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Impact of Protein Internal Dynamics, Process and Formulation Variables on Freeze-Dried Protein Quality Attributes

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Impact of Protein Internal Dynamics, Process and Formulation Variables on Freeze-Dried Protein Quality Attributes

Rui Fang
University of Connecticut, 2018

Abstract

Lyophilization has been used to improve the quality of pharmaceutical proteins when sufficient stability in the aqueous state cannot be achieved. However, due to inherent challenges in both the freeze-drying process and formulation of proteins for freeze drying, efficient delivery of drug products with uniformly improved stability is not trivial. The overall objective of this work was to investigate freeze-dried protein quality attributes affected by stress factors arising from process and formulation.

The natural variation in ice nucleation temperature (T_n) necessitates the control of ice nucleation to ensure uniformity in product quality across a batch. Further, it is less well understood what critical factors are attributed to protein stability after ice nucleation. Freezing parameters including T_n, shelf ramp rate and isothermal hold after ice nucleation were studied. Controlling ice nucleation at a higher temperature was found to not only improve average protein stability, but also improved batch uniformity on in-process stability. Additionally, the findings suggested that a long residence time in the freeze-concentrate, at a temperature above T_g’ of the formulation can result in significant protein aggregation.

Further, to address whether there is a shelf location-dependent mass flow resistance (R_p) during primary drying, differences in R_p between center and edge vials were estimated
from correlations that were established between $T_n$, SSA of the freeze-dried solid, and $R_p$. The findings provide insights into developing methods to accurately measure $R_p$ in individual vials.

Lastly, formulation variables can greatly impact stability during process and storage. Storage below $T_g$ slows molecular mobility of the system on a pharmaceutical relevant timescale and reduces degradation. However, physical and chemical degradation still occurs below $T_g$; other motions, such as internal dynamics within a protein molecule, have been proposed to exist in solid proteins and potentially impacts long-term stability of lyophilized formulations. Solid-state hydrogen/deuterium (H/D) exchange with FTIR spectroscopy was employed to investigate this phenomenon and its potential impact on protein stability in the solid state. The studies demonstrated that the better stability in the rHSA:sucrose formulation correlated with lower extent of H/D exchange, which provides a measure of structure and/or dynamics in the protein formulation.
Impact of Protein Internal Dynamics, Process and Formulation Variables on Freeze-Dried Protein Quality Attributes

Rui Fang

B.S., Dalian Medical University, 2009

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A Dissertation
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Connecticut

2018
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Rui Fang

2018
Doctor of Philosophy Dissertation

Impact of Protein Internal Dynamics, Process and Formulation Variables on Freeze-Dried Protein Quality Attributes

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University of Connecticut
2018
Dedication

To my family for the unconditional love and support
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Summary
Chapter 1

Introduction, Aims and Organization of the Dissertation
Introduction

Therapeutic proteins are a growing class of pharmaceuticals to treat a variety of diseases and the market is expected to increase with a compound annual growth rate of 6.91% during the forecast period between 2012 and 2022 \(^1\). Proteins readily undergo chemical and/or physical degradation by numerous stress factors in solution. In particular, protein aggregates in sub-visible range have attracted great interest due to its potential implication in immunogenicity upon administration \(^2\). To improve stability of this growing category of pharmaceutical products, freeze-drying is commonly used to prepare proteins in the solid state for distribution and storage. According to BCC research, until 2017, 35% of the marketed biotherapeutics are freeze-dried and freeze-drying will soon be required for more than half of the injectable drugs \(^3\). However, due to inherent challenges in both the freeze-drying process and formulation of proteins in the solid-state, efficient delivery of drug products with uniformly improved stability is not trivial. Application of the principles of Quality by Design (QbD) requires control of the entire freeze-drying process for assurance of product quality. Therefore, the understanding, control, and characterization of the process parameters as well as understanding the stabilization mechanism with added excipients in formulations is critical to a better control of the degree of degradation of the protein product.

The effect of freezing parameters on protein stability

The freezing step presents various stresses including cold denaturation, adsorption at ice/freeze-concentrate interface, removal of water, and increase of all solute concentrations through freeze-concentration \(^4,5\). These stresses have been reported to cause irreversible protein damage including structural perturbation, aggregation, and/or
loss of biological activity. Further, the degree of supercooling varies from vial to vial both inter- and intra-batch, which results in variation in ice crystal size and potentially variation in protein damage at the ice/freeze-concentrate interface. It has been suggested that the formation of a large ice/freeze-concentrate interface during fast freezing causes denaturation of surface-active proteins such as lactate dehydrogenase (LDH), catalase, human growth hormone (hGH), bovine IgG, etc. The variation in the ice nucleation step necessitates the control of the freezing parameters for both process economic and product quality reasons. Several controlled ice nucleation techniques have been developed and the technology has been recently listed as an example of an emerging technology by the FDA emerging technology team. In addition to generally improving protein stability, controlled ice nucleation has the potential to improve batch uniformity in product quality. Challenges exist in scaling up the freeze-drying process from the laboratory to manufacturing, due to many factors including the difference in “spontaneous” ice nucleation temperature and heat transfer. If controlled ice nucleation can improve batch homogeneity, its use can improve scale-up from laboratory to manufacturing. Few controlled ice nucleation reports have focused on protein stability; and very little information is available on the effect of controlled nucleation on the stability of proteins formulated with excipients. Investigations of the effect of controlled ice nucleation on average protein stability and batch uniformity in stability will contribute to the applicability and generalizability of controlling ice nucleation in the development of pharmaceutical proteins.

Although the effect of ice nucleation temperature has gained increasing attention, how the freezing protocols after ice nucleation can influence protein stability is less well
understood. Such understanding is critical to designing a process for optimal protein stability. The increasing in the concentration of the solutes (i.e. protein, or any potential reactant) accompanied by ice formation presents a reactive environment for bimolecular reactions such as protein aggregation. This concept was supported by theoretical calculation of bimolecular reaction rate during freezing and experimental observation of increased aggregation of plasmid/linear polyethylenimine polypelex particles arising from significant residence time in the freeze-concentrate. When the proteins are exposed to the freeze-concentrate for a longer time due to a slower ramp rate or longer isothermal hold, aggregation may increase. On the other hand, ice crystals continue grow to larger size and may have the opportunity to remodel to lower a specific surface area, potentially reducing protein adsorption at the ice/freeze-concentrate interface. No experimental data is yet available to support this speculation. With the capability of controlling ice nucleation, it is now possible to study the factors post ice nucleation in practical freeze-drying conditions. A better understanding of whether the freezing protocols after ice nucleation can be advantageous or detrimental to protein stability will contribute to process design for optimal product quality.

**Shelf location-dependence of product resistance during primary drying due to a distribution in ice nucleation temperature**

How the ice crystals nucleate and grow affects the porous structure of the freeze-dried solid. During the primary drying phase of lyophilization, water vapor passes through the porous partially dried layer, experiencing a resistance to mass flow, the so-called product resistance ($R_p$). In a preliminary study, a location-dependent variation in ice nucleation temperature and cake morphology across the shelf was observed even
when ice nucleation was controlled. Existing methods to determine product resistance include Manometric Temperature Measurement (MTM) \textsuperscript{23,24}, utilization of mass flux from Tunable Diode Laser Absorption Spectroscopy (TDLAS) \textsuperscript{25}, and utilization of heat flux from the heat sensors adhered on the shelf surface; however, these approaches measure a batch-averaged $R_p$. Although the heterogeneity in sublimation rate and heat transfer in edge vials compared to center vials has been studied extensively \textsuperscript{26}, little is known about any differences in $R_p$ based on vial location, i.e., between center and edge vials. Improved characterization of the $R_p$ throughout primary drying would aid the optimization of process efficiency while maintaining product temperature below any critical value.

**Implication of protein internal dynamics on protein stability in the solid state during storage**

In addition to the freezing parameters, another critical factor in improving protein stability is to formulate proteins through the use of lyoprotectants such as sucrose or trehalose \textsuperscript{27,28}. The “water substitute hypothesis” through hydrogen bond formation with the protein molecule, and the “glass dynamics hypothesis” through reduced mobility in the rigid matrix are the two main stabilization mechanisms by the addition of disaccharides \textsuperscript{29}. In order to achieve optimal long-term stability, it is recommended that freeze-dried proteins should be stored below the glass transition temperature of the entire system (i.e., an assembly of protein molecules or protein molecules dispersed in a matrix). However, there is an increasing body of work demonstrating that physical and/or chemical degradations still occur during storage at temperatures below the system
glass transition temperature (T\textsubscript{g}) even with good glassy properties\textsuperscript{30-32}. Moreover, there lacks a good correlation between protein stability and T\textsubscript{g} at temperatures below T\textsubscript{g}\textsuperscript{29}. Distinct DSC endotherm events, designated as “pre-T\textsubscript{g}” endotherm, has been reported for insulin, hGH, and bovine serum albumin (BSA) in the solid state\textsuperscript{33-35}. These endotherms occur around 50°C, which is well below the system T\textsubscript{g} for pure solid proteins\textsuperscript{34,35}. Internal dynamic transition on the protein molecule was attributed as the origin of the pre-T\textsubscript{g} endothermic events and was proposed to have stability implications\textsuperscript{30,36}. In principle, effective coupling between the protein internal dynamics and the dynamics of the inert matrix should provide the best stability. However, systematic experimental data is lacking. Understanding the role of excipients on internal dynamics of the protein molecule will contribute to formulation design for optimal storage stability.

**Protein dynamical temperature (T\textsubscript{d})**

The hypothesis of “protein internal dynamics” in dry proteins was adapted from the concept of a glass transition temperature within individual protein molecule, known as the T\textsubscript{d}. T\textsubscript{d} was introduced to describe local fluctuations of protein residues and was well studied in systems with high water/polyol content and in sugar/water glassy matrices, but very little work has been done in dry proteins\textsuperscript{37-39}.

Inelastic neutron scattering has been used to investigate the vibrational and diffusive motions in myoglobin with 36% (w/w) water\textsuperscript{37}. The binding of oxygen to the myoglobin molecule requires higher degree of protein flexibility and density fluctuation. The detected onset of protein motion of myoglobin according to the change in scattering function was above -90°C, which was consistent with the reported onset of oxygen diffusion around -73°C. The temperature at which protein internal motion is activated to
allow effective oxygen binding was referred to as protein dynamical temperature. Protein molecules demonstrate many degrees of molecular motion from smaller-scale local motion such as atomic fluctuations \((10^{15}-10^{-1})\) s, rigid-body motions including helix, domains, and subunit motions \((10^{-9}-1)\) s to larger-scale motions of protein segments such as helix-coil transition, folding and unfolding transitions \((10^{-7}-10^{4})\) s \(^{40}\). Considering protein residues as small molecules, it would be argued that the local motions in protein residues might share glass-like properties. This is evidenced by another study on infrared C=O stretching bands of CO-myoglobin after flash-photolysis, which suggested that proteins and small molecule glasses share common characteristics including meta-stability below glass transition temperature and relaxation behavior following non-exponential Kohlrausch-Williams-Watts (KWW) relaxation \(^{41,42}\).

*The implication of protein internal dynamics on protein stability in solid state*

Given that protein molecules demonstrate glass-like properties including the KWW non-exponential relaxation behavior due to combined relaxation of a number of conformational sub-states, it could be argued that the local non-diffusive movement of the amino acids within proteins may lead to segmental fluctuations, therefore resulting in cooperative larger-scale motion \(^{30,43-46}\). Evidence of loss in protein function at \(T_d\) has also been reported \(^{47-49}\). The potential stability implication of the protein dynamical temperature in solid protein formulations of pharmaceutical significance has been emphasized \(^{43}\). At temperatures exceeding the protein dynamical temperature, there is an increase in local internal motions, which eventually result in larger cooperative motions, and therefore may have significant stability implications.
It is worth noting that protein internal dynamics could be controlled by modulating the dynamical temperature, $T_d$, through the degree of hydration and lyoprotectant content/type $^{38,39,42,43,50,51}$. Hill and co-workers suggested that it is the hydrogen bonding interactions between water and the amino acid residues that facilitates the propagation of fast $\beta$-like motions into larger scale $\alpha$-like motions, which are responsible for protein internal dynamics. Studies on the kinetics of ligand binding to carbon monoxymyoglobin in a dry trehalose system demonstrated that the glassy trehalose suppressed the inter-conversion of protein conformation sub-states, suggesting that protein internal motion is limited or locked in the relatively more rigid matrix $^{52}$. Therefore, effective coupling between protein internal dynamics and the dynamics of the matrices may play an important role in the stability of protein pharmaceuticals.

A full understanding of the effect of the freezing parameters and dynamic coupling of the protein internal dynamics with the dynamics in the formulation matrix will contribute to process and formulation design for optimal protein stability in the solid state.

**Objectives and aims**

The overall objective of this work is to investigate freeze-dried protein quality attributes affected by stress factors arising from the freezing step of the lyophilization process (ice nucleation temperature, residence time in the freeze-concentrate, etc.) and by dynamic coupling of protein molecule and the formulation matrix. Fundamental understanding of the stresses on the protein during freezing and the implication of protein internal dynamics will provide information critical to the efficient formulation and process design.
The specific aims are:

1. To study the effect of two freezing parameters (ice nucleation temperature and residence time in the freeze-concentrate) on stability of protein formulations during the freeze-drying process and long-term storage.

2. To address whether product resistance during primary drying is shelf location-dependent due to an observed distribution of ice nucleation temperature within a batch.

3. To investigate the origin of pre-$T_g$ endothermic events in pure solid proteins and further to explore the implication of the attributed protein internal dynamics on protein stability in the solid state.

Organization of the Dissertation

Large natural variations exist in ice nucleation temperature across the batch. Chapter 2 presents a focused study on the effect of controlling ice nucleation temperature on stability of a model protein known to be sensitive to the ice surface, lactate dehydrogenase (LDH), formulated without any stabilizer. Both average stability of LDH within a batch and homogeneity in stability were evaluated when the samples were frozen at various controlled ice nucleation temperatures as compared to the freezing condition without ice nucleation control. This study provides direct evidence of improved protein stability during freeze-thawing when ice nucleation was controlled at a higher temperature. Interestingly, a higher ice nucleation temperature did not show a vastly superior stability in LDH after the entire freeze-drying process. This work was the first
report that demonstrated improved batch uniformity in protein stability using the controlled ice nucleation technology, ControLyo™.

Very little information is available on the effect of controlled ice nucleation on the stability of proteins formulated with excipients. Moreover, it is less well understood what the critical factors are attributed to protein stability post ice nucleation. Chapter 3 extends the study of the freezing parameters on protein aggregation during the freeze-drying process and storage. Two model proteins were used in the study including intravenous immunoglobulin (IVIG) and recombinant human serum albumin (rHSA) and were formulated with or without the presence of a stabilizer, sucrose. In addition to the ice nucleation temperature, the shelf ramp rate and isothermal hold were varied after ice nucleation. The two opposing effects after ice nucleation (ice surface area and residence time in the freeze-concentration) were proposed to affect protein aggregation. A good correlation was found between residence time and protein aggregation. This study provided experimental evidence in supporting the residence time in the freeze-concentrate as the critical factor in protein aggregation. The results are directly relevant to process design in bulk freezing and thawing when the freezing time is much longer. In addition to stability, characteristics of the final solid including morphology and porous structure were characterized at various freezing conditions using the polymer encapsulation method, mercury intrusion porosimetry method and specific surface area measurements. The findings suggest that it is not only the ice nucleation temperature, but also the thermal history post ice nucleation that define the surface area of ice and the porous structure of the freeze-dried cake.
A distribution in ice nucleation temperature within a batch was observed even when ice nucleation was controlled at a fixed time and shelf temperature. In existing literature, the resistance to mass flow during primary drying, product resistance ($R_p$) was determined as a batch average. **Chapter 4** attempts to address whether there is a shelf location-dependent $R_p$ due to the distribution in ice nucleation temperature. The calculation of real-time $R_p$ in individual vials was described and the challenges using this “direct vial” method were addressed. The correlation between ice nucleation temperature, specific surface area, and $R_p$ in center vials was established to estimate $R_p$ in samples placed at other locations of the shelf (i.e. edge vials). This study filled the gap in the area of understanding whether $R_p$ is dependent on the location of the vial and provides insights in developing methods to accurately measure real-time $R_p$ in individual vials.

Endothermic events were previously reported in DSC thermograms of pure insulin and hGH in the solid state; the events occurred at temperatures well below the glass transition temperature of the system, referred to as pre-$T_g$. **Chapter 5** discusses and studies the origin of the pre-$T_g$ endothermic events. Enhanced internal motion on the protein molecule in that temperature range was proposed to be the underlying cause of the pre-$T_g$ events. This phenomenon, protein internal dynamics, was studied by solid-state hydrogen/deuterium (H/D) exchange in conjunction with FTIR spectroscopy. This work presents a new concept based on existing knowledge about the dynamics on the protein molecule and supported previously reported instability at temperatures lower than the system glass transition temperature.
Chapter 6 is a continued exploration on whether the protein internal dynamics studied in Chapter 5 has any stability consequence in protein formulations in the solid state. Soluble aggregation during storage was evaluated in rHSA freeze-dried with sucrose or trehalose. The sucrose formulation showed better stability than trehalose during storage. To further explore the underlying mechanism of the better stability offered by sucrose, both the protein secondary structure by FTIR spectroscopy and dynamic property by solid-state H/D exchange were evaluated. Both formulations showed similar secondary structure features. The dynamical temperature, $T_d$ appears to not correlate with stability in rHSA systems. However, it found that the lower extent of H/D exchange in the sucrose formulation correlated with its better stability compared to the trehalose formulation.

The overall results and significance of the research was summarized in Chapter 7.
References


Chapter 2

Effect of Controlled Ice Nucleation on Stability of Lactate Dehydrogenase During Freeze-Drying
Abstract

Several controlled ice nucleation techniques have been developed to increase the efficiency of the freeze-drying process as well as to improve the quality of pharmaceutical products. Due to the reduction in ice surface area, these techniques have the potential to reduce the degradation of proteins labile during freezing. The objective of this study was to evaluate the effect of ice nucleation temperature on the in-process stability of lactate dehydrogenase (LDH). LDH in potassium phosphate buffer was nucleated at -4°C, -8°C, and -12°C using ControLyo™ or allowed to nucleate spontaneously. Both the enzymatic activity and tetramer recovery after freeze-thawing linearly correlated with product ice nucleation temperature (n=24). Controlled nucleation also significantly improved batch homogeneity as reflected by reduced inter-vial variation in activity and tetramer recovery. With the correlation established in the laboratory, the degradation of protein in manufacturing arising from ice nucleation temperature differences can be quantitatively predicted. The results show that controlled nucleation reduced degradation of LDH during the freezing process, but this does not necessarily translate to vastly superior stability during the entire freeze-drying process. The capability of improving batch homogeneity provides potential advantages in scaling-up from lab to manufacturing scale.

Keywords:

Freeze drying/Lyophilization; Ice Nucleation; Protein formulation; Solid state stability; Quality by design (QbD)
Introduction

Many therapeutic proteins are freeze-dried to improve their storage stability, since stability in the aqueous solution state is often insufficient for distribution and storage of this growing category of pharmaceutical products. However, freezing can cause irreversible protein damage including aggregation, and/or loss of biological activity\(^1\). There are four major stress factors that reduce protein stability during freezing, including cold denaturation, structural perturbation at the ice/water interface, removal of water, and increase of all solute concentrations through freeze-concentration\(^2,3\). Protein stability can be quite sensitive to the details of the freezing method. Therefore, appropriate control of the freezing process has the potential of providing better control of the degree of degradation of the protein product.

Freezing is normally uncontrolled in the conventional freeze-drying process. The cooling rate of the shelf is controlled; however, shelf cooling rate only controls freezing post ice nucleation. Since ice nucleation is a stochastic process, the degree of supercooling varies from vial to vial both inter- and intra-batch, which results in variation in ice crystal size and potentially in protein damage at the ice/water interface. Improvement in stability of protein formulations has been reported at freezing conditions that reduce ice crystal surface area\(^4-7\) such as slow cooling or annealing, but none of these reports used controlled ice nucleation. It has been suggested that the formation of a large ice/freeze-concentrate interface during fast freezing causes denaturation of surface-active proteins such as lactate dehydrogenase (LDH)\(^2,4,8-13\), catalase\(^4,14\), human growth hormone (hGH)\(^6,7\), bovine IgG\(^15\), etc. Sarciaux and coworkers reported more insoluble aggregates when bovine IgG was rapidly cooled (by quenching in liquid nitrogen) versus
slower cooling at 2°C/min. Moreover, the degree of insoluble aggregate formation was protein concentration dependent, with higher degradation observed at lower protein concentration, suggesting an interfacial phenomenon. Cochran and Nail reported an inverse correlation of LDH (50 µg/mL, pH 7.4) activity recovery after freeze-drying with the degree of super-cooling, suggesting a larger degree of protein degradation at the higher ice/freeze-concentrate interfacial area which results from a greater degree of supercooling. However, there was significant “scatter” in the data, due to large variation in the enzymatic activity assay. Another possible reason for the scatter is lack of ice nucleation control.

Perhaps control of the ice nucleation temperature will provide a clearer picture to evaluate the correlation between ice nucleation temperature and LDH activity recovery. In one study, temperature-controlled sample wells were used, rather than glass vials, in order to achieve rapid cooling (45-60s; i.e., from +5°C to -5°C, -10°C, -15°C, -20°C, or -25°C). Ice formation was initiated by dipping a cold ice covered platinum wire into the solution at the desired final temperature to control the onset of ice formation in this study of the degradation of LDH (5 µg/mL, pH 7.4). The study suggested ice formation as the critical factor in destabilizing LDH. The ice crystals leave fingerprints as pores formed in the freeze-dried solid. The ice surface area, which is the surface area of the freeze-dried solute, can be easily measured by the specific surface area (SSA) of the freeze-dried product. It has been reported that SSA of freeze-dried materials was lower when ice nucleated at a higher temperature. However, in the study by Bhatnagar et al., there was no significant difference in activity recovery of samples when ice was nucleated at -2 versus -9.5°C, which is contrary to what would be expected. A study of a catalase
formulation (1.7 μg/mL) revealed 60% loss of activity at a lower ice nucleation temperature (-10°C) controlled by seeding, compared to 25% activity loss at higher ice nucleation temperature (-2°C) \(^{14}\). More recently, lyophilized hGH was shown to be less stable when a larger fraction of protein was found at the surface of the solid phase, where this fraction varied with the details of the freezing process largely due to variation in specific surface area \(^{6}\). Collectively, these reports suggest that protein stability can be improved by manipulating the freezing parameters, such as ice nucleation temperature and perhaps cooling rate, although other factors specific to the protein may also be important.

