakage of the pore walls, and occurs when the probe penetrates deep into the cake. In the sturdier amorphous cakes, fracture of the pore wall leads to a rapid and significant reduction in the stress resulting in a negative slope periodically, giving rise to the jagged appearance of the curve. In contrast, pore walls in the partially crystalline cakes fracture more easily and lack the jaggedness in the force-displacement curves. The association of jaggedness with amorphous freeze-dried cakes of pure trehalose and sucrose, and its absence in freeze-dried cakes of pure crystalline mannitol has been reported [36, 45]. In the present work on multi-component formulations, the fully amorphous cakes showed the jaggedness as previously reported. Interestingly, the jaggedness was lost in cakes with only a low degree of crystallinity. Additional studies would be required to establish the potential of this mechanical method to qualitatively monitor crystallinity.

Both stage (i) (quantified by crushing resistance) and stage (ii) (qualitatively characterized by jaggedness in amorphous cakes) of the force-displacement curve clearly indicate that the partially crystalline, annealed cakes were more fragile in the dry state when compared to the unannealed, amorphous cakes. Crushing resistance (referred to as crushing strength in the literature) of freeze-dried cakes is known to depend on both cake porous structure (i.e. pore size and porosity) and physical form of the cake solids (amorphous vs. crystalline) [36, 45, 46]. Since the average pore size and porosity of both annealed and unannealed cakes were approximately same (data not shown), the differences in the force-displacement curves can be attributed to crystalline mannitol. However, the mechanism by which crystalline mannitol lowers crushing resistance and improves
reconstitution time is not well understood. Microscopic discontinuities, also called stress raisers, are known to promote fractures by causing stress concentration. These discontinuities, in the form of micro-cracks, voids, notches or corners amplify the applied force leading to more readily fractured structures [47]. Perhaps, mannitol crystals act as stress raisers and provide “weak points” in the lyophilized cakes. Without the “weak points”, the applied compressive force was more evenly distributed over a larger area in the fully amorphous cakes, making them stronger and more difficult to crush. This behavior in the dry state potentially translates to reconstitution, with partially crystalline cakes being more easily dispersed into particles by the reconstitution fluid. As the cake is dispersed into smaller particles, there is more surface area available to the reconstitution fluid thereby improving dissolution.

![Figure 5: Representative force vs. displacement curves for freeze-dried cakes obtained from 100, 117 and 122 mg/ml protein solutions processed using Cycle 1 (unannealed, amorphous cakes) and Cycle 2 (annealed, partially crystalline cakes). Solid arrows indicate the transition point between stage (i) (initial linear portion seen at small displacements) and stage (ii) (pore wall fractures seen at intermediate displacements).]
Effect of freezing rate and primary drying temperature

The previous section showed the effect of annealing on mannitol crystallization to understand the differences between cake attributes of fully amorphous and partially crystalline cakes and relate them to reconstitution time. Using one of the prelyo solution compositions (100 mg/ml protein), the present section explores two other aspects of processing that can affect mannitol crystallization and hence reconstitution – freezing rate and the primary drying product temperature.

Freezing rate did not have a major effect on mannitol crystallization. This is evident from a comparison of the XRPD patterns of Cycles 1 and 3 (slow freezing) with Cycles 5 and 4 (fast freezing) respectively (Figure 6A). Instead, the primary drying conditions had a pronounced influence. The extent of mannitol crystallization was significantly higher (17 and 25%) in aggressively dried cakes (Cycles 3 and 4, respectively) than in the conservatively dried cakes (Cycles 1 and 5) which remained amorphous (Figure 6A). During aggressive primary drying, the product temperatures were 6°C (Cycle 3) and 10°C (Cycle 4) higher than the T_g’. At temperatures above T_g’, the increase in mobility is expected to promote mannitol crystallization.

The difference in the crystallinity of the cakes (amorphous vs. partially crystalline) was also captured by the force vs. displacement plots (Figure 6B). While the amorphous cakes displayed the characteristic jaggedness, the partially crystalline cakes lacked it, suggesting a more fragile cake that offered less resistance to crushing.
Figure 6: (A) XRPD patterns of cakes obtained by lyophilization of 100 mg/ml protein solution using Cycle 1 (slow freezing, conservative drying), Cycle 3 (slow freezing, aggressive drying), Cycle 4 (fast freezing, aggressive drying) and Cycle 5 (fast freezing, conservative drying). (B) Corresponding force vs. displacement curves. Solid arrows indicate the transition point between stage (i) (initial linear portion seen at small displacements) and stage (ii) (pore wall fracture events seen at intermediate displacements).

Figure 7: (A) Reconstitution time (n = 8), (B) contact angle (n = 8), (C) drop penetration time (n = 3), and (D) crushing resistance (n=7) for cakes containing 100 mg/ml protein and obtained from Cycle 1 (slow freezing, conservative drying), Cycle 3 (slow freezing, aggressive drying), Cycle 4 (fast freezing, aggressive drying) and Cycle 5 (fast freezing, conservative drying). All values are represented as mean ± standard deviation. Cakes obtained from Cycles 1 and 5 were amorphous and from Cycles 3 and 4 were partially crystalline.
The reconstitution times for the cakes containing 100 mg/ml protein and obtained from the different freeze-drying protocols are shown in Figure 7A. The conservatively dried, amorphous cakes reconstituted in 9 minutes when slowly frozen (Cycle 1) and in 11 minutes using fast freezing (Cycle 5). However, the differences were not statistically significant. Reconstitution time for the aggressively dried, partially crystalline cakes that were slowly frozen (Cycle 3) was significantly shorter (p < 0.001) than for the fast frozen, aggressively dried, partially crystalline cakes (Cycle 4). Thus, for the 100 mg/ml sample, freezing rate influenced reconstitution time only when the cakes were dried aggressively. The aggressively dried, partially crystalline cakes (Cycles 3 and 4) had lower contact angle (Figure 7B), shorter drop penetration time (Figure 7C) and lower crushing resistance (Figure 7D) than conservatively dried, amorphous cakes (Cycles 1 and 5), consistent with observations made for partially crystalline cakes in the previous section. The cakes obtained from Cycle 4 were surprising because their reconstitution time was long (14 minutes) despite containing crystalline mannitol. The favorable cake attributes associated with partially crystalline cakes, that were shown to promote faster reconstitution in the previous section, did not improve reconstitution in this case. This example points out that mannitol crystallization does not always result in reduced reconstitution times. Other reasons for the long reconstitution time for this sample are further explored below.

There was no obvious correlation of reconstitution time with either degree of crystallinity (Figure 8) or mannitol physical form. At least one report showed a correlation between mannitol physical form and reconstitution time [37]. However, the ranges of physical form (0.05-0.20 mannitol hemihydrate) and reconstitution time (0.7-2 min) were very narrow. In the present study, with only traces of other polymorphs found, no correlations were expected. Reconstitution of all
partially crystalline cakes, irrespective of the freeze-drying protocol, was accompanied by the formation of foam. In contrast to a previous report [37], there was no apparent correlation of the degree of foaming with degree of crystallinity, nor with the reconstitution time. During reconstitution of the 100 mg/ml fast frozen, aggressively dried cakes (Cycle 4), most of the cake dissolved within the first 5 minutes, which was consistent with the reconstitution-enhancing cake attributes associated with partially crystalline cakes. However, there was a small portion of the cake that became entrapped in the foam, which required an additional 9 minutes to reconstitute, producing a long total reconstitution time. Visual observation also revealed that the entrapped cake took a longer time to become wetted by the reconstitution fluid.

![Graph](image.png)

**Figure 8:** Reconstitution time of partially crystalline cakes as a function of the degree of crystallinity of mannitol. The crystallinity was quantified using Eq. (1) and the values are listed in Table 4.

Amongst the partially crystalline cakes, only the 100 mg/ml fast frozen, aggressively dried cake underwent a high degree of supercooling (by inducing ice nucleation at -10°C using the FreezeBooster® ice fog technology) followed by fast freezing to -40°C while all other partially partially crystalline cakes.
crystalline cakes were nucleated at -5°C followed by slow freezing. Fast freezing can lead to the formation of smaller ice crystals which leave behind smaller pores following ice sublimation. The combination of smaller pores and foaming slowed the reconstitution of the 100 mg/ml fast frozen, aggressively dried cake. In previous studies of high concentration proteins, authors have discussed the contribution of cake porous structure to reconstitution time in both partially crystalline [37] and amorphous cakes [16, 17]. However, in only one study was the pore size quantified directly [17]. Quantitative analysis of cake porous structure such as pore size, bulk density and porosity, will improve our understanding of apparent outliers such as the 100 mg/ml fast frozen, aggressively dried cakes, which did not follow the general “rules” of reconstitution based on the measurements made here. The effect of cake porous structure on reconstitution time in addition to the cake attributes discussed here, is currently being explored.

**Conclusions**

In the present study, methods were developed for quantitative characterization of lyophilized cake attributes such as (a) wettability of the cake solids by the reconstitution fluid, (b) penetration of the reconstitution fluid into the cake, and (c) dispersibility or disintegration of the cake. Notably, the last two test methods were performed on intact cakes in vials. This study highlighted the importance of these cake attributes in influencing the reconstitution time of high concentration lyophilized protein formulations.

Good wettability of the cake solids is important for promoting penetration of the reconstitution fluid into the cake interior. However, wettability is not the sole determinant of reconstitution time,
though it is a necessary first step for fast reconstitution. Penetration of the reconstitution fluid into the cake is preferable over wetting and erosion of the intact cake from the outside in. The penetrated fluid not only improves access of the cake interior to the dissolution medium, but also generates pressure inside the cake promoting further cake disintegration. Lastly, cake disintegration prior to hydration and dissolution of the protein matrix promotes faster reconstitution. Crystalline mannitol improved wettability of the cake solids, making the cake interior more accessible to the reconstitution fluid, and also promoted cake disintegration, which are all pre-requisites for rapid reconstitution. The present findings on protein, mannitol, and trehalose are consistent with previous work on reconstitution of partially crystalline cakes containing a protein, mannitol, and sucrose [15], suggesting that the effect of mannitol is essentially the same regardless of lyoprotectant.

Annealing promoted mannitol crystallization and reduced reconstitution time in formulations with protein to sugar ratio less than 2. In the unannealed 100 mg/ml formulation, although freezing rate did not affect mannitol crystallization, primary drying conditions did; aggressive drying promoted crystallization. In these aggressively dried, partially crystalline cakes (100 mg/ml), a slow freezing protocol was shown to reduce the reconstitution time relative to fast freezing. Hence, crystalline mannitol was shown to be necessary but not sufficient to promote rapid reconstitution. Cake porous structure, and foaming during reconstitution also seem to be major contributing factors and are currently being explored.
Mannitol, which is a bulking agent, is not often used in high concentration protein cakes. The current findings suggest a new functionality for this excipient as a ‘reconstitution time enhancer’ in high concentration lyophilized protein formulations. Based on the mechanisms discussed, the reconstitution time enhancement effect is not necessarily limited to mannitol. Any hydrophilic excipient, which can crystallize during lyophilization, should be able to reduce the reconstitution time when compared to cakes in which it remains amorphous. However, this remains to be confirmed. Furthermore, the effect of mannitol crystallinity on reconstitution time appears to be solely related to the presence or absence of crystalline mannitol in the cake and does not seem to depend on the degree of crystallinity. It should be noted that incorporating mannitol in these formulations can also increase the osmolality. Although osmolality around 300 mOsmol/kg is often preferred, especially for intramuscular and subcutaneous injections, the upper osmolality limit for these routes of administration has been suggested to be 600 mOsmol/kg [48], which was achievable in this study.
References


33. Powder Diffraction File, Card # 00-042-1142; a-D-mannitol, Card # 00-022-1793; b-D-mannitol, Card # 00-022-1797; d-D-mannitol, Card # 00-022-1794; Mannitol


Chapter 3

Implications of Formulation Composition and Processing Conditions on Porous Structure of Lyophilized Formulations Containing Highly Concentrated Proteins
Abstract

The porous structure of lyophilized cakes has been reported to influence reconstitution time, physical and chemical stability of the protein, primary drying time and cake mechanical integrity. However, cake porous structure has been mostly described by surrogate measures, such as specific surface area, and qualitative measures, such as scanning electron micrographs. Low pressure mercury intrusion porosimetry (LP-MIP) was developed as an additional tool to provide quantitative measures of the porous structure of intact cakes. The role of formulations and processing in influencing porous structure was also investigated. The glass transition temperature of the maximal freeze concentrate ($T_g'$) and viscosity of the freeze concentrate were formulation dependent factors influencing mobility and hence ice crystal growth. A longer time to reach the $T_g'$ (i.e., a longer residence time in the more “mobile” state above $T_g'$) and lower viscosity of the freeze-concentrate promoted ice crystal growth producing cakes with larger pores. Both these mobility governing factors were not just dependent on the protein concentration but also on the specific protein in the formulation. The Williams-Landel-Ferry (WLF) dependence of freeze concentrate viscosity on temperature below 0°C had implications on the freezing, annealing and primary drying stages of the lyophilization cycle. For formulations containing highly concentrated proteins, controlled ice nucleation at a high temperature (-5°C) produced cakes with largest pores. Annealing at -16°C for 2 hours produced smaller pores in formulations yielding a highly viscous freeze concentrate as compared to formulations with a low freeze-concentrate viscosity. However, formulations were a high freeze-concentrate viscosity were more resistant to cake collapse during aggressive drying. The difference between product temperature ($T_p$) and $T_g'$ was also identified as a critical factor influencing micro-collapse during aggressive drying.
Keywords:
Freeze-drying/lyophilization, proteins, freezing, annealing, primary drying, ice nucleation, porous structure, viscosity, Williams-Landel-Ferry (WLF) kinetics
Introduction

Lyophilization (freeze-drying) is often used to prepare dried biopharmaceuticals, particularly those containing high concentration proteins (≥ 50 mg/ml) to improve stability and extend shelf life. Freeze-drying converts a solution containing the protein and excipients into a dry, highly porous solid, commonly called a ‘cake’. Cake porous structure is widely acknowledged to affect the freeze-drying process and the final product attributes in several ways. During primary drying, the size and shape of the pores or channels resulting from ice sublimation influence the resistance to water vapor flow through the dry layer [1-7]. Larger ice crystals leave behind larger pores and offer lower resistance to water vapor flow, translating in a faster primary drying rate [5, 6, 8]. The porous structure has also been implicated in the long reconstitution times of high concentration proteins [9-11]. Cakes containing open, well connected, larger sized pores and higher porosity are said to facilitate penetration of the reconstitution fluid to the cake interior and improve reconstitution. Porous structure also determines the mechanical integrity of the freeze-dried cake, which is an important attribute for shipping [12-14]. Higher cake porosity and lower pore wall thickness have been shown to reduce the ‘elastic moduli’ of freeze dried cakes, suggesting that these cakes can break more easily. Finally, cake specific surface area (SSA), which serves as a measure of pore size, has been shown to correlate with protein stability in some cases [15-18].

While in some studies, lyophilized cakes with larger specific surface area, corresponding to smaller sized pores, exhibited greater protein degradation owing to a higher protein fraction at the solid-air interface, in some cases no correlation was seen [19, 20]. With additional measures of the porous structure, our understanding of the factors influencing the porous structure of lyophilized cakes will improve. The detailed effects of porous structure on drying rate, reconstitution time, and protein stability can then be explored further.
Current methods for assessing porous structure

The various techniques for analyzing cake porous structure are summarized in Table 1. Scanning electron microscopy (SEM) has been conventionally used for qualitative characterization of freeze-dried cake morphology [3, 9, 12, 13, 21-24]. Images of cake slices cut from the intact cake show detailed cross-sectional surface information (2-D view). This method requires sample preparation which exposes the sample to the atmosphere, potentially altering the microstructure of hygroscopic amorphous cakes. Moreover, sample sectioning is expected to cause sample damage and result in non-representative sections. A related method provides qualitative structural information on a larger length scale. This method involves light microscopy of cakes embedded in paraffin wax, resins or polymers [24-26]. Embedment is meant to protect the cake microstructure from damage during slicing to image the structure of the cake interior and can also minimize moisture pick up. This method also involves a 1-2 day complex sample preparation and exposes the embedded sample to high temperatures (40-60°C) during preparation. Mercury intrusion porosimetry (MIP) on a small portion of the crushed cake has been used to study pore size distribution, bulk density and total pore volume in freeze-dried cakes [9]. The analysis time for MIP is typically 1-4 hours requires little to no sample preparation. However, this method does not interrogate closed pores. Moreover, mercury requires special handling and disposal. Specific surface area (SSA) measurement using powdered cake has been widely used as a surrogate for pore size determination based on the inverse relationship between the two [1, 2].

All of the above techniques that analyze a small cake portion, capture local features of the cake microstructure but do not provide gross structural details. It has been previously shown by embedment and SSA that ice crystal morphology and hence the resulting pore size can vary across
the vial from top to bottom and from the walls to the center [3, 27]. As a result, techniques characterizing the structure of the whole intact cake are expected to be more informative than techniques involving cake sectioning/crushing. More recently X-ray micro computer-aided tomography (micro-CT) has been explored for quantitative characterization of the microstructure of intact cakes [22, 25]. X-ray micro-CT shows promise for providing information about the pore size distribution, porosity, pore wall thickness and pore connectivity. Although a powerful tool for thorough quantitative characterization of intact cakes, this method is associated with long sample analysis time and extensive data processing involving image reconstruction. If not shielded from the atmosphere during long sampling times, moisture uptake can alter the cake during analysis.

Table 1: Techniques for characterization of porous structure of freeze-dried cakes.

<table>
<thead>
<tr>
<th>Method name</th>
<th>Quantitative</th>
<th>Sample</th>
<th>Sample preparation</th>
<th>Analysis time</th>
<th>Measurements obtained</th>
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</thead>
<tbody>
<tr>
<td>SSA</td>
<td>Yes</td>
<td>Powdered cake</td>
<td>Yes</td>
<td>++</td>
<td>• Pore diameter (indirect measurement)</td>
</tr>
<tr>
<td>SEM</td>
<td>No</td>
<td>Thin cake slice</td>
<td>Yes</td>
<td>+</td>
<td>• Images of cake morphology</td>
</tr>
<tr>
<td>Embedment</td>
<td>No</td>
<td>Small cake portion cut out from the intact cake</td>
<td>Yes</td>
<td>+++</td>
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<tr>
<td>MIP</td>
<td>Yes</td>
<td>Powdered cake</td>
<td>Yes</td>
<td>++</td>
<td>• Average pore diameter • Pore size distribution • Total pore volume • Porosity • Bulk density • Tortuosity</td>
</tr>
</tbody>
</table>
### Effects of processing on porous structure

Each of the lyophilization steps can influence the porous structure with the freezing stage being the major contributor [1, 4, 7, 9, 21-23, 28, 29]. The ice crystal structure (size and shape) formed during the freezing step is the primary determinant of the porous structure of the dried product. During freezing, water is progressively removed from the protein-excipient solution as the ice crystals grow, leaving behind a freeze-concentrated solution containing the protein, any lyoprotectant, other non-crystallizable excipients and unfrozen water. The freeze-concentrated solution passes through its glass transition temperature and vitrifies as the amorphous freeze-concentrate. A non-ice crystalline phase may be present in some cases depending on the formulation composition and cooling rate. The parameters of the freezing protocol that affect the ice morphology and eventual porous structure are: (i) the ice nucleation temperature [5, 7] (ii) the freezing rate after ice nucleation [8, 9, 30] and (iii) any annealing treatment [6, 8, 30]. In addition to the freezing protocol, any solid state changes or microstructural movements that might occur during primary and secondary drying can also influence the porous structure.

---

<table>
<thead>
<tr>
<th>X-ray micro-CT</th>
<th>Yes</th>
<th>Intact cake or a small cake portion cut out from the intact cake</th>
<th>Yes</th>
<th>+++</th>
</tr>
</thead>
<tbody>
<tr>
<td>LP-MIP</td>
<td>Yes</td>
<td>Intact cake</td>
<td>No</td>
<td>+</td>
</tr>
</tbody>
</table>

1Analysis time includes both sample preparation and actual measurement time. + = less than an hour, ++ = about 1-4 hours, +++ = a day or longer
The amorphous freeze-concentrate is associated with a characteristic glass transition temperature ($T_g'$) and a collapse temperature ($T_c$). While the freeze-concentrate is ‘glassy’ below $T_g'$ and is considered immobile over the time scale of the drying process, it has increased mobility above the $T_g'$ [31-33]. Primary drying is usually performed at product temperatures below the $T_g'$ (conservative drying) to prevent cake collapse and product instability [34, 35]. Aggressive drying cycles are increasingly being explored for high concentration protein formulations, where the product temperature is maintained above $T_g'$ but below $T_c$, which is often much higher than $T_g'$ [4, 21-23, 36]. Aggressive drying has shown to reduce primary drying time while still maintaining protein stability and acceptable product appearance. In some cases, aggressive drying produced cakes with micro-collapse accompanied by formation of larger pores that further increased primary drying rate. [22, 23]. Lastly, shrinkage and cracking can occur during the secondary drying stage which leads to further changes in the cake structure [14, 27, 37, 38] but is not the focus of the present work.

Effects of formulation on porous structure

Formulation composition such as protein concentration [9, 21, 22] and type of excipients [4, 24] has been shown to affect the porous structure of protein containing lyophilized products. The role of processing in influencing porous structure is much more defined compared to that of formulation. Since ice crystal formation is a kinetic process, formulation factors influencing the mobility of the freeze concentrate such as $T_g'$ and viscosity are important. As discussed earlier, the increased mobility of the freeze-concentrate above the $T_g'$ [30-32] facilitates the growth of ice crystals until the system reaches the $T_g'$. Thus, for a given formulation, a slower shelf cooling rate
allows longer time for ice crystal growth before reaching the $T_g'$. At a constant cooling rate, the time taken to reach $T_g'$ varies with the $T_g'$ of the formulation. Proteins are known to increase the $T_g'$ as a function of concentration [23, 36] and can considerably reduce the time available for crystal growth. Since, the rate of ice growth has profound effects on its morphology [39], the time for ice crystal growth as influenced by the $T_g'$ can impact the porous structure.

An annealing step is often included in the freeze drying cycle to reduce the heterogeneity in ice crystal morphology and size resulting from uncontrolled nucleation [6, 28]. During annealing, the freeze concentrate is maintained at a subfreezing temperature above the $T_g'$ to facilitate growth of ice crystals. The increased mobility above the $T_g'$ facilitates ice recrystallization to achieve a lower energy through a lower ice/freeze-concentrate interfacial area, where the ice crystals with a smaller radii of curvature (higher free energy) melt preferentially and recrystallize on the larger ice crystals [6]. The ice recrystallization process during annealing has been shown to exhibit Williams–Landel–Ferry (WLF) kinetics [6, 31-33]. In fact, it has been suggested that adherence to WLF kinetics indicates that ice recrystallization is controlled by viscosity, via the diffusion of water through the freeze concentrate. As a result, ice crystal growth during annealing is highly dependent on the viscosity at the annealing temperature ($T_a$) because viscosity determines the mobility required in the freeze concentrate for the water molecules to diffuse and rearrange. Lower the viscosity, greater is the mobility. As in the case of annealing, non-Arrhenius (equivalently WLF-like) temperature dependence of viscosity of the amorphous freeze concentrate has been observed during primary drying [40] and the resulting viscous flow can influence pore collapse particularly during aggressive drying. Proteins at high concentration result in a tremendous increase in the viscosity [41-43].
Since both $T_g$ and viscosity of the freeze concentrate are strongly influenced by the formulation composition, the present study focused on a deeper understanding of these factors on porous structure as mediated by the formulation. In this work, the low pressure phase of the mercury intrusion porosimetry analysis was employed to provide an efficient method that can be applied to intact lyophilized cakes in the vial itself or by removing them out of the vial, without disrupting cake structure during measurement. The \textit{in-situ} analysis is expected to reduce the sampling handling artifacts, minimize exposure to the atmosphere and furnish data that is more representative of the intact cake. The present work also aims to study the effect of processing conditions namely, the freezing protocol (ice nucleation temperature, freezing rate after nucleation and annealing) and the primary drying conditions (aggressive vs. conservative) on the porous structure of formulations containing highly concentrated proteins. Previous studies have elucidated the effects of these factors separately; focusing on each step in greater detail. However, there is little literature focusing on the combined effects of variations in the freezing and drying protocol on porous structure. Hence, the current work aims at investigating different combinations of the above conditions to provide comprehensive information about optimal freeze-drying conditions for formulations containing $\geq 100$ mg/ml protein.

\textbf{Materials and Methods}

\textbf{Materials}

Formulation 1-6 were used to study the effect of formulation composition on porous structure while formulations 7 and 8 were used to explore the effect of processing conditions. F1, F2 and F3 contained the same protein but at different concentrations. The amount of lyoprotectant was also varied to maintain the total solids constant at about 20\% w/v. F3, F4, F5 and F6 had the same
composition with respect to excipients and constant total solids at 21% w/v but differed in the specific protein. Formulations 7 and 8 contained mannitol as a crystallizable excipient in addition to the lyoprotectant. F7 and F8 differed in both protein and sugar concentration but total solids were maintained constant at 21% w/v. All formulations contained a buffer at concentration < 0.5% w/v and surfactant at < 0.02% w/v. Since the buffer and surfactant amounts were relatively low as compared to the protein and lyoprotectant, their contribution to the attributes investigated was assumed to be negligible. The formulation details are listed in Table 2.
Table 2: Overview of the composition of formulations used in the study.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Protein concentration (mg/ml)</th>
<th>Type of protein</th>
<th>Molecular weight (kD)</th>
<th>Lyoprotectant (mg/ml)</th>
<th>Mannitol (mg/ml)</th>
<th>Total solids (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1</td>
<td>mAb A</td>
<td>146</td>
<td>200</td>
<td>-</td>
<td>20.4</td>
</tr>
<tr>
<td>F2</td>
<td>50</td>
<td>mAb A</td>
<td></td>
<td>144</td>
<td>-</td>
<td>19.7</td>
</tr>
<tr>
<td>F3</td>
<td>100</td>
<td>mAb A</td>
<td></td>
<td>100</td>
<td>-</td>
<td>20.5</td>
</tr>
<tr>
<td>F4</td>
<td>100</td>
<td>mAb B</td>
<td>150</td>
<td>100</td>
<td>-</td>
<td>20.6</td>
</tr>
<tr>
<td>F5</td>
<td>100</td>
<td>mAb C</td>
<td>146</td>
<td>100</td>
<td>-</td>
<td>20.6</td>
</tr>
<tr>
<td>F6</td>
<td>100</td>
<td>Fusion protein A</td>
<td>284</td>
<td>100</td>
<td>-</td>
<td>20.6</td>
</tr>
<tr>
<td>F7</td>
<td>100</td>
<td>Fusion protein B</td>
<td>73</td>
<td>29</td>
<td>77</td>
<td>20.7</td>
</tr>
<tr>
<td>F8</td>
<td>120</td>
<td>Fusion protein B</td>
<td></td>
<td>34</td>
<td>53</td>
<td>20.8</td>
</tr>
</tbody>
</table>
Methods

**Differential Scanning Calorimetry**

A differential scanning calorimeter (DSC; Model Q1000, TA Instruments, New Castle, DE, USA) was used to determine the glass transition temperature of the freeze concentrate ($T_g'$). Aliquots of each solution (10–15 mg) were hermetically sealed in aluminum pans. Formulations 1-6 were cooled to -50 °C at 0.5 °C/min, held for 15 min and then scanned at 5 °C/min to room temperature. Formulations 7 and 8 were cooled to -50 °C at 1 °C/min, held for 30 min and then scanned at 10 °C/min to room temperature. $T_g'$ values are reported as mean ± standard deviation ($n = 4$) of the midpoint of the transition.

