Mechanisms of Protein Stability in Lyophilized Samples

by

Yemin Xu

B.S., University of Science and Technology of China (USTC), China, 2008

A thesis submitted to the
Faculty of the Graduate School of the
University of Colorado in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy
Department of Biochemistry
2014
This thesis for the degree of Doctor of Philosophy entitled:
Mechanisms of Protein Stability in Lyophilized Samples
written by Yemin Xu
has been approved for the Department of Biochemistry

Dr. Theodore W. Randolph

Dr. John F. Carpenter

Dr. Xuedong Liu

Date___________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
ABSTRACT

Xu, Yemin (Ph.D., Biochemistry)

Mechanisms of Protein Stability in Lyophilized Samples

Thesis directed by Professor Theodore W. Randolph

Lyophilization is often the choice for therapeutic proteins when an aqueous formulation is not sufficiently stable to achieve the desired shelf life. Lyophilization incorporates molecules in rigid and inert glassy matrices by added excipients, where proteins generally exhibit slower degradation kinetics. Unfortunately, both physical and chemical degradations may still occur at unacceptable rates, and the factors that control the rates of these degradations are not well understood. There are a few properties often considered critical to the stability of proteins: a) the extent of native structural retention during lyophilization, b) the dynamic properties of the glassy matrix, c) phase separation, and d) protein adsorption onto glass-air interface.

In this work, we investigated the mechanisms of protein stability in lyophilized sample using two model proteins: recombinant human growth hormone (rhGH) and keratinocyte growth factor-2 (KGF-2). First, we examined formulations of rhGH (helix dominant protein) combined with disaccharides (sucrose or trehalose) and various amounts of hydroxyethyl starch (HES), which upon lyophilization yielded glasses with a wide range of retention of native protein structure and glass transition temperatures, inverse mean square displacements <u2>^-1 for hydrogen atoms (fast β relaxation), and the relaxation time τ^{β}, which correlates with relaxation due to fast
Johari-Goldstein motions. We found that protein structure and local mobility in the glass were strongly correlated with rate constants for aggregation, deamidation and oxidation. Second, we tested lyophilized formulations of KGF-2 (beta trefoil structure). Although degradation rates were generally decreased in formulations with greater native state structural retention and with reduced fast $\beta$ relaxations, these two factors could not account quantitatively for the aggregation and chemical degradation rates of KGF-2 observed. Rather, it appears that the fraction of protein found at the glass solid-air interface is a dominant factor governing protein degradation in freeze-dried formulations. Finally, we lyophilized rhGH using five different methods, which yielded glassy solids with different surface protein contents, glassy state mobilities and degrees of retention of native secondary structure. We found that the extent of rhGH degradation during storage can largely be ascribed to the resulting levels of rhGH found at the solid-air interface after lyophilization.
DEDICATION

To my mother and father, Xiujuan Shen and Jifa Xu, who always love me and trust me!

To my wife Lingling Chen and our newborn daughter Chloe Xu, your smile is my greatest happiness!

To my uncle Xinfa Xu, who has been supporting me through my life!

To my advisors, Ted Randolph and John Carpenter, for preparing me to be a person with scientific integrity and critical thinking!
ACKNOWLEDGMENTS

First of all, I thank my advisor Ted, for supporting me to work on this thesis, for his amazing insights on everything, and for his trust and encouragement all the time! I also want to thank Ted and John, for their tremendous help to start my next career! And a big thank you to the rest of my thesis committees: Xuedong Liu, Amy Palmer, and Joel Kaar! Thank you for the helpful discussions, questions and ideas. I appreciate the time you took to serve on my committee.

I would also like to thank the funding from NIH/NIBIB under grant R01 EB006398-01A1, and thank you to our friendly collaborators: Mikal Pikal, Marc Cicerone and Tom Anchordoquy’s group. Thank you for the stimulating discussion on my manuscripts.

To all of my labmates, colleagues and undergraduate researchers, I am so lucky to have the opportunity to work with you. In particular, thanks to Amber Haynes Fradkin, who has helped me since I first joined Randolph’s group, and has always been helping me with everything.