Several techniques have been developed to control the ice nucleation at fixed time and temperature and thereby minimize primary drying time as well as improve batch homogeneity \(^{17-21}\). Ice nucleation controlling techniques including ControLyo™ \(^{22}\) can potentially reduce the degradation of proteins labile at ice/aqueous interface. However, early on, there were concerns that ControLyo™ would destabilize protein during sudden depressurization that could potentially form small bubbles and/or introduce cavitation, both effects being potentially damaging to protein stability. Preliminary work (Sever and Pikal, unpublished data) showed the stability of hGH in sucrose formulations was in fact improved using ControLyo™ at a higher ice nucleation temperature than uncontrolled ice nucleation.

In addition to generally improving protein stability, controlled ice nucleation has the potential to improve batch uniformity in product quality. It is well known that the ice nucleation temperature is sensitive to the presence of heterogeneous nucleation sites. Fewer and/or different airborne particulates, different vial processing, and therefore fewer
or less effective heterogeneous nucleation sites in manufacturing probably accounts for the lower average ice nucleation temperature observed in production operations relative to laboratory environments \(^{16,23}\). The difference in average “spontaneous” ice nucleation temperatures in lab and production environment presents a serious challenge for scaling up in freeze-drying. If, as is expected, controlled ice nucleation can improve batch homogeneity, its use can improve scale-up from laboratory to manufacturing. Application of the principles of Quality by Design (QbD) requires control of the entire freeze-drying process, including freezing, or at a minimum, demands an understanding of the impact of uncontrolled nucleation on product quality for representative products \(^{24}\). For both product quality and process economic reasons, techniques to control ice nucleation and growth are needed to ensure consistent product quality and lower costs. Such techniques are now available for both laboratory and manufacturing, and have been critically reviewed \(^{25}\).

In the present study, the objective was to investigate the effect of a controlled ice nucleation technique on the average stability and batch uniformity in stability of a protein that shows strong evidence of degradation during freezing due to the presence of ice. Pure LDH at a very low concentration was chosen as the model protein to study the impact of ice nucleation temperature. This formulation is not a representative protein product. However, previous studies clearly implicated interaction with the ice as the primary “stress” during freezing, and LDH satisfies this requirement better than any other system with which we are familiar. ControLyo™ (controlled ice nucleation by depressurization) was used to control the ice nucleation temperature.
Materials and Methods

Materials

L-Lactic dehydrogenase from rabbit muscle (EC 1.1.1.27) was purchased from Sigma-Aldrich (a suspension in 3.2 M ammonium sulfate, St. Louis, MO). All other reagents were reagent grade or higher quality (Sigma-Aldrich, St. Louise, MO or Fisher Scientific, Waltham, MA). Rubber stoppers, (2-prong with outer diameter 20 mm) and 10 mL glass vials (WHEATON, Type I) were purchased from Fisher Scientific (Waltham, MA).

Sample preparation

LDH was dialyzed against 100 mM potassium phosphate buffer (pH 7.3) with a 10K MWCO membrane for 3 hours twice and the third buffer exchange for overnight at 4°C. The concentration of LDH was determined from UV absorbance (extinction coefficient 1.44 mL/mg-cm at 280 nm). The LDH solution was diluted with purified water (0.22 µm-filtered) and potassium phosphate buffer in order to formulate 10 µg/mL of LDH in 5 mM potassium phosphate buffer (pH 7.3). Two milliliters of the sample solution were filled into each 10 mL vial (for a fill depth of 0.6 cm) and were partially stoppered with rubber stoppers.

Freeze-thawing and freeze-drying

The ControLyo™ system (SP Scientific, Gardiner, NY) was used for both freeze-thawing and freeze-drying studies. LDH solution in 10 mL vials was placed at specific locations on the shelf, with three vials in the center, and one each in the front, back, and
edge position of the shelf. Non-sample vials filled with water (2 mL) surrounded the sample vials to partially fill the shelf. The temperatures of the 6 LDH vials were monitored during the freezing process with thermocouples glued with thermal grease to outside of the vials near the vial bottom. For uncontrolled freezing, the LDH solutions were equilibrated in the freeze-dryer at 5°C for 30 minutes, then the shelf was cooled to -40°C at 1°C/min and held for 1 hour. The nucleation of ice in the LDH solutions was controlled at the desired shelf temperatures (-4, -8, -12°C) and frozen according to the recipe shown in Table 1. Preliminary tests showed that the activity of LDH solution was time-dependent after thawing. Therefore, for the freeze-thawing study, products were removed from the freeze-dryer after freezing and allowed to thaw at room temperature, which took approximately 15 minutes. Then the enzymatic activity and tetramer recovery were measured immediately after thawing.

For the freeze-drying study, the LDH solutions were frozen under uncontrolled or controlled ice nucleation at -4°C following the same protocol for freezing as described above. For primary drying, the chamber pressure was set at 100 mTorr and the shelf temperature was heated at 0.5°C/min to -28°C and held constant. When the Pirani gauge reading converged with the capacitance manometer reading, the shelf temperature was raised at 0.2°C/min to 25°C and held for 2 hours with the chamber pressure maintained at 100 mTorr. The freeze-dried product was reconstituted with 2.0 mL of water and the activity and tetramer recovery were measured immediately after reconstitution.

**Enzymatic activity assay**

The activity of LDH was determined by monitoring the decreasing of absorbance at 340 nm with a UV/VIS spectrophotometer (Cary Bio100, Varian Inc., Palo Alto, CA)
resulting from hydrolysis of nicotinamide adenine dinucleotide (NADH). Fifty microliters of each thawed or reconstituted LDH sample (10 µg/mL) were added to a mixture of 30 mM sodium pyruvate and 6.6 mM NADH in 100 mM potassium phosphate buffer at room temperature. Cary WinUV Software in kinetics mode was used for data acquisition and analysis. Results are reported as remaining percent (%) of activity relative to initial values prior to freezing.

**Tetramer recovery by size exclusion chromatography (SEC)**

The LDH solution was filtered (0.45 µm polyvinylidene difluoride filter, Thermo Fischer Scientific, Waltham, MA) and 50 microliters was injected onto a SEC column (TSKgel™ G3000SW XL, 7.8 mm x 30 cm, 5 µm, Tosoh Bioscience, San Francisco, CA) maintained at 25°C. The mobile phase was 100 mM phosphate buffer with 0.2 M NaCl (pH 7.0) delivered at 1.0 mL/min. The HPLC (model 1100, Agilent, Santa Clara, CA) was connected to a diode array detector, with which absorbance was measured at 220 nm (model G1315A, Agilent). Chemstation software (Agilent) was used for data acquisition and analysis. The intensity of the lowest tetramer peak used in the data set was ten times the background noise intensity. Results are reported as a remaining percent (%) of tetramer contents to initial values prior to freezing.

**Statistical analysis**

One-way analysis of variance (ANOVA) was applied to evaluate whether there was statistical significance in stability differences due to the drying step between the two freezing conditions (uncontrolled ice nucleation versus controlled ice nucleation at shelf temperature of -4°C). All variation was expressed as standard deviation.
Results and Discussion

Freeze-thawing study

Using conventional freezing, ice nucleated randomly over a wide temperature range, as seen from the sudden rise in product temperature (Figure 1A), when the exothermic crystallization first occurs. Moreover, often a second “temperature jump” prior/post ice nucleation was observed, representing the heat transferred from neighboring vials undergoing ice nucleation at lower temperatures. In contrast, uniform ice nucleation was observed upon depressurization to initiate nucleation at shelf temperatures of -4 and -8°C (Figure 1B, 1C). However, it was difficult to achieve uniform ice nucleation when attempting control of ice nucleation at lower temperatures such as -12°C; one out of the six vials nucleated spontaneously prior to depressurization (Figure 1D). Controlled ice nucleation was initiated at a given shelf temperature, but the ice nucleation temperature of the LDH solution in the vials was higher (Table 2). Ice nucleation was controlled at average product temperatures of -3.1°C, -6.9°C, and -10.2°C when the solution was equilibrated at shelf temperatures of -4, -8, -12°C, respectively (Table 2). In the case of uncontrolled nucleation, the average ice nucleation temperature was lower (-14.4°C) than for the controlled nucleation conditions (Figure 1, Table 2). Significantly higher average activity of LDH (89.8 ± 0.6%) was retained for solutions in which ice nucleation was initiated across the batch at a shelf temperature of -4°C (product temperature -3.1 ± 0.4°C) compared to uncontrolled nucleation occurring spontaneously in each vial (75.3 ± 2.3%) (Figure 2). The activity improved linearly with an increase in ice nucleation temperature of the LDH solutions (n=24, R²=0.83) (Figure 3).

The loss of LDH activity during freeze-thawing was reported to correlate well with the dissociation process from the native tetramer (142 kDa) to the dimeric subunits,
which have lower activity \(^{11,26}\). Further, Bhatnagar et al. reported that the reassociation from dimer to tetramer occurred upon freeze-concentration at -5°C, and slowly dissociated back to dimer during thawing \(^{27}\). In the present study, the time from thawing to measurement was maintained constant, with the tetramer recovery of LDH solution in vials analyzed immediately after freeze-thawing. Tetramer recovery was 75.8 ± 2.4% for uncontrolled ice nucleation (average ice nucleation temperature -14.4°C). The recovery progressively improved as ice nucleation was controlled at higher temperatures; 76.6 ± 1.0%, 79.8 ± 0.8%, 81.5 ± 1.0%, for control at shelf temperatures of -12, -8, -4°C, respectively. A correlation (R\(^2\)=0.70, n=24) was observed between tetramer recovery measured by SEC and the LDH solution ice nucleation temperature (Figure 4). This observation further supports the strong correlation between the enzymatic activity recovered and the ice nucleation temperature of the LDH solution (Figure 3).

The observed higher recovery of activity of LDH agrees with generally accepted finding of reduced surface area of ice crystals expected at higher ice nucleation temperature. While the current study is focused on LDH, other proteins are also susceptible to increased degradation in formulations that have high SSA of ice \(^{6,14,15}\). However, it should be noted that improvement in the in-process stability is expected to be greatest for low to moderate protein concentrations where a substantial fraction of the protein is likely located at the surface. Formulations with high concentrations of protein or substantial lyoprotectant levels may not show significant loss of activity or increase in aggregates after freeze-drying.

Batch homogeneity is expected to improve with ice nucleation controlled at a higher temperature. An improvement in batch drying rate \(^{12}\) and low standard deviation in
SSA \(^{19}\) have been reported when ice nucleation is controlled, thereby reducing the variation in nucleation temperatures across the batch. Passot et al. demonstrated that the greatest batch homogeneity was obtained by using a nucleation agent in the formulation, followed by precooling the shelf and employing an ultrasound method \(^{12}\). In the present study, a commercially available ice nucleation technology was employed. The variation in the product temperature at which ice nucleated is expressed as the standard deviation of the 6 vials at different locations (Table 2). Ice nucleation control at a particular shelf temperature improved the uniformity of ice nucleation temperatures of the 6 vials, despite the vial locations that are generally considered to have a large position dependence in heat transfer \(^{28}\) – three vials in the center, and one each in the front, back, and edge position of the shelf. The LDH solution in all vials nucleated simultaneously at which ice nucleation was controlled (Figure 1B, C) except that one sample nucleated prior to depressurization in the case of -12°C (Figure 1D); thus, heterogeneity in ice nucleation temperature was significantly reduced compared to the uncontrolled ice nucleation batch (Table 2). To further investigate the potential improvement in batch homogeneity in protein stability, the variation in activity and tetramer recovery of LDH was evaluated in the 6 individual vials frozen at extremes in shelf position under various freezing conditions. The standard deviation of LDH activity recovery after freeze-thawing was lowest under controlled ice nucleation at -4°C compared to uncontrolled nucleation (± 0.6 vs. ± 2.3). Similarly, the standard deviation of tetramer recovery was reduced by about \(\frac{1}{2}\) under controlled ice nucleation (± 1.0 vs. ± 2.4) (Figure 4). In addition, the variability in stability data increases as ice nucleation temperature decreases, even when ice nucleation is controlled (at -4, -8, -12°C). Spontaneous nucleation in some of the vials prior to
reaching the controlled ice nucleation temperature could in principle be the cause of the higher variation at -8 and -12°C. In this case, there was only one vial in the -12°C experiment that nucleated spontaneously prior to depressurization, and none in the case of controlled ice nucleation at -8°C. The improvement in batch homogeneity resulting from controlled ice nucleation at a higher temperature facilitates scale-up and the development of a more robust freeze-drying process, and therefore plays an important role in QbD.

**Freeze-drying study**

In addition to stresses that proteins experience during freezing, the drying step often causes greater protein degradation due to dehydration \(^1\). In the present study, we note further degradation of LDH during the drying stages. A previous study demonstrated a correlation between ice nucleation temperature and activity recovery of reconstituted LDH solution after freeze-drying, but with significant scatter in the data \(^9\). Here, we confirm these earlier findings using controlled ice nucleation and also characterize tetramer recovery of the freeze-dried LDH products for both uncontrolled ice nucleation and nucleation controlled at -4°C. In the case of conventional freezing without control of ice nucleation, there was a wide range in ice nucleation temperature as well as multiple “jumps in temperature” suggesting ice nucleation in a neighboring vial occurred at a later time and lower temperature (Figure 5A). The ice nucleation profile is different from uncontrolled ice nucleation experiments conducted during the freeze-thaw study, suggesting the thermal history of products during freezing is not reproducible inter-batch. However, when using controlled ice nucleation, relatively more homogenous ice nucleation temperature was obtained (Figure 5B and Table 3). The temperature history of
all samples is remarkably consistent. It should be noted that the edge vial placed in the front row facing the door nucleated at the highest temperature indicated by the arrow (Figure 5B), and remained at a higher temperature for a much longer time at the equilibrium freezing point than other vials. The resulting activity recovery is 56.4% for uncontrolled ice nucleation with average product temperature of -14.2°C, and 62.4% for controlled ice nucleation with average product temperature of -2.2°C (Figure 6A). The difference was significant (α=0.01) based on one-way ANOVA analysis. Additionally, the improvement in tetramer recovery was significant for controlled ice nucleation (49.6%) compared to uncontrolled ice nucleation (44.0%) (Figure 6B).

To separate the % activity loss on drying, we assume that the % activity after freeze-thawing (Figure 2) is the % activity after the freezing step of freeze-drying. For example, uncontrolled ice nucleation resulted in 75.3% activity remaining after freeze-thawing. After freeze drying, the % activity based on the initial pre-lyophilized solution was 54.4%. Thus, 18.9 ± 0.6% activity (i.e., 75.3% minus 56.4%) was lost on drying without control of ice nucleation versus 27.4 ± 1.4% (i.e., 89.8% minus 62.4%) for controlled nucleation at -4°C. In fact, the loss in activity during drying was larger for controlled ice nucleation (27.4%) than for uncontrolled ice nucleation (18.9%). The loss in tetramer during drying is the same for the two conditions (31.8 ± 0.8% vs. 31.9 ± 0.2%). Thus, controlled ice nucleation offered less protection during the entire freeze-drying process than during freezing itself. Greater protein damage during the drying step partially erased the activity differences arising from different ice nucleation temperatures during freezing. The question remains: why should a sample produced with a higher ice nucleation temperature (-4°C) show more degradation during drying than the same
composition that begins freezing at a lower temperature and presumably has a higher specific surface area of ice (and solute)? We do not have a satisfactory explanation for this observation. Presumably, this additional degradation occurs during secondary drying since there is little to no evidence that significant degradation occurs during primary drying, even with collapse, and degradation does occur during secondary drying.

**Implications for extrapolating lab data to protein stability at manufacturing scale**

In the manufacturing environment, much smaller ice crystals and higher ice surface area are expected, since ice may nucleate at temperatures lower than -20°C in manufacturing operations. It has been noted that the average ice nucleation temperature in manufacturing can be about 10°C lower than in the laboratory for a given formulation. Further, in the laboratory and presumably also in manufacturing, the range in ice nucleation temperatures for a given product can span 10°C or more (unpublished data in collaboration with Praxair). Therefore, for proteins susceptible to damage at the ice/aqueous interface, both the average and the variation in lyophilized product quality are expected to be significantly different when processed in the lab versus in a manufacturing environment.

The correlation established between ice nucleation temperature and stability, using LDH as a model (Figure 3) can be used to quantitatively estimate protein degradation. Based on laboratory data from Praxair, the ice nucleation temperatures follow a Gaussian distribution with average ice nucleation temperature at -13°C and a span of 10°C (Figure 7). It was suggested that the average ice nucleation temperature is 10°C lower than in the laboratory environment. Assuming the same distribution of ice nucleation temperature (without controlled nucleation) applies in manufacturing, the
average LDH activity after freezing is expected to be 11.4% lower than in lab scale. The activity recovery can be as low as 60% for 5% of the product with the lowest expected ice nucleation temperature of -28°C. The correlation coefficient between stability after freeze-drying and the ice nucleation temperature was low ($R^2=0.60$) after freeze-drying since less difference is seen between the two extreme conditions, controlled ice nucleation at -4°C and uncontrolled nucleation. There is expected to be a ~4% decrease in activity or tetramer recovery for a 10°C decrease in ice nucleation temperature. Significant differences in average product quality between laboratory and manufacturing are likely for any product susceptible to damage at the ice/aqueous interface. Overall, lower variability within a production batch using controlled ice nucleation should be expected for all products.

**Conclusions**

LDH, which is well-known as a protein labile at the ice/freeze-concentrate interface, was frozen using controlled ice nucleation at several temperatures and by spontaneous uncontrolled nucleation. Both higher enzymatic activity and tetramer recovery correlated well with higher ice nucleation temperatures after freeze-thawing. This result is consistent with previous findings that ice nucleation at relatively high temperatures produces larger ice crystals and therefore low ice/aqueous interfacial area. However, since greater degradation occurs during drying for controlled ice nucleation (i.e., higher ice nucleation temperature and presumably less SSA), the total in-process stability was only slightly (~6%) better for controlled ice nucleation. The results indicate that the controlled nucleation technique has the potential to minimize degradation during the freezing process for pharmaceutical protein products that are labile at ice/freeze-
concentrate interface. However, controlled ice nucleation does not necessarily translate to vastly superior stability throughout the entire freeze-drying process, at least in the case of low concentrations of pure LDH. In general, controlling ice nucleation at a higher temperature is most beneficial in improving the average in-process stability for low to moderate protein concentrations without stabilizers. In addition to the impact on protein stability, the improvement in batch uniformity using controlled ice nucleation contributes to better control and development of a more robust freeze-drying process that is easier to scale up to manufacturing, and this result is expected to be general. ICH guidelines require controlling the critical process parameters (CPP) that affects critical product quality attributes (CQA), such as stability. The numerous studies in the literature, as reviewed in the introduction, as well as the current study report that ice nucleation temperature can impact protein stability, indicating that it should be considered a potential CPP unless shown otherwise. However, as noted above, the net effect on stability during the entire freeze-drying process can be complex. Unfortunately, the ice nucleation temperature is currently uncontrolled in nearly all manufacturing plants. Given the availability of ice nucleation control technology for manufacturing, this situation will likely change in the future.

Acknowledgement

Funding for this project from Dane O. Kildsig Center for Pharmaceutical Processing and Research (CPPR) is greatly appreciated.
Pressurization after step 2 (to 28.5 psig with argon gas), depressurization after step 3 (to 2.0 psig)

The shelf temperature was cooled down to the target temperature, -4, -8 or -12°C, at the rate of 1°C/min and was held for 60 minutes. After that, the chamber was pressurized to 28.5 psig with argon gas.

The shelf temperature was held at each target in the pressurized chamber for 15 minutes. After that, the chamber was depressurized to 2.0 psig rapidly. There was a small increase in product temperature (0–0.2°C for control of ice nucleation at -4°C and -8°C, and 0–0.6°C for control at -12°C) during the 15 minutes equilibration after pressurization.

The shelf temperature was cooled down to -40°C at the rate of 1°C/min and was held for 60 minutes.
Table 2. Comparison of product ice nucleation temperatures in each freezing condition

<table>
<thead>
<tr>
<th>Ice nucleation conditions</th>
<th>Uncontrolled</th>
<th>Controlled at shelf temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-4°C</td>
<td>-8°C</td>
</tr>
<tr>
<td>Ice nucleation temperature (°C)</td>
<td>Average&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Maximum</td>
<td>-12.9</td>
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<tr>
<td></td>
<td>Minimum</td>
<td>-17.4</td>
</tr>
<tr>
<td></td>
<td>Max - Min</td>
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</tr>
</tbody>
</table>

<sup>a</sup>Average and standard deviation (Std Dev) were calculated with the values of 6 individual vials located at the center, edge, front and back of the shelf. The thermocouples were placed on the outside of the vials near the vial bottom.
Table 3. Comparison of ice nucleation temperatures in each freezing process of the freeze-drying study

<table>
<thead>
<tr>
<th>Ice nucleation conditions</th>
<th>Uncontrolled</th>
<th>Controlled at shelf temperature  -4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ice nucleation temperature (°C)</td>
<td>Average(^a)</td>
<td>-14.2</td>
</tr>
<tr>
<td></td>
<td>Std Dev(^a)</td>
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<tr>
<td></td>
<td>Maximum</td>
<td>-12.1</td>
</tr>
<tr>
<td></td>
<td>Minimum</td>
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<tr>
<td></td>
<td>Max - Min</td>
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</tbody>
</table>

\(^a\)Average and standard deviation (Std Dev) were calculated with the values of 6 individual vials located at the center, edge, front and back of the shelf. The thermocouples were placed on the outside of the vials near the vial bottom.
Figure 1. LDH temperature profiles during the freezing process of the freeze-thaw study

A) Uncontrolled nucleation        B) Controlled nucleation at shelf temperature -4°C

C) Controlled nucleation at shelf temperature -8°C   D) Controlled nucleation at shelf temperature -12°C
Figure 2. LDH activity recovery after freeze-thawing. Values are expressed as average ± standard deviation of 6 individual vials located at the center, edge, front and back of the shelf. UCN: uncontrolled nucleation.
Figure 3. Correlation between the ice nucleation temperature of the LDH solutions and LDH activity recovery after thawing. Black diamonds represent the values of individual vials. White squares and error bars represent the average and standard deviations of 6 vials in each batch, respectively. Linear regression (dashed line) is the best-fit linear correlation line.
Figure 4. Correlation between the ice nucleation temperature of the LDH solutions and LDH tetramer recovery. Black diamonds represent the values of individual vials. White squares and error bars represent the average and standard deviations of 6 vials in each batch, respectively. Linear regression (dashed line) is the best-fit linear correlation line.
Figure 5. LDH temperature profiles during freezing process of the freeze-drying study.

A) Uncontrolled ice nucleation    B) Controlled ice nucleation at shelf temperature -4°C
Figure 6. LDH recovery after freeze-drying.

A) Activity recovery (%) by enzymatic activity assay, B) Tetramer recovery (%) by SEC.