**Lyophilization**

**Effect of formulation composition**

In most studies, the % total solid content varies along with the other formulation factors, which in itself strongly influences the porous structure [44]. Thus, it has been difficult to understand the separate effects of formulation and % total solids on cake porous structure. Formulations 1-6 were used to investigate the effect of composition on porous structure. All formulations had approximately same total solids at 21% w/v but differed either in protein concentration or the specific protein. The formulations were freeze-dried as a 1.7 ml fill in 3 ml clear tubing glass vials using one of several lab/pilot-scale freeze dryers (Lyostar 3, VirTis Genesis and Hull, SP Scientific, Stone Ridge, NY). Vials were loaded onto the freeze dryer at a shelf temperature ($T_s$) of 5°C followed by a 30 minute hold. The formulations underwent uncontrolled nucleation wherein the shelves were cooled at 0.5°C/min to -5°C and vials were equilibrated at -5°C for 30 minutes.
before cooling the shelf to -40°C at the same rate. The vials were held at -40°C for 120 minutes. All formulations underwent a heat ramp at 0.5°C /min to the annealing temperature (T_a) of -16°C. The formulations were annealed for 120 mins and were cooled back to -40°C at a ramp rate of 0.5°C/min. Following a 120 minute hold at -40°C, the shelf was ramped at 0.3°C/min to the desired primary drying T_s. Primary drying was carried out at a chamber pressure of 100 mTorr with T_s set at -35°C for F1, F2, F3 and at -10°C for F4, F5, F6. End point of primary drying was determined by comparative pressure measurement wherein the difference in pressure recorded by the Pirani gauge and capacitance manometer was within 10 mTorr. Following primary drying T_s was ramped to the secondary drying temperature of 40°C at the rate of 0.3°C/min for F1, F2, F3 and at 0.1°C/min for F4, F5 and F6. Secondary drying was carried out at a chamber pressure of 150 mTorr for 6 hours. At the end of secondary drying, T_s was lowered to 5°C, vials were stoppered, unloaded from the freeze dryer and crimped with aluminum seals.

Effect of processing conditions

Formulations 7 and 8 were used to study the effect of processing conditions on porous structure. The processing conditions investigated were the freezing protocol (ice nucleation temperature, freezing rate after nucleation and annealing) and the primary drying conditions (aggressive vs. conservative). Both freezing protocols investigated in the present study employed shelf-ramp cooling due to its industrial relevance [28]. Both formulations were filled (0.5 ml) in 3 ml clear tubing glass vials (Wheaton Type 1, Fischer Scientific, Waltham, MA, USA). Vials were loaded onto the freeze dryer at a shelf temperature (T_s) of 20°C. Formulation vials were surrounded with surrogate-containing vials to minimize the effect of radiation from the door and walls, thereby ensuring better vial-to-vial uniformity. T-type 36-gauge thermocouples (Omega Engineering, Inc.,
Norwalk, CT) were placed in the bottom center of the vials to record product temperatures during freezing and drying. Formulations were freeze-dried using a lab freeze dryer (Revo, Millrock Technology, Kingston, NY). The freeze-drying cycle details are described in Table 3 and the five cycles can be summarized as:

Cycle 1: slow freezing-aggressive drying
Cycle 2: slow freezing-conservative drying
Cycle 3: fast freezing-aggressive drying
Cycle 4: fast freezing-conservative drying
Cycle 5: slow freezing-annealing-conservative drying

At the end of secondary drying, the shelf temperature was lowered to 20°C, vials were stoppered at 700 Torr and crimped with aluminum seals upon unloading.

Table 3: Description of the freeze-drying protocol for formulations 7 and 8.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Ice nucleation temperature (°C)</th>
<th>Cooling ramp rate (°C/min)</th>
<th>Annealing</th>
<th>Primary dryingshelf temperature (°C) and chamber pressure (mTorr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 1</td>
<td>-5</td>
<td>0.2</td>
<td>-</td>
<td>-10 and 100</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>-5</td>
<td>0.2</td>
<td>-</td>
<td>-35 and 50</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>-10</td>
<td>5</td>
<td>-</td>
<td>-10 and 100</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>-10</td>
<td>5</td>
<td>-</td>
<td>-35 and 50</td>
</tr>
<tr>
<td>Cycle 5</td>
<td>-5</td>
<td>0.2</td>
<td>Yes</td>
<td>-10 and 100</td>
</tr>
</tbody>
</table>

a The shelf temperature ($T_s$) was ramped down from 20°C to the ice nucleation temperature at 1°C/min followed by a 1 hour hold prior to nucleation. Ice nucleation was induced using an ice fog (FreezeBooster nucleation station, Millrock Technology, Kingston, NY).

b Following nucleation the shelf was ramped down to -40°C followed by a 90 minute hold at -40°C.

c The shelf was ramped at 1°C/min to -20°C/min, held for 12 hours, ramped down to -40°C at 1°C/min and held for 90 minutes.

d The end of primary drying corresponded to a difference within 3 mTorr between the Pirani gauge pressure and the pressure measured by the capacitance manometer. The $T_s$ and chamber pressure during primary drying ensured that $T_p > T_g$ for aggressive drying and $T_p < T_g$ for conservative drying. Following primary drying, $T_s$ was raised to 40°C at 0.2°C/min and secondary drying was conducted for 3 hours.
MIP has been extensively used for characterizing porous materials such as pharmaceutical powders [45-47], granules [46, 48] and tablets [49-51]. It has also been used for analyzing the porous characteristics of powdered freeze dried cakes [9]. In the present study, a low pressure (up to 40 psia) mercury intrusion porosimetry (Autopore IV 9500, Micromeritics Instrument Corporation, Norcross, GA, USA) method was developed to determine the pore size distribution, average pore diameter, and porosity of intact cakes. The 3 ml glass vials containing the cakes were scored 1.5 cm from the vial bottom with a tungsten carbide scoring knife. The vial was cracked opened using a hand torch (National, Minneapolis, MN) with propane/oxygen flame (UConn Glass Shop, Storrs, CT, USA) separating the top portion from the bottom 1.5 cm containing the intact cake. The intact cake in the cut vials were sealed with Parafilm® and stored at 5°C in reclosable plastic bags with desiccant canisters.

A 15cc penetrometer with a suitable stem volume was used as the sample holder. The stem volume was selected such that the pore volume of the cake fell within 25-90% of the total stem volume. For cakes that easily slid out of the vial, the intact cake was removed from the vial and placed directly in the penetrometer for analysis. For cakes that were difficult to remove, the cut vial containing the intact cake was placed in the penetrometer. Before sample analysis, the penetrometer was calibrated for volume both with and without an empty cut glass vial. Inclusion of the empty cut glass vial during calibration accounted for the volume occupied by the glass. The sample holder assembly (penetrometer + intact cake/cut vial containing intact cake) was weighed before analysis ($W_1$). The sample holder assembly (penetrometer + mercury + intact cake/cut vial containing intact cake) was reweighed after analysis ($W_2$). Details of the optimized pressure cycle
used for analysis are provided in Table 4. Optimization of the pressure cycle is described in the Results and Discussion section. Pore size distribution was plotted as the log differential intrusion volume of mercury at the pore sizes corresponding to each applied pressure. The reported average pore diameter was the volume-surface average. Total pore volume was obtained by multiplying the % stem reading by the stem volume of the penetrometer. Percent stem is the fraction of total amount of mercury that fills the pores at maximum pressure over the stem volume. The bulk volume of the cake is the difference between penetrometer calibration volume and the volume of mercury at the filling pressure when mercury does not enter the pores. Volume of mercury at the filling pressure was calculated by dividing \( W_2 - W_1 \) by the density of mercury (13.543 g/ml at 21°C). Porosity was calculated by dividing the total pore volume by the cake bulk volume.

*Viscosity*

Formulations were reconstituted in two ways for viscosity measurement: (i) using the full volume of the reconstitution fluid to obtain the pre-lyophilization solute concentration (referred to as ‘full recon’) and (ii) using \( 1/3^{rd} \) of the reconstitution volume such the solute concentration in the reconstituted solution was three times the pre-lyophilization concentration (referred to as ‘1/3rd recon’). Reconstitution with \( 1/3^{rd} \) of the full reconstitution fluid volume yielded solutions with approximately 57% w/v solute concentration. The concentrated solution is expected to better represent the cryo-concentrated formulation at subzero temperature before reaching the \( T_g \) than does the fully reconstituted solution containing 21% w/v solutes. Viscosity of the reconstituted formulations was measured using a rheometer (AR-G2, TA Instruments, New Castle, DE, USA) with the cone and plate configuration (CP-40-2.0), having a diameter of 40 mm with a cone angle
of 2.0° and tip truncation at 56 µm. Samples (600 ± 20 µl) were loaded onto the plate for measurement. A dynamic temperature step and hold (60 seconds) profile was used at a constant frequency of 0.5 rad/s and constant stress amplitude of 0.01 Pa between 20 and -8°C. Previously, mAb solutions have been shown to form shear-sensitive clusters particularly at low temperatures [42]. Hence, oscillation mode was employed to treat the formulations more gently in case clusters were formed at lower temperatures. Viscosity measurements beyond -8°C were not possible because of partial freezing of the sample at lower temperatures. Hence, to find the viscosity of the freeze concentrate at the annealing temperature, $T_a$, (-16°C), a model that fit for the measured viscosity-temperature data (for 1/3rd recon) was extrapolated to the annealing temperature. Two models were explored, Arrhenius and Williams–Landel–Ferry (WLF) equations (Eq. (1) and Eq. (2)), to model the viscosity-temperature relationship during freezing. The Arrhenius equation describes the relationship between viscosity ($\eta$) and temperature $T$ (in kelvin) and consists of 2 parameters namely, $\ln A$ which is the intercept of the $\ln \eta$ versus $1/T$ plot and $E_a/R$ representing the slope where $E_a$ is the activation energy and R is the universal gas constant. The WLF equation also describes the relationship between viscosity ($\eta$) and temperature $T$ (in kelvin) and comprises of 4 parameters; the reference temperature ($T_{\text{ref}}$), viscosity at the reference temperature ($\eta_{\text{ref}}$), $C_1$ and $C_2$ which are coefficients describing the temperature dependence of the relaxation process. To prevent over-parameterization of the WLF equation, $T_{\text{ref}}$ was set at 273K, reducing Eq. (2) to a 3 parameter equation. GraphPad Prism 8 software was used for model fitting and the best fit model was selected by running the F test for model comparison [52, 53]. Additionally, suitability of the model was confirmed by tightness of 95% confidence intervals for the parameter values, a residual plot with low values of residuals randomly distributed about the mean and normality of the residuals determined using the D'Agostino-Pearson normality test (data not shown) [54].
\[ \ln \eta = \ln A + \frac{E_a}{RT} \]  

(1)

\[ \ln \eta = \ln \eta_{ref} - \frac{c_1(T - T_{ref})}{c_2 + (T - T_{ref})} \]  

(2)

Table 4: Optimized pressure cycles used for characterizing the porous structure of formulation 1-8 using low pressure mercury intrusion porosimetry (LP-MIP).

<table>
<thead>
<tr>
<th>Formulations studied</th>
<th>Effect of formulation composition</th>
<th>Effect of processing conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-6</td>
<td></td>
<td>7 and 8</td>
</tr>
<tr>
<td>Filling pressure (psia)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Pressure increments</td>
<td>2.5-4 at 0.25 psia</td>
<td>1-8 at 0.2 psia</td>
</tr>
<tr>
<td></td>
<td>4-6 at 0.2 psia</td>
<td>8-20 at 1 psia</td>
</tr>
<tr>
<td></td>
<td>6-15 at 0.5 psia</td>
<td>20-40 at 4 psia</td>
</tr>
<tr>
<td></td>
<td>15-20 at 1 psia</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20-40 at 4 psia</td>
<td></td>
</tr>
<tr>
<td>Equilibration time (seconds)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Maximum pressure (psia)</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>

Results and Discussion

The porous structure of lyophilized cakes has been reported to influence reconstitution time, physical and chemical stability of the protein, primary drying time and cake mechanical integrity. The porous structure in the lyophilized cake, however, has been mostly described by surrogate measures, such as specific surface area, and qualitative measures, such as scanning electron micrographs. Low pressure mercury intrusion porosimetry (LP-MIP) is an additional tool to provide quantitative measures of the porous structure of intact cakes. Using LP-MIP, the effects of formulation and processing on cake porous structure can be better understood as discussed in
the first section below. In the second section, the underlying factors governing the porous structure are explored in more detail.

Cake porous structure

Low pressure mercury intrusion porosimetry (LP-MIP) for exploring cake porous structure

During analysis using LP-MIP, the penetrometer is first evacuated and then filled with mercury while the entire system is still under reduced pressure. After mercury initially fills the penetrometer without penetrating the sample, the pressure is increased in increments such that mercury penetrates the largest pores first followed by the smaller pores at successive pressure increments. The relationship between the applied pressure and size of pores intruded by mercury at that pressure is given by the Washburn equation Eq. (3), which assumes the pores to be cylindrical.

\[ D = \frac{-1}{p} 4\gamma \cos\theta \]  

where D is pore diameter (cm), P is the applied pressure (dyne/sq.cm), γ is the surface tension of mercury (485 dyne/cm) and θ is the contact angle of mercury (2.26 radians). The size of pores in freeze-dried cakes typically ranges from 1-160 μm, with an average size and distribution that depends on the % total solutes (w/v) of the pre-lyo solution, the protein concentration and the freeze-drying protocol. LP-MIP interrogates pores as small as 4.0 μm. Higher pressures (> 45 psia) are required for interrogating pores smaller than 4.0 μm.

In the current method, a filling pressure of 1 psia, corresponding to a pore size of 180 μm, ensured that no relevant pores were intruded when mercury filled the penetrometer prior to sample
intrusion. Eliminating any intrusion into the cake at the filling pressure enables measurement of the bulk cake volume, which can then be used to calculate the bulk density. The lowest filling pressure recommended by the manufacturer is 0.5 psia which corresponds to a pore size of 362 μm. However, in some cases, it was observed that a filling pressure lower than 1 psia was unable to fill the gap between the glass vial containing the lyophile and the sample, producing a systematic error in the data. Cracks, if present in the freeze-dried cake, are likely to be filled with mercury at the selected filling pressure and therefore cannot be captured by this method.

In order to obtain an accurate pore size distribution, the incremental increase in pressure was adjusted over the pressure range such that more readings were captured in the region of maximal change in the intrusion volume, which may be different for each sample. For example, for F8, the routinely used cycle recorded the intrusion volume at only four pressures (corresponding to four pore sizes) between 40-120 μm (Figure 1A). As a result, a sharp increase is observed in the intrusion volume between 60 and 120 μm. The optimized cycle was able to capture finer details of the larger pore sizes between 40-120 μm where the increase in the intrusion volume is seen to be more gradual. The optimized cycle for F8 was sub-optimal for F1. For F1, maximum intrusion occurred between 12-60 μm (Figure 1B). Hence, a separate optimized pressure cycle was developed for F1. The locally high values of log differential intrusion volume using the optimized cycle for F1 were not reproducible and are suggestive of ‘ink-bottle’ pores, characterized by a narrow pore throat and wide inner cavity. Owing to the narrow radius at the throat, these pores are not intruded by mercury until pressures high enough to intrude the narrow entrance are reached. Once the higher pressure is achieved, both the pore throat and cavity are simultaneously intruded with mercury which is reflected as a local spike in the size distribution plot. Since, F2-F6 were
similar to F1 at least in terms of the fill volume and lyophilization protocol (with an expected similar range of pore sizes), the optimized pressure cycle for F1 was also adequate for analyzing F2-F6 (i.e., for all cakes representing the effect of formulation on porous structure) and the optimized cycle for F8 was extended to F7 (i.e., cakes representing the effect of processing on porous structure).

Insufficient equilibration time at each applied pressure can shift the pore size distribution towards smaller pores particularly at higher pressures [55]. An equilibration time sufficiently long to allow mercury to fill pores without producing a long analysis time is optimal. In the low pressure range employed, equilibration times of 3, 10 and 40 seconds were evaluated on measurements of F8 cakes. There were no meaningful differences in either the pore size distribution (Figure 1C) or the average pore diameter (40, 38 and 41 µm, respectively). Thus, an equilibration time of 10 seconds was considered robust for these formulations and enabled sample analysis to be completed in 30 minutes.

The maximum pressure of 40 psia enabled interrogation of pores down to 4.5 µm. The maximum achievable pressure in the low pressure regime is 45 psia (corresponding to pore size of 4.0 µm). The pressure of 40 psia captured 95% of the pores present in the cake (data not shown). Although higher pressures can be employed for interrogating smaller pores, the minor fraction of those smaller pores is not expected to contribute significantly towards the cake attributes of interest, particularly the reconstitution time. On visual inspection of the cake after LP-MIP, no gross
macroscopic changes to the cake structure were seen, suggesting no alteration in the structure at the applied pressures.

Figure 1: Method optimization for low pressure mercury intrusion porosimetry (LP-MIP) (A) Comparison of routinely used (gray) and optimized (black) pressure cycles for F1 containing 1 mg/ml mAb A. (B) Comparison of routinely used (gray) and optimized (black) pressure cycles for F8 containing 120 mg/ml fusion protein B. Both routinely used and optimized pressure cycles comprised of 10 second equilibration time between consecutive pressures. (C) Comparison of equilibration times of 3 (dotted), 10 (solid) and 40 (dashed) seconds using the optimized pressure cycle for three different cakes of F8 processed using Cycle 2.
Effect of formulation composition (protein concentration and specific protein) on porous structure

Even at a constant % total solids (21% w/v), increasing the protein concentration had a dramatic effect on the porous structure of the dried cake (Figure 2A and Table 6). When comparing formulations containing the same protein (mAb A) at several protein concentrations (F1, F2 and F3), the increasing protein concentration resulted in cakes with smaller sized pores (Figure 3A). The average pore diameter for F1 of 24 µm was significantly higher than F2 and F3 with mean values of 15µm and 12 µm, respectively (Table 6). Although the average diameters for F2 and F3 were not dramatically different, the pore size distribution plots show a higher fraction of larger pores for F2. Even this limited data set shows evidence that protein inhibits the growth of ice during freezing of the pre-lyo solutions.

At the same composition and protein concentration (100 mg/ml), different proteins (F3, F4, F5 and F6) significantly influenced the pore size distribution (Figure 3B). Although the average pore diameters fell within a relatively narrow range of 10-16 µm, the pore size distributions were
dramatically different. However, the total pore volume for formulations F1-F6, all containing constant total solids, was not significantly different. The total pore volume for F5 and F6 appeared to be slightly lower than the other formulations. In the pore size distribution plot, the log differential intrusion for these formulations does not reach the baseline (Figure 3B) at the maximum applied pressure of 40 psia (corresponding to 4.5 µm) in the low pressure regime, suggesting the presence of additional pores < 4.5 µm that were un-interrogated; this would explain the slightly lower total pore volume. However, as pointed out earlier, these smaller pores have a minor contribution towards the pore size distribution and do not play a role in influencing the cake properties of interest (such as reconstitution time and mechanical integrity). The average values for porosity fell within a relatively narrow range of 79.5-82.5%. At constant total solids, measured porosity is not expected to vary unless there is micro or macro collapse.

The foregoing data indicate that average pore diameter is not as discriminating as pore size distribution for describing the relevant porous nature of lyophilized cakes. Similarly, SSA is somewhat less useful as a measure of cake porous structure in that it provides a single value, like average pore diameter from LP-MIP. Assuming cylindrical pores, the pore size within the freeze-dried cake is related to the specific surface area of the cake as shown in Eq. (4), where \( r \) is the pore radius, \( \rho_s \) is the density of the solid and \( \varepsilon \) is the porosity. Based on this equation, specific surface area determined from Brunauer–Emmet–Teller (BET) gas adsorption has been used for many years as an indirect measure of porous structure.

\[
SSA = \frac{2\varepsilon}{\rho_s(1-\varepsilon)} \times \frac{1}{r}
\]  

(4)
From SSA measurements using BET a single “average” pore size can be calculated as per Eq. (4) [1, 2]. However, there is a distribution of different sized pores in the cake and SSA does not provide any information about this distribution. Furthermore, if BET analysis is performed on powdered cakes then the pore size may be biased by the sample preparation.
Table 5: Descriptors of cake porous structure for formulations F1-F6. Results are represented as mean ± range/2 of n = 2.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Protein concentration (mg/ml)</th>
<th>Protein</th>
<th>Average pore diameter (µm)</th>
<th>Total pore volume (µl)</th>
<th>Porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1</td>
<td>mAb A</td>
<td>24.0 ± 0.0</td>
<td>1210 ± 0</td>
<td>82.5 ± 0.5</td>
</tr>
<tr>
<td>F2</td>
<td>50</td>
<td></td>
<td>14.5 ± 2.0</td>
<td>1220 ± 25</td>
<td>82.3 ± 0.5</td>
</tr>
<tr>
<td>F3</td>
<td>100</td>
<td></td>
<td>11.7 ± 0.5</td>
<td>1167 ± 10</td>
<td>81.0 ± 1.0</td>
</tr>
<tr>
<td>F4</td>
<td>100</td>
<td>mAb B</td>
<td>16.0 ± 1.0</td>
<td>1180 ± 20</td>
<td>82.5 ± 0.5</td>
</tr>
<tr>
<td>F5</td>
<td>100</td>
<td>mAb C</td>
<td>9.5 ± 0.5</td>
<td>1090 ± 0</td>
<td>79.5 ± 0.5</td>
</tr>
<tr>
<td>F6</td>
<td>100</td>
<td>Fusion protein A</td>
<td>12.0 ± 1.0</td>
<td>1115 ± 25</td>
<td>80.0 ± 1.0</td>
</tr>
</tbody>
</table>
Effect of processing conditions on porous structure

Both freezing and primary drying steps during lyophilization are known to influence the porous structure of freeze dried cakes [1, 4, 7, 9, 21-23, 28, 29].

Figure 3: Effect of freezing protocol and primary drying conditions on pore size distribution in freeze dried cakes containing fusion protein B at (A) 100 mg/ml (F7) and (B) 120 mg/ml (F8). Data represented here is the average of pore size distributions obtained from 2 vials (for F7) and 4 vials (for F8).

Slow freezing ($T_{nuc}$ of -5°C followed by 0.2°C/min shelf cooling ramp in Cycle 1, 2 and 3) produced cakes with larger pores (Figure 2) with nearly twice the average pore diameter (Table 6) as compared to fast freezing ($T_{nuc}$ of -10°C followed by 5°C/min shelf cooling ramp in Cycles 3 and 4). Moreover, at both 100 mg/ml and 120 mg/ml (F7 and F8 in Figures 3A and 3B, respectively), the average pore diameter was the same, indicating that for a given protein at
constant total solids, pore size was independent of protein concentration, at least within the narrow range studied. The larger pores associated with the “slow freezing” protocol were the result of both the high \( T_{\text{nuc}} \) and the slow freezing rate post ice nucleation. It is well established that a higher nucleation temperature produces larger ice crystals which translate in larger sized pores upon drying [7, 28]. Freezing rate after controlled ice nucleation is also known to affect ice crystal morphology and hence the porous structure [7, 28]. Most previous studies showing a notable influence of freezing rate on porous structure used slow shelf ramp cooling compared to fast quench cooling (liquid nitrogen immersion that produces a freezing rate of approximately 250°C/min) or pre-cooled shelf [9, 25, 28, 30]. However, freezing rates as high as those achieved during quench cooling are not as relevant for pharmaceutical drug products that generally experience much lower cooling rates via controlled shelf cooling ramp. Fang et al. investigated the effect of shelf cooling rate on the porous structure of formulations containing 3 mg/ml rHSA (5.3% w/v total solids) lyophilized as a 1 ml fill in 5 ml vials [20]. The average pore diameter, determined using LP-MIP, was found to increase from 30 to 39 \( \mu \)m when the cooling rate after controlled nucleation at \(-12^\circ\text{C}\) was decreased from 2.5°C/min to 0.1°C/min. In another study, the combined effects of ice nucleation temperature and freezing rate post ice nucleation on the pore size were assessed indirectly by measuring the SSA [29]. At a constant ice nucleation temperature (\(-4^\circ\text{C}\) or \(-8^\circ\text{C}\)), a slower shelf cooling ramp of 0.1°C/min (equivalently a slower freezing rate) produced cakes with lower SSA (larger pores) than a fast cooling ramp of 5°C/min, consistent with the findings by Fang et al. Furthermore, the ice nucleation temperature had a greater effect on the SSA than the cooling ramp rate, i.e., samples nucleated at \(-4^\circ\text{C}\) and cooled at 5°C/min had smaller SSA (larger pores) than samples nucleated at \(-8^\circ\text{C}\) and cooled at 0.1°C/min. It has been suggested that in the case of shelf-ramp cooling, the nucleation temperature is the primary determinant of ice
crystal structure than the cooling ramp rate [28]. Hence, the present study did not investigate the effect of shelf cooling ramp rate following nucleation at high (-5°C) and low (-10°C) temperatures.