Thanks to all the friends who have interacted with me in the other part of my life. It is always nice to share the happiness and stories with you.

Last but not least, the biggest thanks of all goes to my wife Lingling Chen, and our newborn daughter Chloe Xu. Thank Lingling for believing and supporting me all the time; thank my little Chloe, you add tremendous fun into my life. I love my family, and I am so grateful that I have you in my life.
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CHAPTER 1

Introduction

1.1 Motivation and significance

Protein therapeutics offer unique and critical treatments for human diseases. However, to fully realize this potential, protein drugs must be stabilized against physical and chemical degradation during manufacturing, processing, shipping and storage (Carpenter et al., 1997). Protein stability is drawing more and more attention today in the pharmaceutical field and will continue to be as a hot topic as the number of therapeutic protein drugs is increasing every year. Degradation of the protein products may cause loss of protein structure and activity, unexpected immune response and thus compromise their safety and efficacy (Chi et al., 2003; Fradkin et al., 2009; Rosenberg, 2006). The shelf life required for a typical protein pharmaceutical product is 18–24 months. In general, it is challenging to achieve this goal, as proteins are only marginally stable and highly susceptible to physical and chemical degradations. To overcome the instability barrier, one of the most effective strategies for stabilizing therapeutical protein against various degradations is using lyophilization to store protein in a sugar-based dried glass (Carpenter et al., 1997; Chang and Pikal, 2009). Indeed, the importance of lyophilization technology can be appreciated by considering approximately 50% of current marketed biopharmaceuticals are stabilized in sugar-based glasses (Kasper and Friess, 2011).

However, storage of protein in a glassy matrix does not always guarantee an adequate shelf life (Carpenter et al., 1997; Xu et al., 2013). Protein degradations, such
as aggregation or chemical degradation, still occur at unacceptable rates. Despite decades of practical application, the mechanisms that dictate protein stability in the dry, glassy state are still not completely understood (Chang and Pikal, 2009). It is not clear what factors most strongly influence the rates of these degradation processes. In order to rationally design formulations to reduce degradation rates in glassy states, it would be desirable to understand the factors that control the degradation processes. And this outcome will have tremendous benefits to pharmaceutics development and patients’ wellness.

1.2 Backgrounds and competing theories

Solid protein formulations can improve storage-stability and ease shipping and transportation processes, by removing most reactive and abundant species-water (Wang, 2000). There are several techniques that could result in solid-state products, such as lyophilization, spray drying, spray freeze drying, vacuum foam drying and supercritical fluid technology (Ajmer and Scherliess, 2014; Schwegman et al., 2005). However, each technique has its own advantages and disadvantages. Spray drying, which is often used in the food industry, involves atomization of the protein solution through a nozzle into a chamber containing hot gas, forcing the solution to instantly dry into solid (Abdul-Fattah et al., 2008; Ajmer and Scherliess, 2014; Maa et al., 1999). The potential problems with spray drying method are: 1) proteins may come across various stresses when the protein is atomized into fine droplets, and be exposed to the air/liquid interface, possibly causing denaturation; 2) high
temperature when the proteins contact with the hot air possibly induces protein denaturation, aggregation, and loss of protein activity; 3) it is challenging to control the moisture content using this method (Abdul-Fattah et al., 2007b; Ajmer and Scherliess, 2014). Spray freeze drying (SFD) is another widely used method to produce solid formulation for precise control of particle size, in which protein solution is sprayed into a cold vapor over a cryogenic liquid to form droplets before applying a lyophilization cycle (Maa et al., 1999; Webb et al., 2002). In this method, protein adsorption at the air–liquid interface has been shown to result in significant aggregation (Webb et al., 2002). Vacuum foam drying (VFD), which is an established method in the food industry, consists of preconditioning, bubble formation, foam formation and foam desiccation steps (Abdul-Fattah et al., 2007c; Abdul-Fattah et al., 2007d; Pisal et al., 2006). VFD produces foamy cake structure, reduced specific surface area and relaxed glass, which have been proved to effectively improve the protein storage stability (Hajare et al., 2010, 2011). On the other hand, it is also documented that it is still difficult to control the foaming process, especially when the whole batch contains various formulations (Hajare et al., 2011; Pisal et al., 2006). In addition, the entire VFD process contains potential protein loss, such as air-water interface and cavitation during bubble formation step (Jangle and Pisal, 2012). Supercritical fluid has recently been introduced into the pharmaceutical field to produce dry powder (Jovanovic et al., 2004). Supercritical fluids, such as carbon dioxide (CO₂), are substances maintained above their critical temperature and pressure (Randolph et al., 1993; Sellers et al., 2001; Yeo et al., 1993). During the
drying process, supercritical fluid is utilized to form dry powders by rapid expansion of a supercritical solution. This process requires that proteins are directly soluble in supercritical fluid, such as CO2 (Jovanovic et al., 2004).