Results were shown as average ± standard deviation of 6 individual vials.
Figure 7. Distribution of ice nucleation temperature in laboratory at Praxair (private communication with Robert Sever). A total of 42 readings were collected. % Value in the y-axis represents the probability of each ice nucleation temperature in the laboratory environment.
References


Chapter 3

Stability of Freeze-Dried Protein Formulations: Resolving the Contributions of Ice Nucleation and Residence Time in the Freeze-Concentrate
Abstract

ControLyo™ is one of the ice nucleation-controlling techniques to improve process efficiency and product quality. Controlling ice nucleation at a fixed higher temperature results in larger ice crystals, which can reduce the ice/freeze-concentrate interface where proteins can adsorb and degrade. Moreover, protein stability seems to suffer from opposing effects during ice crystal growth post ice nucleation, and limited work has been done to address any effects on protein stability due to a slow ramp or long isothermal hold after ice nucleation. The objective was to evaluate the impact of the ice nucleation temperature and residence time in the freeze-concentrate on in-process and storage stability of representative proteins, intravenous immunoglobulin (IVIG), and recombinant human serum albumin (rHSA). In addition to stability, characteristics of the final solid including morphology and porous structure were characterized at various freezing conditions using the polymer encapsulation method, mercury intrusion porosimetry method and specific surface area measurements. The findings suggest that it is not only the ice nucleation temperature, but also the thermal history post ice nucleation that define the surface area of ice and the porous structure of the freeze-dried cake. Soluble aggregation of IVIG and rHSA formulations was assessed from size exclusion chromatography (SEC). Subvisible particulate formation was determined using Microflow imaging (MFI) and FlowCam. A trend of better stability of IVIG and rHSA with regard to soluble aggregation was observed when ice nucleation was controlled at -5°C compared to -12°C. The results suggest that controlled nucleation has the potential to minimize degradation of protein pharmaceuticals, which are labile at ice/aqueous interface. Apart from the ice nucleation step, the present study identified the residence
time in the freeze-concentrate as the critical factor that influences protein stability post ice nucleation. A long residence time in the freeze-concentrate, at a temperature where enough mobility exists (i.e. above $T_g'$ of the formulation) can result in significant protein aggregation.

**Keywords:**

Freeze-drying; protein stability; ice nucleation; residence time; freeze-concentrate; $T_g'$; cake morphology
**Introduction**

Therapeutic proteins are generally freeze-dried to improve their chemical and/or physical stability when they are insufficiently stable in solution for commercial distribution and storage. However, the freeze-drying process itself stresses protein molecules. In particular, during freezing, protein may adsorb on to the interface between the growing ice and the freeze-concentrate suffering an alteration in conformation. Also during freezing, the concentration of the solutes increases as water is removed to form ice, resulting in a system with higher viscosity but increased reaction rate for bimolecular reactions until it approaches the glassy state. Whatever the mechanism, the freezing step alone has been reported to cause protein structural perturbation, aggregation or loss of activity \(^{1-9}\).

The freezing step starts with ice nucleation and rapid growth of a portion of the ice until the temperature of the slush reaches the equilibrium freezing point of the remaining freeze concentrate; as the shelf of the freeze-dryer is cooled, there is continued crystallization of ice until the freeze-concentrate reaches its glass transition temperature. There exists natural variation in the temperature at which ice nucleates, producing variation in product thermal history. The variation in product thermal history is due not only to the variation in the ice nucleation temperature, but also from additional heating from a neighboring vial that undergoes ice nucleation at a later time. To reduce the variation in freezing for both process economic and product quality reasons, controlling the ice nucleation step has gained increasing attention and was recently listed as an example of an emerging technology by the FDA emerging technology team \(^{10}\). With the recognition and emphasis in adopting such innovative technologies, one might expect that
controlled ice nucleation technology will be routinely implemented in pharmaceutical manufacturing in the near future. Control of ice nucleation at higher temperatures can improve process economics and product quality; it leads to increased drying rate \(^{11-15}\) and improved protein stability \(^7,16\). Moreover, improvement in homogeneity of specific surface area (SSA), drying rate, and residual moisture by controlled ice nucleation was recently critically reviewed \(^17\). Using a controlled ice nucleation technique to synchronize the nucleation of ice in all vials within a batch, inter-vial protein stability has recently been shown to become better and more uniform \(^16\). The individual contribution of each freezing stress (i.e., ice formulation, solute concentration, temperature, and time) on LDH activity was investigated and the ice formation was found to be the dominant factor for irreversible degradation of LDH \(^2\). Rather than typical glass vials and process conditions, the study used a temperature-step approach, where 5 µg/mL of LDH solutions were frozen by seeding in hemi-spherical wells in a thermally conductive slab to achieve very rapid cooling. With controlled ice nucleation technology, it is now possible to study the stresses post ice nucleation in more practical freeze-drying conditions.

As ice is formed, the concentration of the solutes (i.e. protein, or any potential reactant) increases in the regions between the growing ice crystals. Because of the freeze concentration and reduction in temperature, viscosity increases until it reaches the glassy state in amorphous systems. Ice crystal size and residence time in the freeze-concentrate have been proposed as factors of protein degradation post ice nucleation \(^18\). The effect of isothermal hold and shelf ramp rate post ice nucleation on ice crystal size and whether these variables can have an impact on protein stability was discussed theoretically \(^17\); however, there were no data supporting their speculations. In one case, the rate of a
bimolecular reaction in a small amount of drug in 3% sucrose was calculated theoretically assuming the activation energy is 20 kJ/mol (chapter 8, page 198-232)\(^\text{18}\). Despite the Arrhenius effect of low temperatures during freezing, the reaction rate increased about one order of magnitude whether the degradation reaction was completely uncoupled or coupled (i.e. diffusion-controlled) to the system viscosity\(^\text{19}\). In another example, the stability of polyplex particles during freezing was investigated by varying the ice nucleation temperature and shelf ramp rate after the ice nucleation step\(^\text{20}\). Aggregation of the polyplex particles was found to arise from significant residence time in the partially frozen system, when the freeze-concentration increased greatly but still in a low viscosity state. In that study, a residence time was defined as the time from the ice nucleation event to the point when the system reached the maximum freeze-concentration, around \(T_g'\). The concept of residence times can be extended to the study of protein formulations. During that residence time above \(T_g'\), ice crystals continue grow to larger size and may have the opportunity to remodel to lower a specific surface area, potentially reducing protein adsorption at the ice/freeze-concentrate interface. At the same time, however, increased residence time in the increasing concentration of the remaining solution where bimolecular reaction rates are higher may produce protein aggregation. Little experimental work exists in the literature to explore the contributions of these mechanisms in protein systems and identify the critical factors after the initial ice nucleation that affect protein stability.

Few controlled ice nucleation reports have focused on protein stability\(^\text{7,16}\); and very little information is available on controlled nucleation’s effect on the stability of proteins formulated with excipients. We are not aware of any studies that systematically
investigate the impact of residence time post ice nucleation on protein stability. In addition to studying the effect of controlling ice nucleation on protein stability, the present work has a focus on the impact of the freezing protocol post ice nucleation (i.e. shelf ramp rate, holding time, etc.) on protein stability. Several representative protein systems were studied, recombinant human serum albumin (rHSA) and intravenous immunoglobulin (IVIG), both formulated with a commonly used stabilizer, sucrose.

In addition to the effect of freezing protocols on protein stability, the ice nucleation temperature and the sample thermal history post ice nucleation can also affect the morphology and porous structure of the final freeze-dried product. The morphology and porous structure of the freeze-dried product are important characteristics, which has been reported to affect process time \(^{12,21}\) and influence critical quality attributes such as residual moisture level and reconstitution time \(^{22}\) (and Kulkarni et al., unpublished). Several methods have been reported to characterize the microstructure of freeze-dried products \(^{23-25}\). Scanning electron microscopy (SEM) is qualitative and requires cakes to be removed from glass vials for analysis. The fragile cakes often break upon removal, and thus may not be representative of the entire cake structure. Therefore, novel methods are needed to characterize the morphology of freeze-dried product, which allow visualization of structural details without breaking the cake apart. A polymer encapsulation method \(^{24}\) helps maintain the intact cake upon removal and allows a cross-section view, thereby providing qualitative information on ice morphology. Low-pressure mercury intrusion porosimetry (LP-MIP) has recently been adapted to provide the pore size distribution of intact freeze-dried cakes (Kulkarni et al., unpublished). In the present study, the porous structure of the freeze-dried samples frozen under various conditions
(i.e. ice nucleation temperature and ramp rate post ice nucleation) were characterized using the polymer encapsulation method, LP-MIP method, as well as SSA measurement by Brunauer, Emmett and Teller (BET) analysis.

Materials and Methods

Sample preparation

Ultrapure rHSA (>99%, 5% (w/v), Albumin Bioscience, Huntsville, AL) and lyophilized IVIG (Gammagard®, Baxter, Bloomington, IL) were dialyzed against 5 mM potassium phosphate buffer (pH 7.0) with a 10K MWCO membrane at 4°C twice for 3 hours and a third time overnight. The concentration of protein after dialysis was determined by absorbance at 280 nm (Cary Bio100, Varian Inc., Palo Alto, CA) using extinction coefficients for rHSA and IVIG of 0.58 mL/mg-cm$^{26}$ and 1.36 mL/mg-cm$^{27}$, respectively. The dialyzed protein was formulated with or without sucrose at w/w ratios from 1:1 to 1:50. For the study of the effect of ice nucleation temperature on protein stability, proteins were formulated at 1 mg/mL. For all the post ice nucleation studies (by varying the ramp rate, or holding time), proteins were formulated at 3 mg/mL. Aliquots (1 mL) of the formulated protein were filled into 5 mL glass tubing vials, which were partially stoppered with Daikyo Fluorotec stoppers (West Pharmaceutical Services, West Whiteland, PA) to minimize moisture transfer during storage.
**Lyophilization cycle**

Vials with protein solutions were placed on the center of the shelf of a lab freeze-dryer (LyoStar 3, SP Scientific, Gardiner, NY) surrounded by vials with 5% (w/v) sucrose solution to avoid additional radiation heat transfer from the edge and front of the chamber, and to ensure comparable product temperatures among vials containing the same formulation. Product temperature was monitored with a thermocouple glued with heat sink silicone grease (Chemplex 1381, FUCHS lubricants, Harvey, IL) and Kapton tape (Cole-Parmer, Vernon Hills, IL) to the outside of selected vials (2 thermocouples per formulation) near the vial bottom.

**Control of ice nucleation temperature**

Samples were cooled at a shelf ramp rate of 1°C/min to 5°C and equilibrated for 30 min, followed by cooling to the target temperature for ice nucleation (i.e., -5°C or -12°C). Pressurization/depressurization technology (ControLyo™, SP Scientific, Gardiner, NY) was used to control ice nucleation at a fixed time and shelf temperature. For the study of the effect of ice nucleation temperature, the shelf was immediately cooled post nucleation at 1°C/min to -40°C to complete freezing. For the study of residence time post ice nucleation, several shelf ramp rates or hold times post ice nucleation were employed as described in the following section.

**Ramp rate post ice nucleation**

After ice nucleation was induced at a shelf temperature of -12°C, the shelf was directly cooled to -40°C at a ramp rate of 0.1°C/min, 1.0°C/min, or 2.5°C/min. The ramp rate of 2.5°C/min was the fastest well-controlled rate attainable in this unit.
**Hold time post ice nucleation at temperature above $T_g$**

Ice nucleation was initiated at a shelf temperature of -12°C. For pure protein formulation without stabilizer, the $T_g$ was estimated to be -15°C \(^{28}\). The samples were held at -12°C for 3 or 6 hours before being cooled at 1°C/min to -40°C to complete freezing.

**Hold temperature post ice nucleation below $T_g$**

In addition to holding the samples at a temperature just above $T_g$ where there is sufficient mobility for reactions to occur, selected samples were alternatively held at a temperature below $T_g$ for 6 hours where mobility is expected to be limited (i.e. -18°C for pure protein formulation). For these experiments, ice nucleation was also initiated at -12°C followed by cooling the samples at 1°C/min to -18°C for 6 hours and then to -40°C at the same rate to complete freezing.

**Drying**

For primary drying, the pressure was set to 60 mTorr and the shelf was heated at 0.5°C/min to -30°C. After the Pirani and capacitance manometer readings converged, the shelf temperature was raised at 0.1°C/min to 40°C and held at a pressure of 60 mTorr for 6 hours. When the vials were stoppered under full vacuum, there was “blow-out” of the dried material upon opening the vial for analysis. To avoid this, vials were stoppered when vacuum was released to 600 Torr with dry nitrogen to maintain a dry headspace as well as a good seal in the vial.
**Post lyophilization evaluation of protein degradation**

The freeze-dried samples were reconstituted with 1 mL of water (18 MΩ, distilled and deionized, Barnstead™ GenPure™, Thermo Scientific, Waltham, MA). The freeze-dried samples were stored at 40 or 50°C for 3 months. Soluble aggregates and/or subvisible protein particulates were measured immediately after reconstitution as described below.

**Soluble aggregation by size exclusion chromatography (SEC)**

The entire volume (1mL) of the protein solution was filtered through a 0.22 μm polyvinylidene difluoride (PVDF) low protein binding filter (33mm in diameter) and 50 μL of each sample were injected onto a column (TSKgel™ G3000SWXL, 7.8 mm x 30 cm, 5 μm, Tosoh Bioscience, San Francisco, CA) maintained at 25°C. The mobile phase (100 mM sodium phosphate buffer with 0.2 M NaCl at pH 7.0) was delivered at 1.0 mL/min by an HPLC system (Model 1100, Agilent, Santa Clara, CA). The absorbance of the effluent was measured at 280nm (variable wavelength detector, G1315A, Agilent). Absorbance data were acquired and analyzed (ChemStation, Agilent). Monomer, dimer, higher order aggregate and fragment peaks were identified. Percent soluble aggregate is reported as the areas under the dimer and higher order aggregate peaks relative to total area of the identified peaks.

**Subvisible particulate measurement**

Microfluidic-assisted single particle analysis (MicroFlow Imaging (MFI); Model 5100, ProteinSimple, San Jose, CA) and dynamic imaging particle analysis (Model PV100, Flowcam, Fluid Imaging Technologies, Scarborough, ME) were used to count
protein subvisible particulates. Using MFI, 0.9 mL of sample solution was injected at a flow rate of 0.1 mL/min. The particulates with an equivalent circular diameter (ECD) from 1 to 100 µm were counted. Using Flowcam, 0.9 mL of the sample solution was injected with flow rate controlled at 0.15 mL/min, and auto image rate at 32 frames/sec (dark and light threshold were both set to 15 for particle segmentation). The particulates with an equivalent spherical diameter (ESD) from 2 to 100 µm were counted.

Characterization of Freeze-Dried Cakes

Specific surface area (SSA)

The SSA of freeze-dried 10% (w/v) sucrose and rHSA formulated with 5% (w/v) sucrose was determined in triplicate by BET analysis of the gas adsorption isotherm method (Flowsorb II 2300, Micromeritics Instrument Corporation, Norcross, GA). The instrument was calibrated with 100% krypton before analysis. He-Kr gas mixture (0.10 mole% krypton) was used to improve sensitivity for samples with low SSA. Sample preparation and other experimental conditions were previously described 29.

Cake morphology characterization using polymer encapsulation method

Qualitative morphology of intact cakes of the freeze-dried 10% (w/w) sucrose was captured using a method adapted from Patapoff et al 24. The sucrose samples were freeze-dried utilizing controlled ice nucleation at a shelf temperature of -5°C or without ice nucleation control. Conservative drying conditions used, with primary drying at a shelf temperature of -30°C and secondary drying at 40°C, at a chamber pressure of 60 mTorr. To encapsulate the freeze-dried cakes, silicone (a degassed mixture of vinyl-terminated polydimethylsiloxane and curing agent [Sylgard184® silicone elastomer kit,
Ellsworth, Germantown, WI) was slowly infused into the intact freeze-dried cakes and allowed to crosslink at 40°C for 2 days. The glass vials were easily broken using a laboratory press (Carver Inc., Wabash, IN). Intact cakes encapsulated with the polymer were sliced with a custom-made guillotine-like slicer. The sliced cakes were stained with a yellow highlighter pen, which fluoresced under UV light for better contrast. Images were captured under a stereo microscope (Model SZ61, Olympus, Center Valley, PA).

*Low pressure mercury intrusion porosimetry (LP-MIP)*

The average pore size of freeze-dried rHSA samples formulated with 5% (w/v) sucrose was determined by mercury intrusion porosimetry (AutoPore IV 9500, Micromeritics Instrument Corporation, Norcross, GA). The top of a vial containing intact cake was cut off 1.5 cm from the bottom of the vial. The intact cake together with the bottom portion of the vial was placed directly, with no further sample preparation, into a 15 mL penetrometer with stem volume of 1.13 mL. A low filling pressure (1 psi) and optimized pressure increments up to 40 psi were utilized for the analysis as previously described (Kulkarni et al., under review).

*Statistical analysis*

Two-way ANOVA was applied to evaluate the significance of the differences in protein aggregation due to different freezing protocols (i.e. ice nucleation temperature or ramp rate).
Results and Discussion

The ice nucleation temperature has been reported to affect physical stability of proteins sensitive to the ice/freeze-concentrate interface, especially for formulations at low concentration and/or without stabilizers $^4,16,30$. In a recent report, higher activity recovery and tetramer recovery of LDH after freeze-thawing linearly correlated with higher ice nucleation temperature $^{16}$. However, controlling ice nucleation at a higher temperature ($-4^\circ C$) did not show vastly superior stability of freeze-dried LDH. In the present study, two additional proteins, IVIG and rHSA formulated with and without stabilizers were explored to further understand the role of ice nucleation control on physical stability of the protein formulations. In addition to controlling the ice nucleation temperature, the residence time of the proteins in the freeze concentrate above the $T_g$’ of the formulation was varied.

1. Effect of ice nucleation temperature on protein stability and cake morphology

IVIG was formulated at 1 mg/mL with or without sucrose at several ratios (1:3, 1:6, 1:50, w:w). IVIG alone (i.e., without sucrose) was considerably less stable than IVIG with sucrose during long-term storage at 40°C. IVIG was more stable when freeze-dried using the higher ice nucleation temperature of -5°C than -12°C (two-way ANOVA, $p \leq 0.05$) (Table 1). The ice nucleation temperature showed marginal impact on the long-term stability of IVIG formulations with sucrose; there was no significant difference in the stability of IVIG in the 1:3 or 1:50 sucrose formulations. It should be noted that the stock solution of the protein was ~90% monomer, with the remaining including
aggregates and fragments, which might have blurred the ability to determine small, but meaningful differences in stability.

Unlike IVIG, the rHSA used for the present study consisted of >99.5% monomer prior to freeze-drying, which was expected to make detection of small changes in purity easier. rHSA was formulated at 1 mg/mL with or without sucrose at several ratios (1:1, 1:6, w:w). As expected, the rHSA formulation alone (without sucrose) showed the most soluble aggregation. After freeze-drying, only dimer formation were detected in all formulations; on storage at 50°C, higher order aggregates were formed. Stability against aggregation increased as the ratio of sucrose increased in the formulation from 1:1 to 1:6 (rHSA:sucrose, w:w). At each storage time and formulation, there was less aggregation when ice nucleation was controlled at -5°C compared to -12°C (Table 2), but the difference was not always significant. Ice nucleation at -5°C provided significantly greater stability for the rHSA:sucrose 1:1 (w:w) at 1 month and 3 month storage at 50°C and for the rHSA:sucrose 1:6 (w:w) immediately after freeze-drying (p\leq0.05). However, two-way ANOVA of the entire data set (Table 2) showed significantly better protein stability at the ice nucleation temperature of -5°C (p \leq 0.05).

The degree of improvement in protein stability from controlling ice nucleation at a higher temperature appears to be protein-dependent. In a recent report, a ~8% difference in LDH activity was observed after freeze-thaw for a 7°C difference in ice nucleation temperature 16. Although the improvement in storage stability of IVIG and rHSA formulations at the higher ice nucleation temperature was statistically significant, the improvement was small and may not have practical significance, at least from a potency viewpoint. However, at least in principle, even a slightly higher level of protein
aggregates may have a higher propensity to stimulate immune response \(^{31}\). Preliminary data showed no increase in soluble aggregation by SEC after freeze-thaw in both IVIG and rHSA formulations. The data suggested that the damage on the proteins during the process occurred primarily during drying, which is not unusual.

The morphology of the freeze-dried product is another characteristic of the final solid, which can influence product quality attributes such as residual moisture level and/or reconstitution time. The morphology can be affected by the freezing protocol \(^{23}\). Therefore, in addition to investigating the effect of ice nucleation temperature on protein stability, the morphology of freeze-dried cakes at various ice nucleation conditions was characterized. We find morphology is impacted by both ice nucleation temperature and thermal history post nucleation. The lyophilized solid from the 1 mg/mL protein formulation was too fragile to use the polymer encapsulation procedure. Instead, the morphology of freeze-dried 10% (w/v) sucrose samples was characterized using the polymer encapsulation method (Figure 1). The yellow color in the cross-section of the encapsulated cake represents the sucrose material, whereas the black areas correspond to the pores within the cake or gap between the glass vial and the cake occupied by the polymer. Without controlled ice nucleation, the cake had a finer porous structure, corresponding to large number of smaller ice crystals formed by spontaneous ice nucleation of \(-12^\circ\)C measured by thermocouple during freezing. When ice nucleation was controlled at a shelf temperature of \(-5^\circ\)C (when the product temperature was \(-2^\circ\)C), generally larger pores were seen as expected, but also large channels were observed, consistent with previously reported faster primary drying at a higher ice nucleation temperature \(^{21,32}\). The SSAs of the cakes (Figure 1) were 0.28 and 0.58 m\(^2\)/g for the ice
nucleation temperatures of -2°C and -12°C, respectively, measured by thermocouple in
the bottom center of the vial. The SSA of 10% sucrose increases linearly as ice nucleation
temperature decreases (SSA = -0.03×T_{ice	nucleation} + 0.26, R^2=0.92) (Fang et al.,
under review). When the ice nucleation temperature of the two cakes in Figure 1 were
fitted in the above equation, the SSAs were found to be 0.32 vs. 0.62 m^2/g, very close to
the experimental values.