For both F7 and F8, the average pore diameters were same for the slowly cooled samples whether they were unannealed (Cycle 2) or annealed (Cycle 5) (Table 5). This observation is consistent with prior work by Searles et al. who reported that the effects of annealing were much less apparent following controlled ice nucleation at -2°C with subsequent slow cooling [6]. In fact, in the present study, annealing for as long as 12 hours did not have any effect on the pore size. Though industrially impractical, an annealing time of 12 hours was used in the present study to assure that all changes in the partially frozen cake took place and the time for annealing was not a relevant variable as has been previously reported [6, 8]. Although annealing was shown to be redundant with respect to changes in average pore size for cycles with high T\text{nucl}, it has been shown to increase pore size in formulations undergoing uncontrolled nucleation [6, 8, 9, 25] and is expected to do the same for cycles with a low T\text{nucl}. However, even in that case, the pore size post annealing is still expected to be less than that obtained by controlled nucleation at high T\text{nucl}, particularly for cakes containing proteins at high concentrations.

The effect of the drying conditions on porous structure was much smaller than the effect of freezing and was case-specific. For the slowly frozen samples of both F7 and F8 (Cycles 1 and 2), aggressive drying did not result in an increase in either the average pore diameter or total pore volume compared to conservative drying. For the fast frozen samples (Cycles 3 and 4), aggressive drying had an effect only on the lower protein concentration formulation i.e., F7. The fast frozen
and aggressively dried cakes of F7 had larger average pore diameter and higher total pore volume than conservatively dried cakes. Perhaps more importantly, the pore size distribution also shifted towards the right (towards larger pores) in the aggressively dried cakes of F7 (Figure 3). Aggressive drying has been previously shown to increase pore size based on SEM images [4, 21] and X-ray micro-CT [22]. The increased mobility of the freeze concentrate above $T_g'$ [40] during aggressive drying can potentially result in consolidation of the smaller pores to form larger ones. In one study, hole formation was reported in aggressively dried protein formulations and was found to be dependent on the difference between the primary drying product temperature and collapse temperature [4]. In the present study, for the slow frozen samples, the $T_p - T_g'$ during aggressive drying was 6.4°C for F7 and 3.4°C for F8. For the fast frozen samples the temperature difference was 10.1°C for F7 and 6.5°C for F8. A higher $T_p - T_g'$ presumably resulted in greater mobility in fast frozen F7 cakes during primary drying which was reflected in an altered cake structure. It is likely that the mobility in the other aggressively dried cakes was not sufficient (due to lower $T_p - T_g'$) to translate into detectable changes in the porous structure. Pore consolidation is a possible explanation for the higher total pore volume of fast frozen, aggressively dried cakes of F7 compared to fast frozen, conservatively dried cakes. It is likely that conservatively dried cakes have pores < 4.5 µm which are below the detection limit but consolidation of these pores during aggressive drying leads to formation of pores > 4.5 µm that are now detected, resulting in an increased total pore volume. When compared to conservative drying, aggressive drying significantly lowered the primary drying time from 27 hours to 7 hours for slow frozen samples and from 38 hours to 9 hours for fast frozen samples, thereby increasing the cycle efficiency.
Table 6: Descriptors of cake porous structure for formulations containing fusion protein B at 100 mg/ml (F7) and 120 mg/ml (F8) and freeze-dried using Cycles 1-5. Results are represented as mean ± range/2 of n = 2 for F7 and as mean ± SD of n = 4 for F8.

<table>
<thead>
<tr>
<th>Cycle No.</th>
<th>Cycle description</th>
<th>Average pore diameter (µm)</th>
<th>Total pore volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>F7</td>
<td>F8</td>
</tr>
<tr>
<td>1</td>
<td>slow freezing-aggressive drying</td>
<td>39</td>
<td>39 ± 1</td>
</tr>
<tr>
<td>2</td>
<td>slow freezing-conservative drying</td>
<td>37 ± 0</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>fast freezing-aggressive drying</td>
<td>18 ± 1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>fast freezing-conservative drying</td>
<td>14 ± 0</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>5</td>
<td>slow freezing-annealing-conservative drying</td>
<td>37 ± 0</td>
<td>43 ± 3</td>
</tr>
</tbody>
</table>
Underlying factors governing porous structure

The previous sections discussed both formulation and processing influences on cake porous structure. Ice crystal growth during the freezing and annealing stages is particularly relevant in governing the final porous structure. Since ice crystal growth is kinetically driven, mobility of the freeze concentrate using measures such as $T_g'$ and viscosity at sub-zero temperatures are expected to influence ice crystal growth and the resulting porous structure of the dried cake. Both $T_g'$ and viscosity are highly formulation dependent. The present section discusses the influence of $T_g'$ and viscosity on cake porous structure as mediated by the formulation.

Glass transition temperature of the freeze concentrate ($T_g'$) and time to reach $T_g'$

The frozen matrix has higher mobility above the $T_g'$ [30-32]. Owing to the greater mobility, ice crystals have the opportunity to grow until the system reaches the $T_g'$, below which the mobility is essentially zero at least during the time scales involved in the freeze-drying process. Given a constant cooling rate, the time to reach $T_g'$ following ice nucleation or annealing will depend on the $T_g'$ of the formulation. As a result, when formulations are being cooled, a longer time to reach $T_g'$ is expected to produce larger ice crystals and therefore larger pores.
Table 7: Glass transition temperature of the freeze concentrate ($T_g'$) determined using differential scanning calorimetry and expressed as mean ± standard deviation for $n = 4$.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Protein concentration (mg/ml)</th>
<th>Protein</th>
<th>$T_g'$ (°C)</th>
<th>Time to reach $T_g'$ (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1</td>
<td>mAb A</td>
<td>-33.9 ± 0.5</td>
<td>36</td>
</tr>
<tr>
<td>F2</td>
<td>50</td>
<td></td>
<td>-33.2 ± 0.9</td>
<td>36</td>
</tr>
<tr>
<td>F3</td>
<td>100</td>
<td></td>
<td>-27.5 ± 1.2</td>
<td>24</td>
</tr>
<tr>
<td>F4</td>
<td>100</td>
<td>mAb B</td>
<td>-35.0 ± 1.2</td>
<td>20</td>
</tr>
<tr>
<td>F5</td>
<td>100</td>
<td>mAb C</td>
<td>-32.7 ± 1.1</td>
<td>38</td>
</tr>
<tr>
<td>F6</td>
<td>100</td>
<td>Fusion protein A</td>
<td>-26.3 ± 0.6</td>
<td>34</td>
</tr>
</tbody>
</table>

'Represents time to reach $T_g'$ following nucleation and/or annealing. The cooling rate for all formulations was 0.5°C/min. Nucleation temperature was assumed to be -15°C for all formulations and annealing temperature was -16°C.

The $T_g'$ is influenced by the composition of the freeze concentrate at $T_g'$ $i.e.$, by the nature of the glass formers and their concentrations in the freeze concentrate [33, 56]. For formulations containing mAb A, $T_g'$ was unaltered in the range of protein concentration from 1 to 50 mg/ml (F1 vs. F2), but increased when protein concentration was raised from 50 to 100 mg/ml (F2 vs. F3) (Table 7). The main components of the formulations were protein, lyoprotectant, buffer salts and water. Based on the Fox equation, the $T_g'$ of the formulation is the weight average of the $T_g'$ of the pure components [33]. Hence, the $T_g'$ of formulations is expected to increase with protein concentration when the composition of other components remains constant [22, 23, 36]. However, when the fraction of lyoprotectant is reduced as protein fraction increases to maintain a constant % total solids, as in the present scenario, the contribution of the protein fraction in the formulation becomes important. The relative change in $T_g'$ values as a function of protein concentration can be different in lyoprotectant rich formulations as compared to protein rich formulations [21, 22, 36]. In general, for formulations containing no (i.e., a placebo containing lyoprotectant and buffer) or very low amounts of protein ($\leq 5$ mg/ml) such as F1, the $T_g'$ has been shown to range between -32 to -34°C irrespective of the type of protein. In formulations where the protein is 25% of the
total solids, such as in F2, the composition of the freeze concentrate is still dominated by the lyoprotectant and \( T_g' \) has been found to range between -30 to -33°C, which is not significantly different from formulations containing < 5 mg/ml protein. However, when the protein comprises about half of the solids such as in F3, the protein has been shown to significantly raise the \( T_g' \) to as high as -26°C.

Interestingly, \( T_g' \) values for formulations with the same composition but different protein (F3 through F6) ranged from -28 to -35°C (Table 7). Since \( T_g' \) values ranging from -23 to -29°C are more common for protein formulations containing > 50 mg/ml protein [22, 23, 36], the low values for F4 and F5 were unexpected. The \( T_g' \) is greatly influenced by the amount of unfrozen water in the freeze concentrate [31, 32]. Although the amount of unfrozen water in the freeze concentrate typically ranges from 15-20% w/w [28], it can be as high as 50% [56]. Water is a potent plasticizer and lowers the \( T_g' \). The ice melting endotherm in a DSC scan is frequently employed to estimate the amount of unfrozen water in the freeze concentrate [57]. Higher melting enthalpy indicates less amount of unfrozen water in the freeze concentrate which would correspond to a higher \( T_g' \) value. The average enthalpy of melting for F3 and F6 was 222 J/g of pre-lyo solution and was significantly higher \((p < 0.01)\) than the average melting enthalpy of 196 J/g for F4 and F5. The lower fraction of unfrozen water in F3 and F6 is reflected in the higher \( T_g' \) values compared to F4 and F5. Such protein-specific differences in the \( T_g' \) values of formulations with the same composition have the potential to alter the porous structure of the lyophilized cakes. The reasons for the differences in the \( T_g' \) values of the proteins were not explored as that was not the focus of this study. Differences have been noted in frozen-state protein-water interactions for monoclonal
antibodies and lysozyme [58]. In fact, $T_g'$ values of a wide variety of pure proteins have been reported to range between -9 and -15°C [33].

The time to reach $T_g'$ (equivalently the time spent in the “mobile” state) was the same for F1 (1 mg/ml) and F2 (50 mg/ml), but was reduced by 33% for F3 (100 mg/ml) owing to a higher $T_g'$ of the latter mAb A formulation (Table 7). The shorter time to reach $T_g'$ may explain the smaller sized pores in F3 (12 µm). However, despite the same time to reach $T_g'$ as F1, the pore size of F2 (15 µm) was significantly lower than F1 (24 µm) (Figure 2A). Thus, the time to reach $T_g'$ is not sufficient to explain differences in pore size.

When comparing formulations containing different proteins (i.e., F3-F6), the relationship between time to reach $T_g'$ and pore size distribution was contradictory to what was expected. F4 and F5 had a significantly longer time between nucleation/annealing and $T_g'$ as compared F3 and F6. Yet, F3 and F6 cakes had larger pores than F5 (Figure 2B). Furthermore, despite similar times to reach $T_g'$, the pore size distribution plot showed much larger pores in F4 (average 16 µm) compared to F5 (average 10 µm). Hence, time between the start of freezing and the time to reach $T_g'$ is not the sole factor influencing ice crystal growth and mobility related parameters such as viscosity also seem to be contributing factors.
Viscosity-temperature profile during freezing

Figure 4: Viscosity-temperature profiles for formulations F1-F6 reconstituted using full volume (A) and 1/3rd volume (B) of reconstitution fluid. Data are represented as the mean of 3 vials. The error bars denote standard deviation and if not visible are smaller than the symbols used. The line connects the data points to guide the eye and is not a result of model fitting to the data.

Ice crystal growth is expected to be influenced by the local viscosity. Viscosity is known to depend on both temperature and concentration of solutes. The decrease in temperature during freezing and the resulting freeze concentration both contribute to a dramatic rise in the viscosity of the remaining solution until the system reaches its $T_g$ [15, 59]. Hence, viscosity of formulations F1-F6 was measured as a function of temperature from 20 to -8°C, beyond which measurement was not possible due to sample freezing. Although complex viscosity is reported here, the $G'$ values (elastic component) were close to zero for both ‘full recon’ and ‘1/3rd recon’ samples within the temperature range examined. Since $|G'/G''| \leq 0.1$ (data not shown), it can be concluded that the solutions exhibited very low elasticity and the fluid flow and rheological behavior was predominantly determined by the viscous component, $G''$ [42].

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The viscosities of the fully reconstituted formulations (F1-F6) at room temperature (20°C) fell within a relatively narrow range of 5-15 cP (Figure 4A). However, at lower temperatures (-8°C), the viscosity of the formulations increased in the order F1 = F2 < F6 < F4 < F3 < F5. The increase in viscosity at was about 1.3 times for F1 and F2, 2 times for F6, 2.5 times for F4, 3 times for F3, 184 times for F5 than that at room temperature. The sharp rise in viscosity of F5 as compared to F3, F4 and F6 during cooling (particularly at subzero temperatures) potentially indicates formation of clusters at lower temperatures. The cluster forming propensity of proteins depends on a number of factors such as nature of intermolecular interactions, net charge and charge distribution, size, shape and effective volume of the protein [41-43, 60]. Although a detailed investigation of these factors would be helpful, it was outside the scope of the present study. Nonetheless, the point to be highlighted is that the viscosity of high concentration protein formulations during freezing is highly protein-specific and may not be evident at room temperature. Viscosity at subzero temperatures may be more relevant to the growth of ice during freezing and the resultant porous structure of lyophilized cakes.

The viscosity of fully reconstituted solutions (equivalent to pre-lyo solutions) is relevant to ice crystal growth at and immediately following ice nucleation. However, as freezing proceeds, higher formulation concentrations are more relevant, due to freeze-concentration. The solutions obtained by reconstituting cakes with 1/3rd the volume of reconstitution fluid yielded solutions with approximately 57% w/v solute concentration. These “concentrated” solutions are expected to be more representative of the freeze concentrate than the fully reconstituted samples containing 21% w/v solutes. The viscosity at ‘1/3rd recon’ was significantly higher than the corresponding ‘full recon’ viscosity, as expected (Figure 4B). However, unlike the viscosities of the fully reconstituted
protein solutions, significant differences in room temperature viscosity between formulations were observed at ‘1/3rd recon’ with the viscosity rank order from lowest to highest being F1 < F2 < F6 = F3 ≈ F4 < F5. The viscosities of F3, F4 and F6 were practically the same and significantly higher than F1 and F2, but dramatically lower than F5. The same rank order of viscosity was observed at -8°C. The viscosity-temperature profiles for F1 and F2 were similar to each other at ‘full recon’ but showed significant difference at ‘1/3rd recon’. Similarly, the differences in the viscosity-temperature profiles between F2 and F3 were more prominent at ‘1/3rd recon’ than at full recon. These examples point out that increasing solute concentration can influence the viscosity-temperature behavior of formulations with different composition to varying extents, particularly in this case where % total solids constant was constant. However, when comparing the viscosity-temperature profiles of the four proteins in identical formulation matrix (F4-F6), the same general rank order was observed at both full and 1/3rd recon. This indicates that merely increasing the solute concentration does not influence the viscosity rank order for different protein as long as the formulation composition is maintained constant.

The goal is to relate these temperature dependent viscosity differences to the porous structure. However, the ice continues to grow well below the -8C at which the concentrated formulation viscosity (‘1/3rd recon’) was measured. To extrapolate to temperatures as low as $T_g$, both linearized Arrhenius and WLF equations were fitted to viscosity-temperature data. The model of best fit (Table 8) based on the F test for model comparison ($p < 0.0001$) was used for extrapolation. For ‘full recon’ samples at low protein concentration of 1 and 50 mg/ml (F1 and F2), the temperature dependence of viscosity displayed Arrhenius behavior while at high protein concentration of 100 mg/ml (F3-F6), viscosity was better described by WLF. In the case of ‘1/3rd
recon’, all formulations exhibited WLF behavior, as expected from previous WLF temperature dependence for concentrated aqueous solutions at subzero temperatures [31, 32, 40, 59]. In the case of F1, the fit was ambiguous, so the simpler Arrhenius model was used. In the case of F2, the WLF (with a greater number of parameters) did not provide a significantly better fit, the Arrhenius model was used. The low dependence of the viscosity of F1 and F2 on temperature is also reflected in the low R² values for the Arrhenius fit to those data (Table 8).

The results for ‘full recon’ samples of F3-F6 were quite interesting. Viscosity of high concentration protein solutions containing up to 200 mg/ml protein have been previously shown to follow Arrhenius behavior in the 20°C to 5°C temperature range [42, 61, 62]. When the ‘full recon’ viscosities in the 20°C to 5°C range (Figure 4) were fit to Arrhenius and WLF equations, the Arrhenius model provided a better fit (data not shown), consistent with previous findings. However, upon extending the temperature range to -8°C to include the lower temperatures, viscosity showed WLF dependence.

The WLF equation is applicable in the ‘non-glassy’ region typically between T_g and the melting temperature (T_m). Within the WLF region, viscosity and the related mobility phenomena in the freeze concentrate show a steep temperature dependence just above T_g that becomes a more shallow Arrhenius type temperature dependence on approaching T_m. The WLF temperature dependence has profound implications on the process design and product characteristics including porous structure resulting from the ice crystal growth. The ‘full recon’ viscosity represents the viscous behavior of formulations as they undergo cooling before ice nucleation. For example,
when not controlled, ice nucleation can occur spontaneously between -8°C to -20°C in a clean laboratory environment [2, 63]. In contrast, in an ISO 5 environment, the $T_{\text{nuc}}$ can be as low as -25°C [2]. A high degree of supercooling and the consequent fast ice crystal growth rate (due to the lower shelf temperature) is known to result in smaller ice crystals [7, 28]. The dramatically higher viscosity at these low temperatures near the $T_g'$, particularly for formulations containing high concentration proteins, reduces mobility in the freeze-concentrate surrounding the growing crystals, hindering ice crystal growth, which is reflected as smaller pores. Hence, controlling $T_{\text{nuc}}$ at low degree of supercooling (higher shelf temperatures such as -5°C) potentially allows slower growth of ice crystals to a larger size, yielding a higher porosity cake. Based on the WLF equation, the viscosity at this temperature would be approximately 4-5 orders of magnitude lower than that at -25°C. As pointed out earlier, ‘1/3rd recon’ samples are roughly expected to mimic the freeze concentrate due to the high solute concentration. In the case an annealing step is included in the freeze-drying cycle, the freeze concentrate is maintained above the $T_g'$ where the WLF equation applies. Therefore, the viscosity-temperature data for the more highly concentrated ‘1/3rd recon’ samples can be used for understanding the size distribution of ice crystals during annealing. These effects are considered in greater details in the next section.
Table 8: Results obtained by fitting the viscosity-temperature data for ‘full recon’ and ‘1/3rd recon’ samples of F1-F6 to linearized Arrhenius and WLF models (Eq. (1) and Eq. (2) respectively).

<table>
<thead>
<tr>
<th>Sample description</th>
<th>Best fit model</th>
<th>Model parameters</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( E_a/R )</td>
<td>ln A</td>
</tr>
<tr>
<td>F1 full recon</td>
<td>Arrhenius</td>
<td>366</td>
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<tr>
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<td></td>
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<td>-0.9</td>
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<td></td>
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<td></td>
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<td>51</td>
</tr>
<tr>
<td>F1 1/3rd recon</td>
<td>WLF</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>F2 1/3rd recon</td>
<td></td>
<td>0.9</td>
<td>27</td>
</tr>
<tr>
<td>F3 1/3rd recon</td>
<td></td>
<td>3.3</td>
<td>65</td>
</tr>
<tr>
<td>F4 1/3rd recon</td>
<td></td>
<td>5.3</td>
<td>62</td>
</tr>
<tr>
<td>F5 1/3rd recon</td>
<td></td>
<td>7.8</td>
<td>121</td>
</tr>
<tr>
<td>F6 1/3rd recon</td>
<td></td>
<td>13.7</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.4</td>
<td>55</td>
</tr>
</tbody>
</table>

Implications of WLF kinetics in the freeze concentrate on cake porous structure

The WLF equation describes the temperature-dependent behavior of the freeze concentrate at temperatures above the glassy domain, where molecular relaxation and the related viscosity and mobility phenomena depend on the magnitude of the temperature difference above the \( T_g \). During annealing, mobility in the freeze concentrate is a function of the difference between the annealing temperature, \( T_a \), and \( T_g \) (\( T_a - T_g \)). The larger the temperature difference, the lower the viscosity and greater the mobility. The higher mobility at \( T_a \) facilitates restructuring of ice wherein there is a movement of molecules from higher energy sites (i.e., more dendritic ice formed rapidly at high degree of supercooling) to lower energy sites (i.e., smoother more planar surfaces formed at low degree of supercooling) and smaller ice crystals melt and recrystallize on larger crystals [6, 28, 39]. The mobility during the restructuring of ice during annealing is related to the viscosity of the freeze concentrate. As seen in the previous section, the viscosity of the freeze concentrate is highly dependent on both protein concentration and the protein itself. As a result, even formulations with
the same value of $T_a - T_g'$, may have different mobilities at the annealing temperature due to the viscosity. The formulation dependent viscosity differences explains why the time taken to reach $T_g'$ was unable to explain some of the observed differences in the porous structure of the different formulations. Viscosity at $T_a$ ($\eta_{\text{ref}}$) was estimated by extrapolation of the viscosity-temperature data for the ‘1/3rd recon’ samples to -16°C ($T_{\text{ref}}$) using the WLF model parameters from Table 8.

Figure 5: Natural log of viscosity at the annealing temperature ($T_a$) of -16°C for formulations F1-F6. Formulations F1-F3 contained mAb at different protein concentrations; F1 - 1 mg/ml, F2 - 50 mg/ml and F3 - 100 mg/ml. Formulations F3-F6 had the same composition but contained different proteins at 100 mg/ml; F3 - mAb A, F4 - mAb B, F5 - mAb C and F6 - fusion protein A. Viscosity values were estimated by extrapolation of the viscosity-temperature data -16°C using the WLF model parameters from Table 8.

The In (viscosity) of the more highly concentrated ‘1/3rd recon’ solution of F3 extrapolated to the annealing temperature (-16°C) was 2 times that of F1 and about 1.6 times of F2 (Figure 5). The higher viscosity at $T_a$ coupled with the shorter time to reach $T_g'$ resulted in smaller ice crystals, which translate to smaller sized pores. Interestingly, natural log of viscosity of F2 at $T_a$ was only 1.2 times that of F1, yet F1 had significantly larger pores than F2 which is not expected to result solely from the viscosity differences. The time taken to reach $T_g'$ was practically same for these
two formulations (Table 7). On visual observation of the cakes, F1 showed areas of cake at the vial bottom that were pulled away from the walls indicating minor collapse (Figure 6). Similar collapse was seen in placebo cakes obtained by freeze drying the formulation buffer without protein in the same cycle as F1 and F2 (data not shown). The placebo formulation, F1, and F2 were all dried conservatively, with Tₛ of -35°C during primary drying. It is possible that at the primary conditions the product temperature exceeded Tg’ for the formulation buffer and F1 but not for F2; in this case, the higher mobility during primary drying may have resulted in micro-collapse, producing larger pores. Even if the Tg’ of F2 were exceeded by chance, the higher viscosity (due to higher protein concentration) can possibly limit the mobility, thereby preventing collapse.

The ln (viscosity) of F3, F4 and F6 at Tₐ were 8.6, 8.2 and 7.6, respectively (Figure 7B). Despite similar viscosities, the pore size distribution of F4 displayed significantly larger pores than F3 and F4. Although the viscosities were similar, the time to reach Tg’ was greater F4 than F3 and F6, providing a longer time for the ice crystals to restructure, resulting in larger sized pores. When comparing F4 and F5, although the time to reach Tg’ was the same, ln (viscosity) of F5 at Tₐ was 1.5 times that for F4, offsetting the additional time and resulting in smaller pores. Thus, it appears that both residence time in the more mobile state and viscosity at Tₐ influence the size distribution of ice crystals and hence the final pore size distribution in the freeze-dried cake.

According to WLF behavior, the viscosity of the freeze concentrate are much more sensitive to temperature near Tg’ than would be expected from Arrhenius behavior. Therefore, merely increasing the Tₐ by 5-10°C can reduce the viscosity by several orders of magnitude. Not only the
annealing temperature and the viscosity of the freeze-concentrate at that temperature, but the annealing time is also expected to influence the size of ice crystals. The cubic mean size of ice crystals has been shown to increase linearly with time [6]. Hence, annealing at a temperature well above the T_g’ (maximizing T_a - T_g’) for longer durations are expected to increase the pore size, particularly for high viscosity formulations such as F5. Just as the viscosity at T_a influences the porous structure during annealing, the viscosity at the product temperature during primary dry is expected to influence porous structure during aggressive drying. This behavior was evident in F1 compared to F2. F2 having a higher viscosity seemed to be more resistant to collapse compared to F1. In fact, viscosity during primary drying can also explain the significantly different pore size distribution in F4. F4, F5 and F6 were dried aggressively at a shelf temperature of -10°C. It is likely that the mobility in F4 was greater than that for F6 due to the higher difference between the product temperature and T_g’. Furthermore, very viscous F5 was more resistant to micro-collapse than F4 despite a comparable T_g’. Thus, the occurrence and extent of micro-collapse during primary drying may also depend on the viscosity of the freeze concentrate which is highly formulation specific.

Figure 6: Representative mAb A cakes after lyophilization of F1 (1 mg/ml protein) and F2 (50 mg/ml protein). Area of minor collapse in F1 is circled.
Conclusions

Low pressure mercury intrusion porosimetry proved to be a promising technique for fast, in-situ characterization of intact cakes, providing quantitative measures of porous structure such as average pore diameter, pore size distribution, total pore volume and porosity. Pore size distribution was found to be most discriminating in terms of describing the relevant porous structure of freeze dried cakes containing highly concentrated proteins.

The present study highlights the dependence of cake porous structure on both formulation composition and processing conditions. In fact, the choice of processing conditions is highly dependent on the formulation, particularly at high protein concentrations. The $T_g'$ and viscosity of the freeze concentrate were formulation dependent factors influencing mobility and hence ice crystal growth. A longer time to reach the $T_g'$ (i.e., a longer residence time in the more “mobile” state above $T_g'$) and lower viscosity of the freeze-concentrate promote ice crystal growth producing cakes with larger pores. Both these mobility governing factors were not just dependent on the protein concentration but also on the specific protein in the formulation.

WLF temperature dependence of freeze concentrate viscosity was observed at subzero temperatures. The WLF dependence has implications on both the annealing and primary drying stages of the lyophilization cycle. Formulations yielding a highly viscous freeze concentrate would require a combination of higher annealing temperature and longer annealing duration for promoting ice crystal growth (by increasing mobility) to obtain larger pores. However, these
formulations would be more resistant to cake collapse during aggressive drying as compared to formulations with a low freeze-concentrate viscosity.