Among various drying methods, lyophilization, also known as freeze-drying, has been the popular method for products intended for parenteral administration because of several advantages, such as: 1) lyophilization is a low temperature process, thus is expected to have less thermo-denaturation risk, especially compared to the vacuum drying method, 2) lyophilization allows one to well control the moisture level, as well as the headspace gas composition in vials. And this is especially vital for those product whose stability largely depends on moisture level and headspace gas, like O₂, 3) based on decades practice, lyophilization scale-up is relatively easy, 4) last by not least, ample studies have shown that lyophilization results better stability for protein products, compared to aqueous formulation. (Arakawa et al., 1993; Carpenter et al., 1997; Chang and Pikal, 2009; Schwegman et al., 2005; Tang and Pikal, 2004; Wang, 2000; Xu et al., 2013).

On the other hand, lyophilization also has some limitations, such as it can be harmful to proteins due to stress caused by freezing and also drying, which can induce conformational instability (Abdul-Fattah et al., 2007c; Chang and Pikal, 2009; Kasper and Friess, 2011). In order to understand the causes of protein degradation in the lyophilized state and rationally design a sound lyophilization cycle, a good understanding of the stabilizing mechanisms during each step of the lyophilization process as well as in the solid state is a must. Conventional lyophilization consists of
three steps: freezing, primary drying and secondary drying (Carpenter et al., 1997; Tang and Pikal, 2004). In the following sections of this chapter, each step of the lyophilization process will be elucidated, and the emphasis will be on what each step does, what parameters can be tuned to improve the stability of the final products, and some recent highlights for lyophilization cycle optimization. At the end, the prevailing theories for stabilizing mechanisms in the solid samples will be introduced.

1.2.1 Freezing

The freezing step is of paramount importance; it is the first step in lyophilization and it is typically over within a few hours. During the freezing step, most of the water in the formulation is separated from the solutes to form a pure ice phase. The solute phase leftover becomes highly concentrated-containing only about 20% water by the endpoint of freezing, or less than 1% of the water in the original formulation and this concentrated solute phase is usually called “freeze concentrate” (Tang and Pikal, 2004). Because the freezing step poses various stresses onto protein (cold denaturation, precipitation, aggregation at the interface, etc.), it is desirable to optimize the freezing step to minimize protein damage (Kasper and Friess, 2011). To date, several critical processing parameters during the freezing step can be tuned to optimize the freezing step, including freezing rate, controlled ice nucleation and annealing after initial liquid is frozen, and these will be discussed more in Chapter 5 (Kasper and Friess, 2011; Xu et al., 2014). In general, a faster freezing rate generates small ice crystals. This is because water is super-cooled and crystallization into ice
occurs rapidly, producing small ice crystals. In turn, slower freezing generates larger ice crystals. The size of ice crystal matters not only for the pore size in subsequent drying step (large ice crystals create large pores, leading to faster water sublimation during primary drying) (Searles et al., 2001), but also for the amount of proteins on the interface (ice-water interface in freezing step, and solid-air interface at the end of lyophilization). Ice nucleation temperature determines the ice crystals’ size, number, and morphology during the freezing step; the primary drying rate of lyophilization; and the reconstitution time for the final products (Geidobler et al., 2013; Geidobler et al., 2012; Geidobler and Winter, 2013; Searles et al., 2001). Therefore, techniques for controlled ice nucleation have attracted more and more attention to obtain a controlled and homogeneous freezing process. Methods such as ice fog technique (Rambhatla et al., 2004), vacuum induced surface freezing (Kramer et al., 2002), gap freezing (Kuu et al., 2013) etc. have been proved to effectively produce controlled ice nucleation. Annealing offers an alternative way to statistically reduce size heterogeneity and number of ice crystals, decrease surface area of ice-water interface during freezing step, and shorten the primary drying phase. Annealing is usually performed at elevated temperatures above glass transition temperature ($T_g$'), after initial freezing, to allow for complete crystallization of crystalline compounds and to decrease heterogeneity between vials due to the Ostwald ripening process, therefore, annealing can eliminate the interdependence between the ice nucleation temperature and ice crystal size and morphology (Kasper and Friess, 2011; Tang and Pikal, 2004). Furthermore, annealing can be considered advantageous for interfacial-sensitive
proteins, because the ice/water interface area will be dramatically reduced after annealing, compared to other fast freezing methods.