The observation of large channels and pores at a higher ice nucleation temperature
correlated with a lower SSA, which is expected to improve protein stability by
reducing protein adsorption at the ice/freeze-concentrate interface. A 10°C decrease in
ice nucleation temperature (-12 vs. -2°C) resulted in twice the SSA in the 10% sucrose
(w/v) samples. Degradation of another protein, hGH, on storage was largely due to
protein at the surface; that is, nearly all of the degradation occurred in the fraction of
protein at the surface. Therefore, when the SSA of a protein sample is varied due to the
process conditions (i.e. ice nucleation, cooling rate, annealing, or drying), both physical
and chemical stability of the protein formulation can be significantly affected,
particularly for excipient rich formulations. To better understand the surface phenomena
that contributed to degradation of IVIG during freezing, the fraction of protein on the
surface of freeze-dried IVIG:sucrose 1:50 (w:w) formulation was studied; this
formulation provided an excipient-rich system where a significant fraction of the total
protein is expected to be at the surface. The mass fraction of the protein on the surface
was calculated from the SSA measurement and % elemental nitrogen determined by X-
ray photoelectron spectroscopy (XPS) analysis of the outermost ~100 Å layer as
previously described. XPS analysis found 0.7% elemental nitrogen (corresponding to
protein) in samples prepared using both ice nucleation temperatures. The SSAs of the samples were 0.64 ± 0.02 m$^2$/g (-5°C) and 0.90 ± 0.01 m$^2$/g (-12°C), a difference of about 41%. Thus, the % protein at the surface is about 3% and 4% for -5°C ice nucleation and -12°C ice nucleation, respectively. If the degradation rate were directly proportional to the fraction of protein at the surface, the rate would be 41% higher for the lower ice nucleation temperature. However, it appears that the degradation rates on storage were only marginally lower at a higher ice nucleation temperature (Table 1).

In addition to better average protein stability due to the reduction in SSA at a higher ice nucleation temperature, controlling ice nucleation has been shown to improve the batch uniformity of protein stability 16 presumably by producing greater vial-to-vial uniformity in ice structure. The more uniform ice structure across the batch produces more uniform product resistance and therefore more uniform sublimation rates. Uniformity of primary drying rates can be seen from process data. The magnitude of the slope of Pirani gauge reading versus time was previously reported to reflect the uniformity in drying rate across the batch; a larger slope indicates more uniform drying 7. In the present work, the uniformity in drying rate was evaluated with a full shelf of 10% (w/v) sucrose solutions freeze-dried under various ice nucleation conditions (Figure 2). The magnitude of the slope at the inflection point followed the rank order: -5°C (24 mTorr/hr) > -8°C (12 mTorr/hr) > -12°C (7 mTorr/hr) ≈ uncontrolled nucleation (6 mTorr/hr). However, the point where the Pirani signal starts to decrease indicates the point at which primary drying is over in approximately 97% of the vials 34. The higher slope suggested improved uniformity in drying rate for the remaining 3% vials, not the
entire batch. The reason why a lower slope was observed when controlling ice nucleation at lower temperatures (-8°C and -12°C) compared to -5°C was not clear. If, as has been proposed, the uniformity in drying rate is due to the uniformity in SSA from vial-to-vial, such an improvement in slope should also be accompanied by more uniformity in protein stability across the batch.

The above results show that controlled nucleation at a higher temperature improves the storage stability of the two model proteins investigated here, which is consistent with previous reports 7,16. Furthermore, the data on the slope of the Pirani gauge reading with time suggests that controlling ice nucleation at higher temperatures should improve the uniformity of protein stability as was demonstrated in an earlier report 16. However, ice nucleation control only controls the first step in freezing. The completion of freezing occurs during the ramp down to below the glass transition temperature of the freeze concentrate. The effects of the ramp rate and isothermal hold after ice nucleation on protein stability are less well understood. In the next section, shelf ramp rate and the holding duration and temperature were varied to identify the critical factors for protein stability.

2. Effect of residence time (post ice nucleation) on protein aggregation

After ice nucleation, crystal growth continues as the shelf cools the vial contents. On cooling the shelf, the dendritic ice often formed during the nucleation step 35 may have the opportunity to re-crystallize, resulting in fewer, but larger crystals, leading to smaller SSA; conversion to smaller SSA is expected to occur more at slower cooling
rates. The smaller SSA should yield better protein stability due to reduced adsorption at the ice/freeze-concentrate interface. On the other hand, on cooling the shelf, the solutes (i.e. buffer components, excipients, and proteins) become more concentrated as water leaves the solution to form ice. In the remaining freeze-concentrated solution, the rate of bimolecular reactions, such as protein aggregation, is expected to increase, at least until the freeze-concentrate reaches a glassy state in which mobility is sufficiently low to slow the rate of reactions.

Slow cooling increases the time in which the protein is in this more reactive freeze-concentrated state above its glass transition temperature, $T_g'$. Thus, while slow cooling is expected to decrease the surface area of ice to which protein can adsorb and unfold, the slower rate is also expected to increase the time in which the protein resides in the reactive freeze-concentrate. Therefore, slow cooling provides at least these two opportunities for protecting or destabilizing the protein. Conversely, rapid freezing would destabilize by producing a higher ice surface area for protein adsorption, but reduce the time of residence in the reactive environment of the freeze-concentrate. Here, we define the residence time as the time from ice nucleation to when the product temperature reaches $T_g'$. To investigate the contributions of ice surface area and residence time to protein stability, the shelf ramp rate post ice nucleation or the holding temperature and duration were varied.

2.1 Effect of ramp rates post ice nucleation on ice crystal size and protein aggregation

rHSA:sucrose 3:50 (w:w) cooled at the slow rate of 0.1°C/min produced cakes with a large average pore size of 39 µm; with a size range of 28 - 60 µm corresponding to 10% to 90% of the total volume of mercury that was intruded into the sample. Cooling at
2.5°C/min produced a much smaller pore size (30 µm; range of 22 - 48 µm), even though both were nucleated at -12°C (Table 3). The larger pore size in the freeze-dried samples correlated with a lower SSA when cooled at a slower rate (Table 3). About 19% reduction in SSA was observed when the cooling rate decreased from 2.5 to 0.1°C/min. Thus, it is not only the ice nucleation temperature, but also the ramp rate post ice nucleation that defines the SSA of ice and the size of pores in the freeze-dried cake.

Freeze-thawing did not produce soluble aggregates in either rHSA (3 m/mL) alone or rHSA:sucrose 3:50 (w:w) (data not shown). Additionally, storage for 3 months at 40°C did not produce soluble aggregates in lyophilized rHSA:sucrose 3:50 (w:w) (Table 4). However, lyophilized rHSA alone showed an increase in protein aggregation during freeze-drying as well as during storage (Table 4). Though the residence time of rHSA in the freeze-concentrate was twice as long (25 versus 12 min) at the lowest shelf ramp rate of 0.1°C/min versus at 2.5°C/min, there was no significant difference in protein soluble aggregation using these two ramp rates. The SSA was reduced by 19% at a slower ramp rate (0.1 vs. 2.5°C/min). Thus, the lack of difference in protein aggregation between the two ramp rates could be due to the longer residence time at the slower ramp rate that cancelled the stabilization effect arising from the reduction in SSA.

It is possible that protein aggregates larger than the soluble aggregates are sensitive to the cooling rate even when soluble aggregation is not. In a previous study, no soluble aggregation of IgG was observed either after freeze-thawing or after freeze-drying; however larger insoluble aggregates were detected after freeze-drying. More insoluble protein aggregates were reported at a much faster cooling rate (i.e. quench cooling in liquid nitrogen) than slow cooling at 2°C/min in the example of IgG, and the
same observation was made for hGH\textsuperscript{37} comparing cooling rates of 0.5 and 50°C/min after freeze-drying. In summary, insoluble aggregation of protein (i.e., subvisible aggregates) was reported to be influenced by the freezing conditions.

In the present work, the IVIG samples were pulled out of the freeze-dryer using a sample-thief after freezing, primary drying or secondary drying. Subvisible particulate formation was analyzed with MFI in the pre-lyo solutions, thawed, and reconstituted solutions at various stages of freeze-drying. There was little increase in subvisible particulates in the samples after freeze-thawing or primary drying, whereas significant subvisible particulates formed during secondary drying (Figure 3). It could be that proteins are susceptible to elevated temperature in secondary drying, at least when the moisture content is relatively high. Another possible explanation is that proteins generally exhibit best stability at intermediate level of moisture\textsuperscript{38}; therefore, more degradation is expected near the end of secondary drying when the sample has low moisture but still sufficient mobility exists to allow unfolding. Examples of optimal protein stability have been reported at intermediate moisture content (≈ 2-4%) in both pure protein and proteins formulated with disaccharides\textsuperscript{39-41}. However, one must also recognize that the additional stability at intermediate water content, relative to zero water content, was quite small, so other effects may be driving the instability.

Based on the observation of significant increase in subvisible protein particulates after freeze-drying in IVIG, lyophilized rHSA formulations were similarly analyzed using Flowcam at the two ramp rates, 2.5°C/min or 0.1°C/min (Figure 4). There was an increase in subvisible particulates after freeze-drying compared to after freeze-thawing both in the presence and absence of sucrose. The presence of sucrose stabilized the
protein against forming subvisible aggregates relative to rHSA alone. There was a reduction in subvisible particulate formation in those samples cooled at 2.5°C/min compared to 0.1°C/min, but this difference is not statistically significant due to the large variability in particle counts. However, the consistent higher particle levels after drying compared to after freeze-thaw (p<0.05) and the consistent higher particle levels with the slower ramp (p<0.3) suggest the trends may be real.

When the shelf ramp rate was slowed from 2.5°C/min to 0.1°C/min after ice nucleation, the SSA and pore size indicates that ice crystals were substantially larger and/or smoother, reducing the ice/freeze-concentrate interface where proteins can adsorb and unfold. On the other hand, the residence time for the rHSA formulation without excipients increased from 12 minutes to 25 minutes, which potentially promotes protein aggregation due to the longer residence time in the freeze-concentrate. However, it must be acknowledged that only a narrow range of residence time (both within 25 minutes when the shelf ramp rate varied from 0.1 to 2.5°C/min) was evaluated and there may not have been a sufficiently different residence time to produce a measureable difference in either soluble or insoluble aggregates. To further explore the possible effect of residence time, much longer residence times were investigated, and these results are discussed in the following section.

2.2 Effect of holding time post ice nucleation on protein aggregation

For pure rHSA (3 mg/mL) without stabilizers, the residence time in the freeze concentrate until $T_g'$ was reached increased from 12 minutes to 18 minutes to 25 minutes when the shelf ramp rate decreased from 2.5°C/min to 1°C/min or 0.1°C/min. The level of soluble aggregation was comparable within this wide range of ramp rates (Figure 5).
Only dimers were detected by SEC in the reconstituted rHSA samples after freeze-drying. Generally, the $T_g'$ of a frozen solution can be measured with modulated differential scanning calorimetry (MDSC). However, it is difficult to measure $T_g'$ for the freeze-concentrate of a pure protein because the transition is not sufficiently strong or sharp enough to detect even at high protein concentrations >50 mg/mL. In the absence of a measured $T_g'$ for rHSA, the $T_g'$ was estimated to be $-15^\circ$C. rHSA was held at $-12^\circ$C for 3 hours or 6 hours to determine the effect of a long residence time above $T_g'$ on protein stability. Soluble aggregation increased from 1.26% aggregates when the sample was held for 3 hours to 1.90% for a 6-hour hold at $-12^\circ$C; both were significantly higher than the level of soluble aggregates (0.90%) when the residence time was less than 30 minutes at the various ramp rates (Figure 5). The level of soluble aggregate linearly correlated with the residence time in the freeze-concentrate ($R^2=0.97$, Figure 5). Although the residence time during a wide range of practical cooling rates used during freeze-drying does not differ sufficiently to have stability consequences, the hold time experiments show a role for residence times as a factor in protein stability if for some reason, unusually long holding times were used. Indeed, long times are used when annealing the frozen material between the $T_g'$ and the onset temperature of ice melt. However, such annealing procedures are normally only used with proteins formulated with stabilizers.

2.3 Effect of holding temperature post ice nucleation on protein aggregation

As the samples continue to cool to temperatures near and then below $T_g'$, viscosity and freeze-concentration increases to maximum until it reaches a glassy state.
Less soluble aggregate is expected when held at temperatures below $T_g'$ compared to temperatures above $T_g'$ for the same holding duration, since mobility is reduced in a glassy state. To test this expectation, pure rHSA was cooled at 1°C/min post ice nucleation to -18°C (i.e., below $T_g'$), and held for 6 hours followed by cooling the shelf to -40°C to complete freezing. Much less soluble aggregation was found in rHSA when held for 6 hours at -18°C (1.13%) compared to at -12°C (1.90%) (Figure 6). We note that here the time calculated from the ice nucleation event to when the product temperature reached $T_g'$ (-15°C) was only about 20 minutes.

The data in the present study showed an increase in aggregation of rHSA at a long holding time at a temperature above $T_g'$. It is not just the time that the proteins are exposed to in the freeze-concentrate, but rather the freeze-concentration accompanied by ice formation, the temperature at which the system is highly mobile for reactions to occur, and the residence time in the mobile, more concentrated environment that together determine protein degradation post ice nucleation. Cautions are needed in designing a freezing process post nuleation. Protein aggregation was not affected within normal ramp rates (i.e. 0.1-2.5°C/min); however, a long holding time post ice nucleation at temperatures above $T_g'$ does increase degradation and therefore is not desirable. In the case of bulk freezing of active pharmaceutical ingredients (normally pure proteins) for storage and transportation, the freezing time can be as long as 17 hours in a 10 liter container \(^{42}\). The findings in the present study addressed the effect of residence time in the freeze-concentrate on protein aggregation and the results are directly relevant to process design in bulk freezing and thawing.
Summary and Conclusions

In the present work, ControLyo™ was used to study the effect of ice nucleation temperature, and residence time in the freeze-concentrate on aggregation of the protein formulations after freeze-drying. Two proteins, rHSA and IVIG, formulated in various ratios with sucrose were studied. In addition to stability, characteristics of the freeze-dried samples including morphology and porous structure of intact cakes were characterized using polymer encapsulation and mercury intrusion porosimetry methods at various freezing conditions. When ice nucleation was controlled at a higher shelf temperature (i.e. -5°C) compared to spontaneous ice nucleation, not only larger pores but also large channels were observed in the 10% sucrose sample, consistent with a reduced ice surface area which potentially reduces protein adsorption. Average pore size determined by LP-MIP correlated well with SSA of the freeze-dried samples. Larger pore size was found at a slower ramp rate (0.1 vs. 2.5°C/min) even though both were nucleated at -12°C. Thus, it is not only the ice nucleation temperature, but also the thermal history post ice nucleation that define the SSA of ice and the size of pores in the freeze-dried cake. There was a trend of better protein stability (i.e. less aggregation) at a higher ice nucleation temperature (-5 vs. -12°C) in both proteins. One may expect more degradation in manufacturing in nominally the same process as run in the lab since the ice nucleation temperature in manufacturing appears to be about 10°C lower than in the lab. Efforts to reduce ice surface area by controlling ice nucleation at a higher temperature may not be as beneficial for proteins that are stable during freezing or when formulated at a much higher concentration such as with monoclonal antibody formulations. Of course, the advantages of faster primary drying would still apply. Apart
from the ice nucleation step, the present study suggested the residence time in the freeze-concentrate post ice nucleation can be a critical factor for protein stability. A long residence time in the freeze-concentrate, at a temperature where enough mobility exists (i.e. above $T_g'$ of the formulation) may result in significant protein aggregation.

Acknowledgement

Funding for this project from Dane O. Kildsig Center for Pharmaceutical Processing and Research (CPPR) is greatly appreciated. The authors would like to thank Shreya Kulkarni for some experimental help with the MIP measurements.
Table 1. % Degradation (formation of soluble aggregates and fragments) determined by SEC in IVIG after freeze-drying (0 month) and storage for 1 and 3 months at 40°C. The concentration of IVIG was 1 mg/mL in all formulations. The samples were freeze-dried with controlled ice nucleation (CN) at -5°C or -12°C.

<table>
<thead>
<tr>
<th>Freezing condition</th>
<th>IVIG</th>
<th>IVIG:sucrose 1:3 (w:w)</th>
<th>IVIG:sucrose 1:6 (w:w)</th>
<th>IVIG:sucrose 1:50 (w:w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>CN-5°C</td>
<td>10.6</td>
<td>26.9</td>
<td>29.5</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.9</td>
<td>0</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>CN-12°C</td>
<td>11.4</td>
<td>28.6</td>
<td>31.7</td>
<td>11.6</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>SE</td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Table 2. % Soluble aggregation determined by SEC in rHSA after freeze-drying (0 month) and storage for 1 and 3 months at 50°C. The concentration of rHSA was 1 mg/mL in all formulations. The samples were freeze-dried with controlled ice nucleation (CN) at -5°C or -12°C.

<table>
<thead>
<tr>
<th>Freezing condition</th>
<th>rHSA</th>
<th>rHSA:sucrose 1:1 (w:w)</th>
<th>rHSA:sucrose 1:6 (w:w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Storage (month)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>CN-5°C</td>
<td>1.10</td>
<td>25.03</td>
<td>42.95</td>
</tr>
<tr>
<td></td>
<td>0.78</td>
<td>8.06</td>
<td>16.00</td>
</tr>
<tr>
<td></td>
<td>0.44</td>
<td>0.98</td>
<td>2.63</td>
</tr>
<tr>
<td>SE</td>
<td>0.04</td>
<td>0.40</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.03</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>0.04</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.08</td>
<td>0.10</td>
</tr>
<tr>
<td>CN-12°C</td>
<td>1.12</td>
<td>25.29</td>
<td>43.40</td>
</tr>
<tr>
<td></td>
<td>0.82</td>
<td>8.65</td>
<td>17.78</td>
</tr>
<tr>
<td></td>
<td>0.59</td>
<td>1.14</td>
<td>2.90</td>
</tr>
<tr>
<td>SE</td>
<td>0.05</td>
<td>0.04</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.11</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0.08</td>
<td>0.10</td>
</tr>
</tbody>
</table>


Table 3. Pore size (by MIP) and SSA (by BET) in rHSA samples formulated with 5% sucrose.

<table>
<thead>
<tr>
<th>Freezing condition</th>
<th>Pore size (µm)</th>
<th>SSA (m²/g), Average ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average ± SE</td>
<td>Range (corresponding to 10% - 90% mercury intrusion volume)</td>
</tr>
<tr>
<td>CN-12°C, 2.5°C/min</td>
<td>30 ± 0</td>
<td>48 - 22</td>
</tr>
<tr>
<td>CN-12°C, 0.1°C/min</td>
<td>39 ± 0</td>
<td>60 - 28</td>
</tr>
</tbody>
</table>
Table 4. % Soluble aggregation in reconstituted rHSA solutions, rHSA alone or rHSA:sucrose 3:50 (w:w) after freeze-drying (time 0) and storage for 1 and 3 months at 40°C. The concentration of rHSA was 3 mg/mL in each formulation. Ice nucleation was initiated at -12°C using ControLyo™ in all experiments. The shelf cooling rate post ice nucleation was controlled at 2.5°C/min or 0.1°C/min.

<table>
<thead>
<tr>
<th>Ramp rate (°C/min)</th>
<th>rHSA</th>
<th>rHSA:sucrose 3:50 (w:w)</th>
<th>Storage (month)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>2.5</td>
<td>0.96</td>
<td>11.90</td>
<td>21.06</td>
</tr>
<tr>
<td>SE</td>
<td>0.02</td>
<td>0.17</td>
<td>0.12</td>
</tr>
<tr>
<td>0.1</td>
<td>0.90</td>
<td>12.35</td>
<td>21.21</td>
</tr>
<tr>
<td>SE</td>
<td>0.02</td>
<td>0.54</td>
<td>0.14</td>
</tr>
</tbody>
</table>

The residence times were 12 and 25 minutes when the shelf ramp rates were 2.5 and 0.1°C/min, respectively.
Figure 1. Morphology of 10% (w/v) sucrose cakes freeze-dried without nucleation control (left), and with controlled ice nucleation at a shelf temperature of -5°C (right). Both cakes were from vials located on the edge of the shelf. The product ice nucleation temperatures from thermocouple measurement were -12°C (left), and -2°C (right). The SSAs were 0.58 (left) and 0.28 m²/g (right) from BET measurement.
Figure 2. The pressure profile sensed by the Pirani gauge and the capacitance manometer (CM). One full tray (total 264 vials) of 10% sucrose solutions (5 mL fill in 10 mL vial) were freeze-dried under four different conditions, controlled ice nucleation at shelf temperature -5, -8, and -12°C and uncontrolled ice nucleation (UCN) (from right to left in the figure), respectively. To determine the slope (1st derivative) of the Pirani profile with time, the Pirani readings were smoothed from the data collected every 30 seconds. The magnitude of the slope at the inflection point followed the rank order: -5°C (24 mTorr/hr) > -8°C (12 mTorr/hr) > -12°C (7 mTorr/hr) ≈ uncontrolled nucleation (6 mTorr/hr).
Figure 3. Subvisible particulate formation in IVIG solutions (1 mg/mL) measured with MFI. The samples were removed from the freeze-dryer after freezing, primary drying or secondary drying.
Figure 4. Subvisible particulate formation in rHSA formulations (rHSA alone, or rHSA:sucrose (3:50)) measured with Flowcam. The samples were analyzed after freeze-thaw or reconstitution of the freeze-dried samples. Ice nucleation was initiated at -12°C using ControLyo™ in all experiments. The shelf cooling rate post ice nucleation was controlled at 2.5°C/min (white bar) or 0.1°C/min (dotted bar).
Figure 5. % Soluble aggregation of rHSA (3 mg/mL) measured with SEC as a function of residence time at various process conditions (indicated on the left). The data was fitted to a linear function ($R^2 = 0.97$, indicated on the right). In all experiments, ice nucleation was controlled at a shelf temperature of -12°C. Shelf ramp rate post ice nucleation varied from 2.5°C/min to 1, or 0.1°C/min. The residence time in the freeze-concentrate was calculated from when ice nucleated to when the product temperature reached -12°C ($T_g$ of pure rHSA was estimated to be -15°C). N=3 for each experiment.
Figure 6. % Soluble aggregation of rHSA (3 mg/mL) measured with SEC for a 6-hour hold at -12°C or -18°C (T_g’ of pure rHSA was estimated to be -15°C). In all experiments, ice nucleation was controlled at a shelf temperature of -12°C. N=3 for each experiment.
References


Chapter 4

Distribution of Ice Nucleation Temperature in a Batch and Its Implication on Product Resistance During Primary Drying
Abstract

Existing methods have been used to determine average product resistance ($R_p$) during primary drying across a batch. However, little work has been done to address the distribution in $R_p$ at various locations on the shelf. The objective of this study was to determine $R_p$ in 10% (w/v) sucrose samples placed on the edge and center of the shelf. A location-dependent ice nucleation temperature was observed with highest ice nucleation temperature in the samples on the left edge of the shelf followed by comparable ice nucleation temperatures in the right edge and center vials. Product resistance in individual vials was calculated utilizing the cycle data, namely, the “direct vial” method. The challenges in this method were addressed. Finally, correlations were established between the product resistance calculated in the center vials with reliable $K_v$ values and the corresponding specific surface area (SSA) or the ice nucleation temperature. The established correlations were further used to estimate $R_p$ in edge vials with known ice nucleation temperature or SSA. The data suggested $R_p$ is lower in the edge vials due to a higher ice nucleation temperature. The findings provide insights into developing methods to accurately measure $R_p$ in individual vials.