In terms of the freezing protocol, controlled ice nucleation at high temperatures produces cakes with larger pores as compared to uncontrolled nucleation followed by annealing at least for the formulations and processing conditions examined in this study. The controlled ice nucleation has also been shown to reduce heterogeneity across the batch and can potentially improve product stability [7, 28]. Hence, controlled nucleation at a high temperature is advantageous. A nucleation temperature of -4°C or -5°C is preferred because there have been reports of not achieving 100% nucleation success within a batch at $T_{\text{nuc}} > -3^\circ\text{C}$ [63]. A slow shelf cooling ramp following controlled ice nucleation can further help in obtaining larger ice crystals (by increasing the time to reach $T_g^*$), translating into larger pores.
References


Chapter 4

Role of Formulation in Mediating Reconstitution Times of Highly Concentrated Lyophilized Proteins Formulations
Abstract
Lyophilized protein formulations containing highly concentrated proteins often have long and variable reconstitution times. The goal was to understand the role of formulation in mediating the reconstitution time. A variety of formulation variables such as % total solids, protein concentration, protein-to-sugar ratio, different proteins and inclusion of a crystallizable excipient were investigated for their effect on cake properties influencing reconstitution namely, cake wettability, penetration of reconstitution fluid into the cake, cake disintegration and cake porous structure. Additionally, several measures of viscosity were also evaluated for their effect on reconstitution time. Reconstitution time was primarily influenced by the “concentrated formulation viscosity” with negligible contributions from % total solids and protein concentration. The “concentrated formulation viscosity” was sensitive to both protein-to-sugar ratio and the protein itself. Partial crystallinity in the final cake also expedited reconstitution. Wettability (assessed using contact angle), ability of aqueous liquids to penetrate into the cake (assessed using drop penetration time), cake disintegration tendency (assessed from the resistance offered by cakes to crushing in the dry state) and cake porous structure were found to be invariant for amorphous cakes and did not correlate with reconstitution time. However, these properties were sensitive to the presence of crystallinity and resulted in faster reconstitution, at least of the partially crystalline cakes. “Concentrated formulation viscosity” strongly correlated with reconstitution times of amorphous cakes, providing insights on the steps involved in the reconstitution of these formulations.

Keywords: Protein formulation, high concentration, lyophilization, freeze drying, reconstitution, viscosity, cake properties, amorphous, crystalline
Introduction

Lyophilization (freeze-drying) is often used to improve the pharmaceutical stability of proteins that are unstable when formulated as liquids. The lyophilization process transforms aqueous protein solutions into solid, porous cylinders known as “cakes” that must be reconstituted back into a solution prior to patient administration. Some lyophilized protein products, especially those containing high protein concentrations (> 50 mg/ml) have long and variable reconstitution times [1-5]. Long reconstitution times can be problematic particularly for self-administered drug products. For these products, long reconstitution times are likely associated with the risk of immunogenicity due to undissolved proteinaceous small particles if the patient tries to self-administer the drug prior to complete reconstitution. Administration of the partially reconstituted product can also lead to incomplete dosing. In addition to being problematic from a patient perspective, long reconstitution times are also inconvenient for busy health care providers such as doctors, nurses and pharmacists.

Properties of the lyophilized cake that have been implicated in influencing reconstitution are the wettability of the solids comprising the cake [1, 6], liquid penetration into the cake [6], cake dispersibility/cake disintegration [1, 6], cake solids rehydration rate [1], and cake porous structure [2, 6, 7]. Each of these properties is known to be affected by the protein concentration, thereby influencing the reconstitution time. At low to moderate protein concentrations (1-30 mg/ml), the wettability of cake solids by an aqueous drop decreased with protein concentration [1]. The same authors reported that the rates of cake disintegration and hydration decreased dramatically with protein concentration. At higher protein concentrations (>100 mg/ml), the aqueous drop remained on the cake surface for at least 24 hours, signaling long reconstitution times [6]. Measures of the cake porous structure – notably the pore size distribution, bulk density and total pore volume –
were found to be markedly different for formulations containing 50 and 200 mg/ml protein, prolonging reconstitution of the latter [2].

Reconstitution time and the cake properties influencing it are also affected by the percentage of total solids in the pre-lyo solution, protein-to-sugar ratio and inclusion of crystallizable excipient in the formulation. The effect of the % total solids in the pre-lyo solution is exemplified by the so-called “partial recon” approach. Briefly, formulations lyophilized from solutions with lower total solids and reconstituted with a smaller volume of the diluent to obtain the desired high solute concentrations post reconstitution dissolved faster than formulations lyophilized at the desired higher total solid concentration [4, 8]. For example, a cake obtained by lyophilizing 2 ml of a solution containing 100 mg/ml solutes and reconstituted to 1 ml with water to yield 200 mg/ml is expected to dissolve faster than a cake obtained by lyophilizing 1 ml of a solution containing 200 mg/ml solutes and reconstituted using 1 ml diluent. It has been suggested that the more porous cake structure resulting from lower total solids facilitates liquid penetration into the cake, significantly lowering reconstitution time. In fact, in one study, the authors attempted to determine whether there was a threshold value for total solids beyond which the reconstitution times increased dramatically [1], but no such threshold was identified. For cakes in which the solids are amorphous, protein-to-sugar (P:S) ratio has also been proposed as a critical factor influencing reconstitution [1, 6]. A comparison of the P:S ratio in protein formulations (5-60 mg/ml) from several studies found that at P:S ratios less than 1, reconstitution time was less than a minute regardless of the specific protein or sugar. At P:S ratios beyond 1, a dramatic increase in reconstitution times was noted. However, in another study, amorphous cakes with P:S ratio of 0.5 and lyophilized from solutions containing 70 mg/ml protein reconstituted in about 6 minutes. As
the P:S ratio increased from 0.5 to 2 (corresponding to protein concentration from 70 mg/ml to 140 mg/ml), the reconstitution time increased sharply in agreement with Sane et al. [1]. Thus, protein-to-sugar ratio and protein concentration cannot be assessed independently for their effect on the reconstitution time.

Reconstitution times are dramatically different for partially crystalline cakes compared to their amorphous counterparts [4, 6]. The partial crystallinity arises from excipients that easily crystallize during the freezing step or for which crystallization can be promoted during an annealing process. Partial crystallinity in a lyophilized cake significantly improves the wettability, liquid penetration into the cake and dispersibility of the cake, when compared to amorphous cakes of the same formulation [6], translating into reduced reconstitution times. Interestingly, no correlation was found between the degree of crystallinity and reconstitution time; any level of quantifiable crystallinity promoted faster reconstitution. However, it should be noted that crystallization of excipients is inhibited at high protein concentrations [4, 6].

Protein-specific cake properties are also expected to influence reconstitution time if all other variables are maintained constant. While there are no reports documenting the effect of different proteins on reconstitution time, Kulkarni et al. found dramatic protein-specific differences in cake porous structure [9]. Since, cake porous structure is one of the factors affecting reconstitution, protein-specific cake characteristics can presumably influence reconstitution. However, whether protein-specific characteristics influence reconstitution solely through their effect on porous structure or by influencing the other cake properties such as wettability, disintegration and hydration has not yet been reported.
In most previous studies [1-3], an increase in the protein concentration was also accompanied by a concomitant increase in both % total solids and protein-to-sugar ratio, because the concentration of all other excipients was maintained constant. Hence, the longer reconstitution times reported for higher protein concentrations could be due to % total solids, protein-to-sugar ratio, protein concentration, or a combination. Isolating the individual contribution of each factor could suggest which strategies are most effective in reducing reconstitution times. With insights from previous studies, the present report aims to further investigate the role of formulation in influencing reconstitution by examining a wider range of formulation variables, specifically, the total solids, protein concentration, protein-to-sugar ratio, different proteins and presence of a crystallizable excipient. The goal was to evaluate these formulation variables both independently and in combination to identify the relative contribution of each in governing reconstitution time through their effect on the cake properties.

**Materials and Methods**

**Materials**

The proteins investigated in the study were provided by MedImmune (Gaithersburg, MD) and GSK (Collegeville, PA). Three monoclonal antibodies, mAb A, mAb B, and mAb C (MW ~150 kDa) and fusion protein A (MW 284 kDa) were provided by MedImmune. Fusion protein B (MW 73 kDa) was provided by GSK. Each of these proteins was formulated in a buffered (< 0.05% w/v) solution with a lyoprotectant and surfactant (< 0.02% w/v) to yield the formulations denoted F1-F10 (Table 1). At such low concentrations, surfactant has been shown to have no effect on reconstitution [4]. Formulations F1-F8 contained sucrose as lyoprotectant, while F10 contained
trehalose. The formulations containing mAb A differed in their total solids, protein concentration and protein-to-sugar (P:S) ratio which ranged from 10-21% w/v, 1-100 mg/ml and 0.01-1 respectively (F1-F5). Formulations F5-F8 contained different proteins, all formulated at 21% w/v solids, and 100 mg/ml protein and P:S ratio of 1. To evaluate the effect of a crystallizable excipient, glycine was included in F9 and mannitol in F10.
Table 1: Overview of formulation compositions

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Type of protein</th>
<th>Protein concentration (% w/v)</th>
<th>Lyoprotectant concentration (% w/v)</th>
<th>Crystallizable excipient concentration (% w/v)</th>
<th>Protein-to-sugar ratio</th>
<th>Total solids (% w/v)</th>
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</thead>
<tbody>
<tr>
<td>F1</td>
<td>mAb A</td>
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<td>20</td>
<td>0</td>
<td>0.005</td>
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<tr>
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<td>10</td>
<td>0</td>
<td>0.01</td>
<td>10.7</td>
</tr>
<tr>
<td>F3</td>
<td>mAb A</td>
<td>5</td>
<td>14.4</td>
<td>0</td>
<td>0.35</td>
<td>19.7</td>
</tr>
<tr>
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<td>mAb A</td>
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<td>0.50</td>
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</tr>
<tr>
<td>F5</td>
<td>mAb A</td>
<td>10</td>
<td>10</td>
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<td>1.00</td>
<td>20.5</td>
</tr>
<tr>
<td>F6</td>
<td>mAb B</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>1.00</td>
<td>20.6</td>
</tr>
<tr>
<td>F7</td>
<td>mAb C</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>1.00</td>
<td>20.6</td>
</tr>
<tr>
<td>F8</td>
<td>Fusion protein A</td>
<td>10</td>
<td>10</td>
<td>0</td>
<td>1.00</td>
<td>20.6</td>
</tr>
<tr>
<td>F9</td>
<td>mAb A</td>
<td>10</td>
<td>0¹</td>
<td>4²</td>
<td>1.67³</td>
<td>16.8</td>
</tr>
<tr>
<td>F10</td>
<td>Fusion protein B</td>
<td>14</td>
<td>4⁴</td>
<td>2.8⁵</td>
<td>2.1⁶</td>
<td>20.9</td>
</tr>
</tbody>
</table>

¹ F9 contained 2% w/v arginine instead of a lyoprotectant.
² Glycine was included as the crystallizable excipient.
³ 1.67 represents the ratio of protein to excipients (arginine+glycine).
⁴ Trehalose was used as the lyoprotectant in F10. All other formulations contained sucrose.
⁵ Mannitol was included as the crystallizable excipient.
⁶ Protein-to-sugar ratio represents ratio of protein to (trehalose + mannitol).
Methods

Lyophilization

All formulations were freeze-dried from a 1.7 ml fill in 3 ml clear tubing glass vials using one of several lab/pilot-scale freeze dryers (Lyostar 3, VirTis Genesis and Hull, SP Scientific, Stone Ridge, NY) or a small batch freeze dryer (MicroFD, Millrock Technology, Kingston). Vials were loaded onto the freeze dryer at a shelf temperature \(T_s\) of 5°C, held for 30 minute before cooling at 0.5°C/min to -5°C and holding for 30 minutes to provide partial thermal equilibration in the solutions. The shelf was then cooled to -40°C at 0.5°C/min and held for 2 hours. Using a heat ramp of 0.5°C/min, the shelf was raised to -16°C for 2 hours for annealing, after which the shelf was cooled back to -40°C at a ramp rate of 0.5°C/min and held for 2 hours. For primary drying, the chamber pressure was lowered to 100 mTorr and the shelf temperature was raised at 0.3°C/min to the target shelf set point of -35°C for F1-F6 and -10°C for F7-F10. When the Pirani gauge pressure converged to within 10 mTorr of the capacitance manometer reading, the shelf temperature was ramped to 40°C at the rate of 0.3°C/min for F1-F6 and at 0.1°C/min for F7-F10. After 6 hours of secondary drying at a shelf temperature of 40°C and a chamber pressure of 150 mTorr for 6 hours, the shelf temperature was lowered to 5°C, and vials were stoppered, unloaded from the freeze dryer and crimped with aluminum seals. The residual moisture content in all lyophilized formulations was found to be < 1% w/w.

Reconstitution

Vacuum in the vial, if any, was released by inserting a 21G needle through the septum, prior to reconstitution. A 3 ml syringe attached to a 1 in. long 21G needle was filled with the appropriate amount of reconstitution fluid and was pierced through the center of the vial stopper until it was
completely inside the vial. The reconstitution fluid was injected onto the walls of the vials in less than 10 seconds. During injection, the vial was gently rotated to ensure uniform distribution of the fluid throughout the vial. The detailed protocol for reconstitution was previously described [6]. The observational pauses (each for 30 seconds) for the purpose of evaluating reconstitution behavior and determination of reconstitution end point were modified from the previous protocol. The first pause was at 30 seconds after fluid addition and swirling was resumed at 1 minute. The next pause was at 7 minutes for 30 seconds. The next swirling periods were 7 minutes, 15 minutes and subsequently 10 minutes until reconstitution was complete. Total reconstitution time included the observational pause periods, because the cake was in contact with the fluid during that time. Reconstitution times are reported as mean ± standard deviation of 8 vials.

**Wettability of cake solids**

Lyophilized solids were compressed into discs as previously described [6]. Wettability of the compressed cake solids was assessed by measuring the contact angle of a sessile drop of a nearly saturated aqueous solution (68% w/w sucrose) using a goniometer (Model 100-00, Ramé-hart Inc., Succasunna NJ, USA). Contact angle values were reported as mean ± standard deviation (n = 8).

**Liquid penetration into cakes**

The drop penetration time was measured to assess the accessibility of the cake core to the reconstitution fluid as previously described [6]. Briefly, a 10 µl drop of 55% w/w sucrose solution, tinted with a blue dye (to aid visibility) was placed on the surface of the intact cake in the vial. The drop was composed of a nearly saturated solution to reduce dissolution of the cake solids during
measurement. The time taken by the drop to ‘disappear’ (i.e., completely drain) from the surface was measured and reported as the drop penetration time. Some formulations were characterized by a ‘volcano’ (small amount of raised solids) [10] at the central portion of the cake surface. The angled surface was expected to confound the measured drop penetration time, so a small amount of material from the surface was gently scraped away using a metal spatula to create a flat horizontal surface. A similar scraping approach was followed for studying drop penetration in porous powder beds of pharmaceutical excipients used in tablets [11]. Drop penetration times on the unscraped cake surface were highly variable as compared to those on the scraped surface and the latter were used for reporting because they seemed to be more characteristic of the bulk cake structure. The test was terminated at 15 minutes, because after 15 minutes the drop remained on the surface for 24 hours. So, a drop penetration time of 15 minutes can be interpreted as > 15 minutes. Drop penetration times were reported as mean ± standard deviation (n = 4).

_Cake crushing resistance_

The resistance of the cake to crushing in the dry state was previously found to provide an assessment of the ease with which the cake disperses or disintegrates upon addition of the reconstitution fluid and was measured as previously described [6]. Briefly, the force necessary for displacement of a stainless steel, cylindrical probe (3 mm diameter) up to 6 mm into the intact cake at a constant rate (2mm/min) was recorded. The cylindrical probe was attached to a calibrated 1 kN load cell (Model 5866, Instron Corporation, Norwood, MA, USA). The slope of the initial linear portion of the force versus displacement curve was reported as cake crushing resistance (in N/mm) and expressed as mean ± standard deviation (n = 4).
Cake porous structure

Quantitative measures of the porous structure of the cake were determined by low pressure mercury intrusion porosimetry (LP-MIP; Autopore IV 9500, Micromeritics Instrument Corporation, Norcross, GA, USA). The volume of mercury intruded into intact cakes at predetermined pressures was measured as previously described [9]. Briefly, the top portion of the vial was cut off (1.5 cm from the vial bottom), the intact cake was removed from the cut vial and placed in the penetrometer (sample holder) for analysis. Mercury was initially filled into the penetrometer at 1 psia. A previously optimized pressure cycle [9] for the pore size range in the lyophilized cakes allowed details of the pore size distribution to be captured at relevant pore sizes. Specifically, the pressure was increased at increments of 0.2 psia from 1-8 psia, followed by 1 psia increments from 8 to 20 psia, and 4 psia increments from 8 to 40 psia with an equilibration time of 10 seconds at each applied pressure. Measurements were made on 2 vials of each formulation. From the volume of mercury intruded at each pressure, the volume-surface average pore diameter and porosity were determined. In addition, the fraction of pores greater than a threshold pore size (or % pores less than the threshold) was calculated. A value of 12 µm was used as the pore size threshold in this work.

Viscosity

Formulations were reconstituted in three ways for viscosity measurement: (i) using the full volume of the reconstitution fluid to obtain the pre-lyophilization (prelyo) solute concentration (referred to as ‘full recon’); (ii) using 2/3rd of the reconstitution volume such that the solute concentration in the reconstituted solution was about 1.5 times the prelyo concentration (referred to as ‘2/3rd recon’) and (iii) using 1/3rd of the volume yielding about 3 times the prelyo concentration (referred
to as ‘1/3rd recon’). Viscosity of the reconstituted formulations was measured using a cone and plate rheometer (AR-G2, TA Instruments, New Castle, DE, USA) with a 40 mm diameter cone with a 2.0° angle and tip truncation at 56 µm (CP-40-2.0). Samples (600 ± 20 µl) were loaded onto the plate for measurement. Viscosities of the samples were measured at shear rates of 0.1 to 1000 second⁻¹. The torque was allowed to reach steady state over 60 seconds at each shear rate. Five data points were acquired per decade of shear rate. Samples were protected from evaporation by using a solvent trap. Temperature was controlled at 25 ± 0.1°C using the Peltier plate with circulating fluid from a water bath. Viscosity of both ‘full recon’ and ‘1/3rd recon’ samples at 25°C was found to be independent of shear rate between 1-1000 second⁻¹ (data not shown). Viscosity was reported at a shear rate of 10 second⁻¹ because the shear rate during manual swirling using the reconstitution protocol was approximated to be on the order of 10 second⁻¹, in agreement with a previously published report [12]. The mean and standard deviation of three measurements of viscosity is reported in cP (1cP = 1 mPa.s).

Results and Discussion

Formulation is known to affect several cake properties that influence reconstitution [1, 4, 6]. After examining the effect of formulation on reconstitution time in the first section, the second section focuses on understanding the relationship between reconstitution time and these cake properties as mediated by the formulation variables. The last section explores the contribution of formulation viscosity towards the reconstitution behavior.
Influence of formulation on reconstitution time

Figure 1: (A) Effect of protein concentration and (B) Effect of protein-to-sugar ratio on reconstitution time. Reconstitution times (bars) of formulations F1-F10 are expressed as mean ± standard deviation for n = 8 and represented on the primary Y axis. The bar colors represent the different type of proteins with gray for mAb A, blue for mAb B, green for mAb C, orange for fusion protein A and red for fusion protein B. The total solids in each formulation are represented on the secondary X axis corresponding to the formulation shown on primary X axis. The protein concentration (black squares) in each formulation is represented on the secondary X axis in 1A and the line connects the data points to guide the eye. The protein-to-sugar ratio in each formulation (black circles) is denoted on the secondary Y axis in 1B and the line connects the data points to guide the eye. All cakes were amorphous except F9 which was partially crystalline as identified using polarized light microscopy. Partial crystallinity is indicated by a star. F9 did not contain a sugar and the protein-to-sugar ratio reflects the protein to excipient (other than buffer and surfactant) ratio.

In general, the reconstitution times for lyophilized cakes were found to increase with an increase in the protein concentration across formulations F1-F10 (Figure 1A), in agreement with previous reports. However, there are notable exceptions to this very general rule. For example, differences were also noted between formulations containing the same protein concentration. Compare F3 with F4, F5 with F9, and F7 with F5, F6 and F8. Moreover, the reconstitution time of F10, with the highest protein concentration (140 mg/ml), was same as F5, F6, and F8, with only 100 mg/ml. Hence, the differences in reconstitution times cannot be explained by protein concentration alone. These formulations also differed in their % total solids in the pre-lyo solution, protein-to-sugar
ratio, specific protein and inclusion of a crystallizable excipient. The effect of each of these variables on reconstitution time is discussed in detail below.

At low protein concentrations (1 mg/ml), reconstitution was completed within 2 minutes irrespective of the protein-to-sugar (P:S) ratio and total solids as seen in the case of F1 and F2. There are two ways to evaluate an increase in protein concentration on reconstitution time: (i) at constant % total solids, which requires a decrease in lyoprotectant as protein concentration increases and (ii) at a constant % lyoprotectant in the pre-Iryo solution, which increases the % total solids. In both cases, the P:S ratio increases with protein concentration, but it increases more sharply in the first case. Formulations F1, F3 and F5, which contain the same protein, have the same % total solids (20%) and the P:S ratio increases from 0 to 1 (i.e., as in the first case above).

In this series of formulations, the higher P:S ratio prolongs reconstitution (Figure 1B) in agreement with a previous report [6]. Longer reconstitution times have also been reported for the second scenario [1], but could be the result of the higher total solids. In the present study, formulations F3 and F4 have the same protein concentration (50 mg/ml), but the protein-to-sugar ratio of F4 is higher than for F3 (Figure 1B). Formulation F3 with higher total solids (19.7% w/v) reconstituted significantly faster in 8 minutes as compared to 15 minutes for F4 which had lower % total solids (15.6%). In this case, in spite of lower total solids, the higher P:S ratio for F4 (0.50 compared to 0.35 for F3) prolonged its reconstitution. The example of F3 and F4 also may also suggest that P:S ratio has a stronger influence on reconstitution than protein concentration since both formulations contained 50 mg/ml protein. Formulations with P:S ratios less than 1 have been reported to reconstitute in typically less than a minute regardless of the type of protein when protein concentration ranges from 5.5 to 60 mg/ml [1]. However, formulations F3 and F4 are exceptions to this previous finding.
Neither protein-to-sugar (P:S) ratio, protein concentration or % total solids are sufficiently dominant factors to fully explain differences in reconstitution time. For example, formulations F5, F6, F7 and F8 (Figures 1A and 1B) are equal with respect to all three characteristics, with a P:S ratio of 1, protein concentration of 100 mg/ml and 21% w/v total solids. Still, reconstitution of F7 was about twice that of F5, F6 and F8. This suggests an important role for aspects specific to the protein, particularly at high protein concentrations. Additionally, the reconstitution time of the formulation with the highest P:S ratio and protein concentration (F10; P:S ratio of 2.1; 140 mg/ml) was comparable to that of F6 and F8, both with P:S ratio of 1 and protein concentration of 100 mg/ml. In fact, at high protein concentrations (typically > 50 mg/ml), reconstitution time seems to be influenced to a greater extent by the specific protein than the % total solids, protein concentration or P:S ratio.

Table 2 provides a summary of the combined effects of protein concentration, protein-to-sugar ratio, % total solids and protein on reconstitution time for amorphous cakes from the present work and four other studies. To account for differences in the fill volume and size of vials used across the different studies, the cake surface area to volume ratio for each formulation was calculated [1]. A higher ratio is indicative of a lower cake volume due to either lower fill volume or larger vial. The cake surface area to volume ratios were comparable for most cakes, ranging from 4.5-6.5 cm⁻¹, except for 9.6 cm⁻¹ which represented a 0.5 ml fill in 3 ml vials. The higher ratio is expected to promote faster reconstitution compared to a cake with the same composition but a lower value for the cake surface area to volume ratio, because of the better agitation when swirling a larger vial with a lower fill. For example, increasing the fill volume from 0.5 ml to 1.7 ml in a 3 ml vial
doubled the reconstitution time for 140 mg/ml fusion protein B formulation, using the same reconstitution protocol. In addition to the vial size and fill depth differing among the studies summarized in Table 2, the reconstitution method also differed across the studies and is expected to influence the time [4]. However, the goal of Table 2 was to identify general trends in reconstitution time as a function of formulation variables and not necessarily predict absolute values of that time. Several generalizations were identified for protein-containing amorphous cakes:

1. At protein concentrations < 50 mg/ml and total solids < 12% w/v, reconstitution was complete in less than 2 minutes. Protein-to-sugar ratio in these formulations was found to be < 1. Reconstitution is fairly instantaneous at protein concentrations < 50 mg/ml and is independent of total solids, protein-to-sugar ratio and type of protein, consistent with previous findings [1].

2. At protein concentration > 50 mg/ml and total solids typically > 12% w/v, reconstitution times were long and variable. Protein-to-sugar ratio in these formulations ranged from 0.3–4.0. A previous report had identified a threshold value of 1 for protein-to-sugar ratio, beyond which a significant increase in reconstitution time was noted [1]. Examples from this work and the study by Kulkarni et al. [6] that encompass a larger data set find that the ratio at which there is a significant increase in reconstitution time may be a protein-dependent range including ratios less than one.

3. At high protein concentrations (typically > 50 mg/ml), reconstitution times were influenced to a greater extent by the specific protein than the % total solids, protein concentration or protein-to-sugar ratio. However, for a given protein at constant % total solids, higher protein concentration corresponding to a higher protein-to-sugar ratio increased the reconstitution time.
Lastly, the cakes obtained from freeze-drying F9, containing 100 mg/ml mAb A were partially crystalline confirmed by birefringence under polarized light (data not shown). The average reconstitution time for F9 cakes (23 min), with a relatively high protein to excipient ratio of 1.7 was significantly lower than F5 (~40 min), an amorphous cake also containing 100 mg/ml of the same protein, but at a lower P:S ratio of 1. Presence of crystalline mannitol in the lyophilized cake was previously shown to dramatically lower reconstitution times [4, 6]. Kulkarni et al. elucidated the mechanisms by which crystalline mannitol improved the reconstitution time of partially crystalline cakes compared to their amorphous counterparts [6]. Based on these mechanisms, the authors surmised that not just mannitol, but any excipient that crystallizes during the lyophilization cycle should similarly reduce reconstitution time. The present example provides evidence corroborating their extrapolation, where crystalline glycine also offered a reduction in reconstitution time. Interestingly, F9 did not contain a sugar and the ratio of 1.7 was protein to excipients (in this case, arginine and glycine). Improved reconstitution in the “sugar-free” partially crystalline cake (F9) is another example suggesting that disaccharides are not necessary to reduce reconstitution of highly concentrated protein formulations. Mannitol-containing amorphous cakes of F10 were slow to reconstitute reinforcing the conjecture by Kulkarni et al. that the simple inclusion of a crystallizable excipient in the formulation does not expedite reconstitution. The reconstitution rate is enhanced only when this excipient actually crystallizes at least in part during lyophilization.