1.2.2 Drying

During primary drying, frozen water is removed by sublimation, typically with a product’s temperature well below $T_g'$ in order to achieve pharmaceutically elegant products (Carpenter et al., 1997). Due to this temperature restriction and low residual water level requirement in the final products (Carpenter et al., 1997), primary drying is the most time and energy consuming step, and it typically lasts for days. Interestingly, recent studies have shown that cake collapse (as a result of drying above $T_g'$), does not necessarily negatively affect product stability, and in some case studies, collapsed samples even present improved stability after lyophilization and during storage, compared to non-collapsed samples (Schersch et al., 2010, 2012, 2013; Wang et al., 2004).

Secondary drying is relatively short (~hours). During this step, non-frozen water is removed by diffusion and desorption, until finally water content level is reduced to an acceptable level for storage stability, which is usually less than 1% (Carpenter et al., 1997; Tang and Pikal, 2004). In order to avoid cake collapse and shorten the secondary drying step, the shelf temperature is usually increased slowly to a moderate high temperature, normally in the range 30 to 50 °C. Interestingly, recent studies of “post-drying anneal”, physical aging of an amorphous sample at an elevated temperature (still below $T_g'$), showed improved storage stability for some
lyophilized protein samples (Abdul-Fattah et al., 2007a; Abdul-Fattah et al., 2007b; Wang et al., 2010; Wang and Pikal, 2010). The proposed mechanism will be discussed further during next section.

1.2.3 Competing stability theories for lyophilized protein samples

Because of much more complex environment in glassy solid, the mechanisms that control the rates of degradation are not completely understood, and there is still substantial debate about the physical properties of lyophilized formulations that govern the rates of degradation. Currently, there are four classes of theories widely discussed for how proteins are stabilized in glassy matrices. They are: a) the extent of native structural retention during lyophilization, b) the dynamic properties of the glassy matrix, c) phase separation, and d) protein adsorption onto interface.

Retention of protein native structure, also known as “water substitute” theory, claims that stabilizers can form hydrogen bonds with proteins after removal of water upon lyophilization. As a result, proteins in the dry solid states are able to keep the native conformations. This hypothesis assumes that keeping the native protein conformation is a critical point for protein stabilization during lyophilization, as well as long-term storage. There are ample studies supporting this hypothesis (Allison et al., 1999; Carpenter et al., 1998; Garzon-Rodriguez et al., 2004). Solid infrared spectroscopy (IR) is a useful tool to examine protein secondary structure in glassy state, and amide I region of IR spectra contains distinguished signature peaks for
different types of protein secondary structures (Carpenter et al., 1998; Dong et al., 1995; Schwegman et al., 2007; Xu et al., 2013).