Keywords:

Freeze-drying; product resistance; ice nucleation; specific surface area; edge vial
Introduction

During the primary drying phase of lyophilization, water vapor passes through the porous partially dried layer, experiencing a resistance to mass flow, the so-called product resistance ($R_p$). Early in primary drying, $R_p$ is low, and ice sublimation rate is at its maximum. This presents a high mass load for the freeze-dryer, but the product temperature is usually lower due to the heat removed by sublimation. Later in primary drying, the dried layer thickness increases, producing higher resistance to vapor flow, which results in lower sublimation rate and higher product temperature. Improved characterization of the $R_p$ throughout primary drying would aid optimization of process efficiency while maintaining product temperature below any critical value. Existing methods to determine product resistance include Manometric Temperature Measurement (MTM) \(^1,2\), utilization of mass flux from Tunable Diode Laser Absorption Spectroscopy (TDLAS) \(^3\), utilization of heat flux from the heat sensors adhered on the shelf surface; however, these approaches measure a batch average $R_p$. One may also estimate product resistance for product in a given vial from the time dependence of product temperature in primary drying, as long as the vial heat transfer coefficient is known or can be reliably estimated from the cycle data. This is the method we employ in this work to obtain the resistance for product in a given vial, which is a simplified version of the Kuu procedure \(^4\). Little is known about any differences in $R_p$ based on vial location, i.e., between center and edge vials, contrary to the well known edge vial effect for the heat transfer coefficient ($K_v$) \(^5\). In the present study, the objective was to quantify any difference in product resistance between center and edge vials.
**Theoretical calculation of $R_p$:**

The resistance provided by the partially dried cake to flow of water vapor from the sublimation front below, $\bar{R}_p$, (area normalized resistance) can be calculated from the ice sublimation rate, $\dot{m}$, and the difference between the vapor pressure of ice at the sublimation interface ($P_0$) and the controlled chamber pressure, $P_c$. (Eqn 1). Either a time-averaged $\bar{R}_p$ or $R_p(t)$ as a function of time or dry layer thickness can be determined.

$$\bar{R}_p = A_p \frac{P_0 - P_c}{\dot{m}} \quad \text{Eqn 1}$$

**Calculation of time-averaged product resistance, $\bar{R}_p$**

To obtain a time-averaged $\bar{R}_p$, the average sublimation rate, $\bar{m}$, can be determined experimentally from the measured mass loss of water, $m_{H_2O}$, within a defined time period. Alternatively, a time-averaged sublimation rate, $\bar{m}$ (Eqn 2), can also be found by dividing the theoretical mass of water lost, $m_{H_2O}$, (Eqn 3) by the time of sublimation, $\Delta t$, taken as the time from the start to the end of primary drying. Assuming ice sublimation from the cylindrical frozen solution is radially and angularly symmetric, the sudden increase in product temperature (Figure 1), measured at the bottom center of the vial corresponds to the end of sublimation and primary drying in that vial.

$$\bar{m} = \frac{m_{H_2O}}{\Delta t} \quad \text{Eqn 2}$$
The theoretical mass of water lost by sublimation during primary drying can be calculated from the solute concentration, \( c \) (w/w), the fill volume, \( V_{\text{fill}} \), and the density of the solution, \( \rho_{\text{soln}} \). The remaining amorphous solute, sucrose, retains unfrozen water at the level of 22% (or 0.18 g of water for each 1 g of sucrose freeze-concentrate)\(^6\)\(^-\)\(^8\); however, about \( \frac{1}{2} \) of the unfrozen water has been removed by the end of primary drying due to early secondary drying. The amount of water lost by the end of primary drying can then be found from Eqn 3.

\[
m_{\text{H}_2\text{O}} = V_{\text{fill}}\rho_{\text{soln}}(1 - 1.1 c)
\]

Eqn 3

Calculation of product resistance as a function of time, \( R_p(t) \)

Product resistance as a function of time, \( R_p(t) \) can be obtained from a real-time measurement of sublimation rate over time, \( \dot{m}(t) \). TDLAS is commonly used to determine real-time ice sublimation rates; however, it measures a batch-average \( \dot{m}(t) \) rather than in individual vials. Alternatively, a “direct vial” method can be used to calculate real-time sublimation rate. This method utilizes the process data directly obtained from the freeze-drying process (i.e., \( P_c, T_s, T_p \)); the step-by-step calculation to determine \( R_p(t) \) in individual vials is explained in the Methods.
The sublimation rate, \( \dot{m} \), is related to the rate of heat loss due to sublimation, \( \dot{q} \), by the enthalpy of sublimation, \( \Delta H_s \) (Eqn 4). The rate of heat loss can similarly be an average over the total time, \( \bar{q} \), or over a corresponding time interval, \( \dot{q}_i \).

\[
\dot{m} = \frac{\dot{q}}{\Delta H_s} \quad \text{Eqn 4}
\]

Due to the quasi-steady state nature of heat flow during primary drying, the heat flow out of the vial due to sublimation can be equated to the heat flow into the vial primarily from the shelf to the frozen solution. A vial heat transfer coefficient, \( K_v \), is used to relate the heat flow into the vial from the shelf at a temperature, \( T_s \), to the inside bottom of the vial where the frozen product is at a temperature, \( T_p \) (Eqn 5),

\[
\dot{q} = A_v K_v (T_s - T_p) \quad \text{Eqn 5}
\]

where \( A_v \) is the cross-sectional area of the vial parallel with the shelf. Combination of Eqn 4 and 5 leads to an expression for sublimation rate, \( \dot{m} \), in terms of the difference in temperatures of the shelf and the bottom of the frozen product at a given time, \( t \) (Eqn 6).

\[
\dot{m}(t) = \frac{A_v K_v (T_s - T_p(t))}{\Delta H_s} \quad \text{Eqn 6}
\]

Equation 6 provides another means of finding the real-time sublimation rate provided that the value of \( K_v \) is accurate.
Combination of Eqn 4 - 6 and rearrangement yields Eqn 7, providing the basis of determining $R_p$, which is calculated from dimensions of the vial ($A_v$, $A_p$), $K_v$, the freeze-drying process condition ($T_s$, $P_c$), the temperature response of the product ($T_p$), and the vapor pressure at the sublimation interface ($P_0$).

$$R_p = A_p \times \Delta H_s \left( \frac{(P_0 - P_c)}{A_v K_v (T_s - T_p)} \right)$$  

Eqn 7

**Methods**

**Sample preparation and lyophilization process:**

Solutions (10% w/v sucrose) were filtered through 0.22µm filter and filled (2 mL) into 10 mL tubing vials, which were partially stoppered with 20 mm two-legged stoppers. A full tray of vials was loaded onto the bottom shelf of a lab freeze-dryer (Lyostar 3 with ControLyo™, SP Scientific, Stone Ridge, NY). Product temperature ($T_p$) was monitored with 11 thermocouples placed in the bottom center of vials; 3 thermocouples each in vials touching the band on the left and right edges, and 5 thermocouples were placed in vials in the center of the shelf. The temperature of shelf surfaces nearest to the inlet and outlet of heat transfer fluid, as well as the temperature of the band used to hold the vials in place were monitored with thermocouples.

The solutions were frozen without ice nucleation control or using controlled ice nucleation at shelf temperatures of -5 and -8°C. For primary drying, the chamber pressure ($P_c$) was 60 mTorr and the shelf temperature ($T_s$) was raised at 0.5°C/min to -30°C where it was held for primary drying. When the Pirani gauge reading converged with the capacitance manometer reading, the shelf temperature was raised at 0.2°C/min to 50°C and held for 2 hours at a chamber pressure of 60 mTorr.
The specific surface areas of the freeze-dried samples were measured by BET analysis of gas adsorption as previously described \(^9\).

**Calculation of \( R_p \) in individual vials:**

Eqn 6 provided the basis for determining \( R_p \) from the sublimation rate using the “direct vial” method \(^10\). Using this method, \( K_v \) and \( P_0 \) are the only two items that need to be calculated and the calculation is explained from Eqn 8 to 16.

**Determination of \( K_v \):**

Accurate calculation of individual vial real-time \( R_p \) (t) from measurement of \( \dot{m} \) (Eqn 6) requires an accurate value of \( K_v \), which can be calculated from Eqn 8. Note that Eqn 8 is a rearrangement of Eqn 6, which has been averaged over the entire primary drying time. Classically, \( K_v \) is calculated from gravimetrically determined values of \( \dot{m} \) in additional runs using pure ice in place of the product at the same shelf temperature and chamber pressure as previously described \(^11\). When ice is used in place of product, the value of \( T_p \) does not change significantly with time. However, when using the product itself rather than pure ice, the product temperature (\( T_p \)) changes with time due to the resistance built up from the partially dried layer. Thus, when using the “direct vial” method to determine \( K_v \) of each vial containing a thermocouple (Eqn 8), \( \dot{m} \) (Eqn 2) is the theoretical amount of water (from Eqn 3) divided by the drying time, \( \Delta t \), taken from initiation of primary drying to the sudden rise in temperature reported by the thermocouple (Figure 1). The temperature difference between \( T_s \) and \( T_p \) is the average, \( (T_s - T_p) \), over this time period (Eqn 7). Once the \( K_v \) for each vial has been accurately determined, the ice sublimation rate at each time point can be calculated from Eqn 6.
\[ K_v = \frac{\Delta H_s (\bar{m})}{A_v (T_s - T_p)} \]  

Eqn 8

**Determination of \( P_0 \):**

In addition to \( \bar{m} \), the calculation of \( R_p \) from Equation 7 requires the vapor pressure of ice at the sublimation interface \( P_0 \), which is an exponential function of the product temperature at the same location \( T_0 \) (Eqn 9).

\[ P_0 = 2.69 \times 10^{10} e^{(-6144.96/T_0)} \]  

Eqn 9

The product temperature at the sublimation front \( T_0 \) can be found from the temperature at the vial bottom measured by thermocouple, \( T_p \). In this work, we described a simplified calculation (Eqn 11) from Eqn 2 in Tang et al. The heat transferred from the shelf to the bottom of product (Eqn 5) approximately equals to the heat flow from the bottom of the product through the thickness of ice to the ice sublimation interface (Eqn 10).

\[ \dot{q} = A_v \frac{k_{ice}}{l_{ice}} (T_p - T_0) \]  

Eqn 10

The temperature at the sublimation interface, \( T_0 \), is obtained by combination and rearrangement of Eqn 5 and 10, (Eqn 11), which depends on the vial heat transfer

---

1 In Eqn 5, the heat transfer comes from all sources including the top and the bottom of the shelf to the vial, reflected in \( K_v \). In Eqn 10, the heat transfer driven by \( \Delta T (T_p - T_0) \) only comes from the bottom shelf to the sublimation interface over a thickness of \( L_{ice} \). The difference in \( \Delta T \) calculation between the simplified form (Eqn 11) and the comprehensive form is small and did not affect the outcome of \( R_p \).
coefficient ($K_v$), the thermal conductivity of ice ($k_{ice}$), thickness of ice ($L_{ice}$), and $T_p$. $K_v$ is found from Eqn 8 as described in the previous section. The value of $k_{ice}$ is $5.9 \times 10^{-3}$ cal·cm⁻¹·K⁻¹·s⁻¹. The calculation of $L_{ice}$ as a function of time is described through Eqns 12 to 14.

$$T_0 = T_p - \frac{L_{ice}K_v(T_s - T_p)}{K_{ice}} \quad \text{Eqn 11}$$

As ice undergoes sublimation, the thickness of the remaining ice, $L_{ice}$, decreases and the thickness of the “dried” porous layer of solute over the sublimation interface, $L_{dry}$, increases. The thickness of the “dried” layer, $L_{dry,t}$, at a given time is related to the initial thickness of the frozen layer, $L_{max}$, and the amount of water lost up to that time point, $M_t$ (Eqn 12).

$$L_{dry,t} = L_{max} \left( \frac{M_t}{m_{H_2O}} \right) \quad \text{Eqn 12}$$

$L_{ice}$ and $L_{dry}$ are related (Eqn 13).

$$L_{ice} = L_{max} - L_{dry} \quad \text{Eqn 13}$$

$L_{max}$ is the initial height of the frozen sample. In the case of high concentration amorphous solute in the solution, the frozen sample consists of the frozen ice and the unfrozen amorphous material, and $L_{max}$ can be calculated (Eqn 14),
\[ L_{\text{max}} = \frac{V_{\text{fill}} \rho_{\text{soln}}}{A_p} \left( \frac{1 - 1.1c}{\rho_i} + \frac{1.1c}{\rho_u} \right) \quad \text{Eqn 14} \]

where \( V_{\text{fill}} \) and \( \rho_{\text{soln}} \) represent the fill volume and density of the solution, respectively, \( \rho_i \) and \( \rho_u \) represents the density of ice and the unfrozen amorphous material. Approximately, \( \frac{1}{2} \) of the unfrozen water originally presented in the freeze-concentrate is lost during primary drying. This amount of unfrozen water lost due to early secondary drying is \(<1\%\) of the frozen water in the case of 2mL of 10\% sucrose solution, and the influence of early secondary drying on water loss is negligible.

The amount of ice sublimed at each of several time intervals can also be calculated (Eqn 15).

\[
\Delta m_t = \frac{(m_{t_i} + m_{t_{i+1}})}{2} (t_{i+1} - t_i) \quad \text{Eqn 15}
\]

The cumulative amount of ice sublimed at each time, \( M_t \), equals the sum of the differential amounts of ice sublimed \( \Delta m_t \) within each time interval, \( \Delta t \) (Eqn 16). The calculation is valid until the thermocouple loses contact with ice at the bottom of the vial, which is usually associated with a sudden rise in temperature.

\[
M_t = \sum_{t=0}^{t} \Delta m_t \quad \text{Eqn 16}
\]

If sublimation occurs with radial and angular symmetry and no significant secondary drying takes place, the value of \( M_t \) at the end of primary drying should be equal to the theoretical amount of water lost to sublimation, \( m_{H_2O} \) (Equation 3).
In summary, the calculation of product resistance in individual vials, at each time, \( R_p(t) \) using the \textit{“direct vial”} method was achieved from the determination of \( \dot{m}(t) \) and \( P_0 \), both of which depends on accurate determination of \( K_v \) for that vial. These calculations were carried out with an MS Excel spreadsheet, which will be made available to interested parties.

\textit{Determination of \( R_p \) in vials at various shelf locations by establishing \textit{“\( R_p \) - SSA” or \textit{“\( R_p \) – ice nucleation temperature”} correlation}}

The ice nucleation temperature largely determines the porous structure in the dried layer, therefore the resistance to mass flow (\( R_p \))\(^{12,13}\). A higher ice nucleation temperature yields larger ice crystals and therefore lowers SSA of the freeze-dried sample. Thus, in principle, one can estimate the \( R_p \) of a particular group of vials (i.e., edge vial vs. center vial), if the ice nucleation temperature or SSAs are known. Previously, linear correlations between \( R_p \) and SSA were established in various formulations including sucrose, mannitol, dextran and hydroxyethyl starch \(^1\). The correlation in the sucrose samples was based on a 5 mL fill of 5% sucrose solution in 20 mL vials, where the average \( R_p \) was calculated over a cake thickness of 1 cm. In the present work, 2 mL of 10% sucrose solution was filled in 10 mL vials with a fill depth 0.6 cm. Therefore, the correlation established in Rambhatla et al., can not be directly used to estimate a true \( R_p \) in samples at a different concentration and fill depth.

In this study, we established similar correlations using the 10% sucrose samples. \( R_p \) in a given vial was calculated using the \textit{“direct vial”} method. There were challenges in
determining \( R_p \) in edge vials (discussed in Section 3 of Results and Discussion). \( R_p \) in center vials were calculated over an \( L_{dry} \) of 0.60 cm (explained in “Attempt to calculate \( R_p \) in edge and center vials” of Results and Discussion), and correlated with the measured SSA and ice nucleation temperature in corresponding vials. Further, the established linear correlation was used to estimate \( R_p \) in edge vials with known ice nucleation temperature or SSA. 90% confidence intervals (CI) for the linear regressions were constructed to find the uncertainty of the estimated \( R_p \) values for a given edge vial.

**Results and Discussion**

1. **Distribution of ice nucleation temperature in a batch**

   When ice nucleation was controlled at shelf temperatures of -5 and -8°C, ice nucleated at the highest temperature in the sucrose solutions on the left edge of the shelf, followed by the right edge; the solution in the center vials had the lowest ice nucleation temperatures (Table 1). This was caused by small temperature differences in the product at fixed nominal shelf temperature. In the absence of an ice nucleation control technology, ice nucleates spontaneously with an average ice nucleation temperature here of -12°C for solutions in vials at all three locations (i.e., center, left edge and right edge) on the shelf (Figure 2). The surface of the shelf near the outlet for heat transfer fluid, which located at the front left corner, had a slightly higher temperature (0.5°C) than near the inlet at the front right corner throughout the freezing steps. Interestingly, the temperature of the left side of the band was substantially higher (3°C) than the right side of the band at the time of ice nucleation. The band temperature is controlled by the
temperature of the product, inner wall, and the shelf. Although the temperature of the inner wall may not be particularly sensitive to the temperature of the outer wall, the observed higher ice nucleation temperature on the left edge was perhaps due in part to higher radiation effect from the wall, behind which the electrical supply may provide additional heat relative to the right-hand wall of the dryer chamber.

2. Correlation between the ice nucleation temperature and specific surface area (SSA)

Smaller ice crystals are formed at lower ice nucleation temperatures, resulting in higher SSA in the freeze-dried cake. The SSA of the sucrose cakes lyophilized from 10% sucrose solutions linearly correlated with ice nucleation temperature of the solution ($R^2=0.92$), with higher SSA at lower ice nucleation temperature (Figure 3). Moreover, there was a shelf position dependence of the SSA. For example, at a nominal shelf temperature of -5°C, the ice nucleation temperature of the solutions in vials on the left edge was significantly higher than those on the right edge (-2.0 ± 0.6°C vs. -4.0 ± 0.0°C). Correspondingly, the SSA of the dried cakes from vials located on the left edge was smaller than on the right edge (0.29 ± 0.01 m$^2$/g vs. 0.38 ± 0.00 m$^2$/g). In addition, the SSA of the cakes in vials at the right edge was comparable to those in the center vials, corresponding to the similar ice nucleation temperatures (-4.1 ± 0.0°C vs. 4.0 ± 0.0°C) when nucleated at a shelf temperature of -5°C. The trend was similar when ice nucleation was controlled at a shelf temperature of -8°C (Table 2). Based on the correlation between ice nucleation temperature and SSA, one may expect the product resistance to be lower when ice nucleates at a higher temperature, due to the formation of larger ice crystals. If the correlation between $R_p$ with either SSA or ice nucleation temperature is established,
one may be able to estimate $R_p$ in a given vial with known SSA or ice nucleation temperature (discussed in the following section).

3. Attempt to calculate $R_p$ for edge and center vials

The calculation of $R_p$ in a given vial relies on accurate determination of $K_v$ (discussed in “determination of $K_v$” in the Methods). Three methods were used for determining $K_v$, including the standard gravimetric method with pure ice or the sample solution, and the “direct vial” method.

Using the “direct vial” method, the assumption was made that all frozen water was sublimed when the thermocouple loses contact with ice and there was a sudden temperature rise (Figure 1). $K_v$ was calculated from $\bar{m}$ (Eqn 8), where $\Delta t$ (Eqn 2) was obtained from the start of primary drying to the time at which there was a sudden rise in temperature. However, in some vials, there was multiple “sudden rise” in temperature (Figure 1), making it difficult to unequivocally determine the drying time, $\Delta t$. Additionally, we note that while the contour of the vial bottom can influence $K_v$ values due to the contribution of gas conduction in the space between the shelf surface and the bottom of the vial, in this case vials were marked so that the same vials were used in the same positions for the $K_v$ determination using all three methods.

In addition to using the standard method to check the $K_v$ determined by the “direct vial” method, a modification of the standard method was explored; values of $K_v$ were determined gravimetrically according to the standard method protocol, except using the solution of interest, 10% (w/v) sucrose, in lieu of pure water. The freeze-drying process was manually stopped after 5 hours into primary drying, corresponding to 20-33% water
loss. As in the standard method, the vials were weighed before and after drying to measure \( \Delta \bar{m} \), which was used to calculate a \( K_v \) for each vial. The edge vial \( K_v \) values determined by this modification of the standard method were larger than when using the pure water gravimetric method but smaller than the “direct vial” method (Table 1).

All methods provided comparable \( K_v \) values for the center vials. However, compared to the “standard” gravimetric method for determining \( K_v \) using pure ice (Table 1), the \( K_v \) for the edge vials, particularly the left edge vial, calculated by the “direct vial” method was seriously overestimated using the cycle data (Eqn 2 and 8), probably because there was still some ice in the vials and the \( \Delta t \) was too small. The “direct vial” method has been used successfully in our lab to determine \( R_p \) in center vials. Since the \( K_v \) values of the edge vials determined from the “direct vial” method were unrealistically large, the corresponding \( R_p \) will be unrealistic. To determine \( R_p \) in edge vials, an alternative approach was proposed and explained in the following section.

### 4. Estimation of \( R_p \) in edge vials from “\( R_p - SSA \)” or “\( R_p – ice nucleation temperature \)” correlation using center vials

Since the \( K_v \) values in the center vials were comparable in the three methods (Table 1), \( R_p \) calculated in those center vials using the “direct vial” method was assumed to be valid. Using these \( R_p \) values, linear correlations were established between \( R_p \) and ice nucleation temperature (n=30, \( R^2=0.77 \), Figure 4) as well as \( R_p \) and SSA (n=10, \( R^2=0.62 \), Figure 5). There is a good agreement in the estimated edge vial \( R_p \) values using either correlation (Table 2). Indeed, the cakes in vials located on the left edge of the shelf had the lowest \( R_p \) relative to the cakes in vials on the right edge and in center vials (Table
3), which is not unexpected. Analysis on 90% confidence interval for the prediction line showed significant lower $R_p$ on the left edge due to a higher ice nucleation temperature (or lower SSA) compared to the center vial. Comparable $R_p$ values were found in samples on the right edge and the center of the shelf. The maximum difference in $R_p$ was found to be 26% between the left edge and center vials in the case when ice nucleation was controlled at -5°C. Estimation of $R_p$ in the edge vials from the correlation established from $R_p$ (calculated from the center vials) and the ice nucleation temperature or SSA provides good information on shelf location-dependence of $R_p$. The extrapolation of $R_p$ in edge vial was based on the assumption that the samples on the edge dry in a similar manner as the center vial. In cases of high solid content with high fill depth undergoing aggressive drying conditions, the ice morphology and drying behavior can be very different between the center and edge vials. $R_p$ may also be affected by other factors such as gaps (i.e. shrinkage) between the glass vial and the sample, which is not reflected in SSA and ice nucleation temperature.