Both, protein and sugar (lyoprotectants such as sucrose and trehalose) have a concentration dependent inhibitory effect on the crystallization of bulking agents like mannitol and glycine. Protein concentrations > 100 mg/ml strongly inhibit mannitol crystallization; an annealing step is
often required to obtain partially crystalline cakes [6]. In formulations containing ≥ 100 mg/ml protein, annealing has been shown to promote mannitol crystallization up to a protein to mannitol ratio of 3, beyond which amorphous cakes were obtained irrespective of annealing [6]. In the present study, annealing for 2 hours at -16°C enabled at least partial crystallization of glycine in F9 (protein to glycine ratio of 2.5), but not mannitol in F10 (protein to mannitol ratio of 5), consistent with the previous findings. In the previous study, annealing of F10 was carried out at -20°C for 12 hours, and still it remained amorphous.
Table 2: Comparison of reconstitution times of amorphous cakes differing in protein concentration, protein-to-sugar ratio, total solids and specific protein.

<table>
<thead>
<tr>
<th>Protein concentration (mg/ml)</th>
<th>Protein-to-sugar ratio</th>
<th>Total solids (%w/v)</th>
<th>Protein type</th>
<th>Reconstitution time (min)</th>
<th>Cake surface area to volume ratio (cm&lt;sup&gt;1&lt;/sup&gt;)</th>
<th>Reconstitution method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.005</td>
<td>20.4</td>
<td>mAb A</td>
<td>&lt; 2</td>
<td>4.8</td>
<td>Manual; continuous swirling</td>
<td>Present study</td>
</tr>
<tr>
<td>1</td>
<td>0.01</td>
<td>10.7</td>
<td>mAb A</td>
<td>&lt; 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>5.6</td>
<td>mAb 1</td>
<td>&lt; 0.5</td>
<td>4.5</td>
<td>Manual; continuous swirling</td>
<td>[1]</td>
</tr>
<tr>
<td>5</td>
<td>0.2</td>
<td>3.0</td>
<td>Lysozyme, BSA, mAb 2</td>
<td>&lt; 0.2</td>
<td>5.8</td>
<td>-</td>
<td>[13]</td>
</tr>
<tr>
<td>11</td>
<td>0.2</td>
<td>6.1</td>
<td>mAb 1</td>
<td>&lt; 0.5</td>
<td>4.5</td>
<td>Manual; continuous swirling</td>
<td>[1]</td>
</tr>
<tr>
<td>17</td>
<td>0.3</td>
<td>6.6</td>
<td>mAb 1</td>
<td>&lt; 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>0.4</td>
<td>7.2</td>
<td>mAb 1</td>
<td>&lt; 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0.80</td>
<td>4.5</td>
<td>Lysozyme, BSA, mAb 2</td>
<td>&lt; 0.2</td>
<td>5.8</td>
<td>-</td>
<td>[13]</td>
</tr>
<tr>
<td>28</td>
<td>0.55</td>
<td>7.8</td>
<td>mAb 1</td>
<td>&lt; 2</td>
<td>4.5</td>
<td>Manual; continuous swirling</td>
<td>[1]</td>
</tr>
<tr>
<td>33</td>
<td>0.66</td>
<td>8.3</td>
<td>mAb 1</td>
<td>&lt; 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>0.77</td>
<td>8.9</td>
<td>mAb 1</td>
<td>&lt; 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.57</td>
<td>11.0</td>
<td>mAb 3</td>
<td>&lt; 2</td>
<td>6.2</td>
<td>Manual; no swirling</td>
<td>[2]</td>
</tr>
<tr>
<td>50</td>
<td>0.7</td>
<td>12.0</td>
<td>BSA</td>
<td>&lt; 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>0.35</td>
<td>19.7</td>
<td>mAb A</td>
<td>8</td>
<td>4.8</td>
<td>Manual; continuous swirling</td>
<td>Present study</td>
</tr>
<tr>
<td>50</td>
<td>0.5</td>
<td>15.6</td>
<td>mAb A</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>1.7</td>
<td>13.3</td>
<td>mAb 1</td>
<td>14</td>
<td>4.5</td>
<td>Manual; continuous swirling</td>
<td>[1]</td>
</tr>
<tr>
<td>100</td>
<td>0.9</td>
<td>20.7</td>
<td>Fusion protein B</td>
<td>9</td>
<td>9.6</td>
<td>Manual; continuous swirling</td>
<td>[6]</td>
</tr>
</tbody>
</table>

<sup>1</sup> Cake surface area to volume ratio is reported as cm<sup>1</sup>.
<table>
<thead>
<tr>
<th>100</th>
<th>1.4</th>
<th>17.0</th>
<th>Fusion protein 1</th>
<th>13</th>
<th>6.0-7.0(^2)</th>
<th>Manual; intermittent swirling</th>
<th>[4]</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>1.0</td>
<td>20.5</td>
<td>mAb A</td>
<td>40</td>
<td></td>
<td>Manual; continuous swirling</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.0</td>
<td>20.6</td>
<td>mAb B</td>
<td>37</td>
<td>4.8</td>
<td>Manual; continuous swirling</td>
<td>[6]</td>
</tr>
<tr>
<td>100</td>
<td>1.0</td>
<td>20.6</td>
<td>mAb C</td>
<td>74</td>
<td></td>
<td>Present study</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1.0</td>
<td>20.6</td>
<td>Fusion protein A</td>
<td>29</td>
<td></td>
<td>Manual; continuous swirling</td>
<td></td>
</tr>
<tr>
<td>122</td>
<td>1.4</td>
<td>20.8</td>
<td>Fusion protein B</td>
<td>13</td>
<td>9.6</td>
<td>Manual; continuous swirling</td>
<td>[6]</td>
</tr>
<tr>
<td>130</td>
<td>2.0</td>
<td>20.0</td>
<td>Fusion protein 1</td>
<td>15</td>
<td>6.0-7.0(^2)</td>
<td>Manual; intermittent swirling</td>
<td>[4]</td>
</tr>
<tr>
<td>140</td>
<td>2.1</td>
<td>20.9</td>
<td>Fusion protein B</td>
<td>32</td>
<td>4.8</td>
<td>Manual; continuous swirling</td>
<td>Present study</td>
</tr>
<tr>
<td>140</td>
<td>2.1</td>
<td>20.9</td>
<td>BSA</td>
<td>18</td>
<td>4.5</td>
<td>Manual; continuous swirling</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>150</td>
<td>2.1</td>
<td>22.0</td>
<td>Fusion protein 1</td>
<td>18</td>
<td>6.0-7.0(^2)</td>
<td>Manual; intermittent swirling</td>
<td>[4]</td>
</tr>
<tr>
<td>200</td>
<td>4.0</td>
<td>27.0</td>
<td>BSA</td>
<td>40</td>
<td>6.2</td>
<td>Manual; no swirling</td>
<td>[2]</td>
</tr>
</tbody>
</table>

\(^1\) Cake surface area to volume ratio reflects differences in the fill volume and size of vials used for lyophilization. A higher ratio is expected to promote faster reconstitution.

\(^2\) Estimated value assuming vial size to be 3, 5 or 10 ml.
Effect of formulation on cake properties implicated in influencing reconstitution

Cake wettability, liquid penetration into the cake and cake disintegration have been implicated in influencing the reconstitution of high concentration lyophilized proteins [1, 6]. The following sections address how the formulation variables affect these cake properties, thereby influencing reconstitution.

Influence of formulation on cake wettability and liquid penetration and their relationship to reconstitution time

![Graph showing reconstitution times and contact angles for formulations F1-F10](image)

Figure 2: Reconstitution times (bars) of formulations F1-F10 expressed as mean ± standard deviation for n = 8 and represented on the primary Y axis. The bar colors represent the different type of proteins with gray for mAb A, blue for mAb B, green for mAb C, orange for fusion protein A and red for fusion protein B. The contact angles of a nearly saturated aqueous drop on compacted cake solids (black squares) are denoted on the secondary Y axis and are expressed as mean ± standard deviation for n = 8. The line connects the data points to guide the eye. All cakes were amorphous except F9 which was partially crystalline. Partial crystallinity is indicated by a star.
The contact angle\(^1\) of an aqueous drop on compacted cake solids is a measure of the wettability of the solids that form the pore walls in the lyophilized cake, with lower contact angles indicative of better wettability. In a previous study, contact angles of amorphous cakes were found to increase with protein concentration up to 30 mg/ml beyond which they plateaued [1]. In that study, an increase in protein concentration from 0 to 30 mg/ml was also accompanied by an increase in protein-to-sugar ratio from 0 to 0.5. In another study, contact angles of partially crystalline cakes were significantly lower than their fully amorphous counterparts with the same formulation composition at protein concentrations \(\geq 100\) mg/ml and protein-to-sugar ratio ranging between 0.9-1.4 [6]. Based on the Wenzel equation for wettability, chemical heterogeneity arising from partial crystallinity on the surface of the compacts was shown to lower contact angles compared to completely amorphous surfaces [6].

In the present study, the contact angle of the saturated aqueous drop on the compacted cake solids of the amorphous cakes (all formulations except F9) increased from 35° to 94° (Figure 2) as the protein concentration increased from 1 to 140 mg/ml and protein-to-sugar ratio increased from 0.005 to 2.1. Thus, the increased protein concentration and/or protein-to-sugar ratio reduced cake wettability. At the low protein concentration of 1 mg/ml (F1 and F2), wettability of cake solids ranked with protein-to-sugar ratio. The contact angle of an aqueous drop on the compacted solids of F1 with a lower protein-to-sugar ratio of 0.005 was lower than for F2 whose protein-to-sugar ratio was 0.01. Therefore, based on the current data and previous reports, at least at lower protein

\(^1\) It is important to point out that contact angles most accurately reflect the characteristics of a smooth, rigid and non-reactive solid surface. Since the compaction process used to prepare planar samples of the cake solids for contact angle measurements reduced, but could not eliminate all pores in the freeze-dried solids, the reported contact angles should be interpreted relative to each other rather than their absolute values relative to other materials.
concentrations (< 25 mg/ml), wettability seems to be a function of both protein concentration and the protein-to-sugar ratio, with a greater influence of the latter. When comparing F3, F4 and F5, all containing mAb A at different protein concentration and/or protein-to-sugar ratio, the contact angles were the same for all practical purposes (between 80° and 87°), confirming that at higher protein concentrations (≥ 50 mg/ml) contact angle was independent of both protein concentration and protein-to-sugar ratio. Formulations F5, F6, F7, F8 and F10, all containing 100 mg/ml protein, had a wide range of reconstitution times (29 - 74 minutes), but no significant protein-specific differences in contact angle (83° - 94°), which are all indicative of poor wetting. Thus, from the present findings and previous reports, it can be concluded that at moderately high protein concentrations (≥ 25 mg/ml), contact angle values are independent of protein concentration, protein-to-sugar ratio and the type of protein, implying no further change in the ability of the aqueous fluid to wet the surface. Wettability was strongly influenced by the presence of crystallinity (e.g., F9). Despite a high protein concentration and protein to excipient ratio, F9 had a significantly lower contact angle of 66° as compared to the amorphous compacts of F3-F10, with contact angles ranging from 80° - 94°.

Partially crystalline cakes of F9 with a lower contact angle reconstituted faster than the amorphous cakes at the same protein concentration. When comparing the low concentration amorphous formulations (F1 and F2) with the high concentration amorphous formulations (F3-F10 except F9), increased contact angle was accompanied by an increase in the reconstitution time. However, within the high concentration amorphous formulations, reconstitution times were dramatically different despite little difference in contact angles. Thus, low contact angle is a good predictor of fast reconstitution, but after a certain contact angle, the difficulty in wetting is accompanied by
other factors that have additional influence on the reconstitution time. Although wettability was not the sole determinant of reconstitution time, the increase in contact angle points out that the relative hydrophobicity of the amorphous protein-rich solids reduces the wettability and subsequent cake dissolution.

For rapid reconstitution, the reconstitution fluid in addition to being able to wet the cake, must also penetrate the porous cake thereby gaining access to the cake core. Rapid liquid penetration facilitates disintegration of the cake and dissolution of the interior of the cake, without requiring erosion from the surface. The drop penetration test applies the concepts of liquid penetration into porous media to understand penetration of the reconstitution fluid into the porous cake [6]. The time for a drop of a constant volume of a given liquid to penetrate a porous solid, completely disappearing from the surface, is inversely proportional to the pore size, porosity and cosine of the contact angle of the liquid on the solids. Hence, a more porous cake structure with larger pores and good wettability of the cake solids will promote rapid drop penetration.

Based on the results of the drop penetration time (Figure 3), the formulations can be classified into two groups. The first group comprising of F1, F2 and F9 where the drop instantaneously penetrated the cake surface. The second group consisting of all the remaining formulations where the drop did not penetrate the cake surface within the test time of 15 minutes and remained on the surface up to 24 hours. Instantaneous drop penetration was expected for the low protein concentration formulations F1 and F2 which had larger sized pores (Table 3) and dramatically lower contact angles (Figure 3) than the high protein concentration formulations. At high protein concentrations, where the drop did not penetrate the cake (F3-F10 except F9), the formulations were characterized
by pores ranging in size from 7.5 to 17 µm (Table 3), porosity between 77% and 86% (Table 3) and contact angles between 80° and 94°. However, these differences did not result in different drop penetration times, which were measured up to 15 minutes for all formulations. In general, at higher protein concentrations, the reconstitution fluid cannot easily access the cake interior, independent of the protein concentration, P:S ratio and specific protein. An exception was F9, where the drop was instantaneously absorbed into the cake. The atypical behavior of F9 is due to its partial crystallinity. The average pore size and porosity of F9 were similar to the other amorphous high protein concentration formulations. In fact, despite smaller sized pores in F9 compared to F4 (same porosity), drop penetration was still faster in F9, due to the significantly lower contact angle (Figure 3). The same behavior has been reported previously where partially crystalline cakes showed faster drop penetration compared to amorphous cakes with the same formulation composition, pore size and porosity [6]. Overall, the present data suggest that drop penetration time for high concentration protein formulations is largely influenced by the contact angle, with negligible contributions from pore size and porosity. At these higher protein concentrations (≥ 50 mg/ml), liquid penetration was found to be much more sensitive to the presence of crystallinity than the other formulation variables studied.

Neither contact angle nor drop penetration time could fully explain the long reconstitution times of the high concentration amorphous protein formulations. However, the inability of the nearly saturated drop to penetrate these cakes suggests increasing resistance of the porous cake to penetration by the reconstitution fluid, with an increasing portion of the cake likely dissolving by erosion.
Figure 3: Relationship between contact angle (light gray bars) and drop penetration time (black squares) for formulations F1-F10. Contact angle values are represented as mean ± standard deviation (n = 8) on the primary Y axis and the mean values (n = 4) for drop penetration time are denoted on the secondary Y axis. The black line connects the data points to guide the eye. The error bars denoting standard deviation for the drop penetration times are smaller than the symbols used and hence not visible. All cakes were amorphous except F9 which was partially crystalline. Partial crystallinity is indicated by a star.

Table 3: Average pore diameter and porosity of formulations F1-F10 determined using low pressure mercury intrusion porosimetry.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Total solids (% w/v solids)</th>
<th>Average pore diameter(^1) (µm)</th>
<th>Porosity(^2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>20.4</td>
<td>24.0 ± 0.0</td>
<td>82.5 ± 0.5</td>
</tr>
<tr>
<td>F2</td>
<td>10.7</td>
<td>22.5 ± 1.5</td>
<td>90.0 ± 1.0</td>
</tr>
<tr>
<td>F3</td>
<td>19.7</td>
<td>14.5 ± 1.5</td>
<td>82.3 ± 0.5</td>
</tr>
<tr>
<td>F4</td>
<td>15.6</td>
<td>16.5 ± 0.5</td>
<td>85.5 ± 0.5</td>
</tr>
<tr>
<td>F5</td>
<td>20.5</td>
<td>11.5 ± 0.0</td>
<td>81.0 ± 1.0</td>
</tr>
<tr>
<td>F6</td>
<td>20.6</td>
<td>16.0 ± 1.0</td>
<td>82.5 ± 0.5</td>
</tr>
<tr>
<td>F7</td>
<td>20.6</td>
<td>9.5 ± 0.5</td>
<td>79.5 ± 0.5</td>
</tr>
<tr>
<td>F8</td>
<td>20.6</td>
<td>12.0 ± 1.0</td>
<td>80.0 ± 1.0</td>
</tr>
<tr>
<td>F9</td>
<td>16.8</td>
<td>13.0 ± 0.0</td>
<td>85.5 ± 0.5</td>
</tr>
<tr>
<td>F10</td>
<td>20.9</td>
<td>8.0 ± 0.5</td>
<td>76.5 ± 2.5</td>
</tr>
</tbody>
</table>

\(^1\) Values represent the volume-surface average and are reported as mean ± range/2 for n = 2

\(^2\) Values are reported as mean ± range/2 for n = 2
Influence of formulation on cake crushing resistance and its relationship to reconstitution time

![Figure 4: Reconstitution times (bars) of formulations F1-F10 expressed as mean ± standard deviation for n = 8 and represented on the primary Y axis. The bar colors represent the different type of proteins with gray for mAb A, blue for mAb B, green for mAb C, orange for fusion protein A and red for fusion protein B. Crushing resistance (black squares) is denoted on the secondary Y axis and expressed as mean ± standard deviation for n = 4. The line connects the data points to guide the eye. All cakes were amorphous except F9 which was partially crystalline. Partial crystallinity is indicated by a star.](image-url)

Resistance of the cake to crushing in the dry state has been previously shown to serve as a measure of the difficulty with which the cake disperses or disintegrates upon addition of the reconstitution fluid [6]. In that study, partially crystalline cakes offered lower resistance to crushing (indicative of easier cake dispersion) compared to their fully amorphous counterparts with the same composition and total solids. Cake dispersion prior to hydration was found to be favorable for achieving rapid reconstitution [1, 6].

In the present study, crushing resistance was found to depend on the % total solids and hence the porosity (Figure 4 and Table 3), consistent with prior findings [14-16]. F2 with the lowest % total
solids (10.7 %) offered the least resistance to. Although not statistically significant, F4 containing 16% w/v solids exhibited slightly lower average crushing resistance than the formulations containing 21% w/v solids. However, F9 which contained 17% w/v solids, showed significantly lower crushing resistance than most formulations containing 21% w/v solids (p < 0.05 compared to F5, F6, F7 and F8, but not F1). The lower crushing resistance of formulation F9, as has been previously reported [8], is consistent with partial crystallinity of the cake. Thus, crushing resistance was found to be strongly dependent on total solids, moderately on crystallinity, and independent of protein concentration, protein-to-sugar ratio and of the specific protein. In summary, crushing resistance did not correlate with reconstitution time in the amorphous cakes in this sample set (Figure 4).

Influence of formulation on porous structure and its relationship to reconstitution time

![Graph]

Figure 5: Reconstitution times (bars) of formulations F1-F10 expressed as mean ± standard deviation for n = 8 and represented on the primary Y axis. The bar colors represent the different type of proteins with gray for mAb A, blue for mAb B, green for mAb C, orange for fusion protein A and red for fusion protein B. The mean values (n = 2) for % pores < 12 µm (black squares) are denoted on the secondary Y axis.
and the line connects the data points to guide the eye. The blue error bars represent the range (upper and lower limit) for % pores and if not visible indicates the range to be zero. All cakes were amorphous except F9 which was partially crystalline. Partial crystallinity is indicated by a star.

It is generally acknowledged that more porous cakes (i.e., greater porosity) with larger sized pores reconstitute faster [7, 8], based largely on scanning electron microscopic images. Previous studies examined the relationship between specific surface area, an indirect measure of porous structure, and reconstitution time [1-4], but found no evident correlation. Beech et al. assessed porous structure more directly by measuring pore size and porosity of powdered cakes using mercury intrusion porosimetry. They concluded that cakes containing larger sized pores with an open, well connected structure facilitate faster reconstitution. Kulkarni et al. developed a low pressure mercury intrusion porosimetry method for characterizing porous structure of intact cakes [9]. The authors alluded to the significance of a variety of measures of “porous” structure for a complete understanding of the influence of cake structure on reconstitution time [9]. In the present work, three such measures of porous structure were investigated namely, porosity, average pore size represented as the volume-surface average pore diameter, and % pores < 12 µm.

Porosity was found to be strongly influenced by the % total solids in the pre-lyo solution (Table 3) in these cakes which were all annealed for 2 hours. When comparing the high protein concentration formulations (F3-F10), the average pore size fell within a relatively narrow range of 7.5-16.5 µm (Table 3) but the % pores < 12 µm spanned a much larger interval between 0.9% and 93.9% (Figure 5). A higher value of % pores < 12 µm indicates a larger percentage of small sized pores in the cakes. The % pores < 12 µm was influenced by both protein concentration and type of protein but was independent of total solids. The dependence of cake porous structure on protein concentration and type of protein for some of these formulations has been thoroughly investigated.
by Kulkarni et al. in a separate study and will not be addressed here [9]. It should be noted that values for both porosity and % pores < 12 µm are based on the interrogated pore volume. Pores smaller than 4.5 µm remain un-interrogated in the current method. Relative to the total pore volume, the contribution of the smaller pores is negligible. The smaller pores are not expected to be involved in the liquid penetration process. As a result, contribution of the smaller pores towards reconstitution time is expected to be far lower than that from the larger pores (typically > 12 µm).

Reconstitution time of the cakes containing ≥ 50 mg/ml protein (F3-F10) did not correlate with either the porosity, pore size (Table 3) or the % pores < 12 µm (Figure 5). For example, F3 which had 19.4% pores < 12 µm and porosity of 82% had a significantly shorter reconstitution time than F4 which had 9.6% pores < 12 µm and higher porosity (85.5%). The lower % pores < 12 µm of F6 compared to F3 (similar pore size and porosity) cannot explain the significantly long reconstitution time of the former. Also, despite similar porosity, pore size and % pores < 12 µm, F7 had a dramatically longer reconstitution time than F10. There must be protein-specific properties that also influence reconstitution time.

**Influence of several measures of formulation viscosity and their relationship to reconstitution time**

As seen in the previous sections, cake properties such as wettability of cake solids (assessed using contact angle), ability of the liquid to penetrate into the cake (assessed using drop penetration time), cake disintegration tendency (assessed using crushing resistance) and porous structure could not explain reconstitution times of the amorphous cakes at high protein concentrations ≥ 50 mg/ml.
(i.e., F3-F10, except F9 which was partially crystalline). At these high protein concentrations, reconstitution time was found to be largely influenced by the specific proteins in the formulation and in some cases by the protein-to-sugar ratio. The cake properties discussed in the previous sections were independent of the protein and protein-to-sugar ratio at protein concentrations beyond 25 mg/ml. Hence, other formulation properties that could explain these reconstitution differences were explored. Viscosity of proteins solutions is known to increase sharply at high concentrations (> 50 mg/ml) and this increase is highly protein specific [17-19]. Furthermore, solution viscosity is known to affect dissolution rate [20]. Viscosity of formulations reconstituted to the pre-lyo solution concentration using the full volume of reconstitution fluid (referred to as ‘full recon’) were investigated for their influence on reconstitution time. During reconstitution of the lyophilized cake, the formulation concentration is expected to be a nearly saturated solution at the dissolving surfaces. Hence, viscosity at the surface of the dissolving solids (i.e. pore walls of the porous cake) is significantly higher than the viscosity obtained post-reconstitution using the full volume of the reconstitution fluid. The elevated concentration at the dissolving cake surfaces is difficult to define, but can be extrapolated from more concentrated solutions prepared by reconstituting the cake with a reduced volume of the reconstitution fluid. Cakes reconstituted with 2/3rd or with 1/3rd of the reconstitution fluid volume yield highly concentrated solutions that are 1.5 or 3 times that of the solution concentration obtained by reconstituting with the full volume.

Viscosity values of fully reconstituted formulations containing ≥ 50 mg/ml protein in the pre-lyo solution (i.e., F3-F10) fell within a relatively narrow range of 2-8 cP (Figure 6). Since these formulations are required to be syringeable, the ‘full recon’ viscosity is expected to be low (typically between 2-30 cP). The ‘2/3rd recon’ viscosity values for formulations containing 50
mg/ml protein in the pre-lyo solutions (i.e., F3 and F4) were between 2-4 cP (Figure 6A), similar to the respective full recon samples, while those for formulations containing ≥ 100 mg/ml protein in the pre-lyo solutions (i.e., F5-F10) ranged from 6-27 cP (Figure 6B). Thus, at 2/3rd recon, viscosity of the concentrated solutions began to show marked differences between formulations containing 50 vs. ≥ 100 mg/ml protein in the pre-lyo solution. However, amongst the formulations with the same composition but containing different proteins (F5-F8), the ‘2/3rd recon’ viscosity values were still relatively low (< 30 cP) and not markedly different from each other. For all formulations containing ≥ 50 mg/ml protein in the pre-lyo solution (F3-F10), a sharp rise in viscosity was observed for the ‘1/3rd recon’ samples (Figure 6). Furthermore, viscosity increased non-linearly with concentration. As a result, substantial differences in viscosities between formulations (F3-F10) were noted at ‘1/3rd recon’ that did not exist at ‘full recon’ or ‘2/3rd recon’. For example, for formulations containing ≥ 100 mg/ml in the pre-lyo solution (F5-F10) the ‘1/3rd recon’ viscosity ranged between 60-500 cP and increased in the order F9 < F5 = F8 = F9 < F6 < F7 (Figure 6 and 7).
The formulations that showed remarkable differences in reconstitution time (F3-F10) showed a poor correlation of the ‘full recon’ viscosity with reconstitution time (Figure 7, $R^2 = 0.685$). The correlation between reconstitution time and ‘2/3rd recon’ viscosity for the same formulations was better ($R^2 = 0.816$). Still, viscosity at ‘2/3rd recon’ could not explain the dramatically long reconstitution time of F7, which has a ‘2/3rd recon’ viscosity similar to F5, F6 and F8. At ‘1/3rd recon’, the viscosity correlated strongly with reconstitution time ($R^2 = 0.935$). An enormously high ‘1/3rd recon’ viscosity explained the dramatically long reconstitution time of F7 compared to F5, F6, F8 and F10 despite the same formulation composition. Similarly, the ‘1/3rd recon’ viscosity provides a reason for the reconstitution time of F10 being similar to that of F5, F6 and F8 despite a double protein-to-sugar ratio. These examples indicate that the viscosity at 1/3rd recon can capture protein specific differences in the reconstitution time. Furthermore, for a given protein, the ‘1/3rd recon’ viscosity increased with protein-to-sugar (P:S) ratio as indicated from the example of F3, F4 and F5 all containing mAb A but with the P:S ratio ranging between 0.35-1. One could not anticipate the rank order of reconstitution from either ‘full recon’ or ‘2/3 recon’ viscosity data. Thus, the data suggest ‘1/3rd recon’ viscosity to be a better indicator of reconstitution behavior than both full and 2/3rd recon viscosity.
Figure 7: Reconstitution times (bars) of formulations F1-F10 expressed as mean ± standard deviation for n = 8 and represented on the primary Y axis. Viscosities at ‘full recon’ (squares), ‘2/3rd recon’ (triangles) and ‘1/3rd recon’ (inverted triangles) are denoted on the secondary Y axis and are represented as mean ± standard deviation of n = 3. The line connects the data points to guide the eye. The error bars if not visible indicates zero standard deviation or values that were smaller than the symbol used. All cakes were amorphous except F9 which was partially crystalline.