Dynamic properties of the glassy matrix, also noted as mobility of glass, assumes that because of coupling between motions of protein to motion of glassy matrix, the rigid and inert glass matrix can slow down the protein motion, thus improving protein stability. The dynamics of this coupling can be $T_g$-related $\alpha$-relaxation, relaxation time related Johari-Goldstein motions, or local and fast $\beta$-relaxation.

$\alpha$-relaxation (also referred to as “global” mobility) associated with $T_g$ (an important and characteristic property of glass samples), and may be directly related to the issues of potential physicochemical instability. Due to the fact that a greater free volume and higher mobility were reported when temperature is above $T_g$, and $\alpha$-relaxation decreases by roughly 10-fold for every 5–10 K change in temperature near $T_g$ (Chang and Pikal, 2009). During the last two decades, much effort has been directed towards studying glass transition and the implications of the underlying molecular mobility in amorphous pharmaceuticals (Chang and Pikal, 2009; Hilden and Morris, 2004; Katayama et al., 2009). However, $T_g$ has not served as a reliable predictor of the stability in some cases (Pikal et al., 2008; Wang et al., 2009; Xu et al., 2013).

Relaxation time related Johari-Goldstein motion ($\beta_{JG}$), is associated with small amplitude motion involving the entire molecule, and it has been implicated as the precursor of the $\alpha$-relaxation (Bhattacharya and Suryanarayanan, 2009; Cicerone
and Douglas, 2012). Therefore, relaxation time related $\beta_{JG}$, as well as its correlation with $\alpha$-relaxation may provide insights into the role of mobility in the lyophilized samples. Relaxation time is often measured by thermal activity monitor (TAM), and reported as relaxation time constant $\tau^\beta$, where $\tau$ is the enthalpy relaxation time and $\beta$ is the stretching exponent (Chang et al., 2005; Chieng et al., 2013). Larger values of $\tau^\beta$ correspond to slower rates of Johari–Goldstein relaxation within the glass (Xu et al., 2013). The aforementioned “post-drying anneal” approach has been reported to increase the structural relaxation times, i.e. relaxation time related Johari-Goldstein motion ($\beta_{JG}$) is decreased. Therefore, to the extent that $\beta_{JG}$ and degradation are coupled, degradation rates should be minimized upon physical aging. There are several studies that support this concept (Luthra et al., 2008; Wang et al., 2010; Wang and Pikal).

Local, fast $\beta$-relaxation ($\beta_{fast}$) is associated with intramolecular reorientations (Cicerone and Douglas, 2012). Recently, Cicerone et al. have introduced theory that $\beta_{fast}$ on the nanosecond time scale may be important for the protein stability in lyophilized samples, because such local relaxation requires low activation energy and low cooperative intramolecular reorientation movements (Cicerone and Douglas, 2012; Wang et al., 2010; Wang et al., 2009). Neutron backscattering provides information about nanosecond scale $\beta_{fast}$. The motions in the glassy sample are recorded by varying the energy of the incident neutrons and measuring the change in energy after neutrons interact with the sample. Debye–Waller factor ($<u^2>$) is analyzed, and it is the hydrogen-weighted mean-square atomic displacement.
Reciprocal of \(<u^2> (<u^2>^{-1})\) is often used as a measure of \(\beta_{\text{fast}}\) motion, and a larger \(<u^2>^{-1}\) means lower local mobility of the system (Cicerone and Douglas, 2012).