The data suggested $R_p$ is lower in the edge vials due to a higher ice nucleation temperature. It should be noted that the results in the present study may not be widely generalizable to other freezing process; the observations were based on controlled ice nucleation cycles using Lyostar freeze-dryers, at relatively high shelf temperatures (i.e. -5 and -8°C). Cautions are needed when controlling ice nucleation at a higher shelf temperature; the temperature of the sample solutions should be monitored since thermal history of the samples on the edge can be quite different from the center vials.
Summary

It is commonly accepted that controlled ice nucleation results in homogeneous ice nucleation temperature across a batch. However, in the present study, a distribution of ice nucleation temperatures was found within batches where ice nucleation was controlled. Even though nucleation was synchronized, the solutions in vials at the left edge of a laboratory freeze-dryer underwent ice nucleation at a higher temperature than in vials at the right edge and center of the shelf. SSA of the cakes in the vials followed the opposite rank order as the ice nucleation temperature for the three locations of vials.

Without the technology available to monitor ice sublimation rate from a single vial in a batch without disrupting the heat flow pattern in the freeze-dryer, there is no direct experimental method for determining any corresponding rank order of $R_p$ with position. A “direct vial” method of calculating $R_p$ from cycle data proved to be a challenge due to difficulties in accurately assessing individual vial $K_v$ values for the edge vials, particularly vials on the left edge. However, the calculated $K_v$ values in center vials are comparable with the values determined using the standard gravimetric method. Correlations were established between $R_p$, SSA and ice nucleation temperature using the center vials, and further were used to estimate $R_p$ in edge vials. Left edge vials with higher ice nucleation temperatures showed lowest $R_p$ to sublimation followed by comparable $R_p$ in the right edge vials and center vials. Thus, even with synchronous ice nucleation also called controlled nucleation, there may be differences in the resistance to sublimation, $R_p$, within a batch due to location-dependence of heat transfer during the freezing step. Note that the “edge vial effect” on $R_p$ is opposite to the “edge vial effect” on $K_v$. 
Table 1. $K_v \times 10^4$ (cal·cm$^{-2}$·K$^{-1}$·s$^{-1}$) values determined from three methods. Ice nucleation temperature was controlled at -5°C. Primary drying was performed at a shelf temperature of -30°C and chamber pressure of 60 mTorr. The values were represented as average ± standard error of the measurements (n=4 for the left and right edge vials, n=8 for the center vials).

<table>
<thead>
<tr>
<th>Vial location</th>
<th>“Direct vial method”</th>
<th>Gravimetric method (with 10% sucrose)</th>
<th>Standard gravimetric method (with pure ice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left edge</td>
<td>9.9 ± 0.9</td>
<td>6.78 ± 0.9</td>
<td>5.7 ± 0.2</td>
</tr>
<tr>
<td>Right edge</td>
<td>6.5 ± 0.6</td>
<td>5.95 ± 1.4</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>Center</td>
<td>3.3 ± 0.1</td>
<td>2.83 ± 0.1</td>
<td>3.2 ± 0.2</td>
</tr>
</tbody>
</table>
Table 2. Estimated $R_p$ ($cm^2 \cdot Torr \cdot hr \cdot g^{-1}$) in each group of vial at various locations of the shelf. Ice nucleation was controlled at shelf temperatures of -5°C or -8°C.

<table>
<thead>
<tr>
<th>Vial location</th>
<th>$T_{ice , nucleation}$ (°C) (Avg ± Std)</th>
<th>SSA (m²/g) (Avg ± Std)</th>
<th>* Estimated $R_p$ (90% CI) From $R_p$- $T_{ice , nucleation}$</th>
<th>* Estimated $R_p$ (90% CI) From $R_p$- SSA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controlled ice nucleation at a shelf temperature of -5°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left edge</td>
<td>-2.0 ± 0.6</td>
<td>0.29 ± 0.01</td>
<td>3.5 ± 0.3</td>
<td>3.0 – 4.0</td>
</tr>
<tr>
<td>Right edge</td>
<td>-4.1 ± 0.0</td>
<td>0.38 ± 0.00</td>
<td>4.4 ± 0.2</td>
<td>4.0 – 4.8</td>
</tr>
<tr>
<td>Center</td>
<td>-4.1 ± 0.4</td>
<td>0.39 ± 0.00</td>
<td>4.4 ± 0.2</td>
<td>4.1 – 4.8</td>
</tr>
<tr>
<td><strong>Controlled ice nucleation at a shelf temperature of -8°C</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left edge</td>
<td>-5.3 ± 0.0</td>
<td>0.43 ± 0.01</td>
<td>5.1 ± 0.2</td>
<td>4.8 – 5.4</td>
</tr>
<tr>
<td>Right edge</td>
<td>-7.1 ± 0.4</td>
<td>0.47 ± 0.00</td>
<td>5.9 ± 0.2</td>
<td>5.6 – 6.2</td>
</tr>
<tr>
<td>Center</td>
<td>-6.8 ± 0.6</td>
<td>0.45 ± 0.02</td>
<td>6.0 ± 0.2</td>
<td>5.7 – 6.3</td>
</tr>
</tbody>
</table>

* The estimated $R_p$ values were presented as the estimated values from the correlation (Figure 4 or 5) ± the error of the estimation.
Figure 1. An example of product temperature and chamber pressure profile of 10% (w/v) sucrose; ice nucleation was controlled at a shelf temperature of -5°C. On the top panel, the red and blue curve represents the pressure in the chamber measured by the Pirani gauge and capacitance manometer (CM), respectively. The curves on lower panel represent the shelf temperature set point and temperature traces of the samples measured by thermocouple placed in the bottom center of the vial. The arrows show the time points when the thermocouple lost contact with ice. Often times, multiple “sudden temperature rise” was observed especially for the edge vials, indicated by the two pink arrows.
Figure 2. Distribution of ice nucleation temperature across a batch under three conditions: uncontrolled ice nucleation (UCN), controlled ice nucleation at shelf temperatures of -5 and -8°C (designated CN-5°C and CN-8°C, respectively).
Figure 3. Specific surface area (SSA) of 10% (w/v) sucrose as a function of ice nucleation temperature measured in the bottom center of the vial.
Figure 4. Correlation between calculated $R_p$ in center vials and the corresponding ice nucleation temperature. The data (n=30, with black symbols) were fitted in a linear function (black line) to estimate $R_p$ in edge vials. 90% confidence interval for the prediction line was constructed to find the range for the estimated $R_p$ values.
Figure 5. Correlation between calculated $R_p$ in center vials and the corresponding SSA measured by BET. The data (n=10 since only 10 measurement of SSA were available,) were fitted in a linear function (black line) to estimate $R_p$ in edge vials. 90% confidence interval for the prediction line was constructed to find the range for the estimated $R_p$ values.
References


Chapter 5

Protein Internal Dynamics Associated with Pre-$T_g$ Endothermic Events:

Investigation of Insulin and hGH by Solid-State Hydrogen/Deuterium Exchange
Abstract
Lyophilized proteins are generally stored below their glass transition temperature ($T_g$) to maintain long-term stability. Some proteins in the (pure) solid state showed a distinct endotherm at a temperature well below the glass transition, designated as a pre-$T_g$ endotherm. The pre-$T_g$ endothermic event has been linked with a transition in protein internal mobility. The aim of this study was to investigate the internal dynamics of two proteins, insulin and human growth hormone (hGH), both of which exhibit the pre-$T_g$ endothermic event with onsets at 50-60°C. Solid-state hydrogen/deuterium (ss-H/D) exchange of both proteins was characterized by Fourier Transform Infrared (FTIR) spectroscopy over a temperature range from 30 to 80°C. A distinct sigmoidal transition in the extent of H/D exchange had a midpoint of 56.1 ± 1.2 °C for insulin and 61.7 ± 0.9°C for hGH, suggesting a transition to greater mobility in the protein molecules at these temperatures. The data support the hypothesis that the pre-$T_g$ event is related to a transition in internal protein mobility associated with the protein dynamical temperature. Exceeding the protein dynamical temperature is expected to activate protein internal motion, and therefore may have stability consequences.
Keywords:
Freeze drying/lyophilization; proteins; protein structure; amorphous; solid state stability; glass transition; mobility; calorimetry (DSC); FTIR

List of non-standard abbreviations

\[ \begin{align*}
\text{d}T: & \text{ width of the sigmoidal transition; } \\
\text{k: } & \text{apparent rate constant of the observable H/D exchange; } \\
T_{\text{mid}}: & \text{temperature midpoint of the sigmoidal transition; } \\
X: & \text{fraction of H/D unexchanged protons on protein molecule; } \\
x_0: & \text{the fraction at time zero; } \\
x_\infty: & \text{the fraction at the apparent plateau level; } \\
x_{\text{ini}}: & \text{initial value; } \\
x_{\text{fin}}: & \text{final value} \\
\end{align*} \]
**Introduction**

Freeze-drying has been widely used to minimize potential chemical and physical degradation of proteins by the conversion of a protein solution into amorphous (or at least partially amorphous) solid material. In order to achieve optimal long-term stability, it is recommended that freeze-dried proteins should be stored well below the glass transition temperature ($T_g$) of the entire system (i.e., an assembly of protein molecules or protein molecules dispersed in a matrix) \(^{1-4}\). The $T_g$ of such a molecularly miscible system marks the onset temperature of viscous flow with higher mobility on a large length-scale, reflecting the movement and collisions between protein molecules or protein molecules with the excipient matrix. However, there is an increasing body of work demonstrating that physical and/or chemical degradations still occur during storage at temperatures below the system $T_g$ \(^{5-7}\). Besides the global glass transition temperature for the system, the concept of a glass transition temperature within an individual protein molecule, known as the protein dynamical temperature ($T_d$), was introduced to describe local fluctuations of protein residues on a smaller length-scale. As the protein temperature increases beyond the “protein dynamical temperature”, the protein molecule experiences larger fluctuations in structure on shorter time scales, (which is analogous to a small molecule glass passing through it’s $T_g$), eventually resulting cooperative changes producing significant mobility on the short time scale of the differential scanning calorimetry (DSC) scan. The protein dynamical temperature ($T_d$) within protein molecule has been well studied in systems with high water/polyol content and in sugar/water glassy matrices \(^{8-10}\), but little experimental work has been done in dry proteins. At temperatures exceeding the protein dynamical temperature, the increase in local internal motions of
protein molecule may have significant stability implications in solid protein formulations.

In amorphous systems consisting of small molecules, the system $T_g$ can be measured by DSC. The glass transition temperature is observed as a sharp shift in the DSC thermogram, due to an increase in heat capacity ($\Delta C_p$). This increase in heat capacity is often accompanied by a non-reversing endotherm, associated with enthalpy recovery. In contrast, the glass transition temperature of pure proteins cannot be detected by DSC, because a pure protein behaves as a strong glass (i.e., characterized by a broad glass transition with a small change in $C_p$ at $T_g$). However, at least in principle, the enthalpy recovery endotherm should still be observable, which is the suspected origin of a pre-$T_g$ endotherm in solid pure proteins. Pre-$T_g$ endothermic events were reported for both crystalline and amorphous insulin, lyophilized human growth hormone (hGH), and bovine serum albumin (BSA). These endotherms occur well below the global $T_g$ for solid pure proteins, which is around $150^\circ C$. The temperature marking the onset of mobility within BSA, as determined by H/D exchange, coincided with the temperature of the pre-$T_g$ endotherm; this supports the concept that the pre-$T_g$ endotherm represents enthalpy recovery corresponding to the “molecular glass transition” within the protein molecule, or “protein dynamical temperature”. It should be noted that pre-$T_g$ thermal events have also been reported for non-protein systems during DSC scans. However, such an endothermic event in those glassy systems was explained by a very broad distribution of enthalpy relaxation times caused by the variation of thermal history and mobility during processing. The pre-$T_g$ endothermic event in pure protein, such as BSA, has a higher activation energy (138 kJ/mol) than the pre-$T_g$ events in non-protein systems.
glassy systems (75 kJ/mol or less in unannealed samples). In this work, we continue the investigation of the pre-\(T_g\) endothermic event in two additional proteins, hGH and insulin, following essentially the same H/D exchange procedure as previously described.

**Materials and Methods**

**Materials**

Insulin and hGH samples were provided by Eli Lilly & Co (Indianapolis, IN). Pure insulin was in the form of crystalline recombinant insulin. Recombinant human growth hormone (hGH) was pure amorphous freeze-dried material, without excipient. Deuterium oxide (99.8 %D), lithium chloride (>99%, ACS grade), and potassium bromide (99%, IR grade) were purchased from Acros Organics (Morris Plains, NJ).

**Methods**

Procedures were essentially equivalent to those previously described and are briefly summarized here.

**H/D exchange process**

Pure insulin or hGH was transferred to glass desiccators where 11% RH was maintained by saturating \(D_2O\) with LiCl. The desiccators were sealed under vacuum and stored at temperatures ranging from 30 to 80°C. Previous experience with such vacuum “humidity chambers” indicates mass transfer equilibrium is established within 10-20 hours for such samples. The samples were removed at 1, 2, 3 and 4 days under dry air atmosphere and dried immediately in a vacuum oven (model 281A, Fisher Scientific, Pittsburgh, PA) at room temperature and “full vacuum” for 24 hours, so as to quench the
H/D exchange reaction. Quenched samples were analyzed by FTIR spectroscopy. All measurements were performed in duplicate.

**FTIR measurement**

Samples of 0.5-0.8 mg of insulin or hGH were gently ground with 150 mg of dried potassium bromide (KBr) in a glove bag purged with dry air. The resulting mixture was compressed at 10,000 psi for 2 minutes using a laboratory press (Carver Inc, Wabash, IN). The compressed pellet was transferred to a sample holder in a FTIR spectrometer (Nicolet Magma 560, Thermo Scientific, Madison, WI). The CO₂ level and water vapor in the spectrometer chamber were controlled by a purging system with moisture and CO₂ traps (Puregas, LLC, Broomfield, CO).

Spectra were recorded in single beam mode with 128 scans and 4 cm⁻¹ resolution over a range of 400-4000 cm⁻¹. All spectra were processed using software provided with the FTIR spectrometer (Grams/AI 8.0, Thermo Electron, Madison, WI). Each spectrum was first converted to the absorbance format by subtracting the background spectrum collected with KBr. The residual water vapor signals, if any, were removed by subtracting the spectrum of gaseous water until a smooth spectrum was obtained in the 2000-1800 cm⁻¹ range. The amide I band with maximum absorbance at around 1658 cm⁻¹ consists of primarily C=O stretching modes, and is not influenced by H/D exchange. Therefore, the intensity of the amide I band in each spectrum was used to normalize the absorbance for direct comparison among spectra.

**Data Analysis**

The extent of H/D exchange was calculated as previously described (Equations 4-6; 16). The equations are listed in the caption of figure 4 and 5. Briefly, the fraction of
protons remaining to be exchanged, X, was evaluated over 4 days in duplicate studies for each protein. Each set of X versus time data were fitted by a first order decay function to find $X_\infty$ and its error. An average of the duplicate values of $X_\infty$ was calculated, along with the associated error determined by propagation of errors. The value of $X_\infty$ at each temperature with its error was fitted by a sigmoidal function to find the temperature midpoint of the transition and its associated error.

**Results and Discussion**

**Observation of the pre-$T_g$ endothermic event**

The glass transition temperature, $T_g$, of pure proteins can be determined by extrapolating the $T_g$ of systems containing protein co-lyophilized with disaccharide to 100% protein using the Fox equation. Using this extrapolation, the system glass transition of pure hGH in solid state was determined to occur at 136°C (Table 1), which is below the denaturation temperature (above 150°C) at which large-scale molecular mobility on the DSC scan timescale is required $^{13}$. Another endothermic signal in a DSC warming scan was also observed for both hGH and insulin, at a temperature well below the system $T_g$ (Table 1) $^{14,15}$. In Figure 1, the DSC thermogram labeled 4.1 (meaning the water content was 4.1%) represents hGH equilibrated at 11% RH, where a small broad endotherm occurs with an onset of $\approx 50{^\circ}C$ and a maximum at 60°C (Figure 1). Similarly, the DSC thermogram of insulin at 11% RH shows an endothermic event with an onset of $\approx 50{^\circ}C$ and a maximum at 70°C (Figure 2).

The pre-$T_g$ endothermic events for both proteins disappeared on rescanning and hence are not reversible on the timescale of DSC scan. This is a characteristic of enthalpy recovery signal superimposed on a system glass transition. However, the glass transition
temperature is much higher than the pre-$T_g$ endotherm. Instead, the pre-$T_g$ endotherms could be the relaxation enthalpy within the protein molecule recovered at the temperature associated with a transition in protein internal mobility as has previously been suggested 16. While the glass transition temperature of the protein molecule, or “dynamical temperature” may be reversible, the accompanying change in mobility is not directly detectible by DSC. However, when the protein is held/stored for a long time at a temperature below the $T_d$, enthalpy relaxation 12 within the protein would be recovered on scanning through the $T_d$. Without a long hold time post first scan, the pre-$T_g$ endothermic events disappeared at the second scan during the time scale of the DSC experiments, which is much shorter than that of the enthalpy relaxation. It is well known that protein molecules demonstrate glass-like properties (i.e., KWW non-exponential relaxation) associated with diffusive mobility; it has been argued that fast local non-diffusive movement of the amino acid residues within proteins lead to side chain rotation and segmental fluctuations 5,11,23-25. These $\beta$-like processes collectively give rise to highly cooperative “$\alpha$-mobility”-related event, responsible for protein internal mobility 11. The activation energy of the pre-$T_g$ thermal event in BSA was found to be 138 kJ/mol 16, which is more characteristic of “$\alpha$-mobility” than of fast $\beta$ or $\gamma$-mobility (~ 50 kJ/mol) 16,26. Therefore, the pre-$T_g$ endothermic events, having similar characteristics with system glass transition, would be associated with $\alpha$-like motion, which is activated at this temperature range.

The broad pre-$T_g$ endotherm became more energetic and moved slightly towards lower temperatures with increasing water content (Figure 1). Samples were equilibrated with a range of relative humidities for the same time period. At higher water contents,
enthalpy relaxation would be faster, ultimately resulting in a greater enthalpy recovery, near the onset of the glass transition of the protein molecule. The shifting to lower temperatures is qualitatively similar to the plasticizing effect of water on the system glass transition. It was suggested that water facilitates the propagation of β-like motion within the protein molecule into α-like motion that is responsible for protein dynamical transition at $T_d$ \(^{11}\). Protein denaturation generally requires large-scale motion involving the entire peptide backbone. Thus, the observation that the water content appears to have a larger effect on protein denaturation and $T_g$ than on the pre-$T_g$ event (presumably the $T_d$) suggests that the pre-$T_g$ event was due to a transition in protein internal mobility at a smaller length scale compared to the system glass transition and protein denaturation.

**Investigation of protein internal dynamics by ss-H/D exchange study**

The function of proteins depends on their well-defined three-dimensional structure and the ability to rearrange its structure in order to respond to the environment such as association/dissociation processes. This adaptability, reflecting protein intrinsic flexibility and dynamics is strongly affected by many factors including temperature and hydration \(^{27}\). As protein internal mobility increases, some amide hydrogens initially buried in the interior of the protein molecule become transiently exposed and accessible to D$_2$O due to this dynamic or breathing motion of protein molecules, resulting in enhanced H/D exchange \(^{28}\). The rate and extent of H/D exchange also reflects the time-average conformation of the protein (i.e., where the exchangeable hydrogens are, on average, located). Therefore, H/D exchange can be a measure of both dynamics and “structure” in the protein molecule, both of which are relevant to stability. Mass spectroscopy, nuclear magnetic resonance (NMR) and FTIR spectroscopy have been used
to monitor the extent of H/D exchange in protein molecules \textsuperscript{28-32}. The degree of exchanges at specific locations in the primary structure of solid proteins can be measured by employing LC-Mass spectroscopy \textsuperscript{33}. In this work, FTIR was used to monitor the extent of H/D exchange, because it is a simpler and faster technique, providing essentially the same information on non-site specific, total exchange with time.

Changes in the FTIR spectra of hGH and insulin equilibrated with D\textsubscript{2}O vapor as a function of temperature are shown in Figure 3. The amide II’ band at 1445 cm\textsuperscript{-1} corresponds to N-D vibrations developed upon H/D exchange, and the peak intensity increases as the reaction proceeds. However, there is an additional contribution to the amide II’ signal from H-O-D vibration in partially deuterated water\textsuperscript{3}. Similarly, the amide II band at 1535 cm\textsuperscript{-1} represents the N-H vibrational mode, the peak intensity of which decreases as hydrogen is replaced by deuterium. However, this amide II band does not contain interfering contributions from absorption bands of H-O-H, H-O-D and D-O-D. The shoulder around 1515 cm\textsuperscript{-1} is a characteristic band of the tyrosine aromatic ring vibration, and is not sensitive to the H/D exchange reaction \textsuperscript{34,35}. In an attempt to resolve the signal at 1535 cm\textsuperscript{-1} from overlapping signals, primarily from tyrosine ring vibration, deconvolution of the individual peaks in the range of 1300-2000 cm\textsuperscript{-1} was attempted. However, the necessary assumption of Gaussian peak shape introduced artificial peaks in some spectra, resulting in more uncertainty in the measurement of the area of the deconvoluted peak than in the height of the peak at 1535 cm\textsuperscript{-1}. The contribution of tyrosine vibration to the intensity at 1535 cm\textsuperscript{-1} was found to be less than 0.5\% and 3\% for hGH and insulin, respectively. Therefore, the intensity of the amide II band at 1535 cm\textsuperscript{-1}.

\textsuperscript{3} Exhibits strong absorbance around 1460 cm\textsuperscript{-1} \textsuperscript{32}. 

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was used to monitor the extent of H/D exchange as a function of time and temperature, as in the previous study \(^\text{16}\). The peak intensity decreased with temperature, indicating enhanced H/D exchange at elevated temperatures (Figure 3).

The fraction of hydrogens remaining un-exchanged was estimated from the ratio of relative peak intensity of the amide II band at any time point to that at time zero \(^\text{16}\). The time course of H/D exchange was approximated as a single first order exponential decay (Figure 4), to a final fraction of hydrogens remaining un-exchanged, \(X_\infty\). H/D exchange kinetics are expected to follow at least double exponential decay, representing fast exchange for the “exposed” protons and slow exchange for the “buried” protons. The mass transfer equilibrium is essentially complete after about 24 hours at room temperature. The actual H/D exchange rate with the protein is not confounded by the mass transfer kinetics after one day. Instead, over the 4 day duration of our experiments, the rate constant for exchange of the “buried” protons is expected to be so slow that the fraction of unexchanged protons, \(X\), appears to approach a constant at long times, \(X_\infty\), representing the fraction of “buried” protons. The value of \(X_\infty\) was obtained by regression analysis using Equation 5 of Mizuno and Pikal \(^\text{16}\). Others have used the rate constant of the H/D exchange process to differentiate protein dynamics in a series of formulations \(^\text{33}\). In the present work, the exchange of available hydrogens occurred too rapidly (i.e., within one or two days) (Figure 4) to accurately evaluate a rate constant. Moreover, any time constant would likely represent a combination of mass transfer kinetics and H/D exchange kinetics. The unexchanged hydrogens, represented by \(X_\infty\), are presumably buried in the interior of the protein folded structure where they are not dynamically accessible by the D\(_2\)O vapor on the time scale of the experiment \(^\text{16}\). Thus,
the final extent of H/D exchange, $X_\infty$, should quantitatively provide information on the temperature dependence of protein dynamics.