The ‘2/3rd recon’ and ‘1/3rd recon’ viscosities were expected to better mimic the high viscosity presumably at the surface of the dissolving cake when compared to ‘full recon’ viscosity. The increasingly strong correlation between reconstitution time and viscosity in the order ‘full recon’ < ‘2/3rd recon’ < ‘1/3rd recon’ (i.e., as a function of solute concentrations) suggests that cake dissolution is strongly influenced by the viscosity at the dissolving cake surface. Dissolution of these cakes potentially results in a viscous region at the cake surface in contact with the reconstitution fluid. This viscous layer seems to be a major barrier to reconstitution, by reducing the rate of erosion of the viscous layer and by providing a longer path length for the diffusion of the reconstitution fluid through the layer into the remaining undissolved cake.
Interestingly, F9 had lower viscosity (full, 2/3rd and 1/3rd recon) compared to the other formulations containing 100 mg/ml protein (i.e., F5, F6, F7 and F8). When specifically comparing F5 and F9 both containing 100 mg/ml mAb A, the “concentrated formulation viscosity” of F9 was half that of F5 despite a higher protein-to-excipient ratio. The low “concentrated formulation viscosity” of F9 could either be due to lack of sugar or presence of arginine, a viscosity lowering excipient [21-23]. Thus, viscosity lowering excipients such as arginine or salts like sodium chloride [21-24] can be explored as a potential approach to expedite reconstitution by modulating the “concentrated formulation viscosity”.

**Conclusions**

For formulations evaluated in the present study, reconstitution time was primarily influenced by the protein-to-sugar ratio and the protein itself. For amorphous cakes containing > 50 mg/ml protein, cake wettability (assessed using contact angle), liquid penetration into the cakes (assessed using drop penetration time), cake disintegration (assessed from the resistance offered by cakes to crushing in the dry state) and cake porous structure were found to be invariant and did not correlate with reconstitution time. For these formulations, “concentrated formulation viscosity” (i.e., viscosity of the solutions obtained by reconstituting the cakes with 1/3rd volume of the reconstitution fluid) was most sensitive to changes in the formulation composition particularly, protein-to-sugar ratio and the protein itself. “Concentrated formulation viscosity” (equivalently ‘1/3rd recon’ viscosity) also showed the best correlation with reconstitution time compared to ‘2/3rd recon’ and ‘full recon’ viscosities. Thus, for protein concentrations > 50 mg/ml, “concentrated formulation viscosity” provides a tool for better understanding the reconstitution behavior of lyophilized protein formulations than viscosity of the fully reconstituted solution.
Although cake properties such as contact angle, drop penetration time and crushing resistance were non-discriminatory for amorphous formulations with protein concentrations > 50 mg/ml, these measurements were very much sensitive to the trace amounts of partial crystallinity in the cake matrix. These measurements also correlated with faster reconstitution at least for the partially crystalline cakes of F9 and amorphous cakes with lower protein concentrations, F1 and F2 (i.e., < 50 mg/ml). Contact angle, drop penetration time and crushing resistance were developed as simple measures to quantify cake properties influencing reconstitution namely, cake wettability, liquid penetration into cakes and cake disintegration respectively [6]. Kulkarni et al. have elucidated the mechanisms by which partial crystallinity in the cake modulates these properties thereby influencing reconstitution. In that study also, although the quantitative measures of cake properties influencing reconstitution were widely different for amorphous and partially crystalline cakes, they did not vary significantly within the amorphous cakes. These results suggest differences in the stages involved in the reconstitution of amorphous vs. partially crystalline cakes (manuscript to follow).

Lastly, it should be noted that both drop penetration time and crushing resistance only serve as surrogates for understanding the ability of the reconstitution fluid to penetrate into the cake and the disintegration tendency of the cake upon fluid addition. The actual reconstitution process in a vial is much more complex than what is captured by these relatively simple measures. Hence, a detailed characterization of the actual reconstitution behavior (both qualitative and quantitative) is necessary for a complete understanding of the key factors governing reconstitution time and subsequently identifying approaches to promote faster reconstitution (manuscript to follow).
References


Chapter 5

Key Factors Governing the Reconstitution Time of Highly Concentrated Lyophilized Protein Formulations
Abstract
Lyophilized protein formulations containing highly concentrated proteins often have long and variable reconstitution times. Reconstitution time is dependent on a number on factors in a complex manner. Furthermore, factors governing the reconstitution of partially crystalline cakes are reportedly different from amorphous cakes. The goals were to identify the key factors governing reconstitution and understand the mechanisms involved in reconstitution of both amorphous and partially crystalline cakes. Partial crystallinity in the final cake, larger pores and low “concentrated formulation viscosity” (i.e., viscosity near the surface of the dissolving cake) were identified as desirable characteristics for expediting reconstitution. Crystallinity and larger pores dramatically improved wettability and liquid penetration in partially crystalline cakes, ultimately resulting in well dispersed small pieces of partially dissolved cake. The smaller disintegrated cake pieces dissolved faster because of the increased surface area. The amorphous cakes exhibited poorer wettability than partially crystalline cakes. Moreover, the ability of the reconstitution fluid to penetrate the pores, and the resulting cake disintegration was much lower than that observed for partially crystalline cakes. In fact, for some of the amorphous cakes, the reconstitution fluid did not penetrate the cake at all. As a result, the undissolved intact cake or a large cake chunk floated on the reconstitution fluid amidst foam or bubbles generated during reconstitution. Dissolution of the floating cake appeared to proceed via gradual surface erosion where reconstitution time was found to be highly correlated with the viscosity near the surface of the dissolving cake solids. A higher viscosity prolonged reconstitution. Based on the above findings, both formulation and processing conditions can be tailored to achieve faster reconstitution. Including a crystallizable excipient proved to be beneficial. Incorporating an annealing step to facilitate crystallization of the crystallizable excipient and to promote larger pores
was also found to be advantageous. A viscosity lowering excipient in the formulation could potentially be helpful but this approach needs to be explored further.

**Keywords:** Protein formulation, high concentration, freeze drying, reconstitution, viscosity, formulation, processing, annealing, crystallinity, porous structure
Introduction

Recombinant DNA technology has enabled the commercialization of many protein therapeutics over the past three decades. Protein therapeutics are used for treating a variety of diseases including cancers and chronic conditions such as arthritic diseases, asthma and psoriasis. Sub-cutaneous delivery is highly preferred for treating indications where home-based self-administration is desirable, particularly for chronic diseases. The typical injection volume for sub-cutaneous administration is limited to 1-1.5 ml. In order to accommodate a high drug dose (especially for monoclonal antibodies) in a low injection volume, these therapeutics are formulated at protein concentrations typically > 50 mg/ml, and some as high as 200 mg/ml. Therapeutic proteins that are unstable as liquid formulations are lyophilized (i.e., freeze-dried) to improve their pharmaceutical stability. However, lyophilized products containing highly concentrated proteins are known to have long and variable reconstitution times [1-6]. Long reconstitution times are undesirable from a drug product administration perspective. Overcoming the long reconstitution times is expected to improve patient outcomes and enable product differentiation.

Reconstitution time of lyophilized formulations containing highly concentrated proteins depends on a number of factors such as partial crystallinity in the cake [1, 2, 7], wettability of the solids comprising the cake [1, 8], cake porous structure particularly pore size and pore size distribution [6, 7, 9], the ability of the liquid to penetrate into the cake [1], cake disintegration tendency [1, 8], cake hydration rate [8] and certain measures of viscosity [10]. Certain of these factors seem to be more predictive of reconstitution time than others. Furthermore, some of the factors are known to co-vary. For example, resistance of the cakes to crushing (a measure of cake disintegration) has been found to depend on both crystallinity and pore size [11, 12]. The ability of a liquid to penetrate
the pores is related to the wettability of the solids comprising the pore walls and the pore size [1, 13, 14]. Hence, the first goal of the present work was to identify the correlations between the cake properties influencing reconstitution, understand the reasons behind those correlations and subsequently reduce the set of factors governing reconstitution time. The factors affecting reconstitution of partially crystalline cakes are reportedly different from those for fully amorphous cakes. Kulkarni et al. elucidated the mechanisms by which crystalline mannitol improved the reconstitution times of partially crystalline cakes compared to their amorphous counterparts [1]. The authors concluded that phase separation of crystalline mannitol from the amorphous, protein rich matrix improved cake wettability, making the cake interior more accessible to the reconstitution fluid. Furthermore, partially crystalline cakes offered less resistance to crushing in the dry state, indicating that crystalline mannitol potentially enabled easier cake disintegration upon addition of the reconstitution fluid. Hence, crystallinity improved reconstitution times by favorably affecting the pre-requisites for faster reconstitution – rapid liquid penetration into the cake and disintegration of the cake into small pieces that could separately rehydrate and dissolve. The authors also pointed out that although necessary, mannitol crystallinity was not a sufficient condition for improving reconstitution time, but that cake porous structure played an important role. Cake porous structure has also been implicated as a contributor to the reconstitution time of amorphous cakes [6, 8]. Kulkarni et al. have reported reconstitution time of amorphous cakes to be strongly influenced by a measure of the formulation viscosity and to a lesser extent by the porous structure, with negligible contributions from wettability, liquid penetrability of the cake and the cake disintegration tendency [10]. Owing to the differences in the factors influencing reconstitution of partially crystalline vs. amorphous cakes, the present study is divided into two parts. The first part focuses on reconstitution of formulations that yield process-dependent fully
amorphous or partially crystalline cakes. The second part focuses on reconstitution of formulations varying in composition using a process that yields only amorphous cakes. The key predictors of reconstitution time of each set of formulations are explored separately.

Many studies on reconstitution in the literature simply report the total time for reconstitution. The details of cake behavior during reconstitution may be more helpful in defining specific barriers to rapid reconstitution. Understanding these details are more likely to suggest specific approaches to overcoming the barriers in each case. For example, oftentimes, the majority of the cake dissolves rapidly, and a small portion of the cake becomes entrapped in the foam generated during reconstitution. This small piece takes a long time to reconstitute prolonging the total reconstitution time. Previous studies found that this type of two-phase reconstitution behavior was exhibited by some cakes but not by others [1, 8]. The reasons underlying the various types of reconstitution behavior are not yet fully understood. Furthermore, while some reports have shown a positive correlation between foam height and reconstitution [7], others have alluded to the decrease in reconstitution rate due to the foam that is generated during the rapid reconstitution phase of some high concentration proteins [1]. Hence, another objective of the present work was to undertake a detailed investigation of the reconstitution behavior with respect to characteristics such as, i) time taken to reconstitute approximately first 80-90% of the cake solids versus time for reconstituting the last 20-10% of cake, and ii) presence or absence of foam during reconstitution. An attempt was made to relate the cake properties to reconstitution of both first 80-90% and last 20-10% cake. The additional information is expected to improve the understanding of the barriers to reconstitution, such that they can be addressed.
Finally, based on the reconstitution behavior and the key factors identified to influence reconstitution, the present work proposes the different stages involved in the reconstitution of both partially crystalline and amorphous cakes. Furthermore, formulation and processing approaches to expedite reconstitution have also been suggested.

**Materials and Methods**

**Materials**

Fusion protein A (molecular weight, MW = 73 kDa) was provided by GSK (Collegeville, PA) formulated at 140 mg/ml with 40 mg/ml mannitol, 28 mg/ml trehalose, 0.01% w/v polysorbate 80 and 10 mM sodium phosphate buffer (pH 7.0). Fusion protein B (MW = 284 kDa) was provided by MedImmune (Gaithersburg, MD). Three monoclonal antibodies that are designated mAb A, mAb B, and mAb C, with molecular weights of approximately 150 kDa, were also provided by MedImmune. The proteins provided by MedImmune were formulated in buffered solutions (pH 6.0) with sucrose and 0.02% w/v surfactant. Bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO) was used to prepare a formulation containing 140 mg/ml BSA, 40 mg/ml mannitol (β-D-Mannitol, Sigma-Aldrich, St. Louis, MO) and 28 mg/ml trehalose (α,α trehalose dihydrate, USP, high purity, low endotoxin, Pfanstiehl, Waukegan, IL). Mannitol was also used to prepare a 20% w/v solution that was filtered through a 0.22 μm syringe filter (Millex®, MilliporeSigma, Burlington, MA). Clear tubing glass vials (Type 1 glass, 3 ml capacity, Wheaton, Millville, NJ) and stoppers (V50 lyo 4432/50 Gray 13mm, West Pharmaceutical Services, Exton, PA) were used for lyophilization.
Methods

Protein formulations

Formulations yielding process-dependent fully amorphous or partially crystalline cakes

Protein formulations evaluated in this part of the study contained a single protein, but differed in protein-to-sugar ratio to influence mannitol crystallization, all at the same total solids content, 21% w/v. Fusion protein A served as the model protein for this part. The formulation supplied by GSK (designated Formulation A) was diluted with the filtered 20% w/v mannitol to obtain four additional formulations (Table 1). The low surfactant concentrations upon dilution were not expected to influence reconstitution [2]. Sodium phosphate buffer was not expected to crystallize at the low concentrations used in this study [15]. Trehalose crystallization was also not expected owing to the high protein concentration [16]. Since, all the solutions had same total solids at 21% w/v, an increase in protein concentration was accompanied by an increase in protein-to-sugar ratio.

Table 1: Pre-lyo solution compositions of formulations yielding process-dependent fully amorphous or partially crystalline cakes. All formulations contained the same protein but differed in protein-to-sugar ratio.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion protein A (mg/ml)</td>
<td>140</td>
<td>122</td>
<td>117</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>Trehalose (mg/ml)</td>
<td>40</td>
<td>35</td>
<td>33</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>Mannitol (mg/ml)</td>
<td>20</td>
<td>50</td>
<td>57</td>
<td>77</td>
<td>106</td>
</tr>
<tr>
<td>Sodium phosphate buffer (mg/ml)</td>
<td>1.3</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Protein-to-sugar ratio</td>
<td>2.1</td>
<td>1.4</td>
<td>1.3</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Total solids (% w/v)</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>21</td>
</tr>
</tbody>
</table>

Formulations yielding only amorphous cakes

The formulations evaluated in the second part of the study contained different proteins and/or varied in the protein-to-sugar ratio (Table 2). mAbs A, B, and C, fusion proteins A and B, and BSA were formulated in buffered (< 0.05% w/v) solutions with a lyoprotectant and surfactant (<
0.02% w/v) to yield F1-F8. The formulations consisted of 21% w/v total solids, except F2 which had 16% w/v solids. The lyoprotectant in formulations F1-F6 was sucrose, while F7 and F8 contained trehalose. F7 and F8 also contained mannitol. Cakes F1-F6 were lyophilized at MedImmune.
Table 2: Composition details for formulations yielding only amorphous cakes. Formulations contained different proteins and/or varied in protein-to-sugar ratio.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Type of protein</th>
<th>Protein concentration (%w/v)</th>
<th>Lyoprotectant concentration (%w/v)</th>
<th>Other excipient (% w/v)</th>
<th>Protein to sugar ratio</th>
<th>Total solids (%w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>mAb A</td>
<td>5</td>
<td>14.4</td>
<td>-</td>
<td>0.35</td>
<td>19.7</td>
</tr>
<tr>
<td>F2</td>
<td>mAb A</td>
<td>5</td>
<td>10</td>
<td>-</td>
<td>0.50</td>
<td>15.6</td>
</tr>
<tr>
<td>F3</td>
<td>mAb A</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>1.00</td>
<td>20.5</td>
</tr>
<tr>
<td>F4</td>
<td>mAb B</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>1.00</td>
<td>20.6</td>
</tr>
<tr>
<td>F5</td>
<td>mAb C</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>1.00</td>
<td>20.6</td>
</tr>
<tr>
<td>F6</td>
<td>Fusion protein B</td>
<td>10</td>
<td>10</td>
<td>-</td>
<td>1.00</td>
<td>20.6</td>
</tr>
<tr>
<td>F7</td>
<td>Fusion protein A</td>
<td>14</td>
<td>40</td>
<td>28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.9</td>
</tr>
<tr>
<td>F8</td>
<td>BSA</td>
<td>14</td>
<td>40</td>
<td>28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> contained mannitol  
<sup>b</sup> protein to sugar reflects ratio of protein to trehalose and mannitol
**Lyophilization**

*Formulations yielding process-dependent fully amorphous or partially crystalline cakes*

The pre-lyo solutions (0.5 ml) were freeze-dried (Revo®, Millrock Technology, Kingston, NY) in 3 ml vials. Vials were loaded into the freeze dryer at a shelf temperature of 20°C. Formulation-containing vials were surrounded with surrogate (same composition as Formulation A with BSA replacing fusion protein A) containing vials to minimize the effect of radiation from the door and walls, thereby improving vial-to-vial uniformity. The formulations were lyophilized using several freeze-drying protocols described previously [1] designed to alter the crystallization of mannitol. The freeze-drying cycles (with details provided in Table 3) are referred to in the text as:

Cycle 1: slow freezing-aggressive drying
Cycle 2: slow freezing-conservative drying
Cycle 3: fast freezing-aggressive drying
Cycle 4: fast freezing-conservative drying
Cycle 5: slow freezing-annealing-conservative drying

While formulations B, C and D were processed using all 5 cycles, formulations A and E were subjected only to cycles 2 and 5. T-type 36-gauge thermocouples (Omega Engineering, Inc., Norwalk, CT) placed in the bottom center of the vials were used to record product temperatures during freezing and drying; these vials were not used for any characterization. The primary drying conditions were designed to maintain the product temperatures of each formulation above its respective T<sub>g</sub> for the ‘aggressive’ cycles (Cycles 1 and 3) and below the T<sub>g</sub> for ‘conservative’ cycles (Cycles 2, 4 and 5). At the end of secondary drying, the shelf temperature was lowered to 20°C. The vials were stoppered at 700 Torr, crimped with aluminum seals and stored at 5°C until
further use. Residual water content (measured using Karl Fischer titration) was found to be between 0.2-1.2% w/w in all formulations.
Table 3: Details of the freeze-drying protocols used to process formulations A-E.

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Cycle description</th>
<th>Ice nucleation&lt;sup&gt;a&lt;/sup&gt; temperature (°C)</th>
<th>Freezing&lt;sup&gt;b&lt;/sup&gt; ramp rate (°C/min)</th>
<th>Annealing&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Primary drying&lt;sup&gt;d&lt;/sup&gt; shelf temperature (°C) and chamber pressure (mTorr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle 1</td>
<td>slow freezing-aggressive drying</td>
<td>-5</td>
<td>0.2</td>
<td>-</td>
<td>-10 and 100</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>slow freezing-conservative drying</td>
<td>-5</td>
<td>0.2</td>
<td>-</td>
<td>-35 and 50</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>fast freezing-aggressive drying</td>
<td>-10</td>
<td>5</td>
<td>-</td>
<td>-10 and 100</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>fast freezing-conservative drying</td>
<td>-10</td>
<td>5</td>
<td>-</td>
<td>-35 and 50</td>
</tr>
<tr>
<td>Cycle 5</td>
<td>slow freezing-annealing-conservative drying</td>
<td>-5</td>
<td>0.2</td>
<td>Yes</td>
<td>-10 and 100</td>
</tr>
</tbody>
</table>

<sup>a</sup>The shelf temperature was ramped down from room temperature to the ice nucleation temperature at 1°C/min followed by a 1 hour hold prior to nucleation. Ice nucleation was induced using an ice fog (FreezeBooster nucleation station, Millrock Technology, Kingston, NY).

<sup>b</sup>Following nucleation the shelf was ramped down to -40°C followed by a 90 minute hold.

<sup>c</sup>The shelf was ramped at 1°C/min to -20°C/min, held for 12 hours, ramped down to -40°C at 1°C/min and held for 90 minutes.

<sup>d</sup>The end of primary drying corresponded to a difference within 3 mTorr between the Pirani gauge pressure and the pressure measured by the capacitance manometer. Following primary drying, the shelf temperature was raised to 40°C, at 0.2°C /min, and secondary drying was conducted for 3 hours.
Formulations yielding only amorphous cakes

Formulations F1-F6 (Table 2) were freeze-dried from 1.7 ml pre-lyo solutions in 3 ml vials; these formulations were freeze-dried in batches using one of several dryers (Lyostar 3, VirTis Genesis or Hull, SP Scientific, Stone Ridge, NY). Formulations F7 and F8 were also freeze-dried from 1.7 ml of pre-lyo solutions in 3 ml vials, but in a small-batch freeze-dryer (MicroFD, Millrock Technology, Kingston, NY). Vials were loaded into the freeze dryer at a shelf temperature of 5°C and held at that temperature for 30 minutes. For all formulations (F1-F8), the shelves were cooled at 0.5°C/min to -5°C and held at -5°C for 30 minutes before cooling at 0.5°C/min to -40°C and holding at -40°C for 2 hours. Using a heating rate of 0.5°C/min, the shelves were raised to the annealing temperature of -16°C and held for 2 hours. After cooling to -40°C at 0.5°C/min and holding for 2 hours at -40°C, the shelf was raised to the target primary drying shelf temperature at 0.3°C/min. Primary drying was carried out at -35°C for F1-F3 and at -10°C for F4-F8, using a chamber pressure of 100 mTorr for all formulations. The end point of primary drying was determined by convergence of the Pirani gauge pressure reading with capacitance manometer reading to within 10 mTorr. Following primary drying, shelf temperature was raised to 40°C at 0.3°C/min for F1-F3 and at 0.1°C/min for F4-F8. Secondary drying at 40°C was carried out at 150 mTorr for 6 hours. At the end of secondary drying, shelf temperature was lowered to 5°C, and vials were stoppered, unloaded from the freeze dryer and crimped with aluminum seals. Residual water content was < 1 % w/w in all formulations.

Reconstitution behavior

Reconstitution behavior of 8 vials of each formulation was characterized. The total reconstitution time was measured using protocols developed separately for formulations A-E [1] or F1-F8 [10]. Additionally, the time to reconstitute approximately the first 90% (for formulations A-E) or 80%
(for F1-F8) of the cake (based on visual observation) was recorded. The time required to reconstitute the remaining 10% (or 20%) of the cake was found by subtracting the time to reconstitute the first 90% (or 80%) from the total reconstitution time.

The presence of foam or bubbles during reconstitution was also noted. The term, foam, is used for multitudinous tiny aggregated bubbles, which usually formed immediately on adding the reconstitution fluid and did not subside or coalesce immediately following the completion of reconstitution. The term, bubble, is reserved to describe larger bubbles that formed gradually during the course of cake dissolution and subsided relatively rapidly following reconstitution. It was usual for a few (4-5) larger air bubbles to be interspersed with the foam.

**Characterization of factors influencing reconstitution**

**Crystallinity**

X-ray powder diffractometry (XRPD) was employed for identification of mannitol polymorphs and to quantify the degree of crystallinity in the partially crystalline cakes, as previously described [1]. Briefly, the sum of the areas of the XRPD peaks corresponding to δ-mannitol (the predominant polymorph in the cakes) were plotted as a function of degree of crystallinity to obtain a standard curve [1]. Degree of crystallinity (*i.e.*, percent crystalline mannitol relative to total solids) was reported as the mean of *n* = 3.

**Wettability of cake solids**

Wettability of the cake solids was assessed by measuring the contact angle of a nearly saturated sessile drop on a disc of compressed lyophilized solids using a goniometer (Model 100-00, Ramé-
Contact angle values were reported as the means for n = 8.

**Drop penetration into cakes**
A nearly saturated aqueous drop (with the same composition as for the wettability study) was placed on the surface of the intact cake in a vial. The time for the drop to disappear from the surface was reported as the drop penetration time. The test was terminated at 15 minutes, because after 15 minutes, the drop remained on the surface for at least 24 hours. Drop penetration times are reported as mean values of 3 measurements on intact cakes from each formulation.

**Cake crushing resistance**
The resistance of intact cakes to crushing in the dry state was evaluated to potentially serve as a measure of the ease with which the cake disintegrates upon addition of the reconstitution fluid as previously described [1]. Briefly, the force necessary for displacement of a stainless steel, cylindrical probe into an intact cake at a constant rate was recorded using a tensile tester (Model 5866, Instron Corporation, Norwood, MA) [1, 10]. The slope of the initial linear portion of the force versus displacement curve was reported as cake crushing resistance. The mean value of 4 measurements on each formulation was reported.

**Cake pore size**
The volume-surface average pore diameter of the dried cakes of all formulations was determined by low pressure mercury intrusion porosimetry (Autopore IV 9500, Micromeritics Instrument
Corporation, Norcross, GA) as previously described [17] and reported as mean values of 2 measurements.

Concentrated formulation viscosity
Viscosity of the solutions prepared by reconstituting the cake with 1/3rd of the full reconstitution fluid volume was measured as previously described [10]. The solute concentration in these solutions was about three times the pre-lyo concentration. The viscosity, referred to as “concentrated formulation viscosity”, was reported as the mean of 3 measurements.