Phase separation is another possible attribute to protein degradation in lyophilized samples (Randolph, 1997). In order to protect protein through the lyophilization process, addition of protective excipient and bulk agent is generally required. It is clear that one of the possible effects of phase separation during lyophilization is crystallization of buffer component and/or a bulking agent from the amorphous phase, or the formation of two different amorphous phases (Heller et al., 1997, 1999a, b; Katayama et al., 2009; Randolph, 1997). As a result, protein won’t be able to receive the expected protection as original designed formulation. On the other hand, the non-protein phase also may be adversely detrimental to protein stability, due to shifted pH, high water content and lower \(T_g\) (Heller et al., 1996, 1999a). Techniques, such as electron microscopy and differential scanning calorimetry are commonly used to monitor phase separation (Heller et al., 1996). Strikingly, recent studies showed that in highly concentrated disaccharide and protein mixed frozen solution, annealing (heat treatment) caused two distinguished \(T_g\)’s, suggesting the separation of the solutes into concentrated protein–disaccharide mixture phase and disaccharide phase (Izutsu et al., 2013). Similar \(T_g\)’s splitting phenomena was also seen in highly concentrated disaccharide and polymer frozen solutions (Izutsu et al., 2011). Certainly, this annealing-induced phase separation should affect the stability of protein after lyophilization in various ways, especially for high protein formulations, like therapeutic antibodies. On the other hand, for the low
concentration system, so far there is no evidence showing phase separation between disaccharide and protein has an impact on protein stability after lyophilization. We speculate that given existence of protein-excipient phase and excipient-rich phase, the excipients still provide sufficient interaction to protect the protein throughout the lyophilization process and during storage.

Protein adsorption onto interface is frequently reported (Bee et al., 2010; Bee et al., 2012; Hsu et al., 1995; Webb et al., 2003; Webb et al., 2002). During the lyophilization process, proteins are likely absorbed onto ice-glass interface at freezing step, and glass-air interface after ice sublimation during the drying phase (Webb et al., 2003; Xu et al., 2014). Because surface proteins are likely to have perturbed structure (Chang et al., 1996) and experience different environment than bulk protein: less excipient protection (Abdul-Fattah et al., 2008), one way to improve protein stability is to minimize the surface area of these interfaces. Studies have showed that collapsed lyophilized product, which has smaller surface area, presented equivalent or even better stability than non-collapsed ones after lyophilization process (Schersch et al., 2010). Vacuum foam drying, an alternative for lyophilization, has shown substantially reduced surface area products than lyophilized samples, and better processing and storage stability (Hajare et al., 2010; Jangle and Pisal, 2012; Pisal et al., 2006). Another way to reduce surface area is addition of annealing step after initial freezing (Webb et al., 2003). Due to the Ostwald ripening effect, larger ice crystals are generated after annealing treatment, which in turn results in
lyophilized products with reduced surface area (Kasper and Friess, 2011; Xu et al., 2014).

1.3 References


CHAPTER 2

Objectives

In this thesis, the overall objective was to develop a systematic approach for the stabilization of therapeutical protein using rationally designed lyophilization process. We utilized recombinant human Growth Hormone (rhGH) and Keratinocyte Growth Factor-2 (KGF-2) as model proteins, and attempted this objective through four specific steps as follows:

I. Produce gram-scale quantities of highly pure rhGH

II. Use rhGH as a model in lyophilized formulation to investigate how rhGH degradation are linked to glassy state dynamics and the structure of protein immobilized in glassy matrices

III. Use KGF-2 as a model to investigate how protein stability correlates with glass dynamics, protein structure, as well as the amount of protein absorbed at solid-air interface

IV. Use rhGH as a model to study the correlation between the extent of rhGH degradation during storage and amount of rhGH found at the solid-air interface after lyophilization by employing lyophilization cycles that incorporated various freezing and annealing procedures

The first step of this thesis was to obtain a large quantity of rhGH. Due to the large amount of rhGH we needed for the experiments, it was necessary and economical to produce the proteins in the lab. *E. coli* is a robust host to produce large
quantities of recombinant proteins. However, when overexpression of recombinant protein occurs, the result is the production of insoluble protein inclusion bodies. Here we first used urea as denaturant to denature and solubilize the rhGH inclusion bodies, then refolded proteins overnight by adding appropriate amount of cysteine. After this refolding process, all the inclusion bodies were solubilized and rhGH proteins were in correct folded conformation. Using two-step fast protein liquid chromatography (FPLC) purification process, we achieved purified rhGH with over 99% purity, with correct folded monomer conformation. The overall yield from initial inclusion body to final purified monomeric rhGH was between 50-60%, and we have purified over 2 grams rhGH with consistent purity and yield. The detailed protocol for rhGH fermentation, purification and characterization will be described in Appendix A section.