A sigmoidal function was fitted to the temperature dependence of the plateau level of ss-H/D exchange (i.e., $X_\infty$) for hGH and insulin (Figure 4). The mid-point of the sharp decrease in $X_\infty$ obtained from the sigmoidal function fell in the same temperature range as the DSC pre-$T_g$ transitions $^{14,15}$. For hGH, the mid-point of this transition in protein dynamics was at $61.7 \pm 0.9^\circ C$ compared to the pre-$T_g$ endothermic event at 50-70$^\circ C$ with a maximum at 60$^\circ C$. Similarly, the mid-point of the protein internal dynamical transition for insulin, by H/D exchange, was found to occur at $56.1 \pm 1.2^\circ C$, corresponding to the broad pre-$T_g$ event in DSC between 50-80$^\circ C$ with a maximum at 70$^\circ C$. In BSA, the low temperature pre-$T_g$ DSC endothermic event was suggested to result from a mobility transition within the protein molecule itself, since it occurred over the same temperature range as the sharp increase in extent of H/D exchange $^{16}$. Alternatively, it could be argued that the pre-$T_g$ endotherm and the temperature dependence of the H/D exchange correspond to a protein conformational change, such as a partial unfolding. However, at least from the FTIR spectra, there were no detectable changes in secondary structure with insulin or hGH, although a tertiary structural change, such as a transition to a “molten globule” state cannot be ruled out from the available data. Whether the temperature dependence of H/D exchange is attributed to a transition in internal protein dynamics or to a conformational change may not affect its implications for protein stability. Just as the glass transition results in a state of higher entropy relative to the structure below $T_g$, the protein structure above the protein molecule’s glass transition (dynamical transition) would likely be a structure of higher entropy, as well as a state of greater configurational
mobility. For example, if the protein transitions to a “molten globule” type of structure, both the time-average tertiary structure would change (i.e., partially lost) and the protein internal mobility would increase, both of which have potentially adverse stability consequences. In summary, the H/D exchange data suggest that there is a transition in protein mobility, which is the origin of the pre-$T_g$ endothermic events detected by DSC. Both the DSC endotherm and the H/D exchange transition result from collective small-scale motions within the protein molecules, which increase at the protein dynamical temperature.

**Stability consequences of protein internal dynamics**

Solid protein formulations are normally stored at a temperature, $T$, well below $T_g$ to improve stability during storage. However, often there is no correlation between protein stability and the temperature difference, $T-T_g$, at storage temperatures well below $T_g$. Proteins in solution, in membranes and in crystalline states lose function at or near the protein dynamical temperature ($T_d$). Therefore, more recently, it has been argued that protein formulations should be stored below the dynamical temperature, $T_d$, representing the temperature at which protein molecules gain internal mobility.

The protein dynamical temperature $T_d$ can be modulated through dehydration and lyoprotectants. For example, analysis of the fluctuation in the internal structure of hydrated proteins suggested that changes within the hydration layer, which are coupled to the protein, determine the protein dynamical temperature $T_d$. Additionally, studies of the kinetics of ligand binding of carbon monoxymyoglobin in a dry trehalose system demonstrated that glassy trehalose suppressed the inter-conversion of protein conformational substates in the protein molecule, meaning that protein internal motion...
became limited or locked in the more rigid trehalose matrix \(^4\). Based on these findings, Hill and co-workers suggested that there is a network of hydrogen bonding that connects the protein surface with water and the disaccharide (such as sucrose and trehalose); the composition of the dried product including residual moisture plays an important role in coupling protein internal dynamics with that of the surrounding matrix \(^5\). Therefore, effective coupling between protein internal dynamics and the dynamics of the matrices (potentially evaluated by H/D exchange) should be significant in predicting the long-term stability of protein pharmaceuticals in the solid state. Recently we have started one study on formulated recombinant human serum albumin.

**Conclusion**

Solid-state hydrogen/deuterium (ss-H/D) exchange monitored by FTIR spectroscopy can provide a useful tool to investigate the dynamical transition in protein molecules. The current study extends the set of data using this method to two additional proteins, insulin and hGH. Data and previous interpretations suggest that such studies may well play an important role in quickly predicting differences in storage stability in a series of trial formulations. Clearly, there is a need for extending such studies to formulated freeze dried proteins.

**Acknowledgement**

The authors would like to thank Professor Aichun Dong at the University of Northern Colorado for the helpful discussion on the interpretation of FTIR spectra.
# Tables

Table 1. Summary of transition temperatures of hGH, insulin and BSA as a function of water content.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Physical State</th>
<th>% RH</th>
<th>% H₂O</th>
<th>DSC</th>
<th>H/D Exchange</th>
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<td>°C</td>
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<td>75&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>52</td>
<td>13.6</td>
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<td>2.5</td>
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<sup>a</sup> Obtained from<sup>15</sup> except where noted.

<sup>b</sup> Obtained from<sup>14</sup> except where noted.

<sup>c</sup> Obtained from<sup>16</sup> except where noted.

<sup>d</sup> Temperature midpoint of the thermal events.

<sup>e</sup> Obtained from<sup>13</sup>.

<sup>f</sup> The T<sub>d</sub> values were obtained in the H/D exchange experimental data (Figure 5) from this work.
Figure 1. DSC thermograms for freeze-dried hGH as a function of water content, indicated for each sample in weight percent. The solid samples were hermetically sealed and scanned at 10°C/min from 20 to 180°C. N.B. On re-scanning from room temperature past the end of the pre-T_g endotherm (about 90°C), but well below either the estimated system T_g or the denaturation temperature, the endothermic event was not detectible. (Reproduced from 15)
Figure 2. DSC thermograms of amorphous (solid line) and crystalline (dashed line) insulin equilibrated at 11% RH and 75% RH. The solid samples were hermetically sealed and scanned at 10°C/min from 20 to 180°C. N.B. On re-scanning from room temperature past the end of the pre-$T_g$ endotherm (about 90°C), but well below either the estimated system $T_g$ or the denaturation temperature, the endothermic event was not detectible. (Reproduced from 14)
Figure 3. Selected FTIR spectra of pure hGH (a) and insulin (b) recorded from 30 to 80°C during the H/D exchange process. The amide I band was normalized for comparison among spectra. The intensity decay of the amide II band representing N-H vibrational modes was monitored to estimate the extent of the H/D exchange reaction.
Figure 4. First order decay plot of the ratio of un-exchanged hydrogen (X) at various temperatures for pure hGH (a) and insulin (b) equilibrated in D$_2$O at 11% RH. The first order kinetics of the reaction was quantitatively analyzed by non-linear curve fitting using the following exponential model: $X = X_\infty + (X_0 - X_\infty)\exp(-kt)$, where $t$ is time, $k$ is an apparent rate constant for the observable exchange, $X_\infty$ and $X_0$ are H/D un-exchanged fractions at the apparent plateau level (i.e., nominally infinite time) and $t=0$ ($X_0=1.0$), respectively.
Figure 5. Temperature dependence of the plateau level of H/D exchange ($X_\infty$) in pure hGH (a) and insulin (b), fitted to a sigmoidal function: 

$$X_\infty = (X_{\infty i} - X_{\infty f})/(1 + \exp ((T - T_{mid})/dT) + X_{\infty f},$$

where $X_{\infty i}, X_{\infty f}, T_{mid}, dT$ are $X_\infty$ initial value, $X_\infty$ final value, temperature midpoint of the sigmoidal transition, and $1/4$ width of the transition, respectively. The mid-point of the sharp transition ($T_{mid}$) in pure hGH was $61.7 \pm 0.9^\circ C$, with $dT 6.3 \pm 1.0^\circ C$. The mid-point of the sharp transition ($T_{mid}$) in pure insulin was $56.1 \pm 1.2^\circ C$, with $dT 5.9 \pm 1.3^\circ C$. 
References


Chapter 6

Apparent Protein Internal Dynamics: Investigation of its Implication on Protein Stability by Solid-State Hydrogen/Deuterium Exchange with FTIR Spectroscopy
Abstract

Recent hydrogen/deuterium (H/D) exchange studies suggest that a structural and/or mobility transition in the protein molecule in the solid state, similar to the protein dynamical temperature \( T_d \), exists in a temperature range below the system glass transition temperature \( T_g \). If this transition does indeed represent a protein dynamical temperature in the solid state, one might expect protein stability in the solid to correlate with the difference between storage temperature and this apparent \( T_d \). Solid-state H/D exchange in conjunction with FTIR spectroscopy was used to study this transition in both pure proteins and proteins formulated with disaccharides, and further to evaluate whether this apparent \( T_d \) of the protein molecule can be modulated by coupling with the inert matrix. The hypothesis is that the formulation that provides effective coupling of \( T_d \) with the dynamics of the matrix should demonstrate better long-term stability in the solid state. That is, a formulation with a higher \( T_d \) should show better stability. In this work, we study the impact of formulation on \( T_d \) in recombinant human serum albumin (rHSA) and on stability in freeze-dried sucrose and trehalose formulations. We find rHSA:sucrose showed superior long-term stability at 40°C by size exclusion chromatography (SEC) than the trehalose formulation, but the apparent \( T_d \) was much higher in the trehalose formulation. Both sucrose and trehalose showed equivalent protection of protein secondary structure by FTIR spectroscopy. On the other hand, a lower extent of H/D exchange \( (X_\infty) \) was observed in the sucrose formulation at the temperature of the stability studies (40°C) than found for the trehalose formulation, which was consistent with better stability in the sucrose formulation. Thus, while apparent \( T_d \) did not correlate with protein stability for rHSA, the extent of H/D exchange, \( X_\infty \) did.
Keywords:

Freeze-drying; protein stability; solid-state H/D exchange; protein internal dynamics; protein dynamical temperature; FTIR
Introduction

Freeze-drying is commonly used to improve the stability of proteins, which undergo significant physical and/or chemical degradation in solution. In order to achieve optimal long-term stability, it is recommended that freeze-dried solid proteins should be stored below the glass transition temperature (\(T_g\)) of the entire system (i.e., an assembly of protein molecules or protein molecules dispersed in a matrix). The \(T_g\) of such a molecularly miscible system marks the onset temperature of viscous flow with higher mobility on a large time scale (i.e. 10-10^4 s). However, there is an increasing body of work demonstrating that physical and/or chemical degradations still occur during storage at temperatures well below the system \(T_g\) \(^1\-^3\). Moreover, there is no correlation between protein stability and \(T_g\) at temperatures well below \(T_g\) \(^4\).

Previous studies on pure solid bovine serum albumin (BSA) \(^5\), insulin and human growth hormone (hGH) \(^6\) suggested onset of significant mobility in the protein molecule at temperatures below the system \(T_g\). This mobility was suggested to represent protein internal dynamics, which implies that the temperature at which this transition occurs represents the protein dynamical temperature, \(T_d\), in the dry solid state. The low temperature motion arising from the protein molecule was suggested to be associated with local fluctuations of protein residues on a smaller time scale (i.e. \(10^{-15}\)-\(10^{-1}\) s) \(^1\). Thus, at temperatures exceeding the protein dynamical temperature, there is an increase in local internal motions, which eventually result in larger cooperative motions, and therefore may affect stability of the protein formulations. Evidence of loss in protein function at \(T_d\) has also been reported \(^7\-^9\). \(T_d\) was well studied in systems with high water/polyol content and in sugar/water glassy matrices, but very little work in dry
proteins \textsuperscript{10-12}. The potential stability implication of the protein dynamical temperature in solid protein formulations of pharmaceutical significance has been recently emphasized \textsuperscript{13}.

The $T_d$ can be modulated through the degree of hydration and addition of lyoprotectant \textsuperscript{11-16}. Hill and co-workers suggested that it is the hydrogen bonding interactions between water and the amino acid residues that facilitates the propagation of fast $\beta$-like motions into larger scale $\alpha$-like motions, which are responsible for protein internal dynamics. Studies on the kinetics of ligand binding to carbon monoxymyoglobin in a dry trehalose system demonstrated that the glassy trehalose suppressed the inter-conversion of protein conformation sub-states, suggesting that protein internal motion is limited or locked in the relatively more rigid matrix \textsuperscript{17}. Therefore, effective coupling between protein internal dynamics and the dynamics of the matrices may play an important role in the stability of protein pharmaceuticals.

Hydrogen deuterium (H/D) exchange has been employed extensively to gain a better understanding of protein structural features and conformational dynamics \textsuperscript{18-22}. The rate and extent of H/D exchange can provide a measurement of protein structure and/or dynamics in the protein formulation, which are relevant to stability. Solid-state H/D exchange in conjunction with FTIR was applied previously to study the apparent protein dynamical transition as signaled by the sharp transition in H/D exchange over a small temperature range \textsuperscript{5,6}. In the present study, we applied the same approach and extended the study on protein internal dynamics to proteins formulated with lyoprotectants. Recombinant human serum albumin (rHSA) was used as the model protein. Sucrose and trehalose were chosen as the lyoprotectants since both are commonly used to stabilize
proteins in the solid state. The ultimate goal was to address the question whether or not the best stability during storage would be the formulation in which the apparent $T_d$ is higher.

**Materials and Methods**

**Materials and sample preparation**

Stock solution of ultrapure rHSA (50 mg/mL, Albumin Bioscience, Huntsville, AL) was dialyzed using a 10K MWCO dialysis cassette against 5 mM potassium phosphate buffer (pH 7.0) at 4°C twice for 3 hours and the third time overnight. The recovered solution was filtered through 0.22 μm polyvinylidene difluoride (PVDF) low protein binding filter (33mm in diameter). The concentration of rHSA after dialysis was determined by absorbance at 280 nm (Cary Bio100, Varian Inc., Palo Alto, CA) using extinction coefficient of 0.58 mL/mg·cm$^{23}$. Sucrose or trehalose (Sigma Aldrich, St. Louis, MO) was added to the formulation at 1:1 weight ratio of disaccharide to protein. The protein was formulated at 3 mg/mL in all three formulations.

D$_2$O (99.8% D), lithium chloride (LiCl) (>99%, American Chemical Society grade), and potassium bromide (KBr; 99%, infrared grade) from Acros Organics (Morris Plains, NJ) were used for the H/D exchange study.

**Freeze-drying procedure**

Aliquots of one mL of each sample solution were filled in 5 mL glass tubing vials and were partially stoppered with Daikyo Fluorotec stoppers (West Pharmaceutical
to minimize moisture transfer during storage. All vials containing the samples were placed on the center of the shelf surrounded by 5% (w/w) sucrose solutions to avoid any radiation effect from the edge and front of the chamber and maintain comparable product temperatures. Product temperature was monitored with a thermocouple glued with heat sink silicone grease (Chemplex 1381, FUCHS lubricants, Harvey, IL) and Kapton tape (Cole-Parmer, Vernon Hills, IL) to the outside of selected vials near the vial bottom. The samples were freeze-dried according to the recipe (Table 1) and then stoppered, sealed and stored in the -20°C freezer for further analysis.

**Physical stability of rHSA formulations during storage by SEC**

Freeze-dried rHSA samples were equilibrated at 11% RH overnight and stored at 40°C for 6 months. The samples after each time point (0, 1, 3, 6 month) were reconstituted with 1 mL of water (18 MΩ, distilled and deionized, Barnstead™ GenPure™, Thermo Scientific, Waltham, MA). No precipitation or visible particle was observed after reconstitution. The entire volume of reconstituted solutions was filtered with 0.22 µm PVDF filters and 50 microliters of each sample were injected onto a column (TSKgel™ G3000SWXL, 7.8 mm x 30 cm, 5 µm, Tosoh Bioscience, San Francisco, CA) maintained at 25°C. The mobile phase (100 mM sodium phosphate buffer with 0.2 M NaCl at pH 7.0) was delivered at 1.0 mL/min by an HPLC system (Model 1100, Agilent, Santa Clara, CA). The absorbance of the effluent was measured at 280nm (variable wavelength detector, G1315A, Agilent). Absorbance data were acquired and analyzed (ChemStation, Agilent). Monomer, dimer, and higher order aggregates were identified. Percent soluble aggregate represents the areas under the dimer and higher
order aggregate peaks relative to total area of the identified peaks.

Protein aggregation follows the square root of time kinetics \(^2\). Therefore, the rate constant of aggregation was determined by fitting the stability data in the equation below.

\[
%P = %P_0 + k\sqrt{t}
\]

%P, %P\(_0\), and k represent the present soluble aggregates at time t, time 0 (after freeze-drying), and the rate constant of aggregation, respectively.

**Secondary structure of the rHSA formulations**

Spectra of the protein samples were recorded with a FTIR spectrometer (Nicolet Magma 560, Thermo Scientific, Madison, WI) in single beam mode with 128 scans and 4 cm\(^{-1}\) resolution over a range of 400-4000 cm\(^{-1}\). All spectra were processed using the software Grams/AI 8.0 (Thermo Electron, Madison, WI). Secondary structure of the rHSA formulations was characterized from the amide I band in the region 1600-1700 cm\(^{-1}\) of the FTIR spectra. This region primarily consists of C=O stretch mode, which is well known to be sensitive to protein secondary structure. Second derivative analysis of the amide I region was carried out to resolve the underlying peak components in this region.

**H/D exchange process to evaluate the dynamics of the protein formulations**

The solid-state H/D exchange process for the freeze-dried rHSA formulations followed the same procedure developed in previous studies \(^5,6\). Briefly, the freeze-dried samples were incubated in a desiccator maintained with 11% RH using saturated LiCl solution in D\(_2\)O. The desiccators were sealed under vacuum and stored at various temperatures from 30 to 80°C. The H/D exchange reaction was quenched by drying the
samples in the vacuum oven at room temperature overnight. The time course (over 4
days) of the H/D exchange process at each temperature was determined from FTIR
measurement. All measurements were performed in duplicate.

0.6 mg of each freeze-dried protein sample was mixed with 150 mg of KBr in a
glove bag where humidity was controlled below 2% by purging dry air. Spectra of the
protein samples were recorded with a FTIR spectrometer. The extent of H/D exchange
was based on the intensity change of the amide II peak at 1535 cm$^{-1}$, which represents N-
H vibration mode. The calculation was based on studies as previously described $^{5,6}$.

**Data analysis on the H/D exchange process**

Regression analysis was performed in the H/D exchange process using the same
approach employed in a previous study $^{6}$. Briefly, the fraction of protons remaining to be
exchanged, X, versus time was evaluated over 4 days in each formulation and fitted by a
first-order decay function as previously described $^{6}$ to find X$_{\infty}$ (the “plateau un-
exchanged fraction” of hydrogens on the protein) and its error. The value of X$_{\infty}$ at each
temperature with its error was fitted by a sigmoidal function $^{6}$ to find the temperature
midpoint of the transition (inflection point) and its associated error.

**Results and Discussion**

*Aggregation stability of the freeze-dried rHSA formulations during storage*
To evaluate any possible correlation between the protein apparent dynamical temperature \( (T_d) \) and the protein stability in the solid state, sucrose or trehalose with distinct glass transition temperatures were added to the rHSA formulation. Physical degradation, specifically formation of soluble aggregates of the rHSA formulations were compared during storage at 40°C and 11% RH. Protein aggregation followed square root of time kinetics. The rate constant for protein aggregation, \( k \) \((\% \cdot months^{-1/2})\), was determined from the slope of the linear fit (Figure 1). As expected, rHSA alone was least stable with a rate constant of 12.5% \( \cdot months^{-1/2} \). Both disaccharides stabilized rHSA reducing the rate constants to 3.6 and 1.2% \( \cdot months^{-1/2} \) for trehalose and sucrose, respectively (Figure 1).

**The effect of sucrose and trehalose on stabilizing protein formulations during freeze-drying**

Both sucrose and trehalose are known to stabilize protein formulations after freeze-drying \(^4\). The “water substitute hypothesis” describes stabilization by the disaccharide through hydrogen bond formation with the protein molecule, whereas the “glass dynamics hypothesis” explains stabilization through reduced mobility in the rigid matrix \(^4\). The addition of sucrose to a protein formulation results in a decrease in \( T_g \) compared to protein alone \(^2,24,25\) which is contrary to its stabilizing effect based on the simple interpretation of the “glass dynamics hypothesis”, which suggests that the system with the higher \( T_g \) will be more stable. The stabilization effect by sucrose is not necessarily in conflict with a more general interpretation of the impact of glass dynamics
on stability. It is possible that rather than the system $T_g$, the protein dynamical temperature ($T_d$) may be a more appropriate measurement to determine stability, which can be modulated by the excipients.

Better solid-state stability in a sucrose formulation compared to a trehalose formulation was also reported for IgG, hGH and lysozyme (Table 2). It was suggested that lower amplitude motions associated with “fast dynamics” in the hGH:sucrose formulation (on a nanosecond scale) as measured by neutron scattering correlated with the better stability compared to trehalose. Micro-phase separation of trehalose formulations was another proposed mechanism of the lower stability in trehalose than in sucrose. In contrast, sucrose and trehalose formulations provided comparable solid-state stability of catalase, interleukin-2, and a recombinant humanized monoclonal antibody (rhu-mAb) when stored at a temperature below the glass transition temperature of the formulation (Table 2). However, better solid-state stability in trehalose than in sucrose was found in hGH and a mAb when stored at a condition that the glass transition temperature of the sucrose formulation was below or equal to the storage temperature (Table 2).

**Secondary structure of the rHSA formulations**

Protein aggregation often requires protein structural perturbation. The rHSA:sucrose formulation showed superior physical stability during long-term storage than the trehalose formulation (Figure 1). To evaluate whether the difference in aggregation is associated with conformational stability (i.e. protein structure) between the
two formulations after freeze-drying, the secondary structure of the freeze-dried samples was characterized using the second derivative of the amide I region in the FTIR spectra. rHSA consists of predominantly α-helix in its secondary structure (1654 cm\(^{-1}\)). Both the sucrose and trehalose formulations protected rHSA from severe perturbation in secondary structure due to the freeze-drying process, in the sense that a much sharper α-helix peak with higher intensity was evident in the disaccharide formulations compared to rHSA alone (Figure 2). However, there were no differences in key features of the native α-helix band such as peak position or intensity of the sucrose and trehalose formulations. Similarly, in freeze-dried hGH:dissachride 1:6 (w:w), where the aggregation rate constant was about a factor of two less in the sucrose formulation than in the trehalose formulation\(^2\), there was no difference in their secondary structure by FTIR spectroscopy. Although there was no difference in secondary structure within the sensitivity of the FTIR spectrometer, it is possible that minor tertiary structural alteration may exist that cannot be captured by FTIR spectroscopy.