Statistical analysis
Data were analyzed using Pearson’s correlation, multivariate regression analysis and principal component analysis (PCA) (SAS Version 9.4; SAS Institute, Cary, NC). Pearson’s correlation and regression analysis were performed to identify correlations between the factors influencing reconstitution and to reduce the factors to the key predictors of reconstitution time. PCA was used as alternate technique to confirm the redundancy among the factors influencing reconstitution and to evaluate if the large number of interrelated variables could be reduced to fewer “reduced” variables.

During regression analysis, stepwise backward regression was used to eliminate all but the most statistically significant variables. Thus, the final models retained the least number of predictor variables yielding a simple model with maximum predictive power. Regression analysis was conducted using SAS and observations with missing values were omitted. The data that was mean-
centered and the variance was normalized before performing PCA using SAS. The values (or component scores) of these new “reduced” variables were calculated by the SAS software (data not shown).

**Results and Discussion**
A variety of factors have been implicated in the long and variable reconstitution times of lyophilized formulations containing high protein concentration (> 50 mg/ml). The goal of the present study was to understand the inter-relationship between these factors and subsequently identify the key factors governing reconstitution. Partially crystalline cakes reconstitute significantly faster than fully amorphous cakes of the same composition [1]. In addition, the factors influencing the reconstitution times of fully amorphous and partially crystalline cakes are reportedly different [10]. Hence, the results and discussion section is divided into four parts. The first section focuses on formulations of the same composition that yield either fully amorphous or partially crystalline cakes depending on the lyophilization process. The second section focuses on a wider variety of formulations, all processed using the same protocol, producing only amorphous cakes. Direct comparison of the two sets of lyophilized formulations was not possible, because the fill depths of the two sets were different and as were the lyophilization cycles. Thus, the key predictors of reconstitution time of each set of formulations were explored separately. Based on the findings, the third section discusses the reconstitution stages for partially crystalline cakes as compared to amorphous cakes. Finally, the fourth section enumerates approaches to reduce reconstitution times of lyophilized formulations containing highly concentrated proteins.
Formulations yielding process-dependent fully amorphous or partially crystalline cakes

Formulations evaluated in this section contained a single protein but differed in their protein-to-sugar ratio to influence the crystallization of mannitol during the various lyophilization cycles employed. As a result, lyophilized cakes were either fully amorphous or partially crystalline.

Reconstitution behavior

Figure 1: Reconstitution time (n=8) for formulations (A-E) processed using five freeze-drying protocols namely, Cycle 1: slow freezing-aggressive drying (gray), Cycle 2: slow freezing-conservative drying (blue), Cycle 3: fast freezing-aggressive drying (red), Cycle 4: fast freezing-conservative drying (green), Cycle 5: slow freezing-annealing-conservative drying (black). The total reconstitution time is divided into the time taken to reconstitute approximately first 90% of the cake solids (pattern fill) and the time taken to reconstitute the remaining 10% of the cake solids (solid fill). Presence of foam during reconstitution is indicated as ‘+’ and absence of foam (equivalently presence of bubbles) as ‘-’.

In general, the total reconstitution time of partially crystalline cakes was lower than fully amorphous cakes (Figure 1), consistent with previous findings [1]. Partial crystallinity was governed by both formulation composition (specifically the protein-to-sugar (P:S) ratio in these cakes prepared from solutions containing the same % solids w/v) and processing conditions, with
a greater contribution from the former. For example, at P:S ratios of 2.1 mannitol did not crystallize despite annealing for 12 hours. Aggressive drying promoted mannitol crystallization in formulation D (P:S ratio = 0.9), but not in formulations B and C with P:S ratios 1.4 and 1.3, respectively. The aforementioned dependence of mannitol crystallization on formulation and processing for the formulations investigated here has been more fully described previously [1] and agrees with findings by other investigators [18-21].

The detailed reconstitution behavior was remarkably different across the formulations in this high (70-140 mg/ml) protein concentration range (Figure 1). For example, total reconstitution time for cakes with P:S ratio < 1 and/or exhibiting partial crystallinity was dominated by slow reconstitution of the last 10% of the cake, which followed rapid reconstitution of the first 90% of the cake (within about 2 minutes). Reconstitution of all of these cakes was accompanied by the formation of foam. In at least one citation, foam height ranked with short recon times in formulations with moderately high protein concentration of 50 mg/ml [7]. This suggests that the low P:S ratio (< 1) and partial crystallinity that facilitate initial cake dissolution also produce a foam that hinders the dissolution of any solids that remain after foam formation. In fact, foaming has been previously implicated in prolonging reconstitution particularly of the cake fragments occluded in the foam [1, 8]. Foam was reported in those studies to entrap a portion of the cake causing it to float in the foam where it is less exposed to dissolution by the reconstitution fluid. Similar behavior was noted in the present study during reconstitution of cakes accompanied by foam formation. Despite the longer time for reconstituting the last 10% of the cake (generally 3-6 minutes), the total reconstitution time for these cakes was generally low, ranging between 5-8 minutes. Exceptions to this were the rapidly frozen (using Cycles 3 and 4) cakes of formulation D,
where the time taken to reconstitute the last 10% cake solids was significantly longer, prolonging the total reconstitution time to about 11-13 minutes. These cakes were frozen using a low ice nucleation temperature followed by a fast cooling ramp compared to the other cakes exhibiting foaming, which were nucleated at a high temperature followed by slow cooling. These differences in the freezing protocol affect pore size, with the latter producing larger pores. Reasons for this “exceptional” behavior will be discussed more later.

Reconstitution of amorphous cakes with P:S ratio > 1 was not accompanied by foam but rather by larger bubbles. Bubbles were generated over time as the cake dissolved slowly by gradual erosion. Unlike the fast dissolving, foam-forming cakes, dissolution of the first 90% dominated the reconstitution of cakes with typically long total reconstitution times. For these cakes, once the majority of the cake solids had dissolved within the first 8-12 minutes (~70% of the total reconstitution time), dissolution of the remaining solids was completed within 3-4 minutes (~ 30% of total reconstitution time). The faster reconstitution of the last 10% solids in these cakes which did not produce foam further implicates the lengthening of total reconstitution time due to cake entrapment in foam.

**Key factors governing reconstitution time of process-dependent fully amorphous or partially crystalline cakes**

Kulkarni et al. demonstrated the complex dependence of reconstitution time on multiple factors such as [1, 10]:

- Protein-to-sugar (P:S) ratio of the solids comprising the lyophilized cake,
- Partial crystallinity in the cake,
• Wettability of the cake solids (assessed using contact angle),
• Ability of the reconstitution liquid to penetrate into the cake (assessed using drop penetration time),
• Disintegration tendency of the cake (assessed from the resistance of the cakes to crushing),
• Cake porous structure (particularly the average pore diameter and pore size distribution in the cake),
• Foaming during reconstitution, and
• Concentrated formulation viscosity (to account for protein-specific differences).

The 19 cakes obtained by processing formulations A-E using Cycles 1-5 all contained the same protein at the same % total solids. Multiple cakes of each formulation were quantitatively characterized for the factors enumerated above (Table 4) and reported along with their reconstitution times. The fast reconstituting cakes (total reconstitution time < 8 minutes) were characterized by partial crystallinity in the cake, lower contact angle, shorter drop penetration time, lower crushing resistance, larger sized pores and foam generated during reconstitution.

Correlations between factors influencing reconstitution

Before studying the combined influence of the above factors on reconstitution time and identifying the key factors predictive of reconstitution, the cake properties were examined for collinearity by evaluating the Pearson’s correlation coefficient (ρ) between paired factors (Table 5). A high absolute value of the Pearson’s correlation coefficient indicates a strong correlation between the two paired attributes. Models relating correlated properties to each other were found by regression analysis (Table 6) as described below.
1. Protein-to-sugar (P:S) ratio correlated with crystallinity, contact angle, drop penetration time and foam ($R^2 > 0.6$, $p < 0.01$, Table 5). An increase in the P:S ratio is accompanied by a decrease in crystallinity (negative $\rho$), which is consistent with previous findings that proteins have a concentration dependent inhibitory effect on the crystallization of excipients during freeze drying [18, 20]. Increased P:S ratio also reduces foaming, seen from the examples of formulations A, B and C which lacked foam during reconstitution. Increased P:S ratio also increases the contact angle due to the relative hydrophobicity of the protein rich matrix. Finally, an increase in P:S ratio is accompanied by an increase in the drop penetration time because drop penetration time is correlated with contact angle (discussed below).

2. Contact angle was most highly correlated with crystallinity ($R^2 = -0.877$, $p < 0.001$, Table 5), followed by drop penetration time and foaming ($R^2 > 0.840$, $p < 0.001$, Table 5), in addition to being correlated with P:S ratio as described above. Regression analysis (Table 6) indicated that cake wettability (assessed using contact angle) depends on both crystallinity and ability of fluid to penetrate into the cake (assessed using drop penetration time). The mechanisms by which crystallinity improves cake wettability have been previously addressed [1] and will not be discussed here.

3. Drop penetration time was highly correlated with foaming ($R^2 = -0.924$, $p < 0.001$, Table 5) in addition to crystallinity, contact angle and P:S ratio. Based on regression analysis, drop penetration time was found to be solely dependent on the contact angle (Table 6). As per the Washburn equation for liquid penetration into porous media, penetration time for a drop of constant volume for a given liquid is also expected to depend on the cake porosity (which was constant for the cakes evaluated) and pore size, Hence, the data suggests that
drop penetration time as measured here was insensitive to pore size, at least in the range of 14-44 µm. This observation is consistent with previous reports by Kulkarni et al. where drop penetration time was found to be independent of pore size in the 9-24 µm range [10], but very sensitive to the presence of crystallinity and therefore contact angle. Based on the findings from the present study and previous work, the drop penetration time test as developed by Kulkarni et al. seems to serve only as an indicator of partial crystallinity in the cake matrix. In this context, it is interesting to point out examples of some of the amorphous cakes of formulations C and D which showed faster drop penetration in spite of higher contact angles. Powder from these cakes showed birefringence (1 particle out of 5 fields of view) under polarized light, indicating crystallinity, which was not detectable by XRPD [2]. Not only was the crystallinity non-quantifiable by XRPD but it also did not result in lower contact angles. Thus, the drop penetration test seemed to be exquisitely sensitive to trace amounts of crystallinity. These correlations indicate that partial crystallinity improves wettability (lowers the contact angle) and the improved wettability facilitates liquid penetration (shorter drop penetration times) in Table 4. When the reconstitution fluid penetrates the cake nearly instantaneously, it can dissolve the cake solids that make up the pore walls before displacing the air from all the adjacent pores. As pore walls dissolve, it is expected that air-filled pores now surrounded by reconstitution fluid give rise to numerous tiny air bubbles or “foam” that sometimes occludes the undissolved cake solids. However, in the case of the slowly reconstituting amorphous cakes, liquid penetration is much slower. As the reconstitution fluid gradually penetrates the pores, it displaces the air in the pores more slowly, which may explain the larger bubbles as small air pockets have the time to coalesce as they are slowly produced.
4. Crushing resistance was found to be highly correlated with pore size ($R^2 > 0.818$, $p < 0.001$, Table 5) followed by crystallinity ($R^2 > 0.646$, $p < 0.01$, Table 5), in agreement with previous reports where crystalline cakes were easier to break than amorphous cakes and cakes with higher porosity and larger sized pores crushed more easily [11, 12, 22]. Based on the regression model, crushing resistance was found to be a function of both crystallinity and pore size (Table 6). In a related study, Kulkarni et al. found crushing resistance to be related only to the presence of crystallinity and independent of pore size [10]. While the average pore size of the freeze-dried cakes in that study ranged between 9-24 µm, in the present study they spanned a wider range of 14-44 µm. The combined data from both studies suggests that crushing resistance is more sensitive to crystallinity than pore size. This is also reflected in the regression coefficient for crystallinity which is twice the regression coefficient for pore size (Table 6). Hence, it is likely that large differences in pore size are required to observe measurable differences in the crushing resistance.

In summary, a strong dependence of contact angle, drop penetration time and crushing resistance on crystallinity suggests that these measures can discriminate between amorphous and partially crystalline cakes. However, they are relatively constant for amorphous cakes, making them less discriminatory for the longer reconstitution times of amorphous cakes as discussed later in the next section.
Table 4: Average values of the total reconstitution time and factors influencing it for the 19 cakes obtained by subjecting formulations A-E (all containing fusion protein A) to different lyophilization protocols.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Cycle</th>
<th>Reconstitution time (minutes)</th>
<th>Protein to sugar ratio</th>
<th>Contact angle (°)</th>
<th>Drop penetration time (minutes)</th>
<th>Crushing resistance (N/mm)</th>
<th>Pore size(^a) (µm)</th>
<th>Crystallinity (%)</th>
<th>Foam(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2</td>
<td>16</td>
<td>2.1</td>
<td>76</td>
<td>15</td>
<td>33</td>
<td>-</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td>A</td>
<td>5</td>
<td>15</td>
<td>2.1</td>
<td>76</td>
<td>15</td>
<td>35</td>
<td>-</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>13</td>
<td>1.4</td>
<td>73</td>
<td>15</td>
<td>31</td>
<td>39</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>13</td>
<td>1.4</td>
<td>72</td>
<td>15</td>
<td>28</td>
<td>39</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>10</td>
<td>1.4</td>
<td>65</td>
<td>15</td>
<td>43</td>
<td>17</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>14</td>
<td>1.4</td>
<td>67</td>
<td>15</td>
<td>44</td>
<td>15</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>5</td>
<td>1.4</td>
<td>54</td>
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<td>14</td>
<td>1.3</td>
<td>70</td>
<td>7.2</td>
<td>37</td>
<td>38</td>
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</tr>
<tr>
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<td>13</td>
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<td>68</td>
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</tr>
<tr>
<td>C</td>
<td>3</td>
<td>12</td>
<td>1.3</td>
<td>66</td>
<td>5.3</td>
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<td>17</td>
<td>0</td>
<td>N</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>12</td>
<td>1.3</td>
<td>67</td>
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<td>56</td>
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<td>N</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>5</td>
<td>1.3</td>
<td>55</td>
<td>0.1</td>
<td>13</td>
<td>41</td>
<td>9</td>
<td>Y</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
<td>8</td>
<td>0.9</td>
<td>56</td>
<td>0.4</td>
<td>33</td>
<td>39</td>
<td>6</td>
<td>Y</td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>9</td>
<td>0.9</td>
<td>67</td>
<td>2.3</td>
<td>34</td>
<td>37</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>13</td>
<td>0.9</td>
<td>55</td>
<td>0.5</td>
<td>35</td>
<td>18</td>
<td>9</td>
<td>Y</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>11</td>
<td>0.9</td>
<td>62</td>
<td>1.9</td>
<td>45</td>
<td>14</td>
<td>0</td>
<td>Y</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>7</td>
<td>0.9</td>
<td>50</td>
<td>0.1</td>
<td>24</td>
<td>37</td>
<td>12</td>
<td>Y</td>
</tr>
<tr>
<td>E</td>
<td>2</td>
<td>6</td>
<td>0.5</td>
<td>55</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>Y</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>7</td>
<td>0.5</td>
<td>53</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>Y</td>
</tr>
</tbody>
</table>

\(^a\) Values represent volume-surface average pore diameter

\(^b\) Presence of foam is indicated with ‘Y’ (Yes) and absence with ‘N’ (No)
Table 5: Pearson’s correlation coefficients for pairwise comparison of factors influencing the reconstitution time of formulations yielding process-dependent amorphous or partially crystalline cakes (formulations A-E).

<table>
<thead>
<tr>
<th>Cake property</th>
<th>PSR</th>
<th>CA</th>
<th>DPT</th>
<th>CR</th>
<th>PS</th>
<th>CY</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-to-sugar ratio (PSR)</td>
<td>1.00</td>
<td>0.745***</td>
<td>0.716***</td>
<td>-0.022</td>
<td>0.054</td>
<td>-0.635**</td>
<td>-0.706***</td>
</tr>
<tr>
<td>Contact angle (CA)</td>
<td>1.00</td>
<td>0.848***</td>
<td>0.410</td>
<td>-0.127</td>
<td>-0.877***</td>
<td>0.844***</td>
<td></td>
</tr>
<tr>
<td>Drop penetration time (DPT)</td>
<td>1.00</td>
<td>0.445</td>
<td>-0.245</td>
<td>-0.750***</td>
<td>-0.924***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crushing resistance (CR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.818***</td>
<td>-0.646**</td>
<td>-0.471</td>
</tr>
<tr>
<td>Pore size (PS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.000</td>
<td>0.341</td>
<td>0.254</td>
</tr>
<tr>
<td>Crystallinity (CY)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.000</td>
<td>0.761***</td>
</tr>
<tr>
<td>Foam(^a) (F)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.000</td>
</tr>
</tbody>
</table>

\(^{**}\) Correlation significant at p < 0.01  
\(^{***}\) Correlation significant at p < 0.001  
\(^a\) Foam was a categorical variable where absence of foam during reconstitution was assigned a value of 0 and presence of foam was assigned a value of 1

Table 6: Multivariate analysis of cake properties influencing the reconstitution of formulations yielding process-dependent fully amorphous or partially crystalline cakes (formulations A-E).

<table>
<thead>
<tr>
<th>Cake property</th>
<th>Variable</th>
<th>Coefficient</th>
<th>Standard error</th>
<th>p value</th>
<th>R²(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact angle</td>
<td>Intercept</td>
<td>62.9</td>
<td>2.11</td>
<td>&lt; 0.0001</td>
<td>0.852</td>
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<tr>
<td></td>
<td>Crystallinity</td>
<td>-0.86</td>
<td>0.23</td>
<td>0.0016</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Drop penetration time</td>
<td>0.52</td>
<td>0.17</td>
<td>0.0084</td>
<td></td>
</tr>
<tr>
<td>Drop penetration time</td>
<td>Intercept</td>
<td>-38.3</td>
<td>6.95</td>
<td>&lt; 0.0001</td>
<td>0.720</td>
</tr>
<tr>
<td></td>
<td>Contact angle</td>
<td>0.72</td>
<td>0.11</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Crushing resistance</td>
<td>Intercept</td>
<td>56.9</td>
<td>3.60</td>
<td>&lt; 0.0001</td>
<td>0.838</td>
</tr>
<tr>
<td></td>
<td>Crystallinity</td>
<td>-1.14</td>
<td>0.32</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pore size</td>
<td>-0.65</td>
<td>0.12</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) All models were significant at p < 0.001
Reduction of the number of independent factors influencing reconstitution

Principal component analysis is another procedure that reduces the dimensionality of a data set containing a large number of inter-related “measured” properties (Table 4) into a new set of “reduced” variables called principal components, which are uncorrelated. These principal components can then be used as predictors in subsequent regression analysis. The principal components are ranked in order of their contribution to the variance of the data set. Although the total number of components is equal to the total number of measured properties, only the first two or three principal components, typically accounting for > 85% of the variance, are retained during analysis. The remainder of the components generally describe only random variance in the data. Thus, the principal components are weighted linear combinations of the measured or input variables where the weights are chosen to account for the largest amount of variation in the data.

For the present data set (Table 4), only the first 2 principal components accounting for 86% of the total variance were retained for further analysis. In interpreting the loadings derived from PCA, a particular property of the lyophilized cakes was said to load on a given component if the loading was 0.70 or greater for that component, and less than 0.70 for the other component. The first component had significant contributions (i.e., loadings in Table 7) from protein-to-sugar ratio, crystallinity, contact angle, drop penetration and foam (Table 7). Recall that these cake properties were found to be highly correlated with each other based on the Pearson’s correlations (Table 5). The grouping of these related properties in the first principal component similarly suggests that they represent a common construct. One commonality among this set of cake properties is their dependence on formulation composition. Hence, the first principal component broadly represents those properties related to formulation composition. The second component had significant
contributions (i.e., loadings in Table 7) from pore size and crushing resistance. Recall that both pore size and crushing resistance were highly correlated with each other (Table 5). For a given protein at concentrations (> 50 mg/ml) pore size is primarily influenced by the processing conditions [17]. Hence, component 2 seems to generally represent those properties influenced by processing conditions. Thus, formulation composition and processing conditions were identified as the new “reduced” variables by principal component analysis and were evaluated as predictors of reconstitution time in the next section.

Table 7: Loadings obtained from principal component analysis (PCA) of cake properties influencing reconstitution of formulations yielding process-dependent fully amorphous or partially crystalline cakes (formulations A-E).

<table>
<thead>
<tr>
<th>Cake property</th>
<th>Loadings$^a$</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Component 1</td>
<td>Component 2</td>
</tr>
<tr>
<td>Protein-to-sugar ratio</td>
<td>0.834</td>
<td>0.270</td>
</tr>
<tr>
<td>Contact angle</td>
<td>0.881</td>
<td>-0.255</td>
</tr>
<tr>
<td>Drop penetration time</td>
<td>0.905</td>
<td>-0.230</td>
</tr>
<tr>
<td>Crushing resistance</td>
<td>0.291</td>
<td>-0.938</td>
</tr>
<tr>
<td>Pore size</td>
<td>-0.016</td>
<td>0.910</td>
</tr>
<tr>
<td>Crystallinity</td>
<td>-0.764</td>
<td>0.483</td>
</tr>
<tr>
<td>Foam</td>
<td>-0.928</td>
<td>0.227</td>
</tr>
</tbody>
</table>

$^a$ The loadings for each cake property represent the contribution of that property to the principal components and are in fact the correlation coefficients between the property and the principal component.

Models relating reconstitution time to the factors identified above

Models were developed for relating the properties identified above with (i) the total reconstitution time, (ii) reconstitution time of the first 90% cake solids and (iii) reconstitution time of the last 10% cake solids (Table 8). These models were based on the properties (Table 4) that were shown to influence reconstitution time. In addition to expressing the total reconstitution time as a function
of the cake properties, an additional model was also developed using the principal components derived in the previous section.

Of the seven cake properties measured, total reconstitution time was found to be primarily governed by crystallinity and pore size ($R^2 = 0.794$) for this data set. Crystallinity was highly correlated with contact angle, drop penetration time and crushing resistance and thus the latter three did not add significantly to the ability of the model to describe the data. These three quantitative measures serve as measures of cake wettability, ability of the fluid to penetrate into the cake and cake disintegration tendency, respectively, which are all pre-requisites for faster reconstitution. Since crystallinity had such a dominating effect on lowering reconstitution time by influencing the three correlated properties, it is clearly a key factor governing reconstitution in this data set.

Larger pores were a common characteristic of all fast (< 8 min) reconstituting cakes (Table 4). Pore size is so significant that despite being partially crystalline, cakes of formulation D obtained from Cycle 3 that had a long reconstitution time (~ 14 minutes), most likely due to the small pore size. In addition to linear coefficients relating pore size and crystallinity to reconstitution time, there is a term in the model describing an additional combined effect of crystallinity and pore size on reconstitution time. This interaction term in the model (Table 8) indicates that crystallinity and pore size do not operate independently i.e., the effect of crystallinity on reconstitution time depends on the value of the pore size and vice versa. For example, partially crystalline cakes with smaller pores have longer times reconstitution compared to partially crystalline cakes with larger pores, as
exemplified by formulation D Cycle 3 samples. Similarly, amorphous cakes with large sized pores will have longer reconstitution times than partially crystalline cakes with pores of the same size, as exemplified by samples obtained from Cycles 2 vs. 5 for formulations B and C. In fact, pore size by itself seems to be less critical in governing reconstitution based on the non-significant p value (p > 0.05).

Total reconstitution time could be also be described using the calculated principal components (Table 8). As discussed in the previous section, principal component 1 generally reflects the contributions from formulation composition and principal component 2 stands for the processing conditions. Both principal components contributed equally and significantly to the reconstitution time as seen from the coefficients and p values for the coefficients respectively (Table 8). The model derived directly from properties (rather than regression after PCA) indicated crystallinity and pore size to be the key factors governing reconstitution. While crystallinity in the final cake depends on the formulation composition (P:S ratio) and processing conditions, pore size is governed by processing. Thus, both models suggest that both formulation and processing are factors in reconstitution time.

Reconstitution time of the first 90% of the cake is governed by the P:S ratio and whether foam was generated during reconstitution (Table 8). Based on the coefficients in the model, the time to reconstitute the first 90% of the cake increased with an increase in the P:S ratio and an absence of foam. While the long reconstitution times were caused by the P:S ratio, foam was a side-effect of rapid reconstitution. The time to reconstitute the last 10% of the cake solids depended on pore size.
and foam. Foam significantly increased the time to reconstitute the last 10% of the cake, particularly for cakes with smaller sized pores.
Table 8: Multivariate analysis of key factors governing the reconstitution times of formulations yielding process-dependent fully amorphous and partially crystalline cakes (formulations A-E).

<table>
<thead>
<tr>
<th>Reconstitution time</th>
<th>Variable</th>
<th>Coefficient</th>
<th>Standard error</th>
<th>p value</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total (predicted using the measured cake attributes)</td>
<td>Intercept</td>
<td>11.5</td>
<td>1.28</td>
<td>&lt; 0.0001</td>
<td>0.794</td>
</tr>
<tr>
<td></td>
<td>Crystallinity</td>
<td>0.88</td>
<td>0.35</td>
<td>0.0304</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pore size</td>
<td>0.02</td>
<td>0.04</td>
<td>0.6429</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crystallinity × Pore size</td>
<td>-0.04</td>
<td>0.01</td>
<td>0.0028</td>
<td></td>
</tr>
<tr>
<td>First 90% of cake solids</td>
<td>Intercept</td>
<td>6.41</td>
<td>1.11</td>
<td>&lt; 0.0001</td>
<td>0.947</td>
</tr>
<tr>
<td></td>
<td>Protein-to-sugar ratio</td>
<td>1.93</td>
<td>0.72</td>
<td>0.0161</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Foam</td>
<td>-5.94</td>
<td>0.60</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>Last 10% of cake solids</td>
<td>Intercept</td>
<td>3.36</td>
<td>1.01</td>
<td>0.0069</td>
<td>0.833</td>
</tr>
<tr>
<td></td>
<td>Pore size</td>
<td>0.01</td>
<td>0.03</td>
<td>0.7830</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Foam</td>
<td>10.4</td>
<td>1.67</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pore size × Foam</td>
<td>-0.24</td>
<td>0.05</td>
<td>0.0006</td>
<td></td>
</tr>
<tr>
<td>Total (predicted using principal components calculated from Eq. (1))</td>
<td>Intercept</td>
<td>10.5</td>
<td>0.56</td>
<td>&lt; 0.0001</td>
<td>0.633</td>
</tr>
<tr>
<td></td>
<td>Principal component 1</td>
<td>1.71</td>
<td>0.60</td>
<td>0.0154</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Principal component 2</td>
<td>-1.73</td>
<td>0.57</td>
<td>0.0108</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ All models were significant at p < 0.001
**Formulations yielding only amorphous cakes**

Formulations evaluated in this section differed with respect to the protein and/or the protein-to-sugar (P:S) ratio. All formulations yielded fully amorphous cakes post lyophilization. It should be noted that formulations F1-F7 had a higher fill volume and were freeze-dried using a different lyophilization cycle than formulations A-E. Hence, it was not possible to compare F1-F7 with the formulations investigated in the previous section.