**Investigation of protein dynamical temperature using H/D exchange**

In previous studies, low-temperature endothermic events (40–60°C) below the \(T_g\) of the protein were observed in differential scanning calorimetry (DSC) in solid pure BSA, insulin and hGH without excipients\(^5,6,26\). It was proposed that the endotherms potentially represented enthalpy recovery of a protein dynamical transition within the protein molecule. The temperature of the enthalpy recovery correlated well with a transition temperature found using H/D exchange monitored by FTIR, supporting the suggestion that the low temperature endotherms were associated with a protein dynamical transition temperature.
Based on extensive literature on the protein dynamical transition in hydrated systems\textsuperscript{1}, Hill, Shalaev and Zografi hypothesized that effective coupling between the internal dynamics within the protein molecule and a stabilizer could provide better the long-term stability of the formulation. However, no experimental studies have tested this hypothesis in the solid state. To explore the impact of this transition on protein stability in solid state, we evaluated $T_d$ of the three formulations, rHSA alone, rHSA:sucrose 1:1 (w:w) and rHSA:trehalose 1:1 (w:w). Pre-$T_g$ events were previously reported in hGH:disaccharide formulations\textsuperscript{26}; however, the events were attributed to the disaccharide itself, which has a pre-$T_g$ signal in the same temperature range, due to a distribution of relaxation times within the non-equilibrium amorphous solid system. Therefore, a pre-$T_g$ endothermic event in the protein molecule, could not be uniquely identified from the DSC scan, at least when the protein formulation contains sucrose or trehalose. However, any change in the protein mobility/structure with temperature, which can be monitored by the H/D exchange at the amide nitrogen would be not be complicated by the presence of a stabilizer such as sucrose or trehalose, at least in theory.

In the present study, a change in the mobility of the protein was monitored by the extent of H/D exchange, not on the rate of H/D exchange. The rate of H/D exchange would potentially be complicated by the presence of the stabilizer. The extent of H/D exchange on the protein molecule was based on the intensity change of the amide II peak at 1535 cm\textsuperscript{-1}, which presents N-H stretch mode. The absorption spectra of sucrose and trehalose were in the range of 1400 to 400 cm\textsuperscript{-1} (C-O stretch), and above 3200 cm\textsuperscript{-1} (O-H stretch); neither overlapped with the protein amide II. To further evaluate any interference of sucrose and trehalose with FTIR monitoring of H/D exchange, the
spectrum of freeze-dried amorphous sucrose was subtracted from the spectrum of rHSA:sucrose. The intensity of Amide I band was used for normalization. The analytical peak (amide II at 1535 cm\(^{-1}\)) was unaffected by subtraction of the sucrose spectrum (Figure 3). Similarly, trehalose did not affect the amide II band (data not shown). Therefore, all analysis was performed using the spectral data without subtraction of the stabilizer spectra, following a previously established methodology.\(^5,6\)

Freeze-dried rHSA samples with or without stabilizer were equilibrated with 11% RH D\(_2\)O over a range of temperature. Samples were taken each day for 4 days to determine the equilibrium extent of H/D exchange. High variability in the preliminary data was observed for the pure rHSA (3 mg/mL) samples taken from the same or different vials. Some of the variations may have come from inter-vial variation in ice nucleation temperature, leading to inter-vial variation in protein structure as reported elsewhere.\(^27\) The high variation was not observed in the sucrose or trehalose formulation. Therefore, to reduce the variation in the pure rHSA sample, a higher concentration of pure protein, (i.e., 25 mg/mL compared to 3 mg/mL in the presence of stabilizers) was freeze-dried such that all pure protein samples used for the H/D exchange were taken from the same vial (well mixed) to ensure uniformity in structure in the pure protein samples.

The H/D exchange was monitored in the FTIR spectra as a function of temperature. Since H/D exchange can be a measure of both protein structure and dynamics, a greater extent of exchange is expected at a higher temperature when the interior of the protein molecule is exposed to the D\(_2\)O vapor, and/or when there is greater fluctuation in protein molecular structure (i.e., faster dynamics). The amide II band at
1445 cm\(^{-1}\) represents the N-D vibrational mode. However, the contribution from H-O-D vibration mode at 1460 cm\(^{-1}\) interferes with this signal\(^{22}\). Instead, the amide II band at 1535 cm\(^{-1}\), representing the N-H vibrational mode, was used to monitor the extent of H/D exchange. The amide I band at 1655 cm\(^{-1}\), which is not influenced by the H/D exchange process was used for normalization of the spectra. At higher temperatures, the intensity of amide II band at 1535 cm\(^{-1}\) decreased, suggesting enhanced H/D exchange due to perturbation in protein structure or increase in mobility within the protein molecule (Figure 4).

The fraction of hydrogens remaining un-exchanged (denoted as X) at any time is the ratio of the intensity of amide II (1535 cm\(^{-1}\)) relative to its intensity before exchange. The time course of the H/D exchange process was treated empirically as a first-order exponential decay (Figure 5). The extent of exchange at infinite time (X\(_\infty\)), was determined from the regression analysis using Equation 5 of Mizuno and Pikal\(^5\). The amide hydrogens remaining unexchanged (X\(_\infty\)) were not dynamically accessible by the D\(_2\)O vapor on the time scale (4 days) of the experiment, as they are presumably buried in the “rigid” interior of the protein-folded structure. Thus, the goal was to use the X\(_\infty\) versus temperature to quantitatively interpret the temperature dependence of protein dynamics.

As in previous studies\(^5,6\), a sigmoidal function was fitted to the temperature dependence of X\(_\infty\). The mid-point of the transition (T\(_{\text{mid}}\)), nominally equivalent to the dynamical transition temperature (T\(_d\)) of the protein molecule, was obtained from the sigmoidal function (Figure 6). According to the hypothesis of Hill, Shalaev and Zografi, the formulation that yields a higher T\(_d\) of the protein would be effectively coupled to the
protein and provide better stabilization. The transition region for \( X_\infty \) with temperature for pure rHSA was broad ±6 °C (compared to the previously reported transition in insulin and hGH \(^6\)) with a midpoint, or nominally \( T_d \) of 56 °C (Figure 6). The transition in \( T_d \) in rHSA:sucrose was at 62.3°C (±3.3°C), higher than that of pure rHSA. This observation was qualitatively consistent with the well-known stabilizing effect of sucrose. However, the \( X_\infty \) versus temperature for rHSA:trehalose was not sigmoid in shape within the temperature range from 30 to 100°C. Perhaps the \( T_d \) of rHSA:trehalose is above this temperature range, beyond what was experimentally accessible, due to the use of the 11% RH chamber. However, if the protein in trehalose does have an inflection point associated with a \( T_d \) or other relevant transition, it is clearly higher than when the protein is formulated with sucrose.

If the hypothesis on protein dynamical temperature (\( T_d \)) were true, the trehalose formulation would be expected to be more stable than the sucrose formulation owing to the presumably higher \( T_d \) of the protein in trehalose. However, clearly the rHSA:sucrose formulation was more stable than the trehalose formulation with a degradation rate constant only 1/3 that of the trehalose formulation (Figure 1). The stability data in the present study is not consistent with the hypothesis that trehalose should provide better stability due to a higher \( T_d \). Perhaps \( T_d \) is not a predictive parameter for stability in the rHSA system. Further studies with other protein systems formulated with other excipients that lead to distinct \( T_d \) values are needed to fully understand this \( T_d \) concept, particularly in the solid state.
The extent of H/D exchange

Neither apparent $T_d$, nor the secondary structure correlated with stability of rHSA:disaccharide formulations during storage. The extent of H/D exchange ($X_e$), which provides another measure of structure or dynamics in the protein formulation was compared between the sucrose and trehalose formulation, which was used in recent H/D exchange studies $^{28,29}$. There was a trend of lower extent of H/D exchange in the rHSA:sucrose formulation at 40°C, suggesting better stability in the sucrose formulation (Figure 7) at the stability storage temperature of 40°C. At temperatures above 60°C, the trend was reversed with less H/D exchange in the trehalose formulation, likely because the temperature was reaching the glass transition region for the sucrose formulation but not the trehalose formulation. We note that sucrose:hGH (1:1) at 11% RH has a $T_g$ of 58°C, but the corresponding trehalose formulation has a $T_g$ of 70°C. Thus, the extent of H/D exchange in the formulation at the lower temperature of 40°C seemed to predict protein stability. Both sucrose and trehalose are well known as very good lyoprotectants in protein formulations, providing essentially identical stability results in some cases and a modest increase in stability in the sucrose formulation for several cases, including hGH $^2$, IgG $^{25}$ and rHSA. H/D exchange seems to have sufficient sensitivity to predict such modest changes in stability. Recently, Moorthy and colleagues demonstrated strong correlation between the deuterium incorporation at time 0 using H/D exchange-mass spectrometry (HDX-MS) and the extent of aggregation during storage in myoglobin $^{28}$ and monoclonal antibody $^{29}$ formulated with excipients with stabilization propensities very distinctive from each other (i.e. sucrose, mannitol, NaCl). Our results are consistent with theirs but with systems with much smaller stability differences. Previously, fast
dynamics by neutron scattering correlated with the stability difference between the sucrose and trehalose formulations in hGH $^2$. Potentially these methods, H/D exchange and “fast dynamics” can be used to capture small differences in proteins formulated with similar stabilization effects, and therefore to predict modest protein stability differences in the solid state.

**Summary and Conclusions**

We have observed what appears to be a protein dynamical temperature ($T_d$) $^{1,13}$ in solid state proteins below the system glass transition temperature $^{5,6}$, which means a cooperative transition in mobility within the protein molecule. Solid-state H/D exchange in conjunction with FTIR spectroscopy was used to study this phenomena in both pure proteins and proteins formulated with disaccharides, and further to evaluate whether the apparent $T_d$ of the protein molecule can be modulated through coupling with the inert matrix (sucrose or trehalose). A higher $T_d$ should mean a more stable protein formulation. However, rHSA:sucrose showed superior long-term stability at 40°C by SEC than the trehalose formulation but exhibited a lower apparent $T_d$. Further, while both sucrose and trehalose showed equivalent protection of protein secondary structure by FTIR spectroscopy, the extent of exchange at nominal time infinity, ($X_\infty$) was less for sucrose than for the trehalose formulation at 40°C, suggesting that at least at 40°C, the protein structure and/or dynamics was more native/rigid in the sucrose formulation. Although the apparent $T_d$ did not correlate with protein stability in rHSA systems, the extent of H/D exchange did correlate, suggesting $X_\infty$ can potentially be a useful predictor in stability of the freeze-dried protein during storage, consistent with data in the literature.
Further studies with other protein systems formulated with excipients that lead to distinct $T_d$ values are needed to fully understand the where or not the apparent $T_d$ is indeed a protein dynamical temperature in the solid state, and if in other systems, this property does indeed correlate with stability.
Table 1. Freeze-drying recipe

<table>
<thead>
<tr>
<th>Step</th>
<th>Freezing</th>
<th>Primary drying</th>
<th>Secondary drying</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>5</td>
<td>-5</td>
<td>-40</td>
</tr>
<tr>
<td>Ramp rate (°C/min)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hold time (min)</td>
<td>30</td>
<td>30</td>
<td>180</td>
</tr>
<tr>
<td>Vacuum (mTorr)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* The process was advanced to secondary drying when the Pirani gauge signal converged with the capacitance manometer at 60 mTorr.
Table 2. Comparison of stability of proteins formulated with sucrose or trehalose.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Process condition</th>
<th>Storage condition</th>
<th>Order of stability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGH</td>
<td>Freeze-drying</td>
<td>40°C</td>
<td>Sucrose &gt; trehalose</td>
<td>2</td>
</tr>
<tr>
<td>hGH</td>
<td>Freeze-drying</td>
<td>50°C, 22% RH</td>
<td>Sucrose &lt; trehalose</td>
<td>$^a$ 2</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Spray-drying</td>
<td>NA</td>
<td>Sucrose &gt; trehalose</td>
<td>30</td>
</tr>
<tr>
<td>IgG</td>
<td>Freeze-drying</td>
<td>50°C</td>
<td>Sucrose &gt; trehalose</td>
<td>25</td>
</tr>
<tr>
<td>rHSA</td>
<td>Freeze-drying</td>
<td>40°C, 11% RH</td>
<td>Sucrose &gt; trehalose</td>
<td>this work</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Freeze-drying</td>
<td>NA</td>
<td>Sucrose ≈ trehalose</td>
<td>31</td>
</tr>
<tr>
<td>Catalase</td>
<td>Freeze-drying</td>
<td>NA</td>
<td>Sucrose ≈ trehalose</td>
<td>31</td>
</tr>
<tr>
<td>mAb</td>
<td>Freeze-drying</td>
<td>40°C</td>
<td>Sucrose ≈ trehalose</td>
<td>32</td>
</tr>
<tr>
<td>mAb</td>
<td>Freeze-drying</td>
<td>60°C</td>
<td>Sucrose &lt; trehalose</td>
<td>$^b$ 32</td>
</tr>
<tr>
<td>Interlukin-2</td>
<td>Freeze-drying</td>
<td>45°C</td>
<td>Sucrose ≈ trehalose</td>
<td>33</td>
</tr>
<tr>
<td>rhu-mAb*</td>
<td>Freeze-drying</td>
<td>30°C</td>
<td>Sucrose ≈ trehalose</td>
<td>34</td>
</tr>
</tbody>
</table>

* rhu-MAb represents recombinant humanized monoclonal antibody

$^a$ $T_g$ of the sucrose and trehalose formulations were 36°C and 50°C, respectively, when stored at 50°C, 22% RH.

$^b$ $T_g$ of the sucrose and trehalose formulations were 59°C and 80°C, respectively, when stored at 60°C.
Figure 1. % Soluble aggregation by SEC in the three formulations, rHSA (circle), rHSA:sucrose 1:1 (w:w) (triangle), and rHSA:trehalose 1:1 (w:w) (square). The samples were equilibrated with 11% RH and then stored at 40°C for 6 month. Error bar represents standard error from 3 measurements.
Figure 2. Second derivative of amide I region in the FTIR spectra of the three formulations after freeze-drying. This process allows the underlying components that overlap in the amide I region to be visually resolved. rHSA consists of predominantly α-helix structure with a peak at 1654 cm$^{-1}$. The remaining structural elements are β-sheet and random turns. rHSA was formulated at 3 mg/mL in three formulations: rHSA alone, rHSA:sucrose 1:1 (w:w), rHSA:trehalose 1:1 (w:w).
Figure 3. FTIR absorption spectra of freeze-dried rHSA:sucrose formulation before (solid line) and after subtraction of the sucrose spectrum (dashed line). The intensity of amide I band (1655 cm$^{-1}$) was normalized for comparison. The subtraction procedure did not affect the intensity of the amide II band at 1535 cm$^{-1}$, which was further used to calculate the extent of H/D exchange.
Figure 4. Representative FTIR spectra of freeze-dried rHSA (left), rHSA:sucrose (middle) and rHSA:trehalose (right) recorded from 30 to 90°C after 4 days of the H/D exchange process. The amide I band was used to normalize the spectra. The decay in peak intensity of the amide II band (1535 cm\(^{-1}\)) representing N-H vibrational modes was monitored to determine the extent of the H/D exchange reaction.
Figure 5. First order decay plot of the ratio of un-exchanged hydrogens (X) at various temperatures for rHSA (left), rHSA:sucrose (middle) and rHSA:trehalose (right) equilibrated in D$_2$O at 11% RH. The first order kinetics of the reaction was quantitatively analyzed by non-linear curve fitting using the following exponential model: \( X = X_\infty + (X_0 - X_\infty) \exp(-kt) \), where \( t \) is time, \( k \) is an apparent rate constant for the observable exchange, \( X_\infty \) and \( X_0 \) are H/D un-exchanged fractions at the apparent plateau level (i.e., nominally infinite time) and \( t=0 \) (\( X_0=1.0 \)), respectively.
Figure 6. Temperature dependence of the plateau level of H/D exchange ($X_{\infty}$) in pure rHSA (left), rHSA:sucrose (middle) and rHSA:trehalose (right), fitted to a sigmoidal function: 

$$X_{\infty} = (X_{\infty i} - X_{\infty f}) / (1 + \exp ((T - T_{mid})/dT) + X_{\infty f},$$

where $X_{\infty i}$, $X_{\infty f}$, $T_{mid}$, and $dT$ are $X_{\infty}$ initial value, $X_{\infty}$ final value, temperature midpoint of the sigmoidal transition, and $1/4$ width of the transition, respectively. The mid-point of the sharp transition ($T_{mid}$) in pure rHSA was $56.2 \pm 6.3^\circ C$. The mid-point of the transition ($T_{mid}$) in the rHSA:sucrose formulation was, $62.3 \pm 3.3^\circ C$. No sigmoidal transition was found in the rHSA:trehalose formulation within the temperature range 30-100^\circ C.
Figure 7. Fraction of un-exchanged hydrogens at infinite time ($X_\infty$) at selected temperatures for the two formulations, rHSA:sucrose and rHSA:trehalose. Error bars represent the standard deviation from two replicates.
References


Lyophilization is a commonly used approach to improve stability of pharmaceutical proteins when they are not sufficiently stable in solution due to physical or chemical degradation. However, there are several challenges in the design of freeze-drying process and formulation approaches inhibiting the ability to deliver final products with uniformly improved stability in an efficient manner. These challenges include the natural variation in ice nucleation temperature, lack of understanding of the critical factors for protein stability after the ice nucleation step, as well as the potential role of protein internal dynamics on stability of proteins formulated with excipients. The goal of this work was to understand how the freeze-dried protein quality attributes could be affected by these stress factors.

Work presented in Chapter 2 and 3 investigated how the freezing protocols can affect protein stability during freeze-drying. Large variations exist in ice nucleation temperature across the batch. A lower ice nucleation temperature results in smaller ice crystal size, and hence larger surface area between the growing ice and the freeze-concentrate where proteins can adsorb and denature. Moreover, the variation in ice nucleation temperature can result in heterogeneity in protein stability. Few controlled ice nucleation reports have focused on protein stability. In Chapter 2, a focused study was performed on the effect of controlling ice nucleation temperature on stability of a model protein known to be sensitive to the ice surface, lactate dehydrogenase (LDH), formulated without any stabilizer. Both average stability of LDH within a batch and homogeneity in stability were evaluated when the samples were frozen at various controlled ice nucleation temperatures as compared to the freezing condition without ice nucleation control. This study provides direct evidence of improved protein stability
during freeze-thawing when ice nucleation was controlled at a higher temperature. This work provides the first report that demonstrates improved batch uniformity in protein stability using the controlled ice nucleation technology, ControLyo™.

Very little information is available on controlled nucleation’s effect on the stability of proteins formulated with excipients. **Chapter 3** extended the study of ice nucleation temperature in proteins formulated with excipients. Two model proteins were used in the study including intravenous immunoglobulin (IVIG) and recombinant human serum albumin (rHSA) and were formulated with or without the presence of a stabilizer, sucrose. The degree of improvement in protein stability when controlling ice nucleation at a higher temperature appeared to be protein-dependent. The second part of the investigation in Chapter 3 was to understand the critical factors attributed to protein stability post ice nucleation. Two opposing effects (ice surface area and residence time in the freeze-concentration) were proposed to affect protein aggregation. The shelf ramp rate and isothermal hold were varied after ice nucleation while maintaining the same ice nucleation temperature. Although the theory has been discussed previously, this study provided experimental evidence in supporting the residence time in the mobile, more freeze-concentrated environment as an important factor in protein aggregation post ice nucleation. The results are directly relevant to process design in bulk freezing and thawing when the freezing time is much longer. In addition to stability, characteristics of the final solid including morphology and porous structure were characterized at various freezing conditions using the polymer encapsulation method, mercury intrusion porosimetry method and specific surface area measurements. The findings suggest that it
is not only the ice nucleation temperature, but also the thermal history post ice nucleation that define the surface area of ice and the porous structure of the freeze-dried cake.

Further, a distribution in ice nucleation temperature within a batch was observed even when ice nucleation was controlled at a fixed time and shelf temperature. Based on this observation, Chapter 4 attempted to address whether there is a shelf location-dependent resistance to mass flow ($R_p$) during primary drying, due to the distribution in ice nucleation temperature. In existing literature, the resistance to mass flow during primary drying, product resistance ($R_p$) was determined as a batch average. The first step was to calculate $R_p$ in individual vials. The calculation using cycle data was described and challenges in using this method were addressed. Finally, correlations were established between ice nucleation temperature, specific surface area (SSA), and $R_p$ in center vials (in which the calculated $K_v$ in the sample solution was found reliable). The correlations were further used to estimate $R_p$ in samples placed at other locations of the shelf (i.e. edge vials). This study filled the gap in the area of understanding whether $R_p$ is dependent on the location of the vial and provides insights in developing methods to accurately measure real-time $R_p$ in individual vials.

Endothermic events were previously reported in DSC scan in pure insulin and hGH in the solid state; the events occurred at temperatures well below the glass transition temperature of the system, referred to as pre-$T_g$. Chapter 5 discussed and studies the origin of the pre-$T_g$ endothermic events. Enhanced internal motion on the protein molecule in that temperature range was hypothesized as the underlying cause of the pre-$T_g$ events. This phenomenon, protein internal dynamics, was studied by solid-state hydrogen/deuterium (H/D) exchange in conjunction with FTIR spectroscopy. The results
showed a sigmoidal transition in H/D exchange in the same temperature range as the pre-\( T_g \) endothermic events. This work presented a new concept in dry proteins based on existing knowledge about the dynamics on the protein molecule well studied in systems with high water/polyol content and in sugar/water glassy matrices.

**Chapter 6** is a continued exploration on whether the protein internal dynamics studied in Chapter 5 has any stability consequence in protein formulations in the solid state. Soluble aggregation during storage was evaluated in rHSA freeze-dried with sucrose or trehalose. Solid-state H/D exchange with FTIR spectroscopy was used to determine the dynamical temperature (\( T_d \)) in each formulation. It was found that rather than \( T_d \), the lower extent of H/D exchange in the sucrose formulation correlated with its better stability compared to the trehalose formulation. Further work using other proteins and/or excipients is needed to fully understand the implication of apparent \( T_d \) on protein stability in the solid state.

**Significance**

With the correlation established between protein stability and ice nucleation temperature in the laboratory, the degradation of proteins in manufacturing arising from ice nucleation temperature differences can be quantitatively predicted. The capability of improving batch homogeneity by controlling uniform ice nucleation provides potential advantages in scaling-up from lab to manufacturing scale. Fundamental understanding of the degradation stresses during freezing, the implication of protein internal dynamics and interactions with the formulation matrix will provide information critical to the efficient formulation and process design.