**Reconstitution behavior**

![Reconstitution Time Graph](image)

**Figure 2:** Total reconstitution time (n=8) for formulations (F1-F7) divided into the time taken to reconstitute approximately first 80% of the cake solids (pattern fill) and the time taken to reconstitute the remaining 20% of the cake solids (solid fill). Presence of foam during reconstitution is indicated as ‘+’ and absence of foam (equivalently presence of bubbles) as ‘-’. The formulations differed with respect to the protein and/or protein-to-sugar ratio.

When comparing the formulations in Figure 2, the total reconstitution time was found to be primarily influenced by the specific protein with negligible contribution from the P:S ratio. However, when comparing reconstitution times of a single protein (mAb A) in formulations with different P:S ratios (F1-F3), a higher protein-to-sugar ratio prolonged the reconstitution time. Formulations in the first section with P:S ratio < 1 showed foam formation while at ratios > 1
bubble formation was observed. In comparison with those data, formulations F1-F7 showed interesting results. As expected F1 and F2 (P:S ratio of <1) generated foam on reconstitution. However, F3 and F6 (P:S ratio of 1) also generated foam, while other formulations at the same P:S ratio, but containing a different protein, did not generate foam (F4 and F5). This broader set of formulations suggests that foaming during reconstitution may be more protein-specific than previously thought [7].

In fact, the reconstitution behavior of amorphous formulations containing different proteins (i.e., F3-F7) was dramatically different (Figure 3), manifesting in what can be separated into at least 3 categories of reconstitution behaviors. In the first category of behavior, displayed by F3 and F6, the reconstitution medium rapidly penetrated the cake upon fluid addition and was accompanied by foam formation. The cake also partially disintegrated into smaller pieces of cake that separated (not visible in the image) from the majority of the undissolved cake which became entrapped in the foam. Reconstitution of the cake floating in the foam proceeded slowly through gradual erosion involving wetting and hydration of the cake surface exposed to the reconstitution fluid. In some cases where it was possible to monitor the reconstitution progress, a translucent viscous layer was visible at the surface of the dissolving cake in immediate contact with the fluid, while an opaque inner cake core was evidence of a dry interior. It should be noted that F1 and F2 also fell in to the first category because their reconstitution behavior was similar to F3 and F6 (images not shown).

The second category of reconstitution behavior was exhibited by F4. Slower penetration of the reconstitution fluid into the cake did not produce foam formation but rather gradual formation of
larger bubbles. Here, too, a portion of the cake disintegrated (as seen by the cloudiness in the bottom part of the vial at time zero in Figure 3). Also similar to the first category of reconstitution behavior, a majority of the undisintegrated cake floated on the reconstitution fluid amidst the bubbles and reconstitution progressed slowly via gradual erosion.

In the third category of reconstitution behavior of amorphous cakes (demonstrated by F5 and F7), the reconstitution fluid did not penetrate the cake. As a result, the cake did not disintegrate, but rather the intact cake detached from the vial bottom and floated on the reconstitution fluid without the formation of foam or bubbles. Reconstitution progressed very slowly with gradual evolution of large bubbles. The reconstitution fluid did not readily penetrate these cakes even at later time points. Dissolution of cake solids proceeded by erosion of the translucent viscous layer surrounding the unhydrated cake core (visible at the 7 minute time point for F5 in Figure 3); similar to the later behavior in categories 1 and 2.
Figure 3: Reconstitution behavior of amorphous cakes of formulations F3, F4, F5, F6 and F7 containing mAb A (F3), mAb B (F4), mAb C (F5), fusion protein A (F6) and fusion protein B (F7) at 0, 7, 15 and 30 minutes after addition of reconstitution fluid. The undissolved cake is pointed out using arrows.

Consistent with formulations in the first section, the time to reconstitute the first 80% cake solids of F1-F3 (accompanied by foam formation) increased with the P:S ratio. Time to reconstitute the last 20% of cake solids also increased with the presence of foam and decrease in pore size across F1-F3 (Table 9), again consistent with the results from the first section. When comparing the formulations containing different proteins (F3-F7), the time to reconstitute the first 80% of cake solids was faster for cakes that dispersed, at least to some extent, due to liquid penetration. For example, F3, F4 and F6 which partially dispersed reconstituted in ~ 15 minutes, whereas F5 and F7 took nearly twice as long (~ 27 minutes) to reconstitute since fluid did not penetrate the cake,
but rather the cake floated where it was less exposed to the reconstitution fluid. The time to reconstitute the last 20% of the cake did not trend with any of the cake properties evaluated in the previous section, possibly suggesting dependence on another property yet to be studied.

**Key factors governing reconstitution time of amorphous cakes**

No differences in the contact angle, drop penetration time and crushing resistance were found in any of the amorphous cakes F1-F7 (Table 9), in agreement with the first set of formulations. In those formulations, contact angle, drop penetration time and crushing resistance were highly sensitive to partial crystallinity, but relatively constant in amorphous formulations. Hence, these measures of cake wettability, liquid penetration and cake disintegration tendency were expected to be non-discriminatory for amorphous cakes. Indeed, they did not capture differences in the ability of the liquid to penetrate into the cake and the cake disintegration tendency of F3-F7 discussed above. This indicates that drop penetration time and crushing resistance, developed as simple measures to quantify liquid penetration and cake disintegration respectively, are not sufficiently sensitive surrogates for characterizing these complex steps involved in the reconstitution of amorphous formulations.

The average pore size of F5 and F7 was <10 µm compared to the other cakes where the pore size range was 11-17 µm. The smaller pores are expected to exclude the reconstitution fluid, especially when the wettability is so poor. Although pore size (Table 9) could explain differences in liquid penetration into cakes, it did not account for the differences in the reconstitution times. “Concentrated formulation viscosity” was the only characteristic that varied across F1-F7. The
so-called “concentrated formulation viscosity”, which is measured on the solution formed by using $1/3^{rd}$ of the full volume of the reconstitution fluid, was previously shown to be better correlated with reconstitution time than the viscosity of the pre-lyo solution [10]. The “concentrated formulation viscosity” is thought to serve as a better surrogate measure of the viscosity near the surface of the dissolving cake, where the major barrier to reconstitution exists. Using this single characteristic, a linear model was developed for reconstitution time of F1-F7 (Eq. (1), $R^2 = 0.935$).

A model using only “concentrated formulation viscosity” described the small 7 formulation data set very well. A new formulation, F8, with the same composition as F7 but substituting a new protein (BSA instead of fusion protein A) was prepared as a test formulation to provide preliminary assessment of the model. The concentrated formulation viscosity of F8 was 36 cP. The reconstitution time predicted by the model was 18 minutes for F8 while the measured reconstitution time was $18 \pm 2$ minutes. This provides preliminary evidence for the usefulness of the so-called “concentrated formulation viscosity”.
Reconstitution time (minutes) = 0.127 × Concentrated formulation viscosity (cP) + 13.44  \hspace{1cm} (1)

Table 9: Average values of factors expected to influence the reconstitution of amorphous cakes obtained by lyophilizing formulations F1-F7.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Type of protein</th>
<th>Protein concentration (%w/v)</th>
<th>Protein to sugar ratio</th>
<th>Contact angle (°)</th>
<th>Drop penetration time (mins)</th>
<th>Crushing resistance (N/mm)</th>
<th>Pore size(^a) (µm)</th>
<th>Concentrated formulation viscosity (cP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>mAb A</td>
<td>5</td>
<td>0.35</td>
<td>81</td>
<td>15</td>
<td>24</td>
<td>14.0</td>
<td>19</td>
</tr>
<tr>
<td>F2</td>
<td>mAb A</td>
<td>5</td>
<td>0.50</td>
<td>87</td>
<td>15</td>
<td>18</td>
<td>17.0</td>
<td>16</td>
</tr>
<tr>
<td>F3</td>
<td>mAb A</td>
<td>10</td>
<td>1.00</td>
<td>83</td>
<td>15</td>
<td>28</td>
<td>11.5</td>
<td>131</td>
</tr>
<tr>
<td>F4</td>
<td>mAb B</td>
<td>10</td>
<td>1.00</td>
<td>91</td>
<td>15</td>
<td>25</td>
<td>16.0</td>
<td>197</td>
</tr>
<tr>
<td>F5</td>
<td>mAb C</td>
<td>10</td>
<td>1.00</td>
<td>90</td>
<td>15</td>
<td>32</td>
<td>9.5</td>
<td>495</td>
</tr>
<tr>
<td>F6</td>
<td>Fusion protein B</td>
<td>10</td>
<td>1.00</td>
<td>94</td>
<td>15</td>
<td>28</td>
<td>12.0</td>
<td>137</td>
</tr>
<tr>
<td>F7</td>
<td>Fusion protein A</td>
<td>14</td>
<td>2.1</td>
<td>83</td>
<td>15</td>
<td>29</td>
<td>8.0</td>
<td>129</td>
</tr>
</tbody>
</table>

\(^a\)Values represent surface-weighted average pore diameter
Stages in the reconstitution of lyophilized cakes containing highly concentrated proteins

From the foregoing analysis of the reconstitution behavior of the amorphous cakes, a major portion of all cakes dissolved via gradual erosion, irrespective of their reconstitution behavior category. During reconstitution, cake that is in immediate contact with the reconstitution fluid is wetted and/or hydrated forming a viscous layer near the surface of the dissolving cake. This viscous layer seems to be a major barrier to reconstitution. Higher the viscosity of this layer (which is approximated by the “concentrated formulation viscosity”), lower is the erosion rate and greater is the path length, which provide resistance to the diffusion of water through the layer; delaying wetting and hydration of the cake core and prolonging the total reconstitution time. Lyophilized cakes with pores typically > 12 µm (F1, F2, F3, F4 and F6) facilitated the penetration of the reconstitution fluid into the cake. Penetration into the cake is expected to promote cake disintegration by partial dissolution of the structure and the pressure caused by the ingress of fluid and the dispersed cake pieces are expected to dissolve more rapidly because of the increased surface area. However, despite liquid penetration and partial disintegration, F3, F4 and F6 displayed the same reconstitution time as F7 which did not disintegrate at all. As seen from Figure 3, although liquid penetration resulted in disintegration to some extent in all amorphous cakes (except F5 and F7), it was insufficient to produce smaller cake pieces. As a result, a major portion of the cake still remained intact and floated as a chunk on the reconstitution liquid, where it was less exposed to the reconstitution fluid. Foam entrapment further slows reconstitution because the fluid is unable to hydrate the occluded cake. Once hydrated, the undissolved chunk can lose buoyancy and become more exposed to the fluid and dissolution progresses as a function of the “concentrated formulation viscosity”. Therefore, irrespective of their reconstitution behavior category, dissolution of all amorphous cakes is solely governed by the “concentrated formulation viscosity”.
viscosity” and most likely involves surface erosion where only the cake surface exposed to the fluid dissolves and the dissolution front moves inwards.

“Concentrated formulation viscosity” is highly dependent on composition, particularly protein-to-sugar ratio and the protein itself [10]. Hence, cakes with same formulation composition but processed differently should still have the same “concentrated formulation viscosity” despite their amorphous or partially crystalline nature. However, for a given composition, partially crystalline cakes reconstitute much faster than their amorphous counterparts [1]. Thus, the model of reconstitution time that depends on the single property -- “concentrated formulation viscosity” for amorphous cakes, is not applicable to partially crystalline cakes. Crystallinity results in remarkably improved wettability and faster liquid penetration, resulting in disintegration of the cake into very many small pieces. In addition to the cake disintegration resulting from an increase in pressure due to liquid penetration, crystallinity in itself has also been shown to produce cakes that break more readily (characterized by lower crushing resistance) at least in the dry state. The reconstitution fluid more efficiently hydrates the solids of the smaller, well dispersed cake pieces and dissolution proceeds more rapidly. Still, there is an effect of the “concentrated formulation viscosity” on dissolution of the dispersed pieces, but the path length is smaller and there is an increase in surface area of the dispersed solids, lowering the barrier provided by the viscous layer.
**Approaches to expedite reconstitution of lyophilized formulations containing highly concentrated proteins**

Based on the key factors influencing reconstitution as identified above and from the proposed mechanisms involved in the reconstitution of lyophilized cakes (both fully amorphous and partially crystalline) containing highly concentrated proteins, the following formulation and processing approaches can be adopted to promote faster reconstitution:

1. **Including a crystallizable excipient in the formulation and adjusting the lyophilization cycle to promote its crystallization:** Both mannitol and glycine have been shown to improve reconstitution upon crystallizing, at least partially, in the final freeze-dried cake [1]. Both excipients are commonly used as bulking agents and hence are not often required in high concentration formulations. However, incorporating them to serve as ‘reconstitution rate enhancers’ can prove beneficial. Based on the present findings and prior work [1, 2, 7], the reconstitution rate enhancement by these excipients seems to be independent of the lyoprotectant (sucrose or trehalose). Furthermore, the effect of crystallinity on reconstitution time was found to be independent of the degree of crystallinity [1]. Trace but quantifiable amount of crystallinity seems to be sufficient for promoting faster reconstitution. However, crystallization of these excipients is inhibited in the presence of protein. Hence, an annealing step may be required in the lyophilization cycle. Aggressive drying has also been shown to induce mannitol crystallization in formulations with moderate protein concentration (≤ 100 mg/ml) and protein to sugar ratio typically < 1 [1].

2. **Adjusting the lyophilization cycle to promote the formation of larger pores:** In general, larger pores aid penetration of the reconstitution fluid and reduce reconstitution time even in the presence of foam. This can be achieved by controlling ice nucleation at higher temperatures
(typically between -4 to -6°C). Alternately, an annealing step can also be incorporated in the cycle. The time and temperature of annealing are both significant in defining the porous structure of cakes containing highly concentrated proteins [17].

3. **Incorporating a viscosity lowering excipient in the formulation:** Reconstitution time is strongly influenced by the “concentrated formulation viscosity”, presumably proportional to the viscosity of the hydrated layer near the surface of dissolving proteinaceous Excipients that can lower this viscosity can potentially enable faster reconstitution. Salts and amino acids are two classes of excipients that have been shown to lower the viscosity of high concentration protein formulations [23-26], with sodium chloride and arginine showing the most promise. Sodium chloride, at certain concentrations, not only lowers viscosity but it can also crystallize during lyophilization potentially providing dual benefit. Likewise, in addition to its viscosity lowering effect, arginine is also known to improve protein stability [27-30]. Hence, it would be interesting to explore these excipients as ‘reconstitution rate enhancers’.

**Conclusions**

Overall, partial crystallinity, larger pores and low “concentrated formulation viscosity” (i.e., viscosity near the surface of the dissolving cake) were identified as desirable characteristics for expediting reconstitution. For the two formulations sets investigated, crystallinity and pore size were found to be the key factors governing the reconstitution of formulations yielding process-dependent fully amorphous or partially crystalline cakes. In the case of fully amorphous cakes, “concentrated formulation viscosity” alone was the key factor influencing reconstitution. Partial crystallinity in the cake tremendously improves wettability, liquid penetration and disintegration of the cake. Larger pores facilitate liquid penetration and
therefore disintegration, resulting in faster reconstitution. Amorphous cakes exhibit poorer wettability, liquid penetration and disintegration. The undissolved cake chunk is surrounded by a viscous proteinaceous layer. Low viscosity of this viscous layer near the surface of the dissolving cake (as indicated by the “concentrated formulation viscosity”) hastens erosion of the undissolved cake solids by allowing greater influx of water and improving the access of the cake core to the reconstitution medium.

Dependence of reconstitution time on the factors enumerated above for the respective cakes could be explained in terms of the stages involved in the complex reconstitution process for partially crystalline vs. amorphous cakes. A detailed analysis of the reconstitution behavior of the cakes particularly, (i) the time to reconstitute the first 80-90% cake solids vs. time taken to dissolve the last 10-20% cake solids and (ii) the formation of foam during reconstitution, enabled distinction between the stages through which reconstitution proceeds in partially crystalline vs. amorphous cakes. In partially crystalline cake, crystallinity improves wettability, liquid penetration and disintegration to such an extent that it generates small enough cakes pieces, increasing the surface area and expediting reconstitution. Foaming, an indicator of rapid liquid penetration and possibly cake disintegration (pre-requisites for fast initial reconstitution), negatively affects total reconstitution time due to cake entrapment. Even in the presence of foam, larger sized pores speed reconstitution. Thus, larger pores in combination with crystallinity facilitate liquid penetration and therefore disintegration, but larger pores by themselves are not sufficient for expediting reconstitution. In amorphous cakes, liquid penetration was highly dependent on pore size. Amorphous cakes with smaller pores did not allow rapid liquid penetration and subsequent disintegration, but rather the air-filled intact
cake floated on the reconstitution fluid. In contrast, amorphous cakes with larger pores disintegrated to a minor extent with only tiny pieces separating from the cake while a major portion of the cake still floated on the reconstitution fluid amidst bubbles or foam formed from the minor disintegration. In both cases, reconstitution proceeded predominantly via erosion from the surface in contact with the fluid. In either case, viscosity of the viscous layer at the surface (represented by “concentrated formulation viscosity”) of the dissolving solids provided the major barrier to reconstitution, reducing the erosion rate and limiting the influx of water to the dry solids it surrounds.

Based on the key factors governing reconstitution and the mechanisms involved, both formulation and processing can be tailored to overcome slow reconstitution. Including a crystallizable excipient in the formulation and facilitating its crystallization by incorporating an annealing step are advantageous. Annealing or controlled ice nucleation at higher temperatures that promote the formation of larger pores are beneficial. Lastly, incorporating a viscosity lowering excipient like arginine or sodium chloride may also prove to be helpful, although further investigation is needed to provide stronger evidence for this approach.
References


Chapter 6

Summary
Lyophilized high concentration protein formulations must be dissolved in a suitable diluent prior to patient administration and often have long and variable reconstitution times. Long reconstitution times can be problematic to patients, particularly for self-administered products. Moreover, long reconstitution times are likely to be associated with risks of immunogenicity due to undissolved proteinaceous small particles and incomplete dosing if the patient tries to self-administer the drug prior to complete reconstitution. Long reconstitution times can also be inconvenient for the health care provider in today’s fast-paced clinical settings. The overall objective of this work was to identify factors governing the reconstitution times of highly concentrated lyophilized protein formulations. Fundamental understanding of these factors will provide information critical to efficient formulation and process design with the end goal of overcoming long reconstitution times.

Properties of the lyophilized “cake” that have been previously implicated in influencing reconstitution are the cake wettability, liquid penetration into the cake, cake disintegration, and cake porous structure. However, current characterization techniques do not adequately quantify these properties without undue sample handling. Novel techniques were developed in Chapters 2 and 3 to analyze properties of intact cakes in the vial thereby reducing sample handling artifacts. These in-situ methods represent a significant improvement over the current experimental methods by overcoming the disadvantages associated with crushing and removing cakes out of the vial for analysis.

Partial crystallinity in the cake had been previously shown to promote reconstitution, but the underlying reasons were not fully understood. Chapter 2 unraveled the mechanisms by which
crystalline mannitol improved reconstitution times. Phase separation of crystalline mannitol from the amorphous, protein rich matrix improved wettability of the cake solids and promoted penetration of the reconstitution fluid into the cake interior. The partially crystalline cakes offered less resistance to crushing in the dry state than the amorphous cakes. The crystalline mannitol in the cakes provided “weak points”, presumably enabling easier cake disintegration upon addition of the reconstitution fluid. Above a quantifiable threshold of crystallinity, there was no evident correlation between the degree of crystallinity and reconstitution time. While crystalline mannitol generally decreased reconstitution time by favorably affecting the cake properties influencing reconstitution, it did not always reduce reconstitution time. Cake porous structure and foaming during reconstitution also seemed to be major contributing factors, as followed up in Chapter 5.

Chapter 3 was a detailed investigation of the implications of formulation composition and processing conditions on the porous structure of lyophilized formulations containing highly concentrated proteins. Low pressure mercury intrusion porosimetry was adapted and further developed for characterizing measures of porous structure (i.e., average pore diameter, pore size distribution, total pore volume, porosity and bulk density) for intact cakes.

The glass transition temperature of the maximal freeze concentrate (\(T_g\)') and the viscosity of the freeze concentrate were formulation dependent factors influencing mobility and hence ice crystal growth. A longer time to reach the \(T_g\)’ (i.e., a longer residence time in the more “mobile” state above \(T_g\)’) and lower viscosity of the freeze-concentrate promoted ice crystal growth producing cakes with larger pores. Both of these mobility governing factors were not just dependent on the protein concentration but also on the specific protein in the formulation. The freeze concentrate
viscosity exhibited a Williams-Landel-Ferry (WLF) dependence on temperature below 0°C, having implications on the freezing, annealing and primary drying stages of the lyophilization cycle. For formulations containing highly concentrated proteins, controlled ice nucleation at a high temperature (-5°C) produced cakes with largest pores. Annealing at -16°C for 2 hours produced smaller pores in formulations yielding a highly viscous freeze concentrate as compared to formulations with a low freeze-concentrate viscosity. However, formulations with a high freeze-concentrate viscosity were more resistant to cake micro-collapse during aggressive drying. The difference between product temperature \( T_p \) and \( T_g' \) was also identified as a critical factor influencing micro-collapse during aggressive drying.

Chapter 4 focused on a detailed understanding of the role of formulation in mediating the reconstitution time. A variety of formulation variables such as % total solids, protein concentration, protein-to-sugar ratio, different proteins and inclusion of a crystallizable excipient were explored for their effect on cake properties influencing reconstitution namely, cake wettability, penetration of reconstitution fluid into the cake, cake disintegration and cake porous structure. Additionally, the effect of formulation on several measures of viscosity and the relationship between these viscosities and reconstitution time were also evaluated.

Reconstitution time was primarily influenced by the viscosity near the surface of the dissolving cake (approximated using the “concentrated formulation viscosity”) with negligible contribution from protein concentration. The “concentrated formulation viscosity” was dependent on protein-to-sugar ratio and the protein itself, thereby emerging as a cake property sensitive to formulation that influenced reconstitution. Partial crystallinity in the final cake also expedited reconstitution.
Wettability (assessed by contact angle), ability of aqueous liquids to penetrate into the cake (assessed by drop penetration time), cake disintegration tendency (assessed from the resistance offered by cakes to crushing in the dry state) and cake porous structure were found to be invariant for the amorphous cakes studied and did not correlate with reconstitution time. However, these properties were sensitive to the presence of crystallinity and resulted in faster reconstitution at least of the partially crystalline cakes. “Concentrated formulation viscosity” strongly correlated with reconstitution times of amorphous cakes, providing insights on the steps involved in the reconstitution of these amorphous formulations.

**Chapter 5** focused on the key factors governing reconstitution and providing an understanding of the mechanisms involved in reconstitution of both amorphous and partially crystalline cakes. Partial crystallinity in the final cake, larger pores and low “concentrated formulation viscosity” (i.e., viscosity near the surface of the dissolving cake) were identified as desirable characteristics for expediting reconstitution. While “concentrated formulation viscosity” was the key factor governing reconstitution of amorphous cakes, crystallinity and larger pores governed reconstitution of partially crystalline cakes.

“Concentrated formulation viscosity” was identified as the major barrier to reconstitution. Cake hydration results in a viscous region at the cake surface in contact with the reconstitution fluid. The higher the viscosity this layer, the lower is the erosion rate and greater is the path length for the diffusion of the reconstitution fluid through the layer into the remaining undissolved cake. Hence, the viscous layer provides resistance to the diffusion of water, delaying wetting and hydration of the cake core and prolonging the reconstitution time. The amorphous cakes exhibited
poorer wettability than partially crystalline cakes. Moreover, the ability of the reconstitution fluid to penetrate the pores, and the resulting cake disintegration was much lower than that observed for partially crystalline cakes. In fact, for some of the amorphous cakes, the reconstitution fluid did not penetrate the cake at all. As a result, the undissolved intact cake or a large cake chunk floated on the reconstitution fluid amidst foam or bubbles generated during reconstitution. Dissolution of the floating cake appeared to proceed via hydration followed by gradual surface erosion where reconstitution time was found to be highly correlated with the “concentrated formulation viscosity”. Partial crystallinity and larger pores tremendously improved wettability and liquid penetration in partially crystalline cakes, ultimately resulting in small, well dispersed cake pieces. Thus, cake dissolution proceeded via wetting and liquid penetration, disintegration followed by hydration. The “concentrated formulation viscosity” is still expected to influence dissolution of the dispersed hydrated pieces, but there is an increase in surface area of the dispersed solids, lowering the barrier provided by the viscous layer. Thus, the stages and mechanisms involved in the reconstitution of amorphous cakes seem to be different from partially crystalline cakes.

Based on these findings, both formulation and processing conditions can be tailored to achieve faster reconstitution. Including a crystallizable excipient and incorporating an annealing step to facilitate its crystallization and to promote larger pores were advantageous. Controlling ice nucleation at a high temperature (e.g., -5°C) provided the most benefit in terms of producing cakes with larger pores. However, controlled ice nucleation technologies are currently rare in the manufacturing setting. In that case, the annealing step is particularly helpful in producing larger pores. The choice of annealing temperature and time is highly formulation specific and is governed by the viscosity of the freeze concentrate at the annealing temperature (as shown in Chapter 2).
Formulations yielding a highly viscous freeze concentrate may require a combination of higher annealing temperature and longer annealing duration for promoting ice crystal growth to obtain larger pores. A viscosity lowering excipient in the formulation is expected to be beneficial in reducing the reconstitution time. However, further investigation is needed to provide stronger evidence for this approach.

**Significance**

The key factors governing reconstitution times identified in the present work will enable pharmaceutical scientists to overcome the challenge of long reconstitution times by efficient formulation and process design. The findings can potentially be extended to spray dried proteins formulations which are also known to have long reconstitution times.