The next step, we utilized purified rhGH (helix dominant structure) as a model protein in lyophilized formulations to investigate how protein degradation pathways of aggregation, deamidation and oxidation are linked to glassy state dynamics and the structure of protein immobilized in glassy matrices. This study examined formulations of rhGH combined with the disaccharides sucrose or trehalose and various amounts of hydroxyethyl starch (HES), which upon lyophilization yielded glasses with a wide range of retention of native protein structure and glass transition temperatures. After storage at temperatures of 313, 323 and 333 K for periods of up to 16 weeks, samples were analyzed for protein secondary structure, the amount of various types of protein degradation, as well as structural relaxation time and the
local mobility. Surprisingly, protein degradation rates were higher in formulations with higher glass transition temperatures and slower global dynamic motions. Formulations in which the protein retained more native-like structure immediately following lyophilization exhibited better stability, and these formulations also showed larger free energy barriers to structural perturbations. Protein structure and local mobility in the glass were strongly correlated with rate constants for aggregation, deamidation and oxidation, but degradation rates had different dependencies on these stability metrics at different temperatures.

In order to further test the conclusions from rhGH study, we used another protein KGF-2 (beta trefoil structure). The magnitudes of degradation were compared the key physical properties of the formulations including retention of protein native secondary structure, glass transition temperature ($T_g$), inverse mean square displacements $<u^2>^{-1}$ for hydrogen atoms (fast $\beta$ relaxation), and the relaxation time $\tau^\beta$, which correlates with relaxation due to fast Johari-Goldstein motions in the glass. In addition, we measured the specific surface areas of various lyophilized disaccharide-HES formulations, and used these data to estimate the amount of KGF-2 present at the glass-air interface. We propose a simple model that accounts for the possibility that the protein molecules adsorbed at interfaces may degrade more rapidly than protein molecules found in the bulk glass.

Because we anticipate that protein molecules located on the surface of lyophilized glassy solids will have significantly faster degradation rates, we hypothesize that the extent of protein degradation during storage can largely be
ascribed to the resulting levels of protein found at the solid-air interface after lyophilization. In order to test this hypothesis, we lyophilized rhGH with various glass-forming stabilizers, employing cycles that incorporated various freezing and annealing procedures to manipulate glass formation kinetics, associated relaxation processes, glass specific surface areas (SSAs), and amount of protein on the solid-air interface measured by electron spectroscopy for chemical analysis (ESCA). Compared with samples lyophilized after rapid freezing, rhGH in samples that had been annealed in frozen solids prior to drying, or annealed in glassy solids after secondary drying retained more native-like protein secondary structure, had a smaller fraction of the protein on the surface of the cake, and exhibited lower levels of degradation during incubation. By calculating degradation rates for both surface layer protein and protein in the bulk using simple first order kinetics, results suggested that the differences in the extent of rhGH degradation during storage in the dried state between different formulations and processing methods could largely be ascribed to the associated levels of rhGH at the solid–air interface after lyophilization.

As a whole, this dissertation seeks to better understand the mechanisms for protein degradation in lyophilized samples. While lots of literatures have proposed different factors which account for the protein degradation in lyophilized state, quantifying and differentiating the effects of each of these factors will require well-controlled and well-characterized glassy formulations, as well as various types of protein degradation pathways. The four aspects of this dissertation are used collectively to evaluate the existing completing theories for explaining protein
stability mechanisms of lyophilized samples. Finally we have proposed a mathematical model to calculate degradation kinetics for proteins on the solid-air interface and in the bulk, which indicates that the amount of protein on the solid–air interface should be a key factor to consider for formulation and lyophilization design.
CHAPTER 3

Contributions of Local Mobility and Degree of Retention of Native Secondary Structure to the Stability of Recombinant Human Growth Hormone (rhGH) in Glassy Lyophilized Formulations


3.1 Introduction

Protein therapeutics offer unique and critical treatments for human diseases. However, to fully realize this potential, protein drugs must be stabilized against physical and chemical degradation during processing, shipping and storage. Degradation of a protein product may compromise its safety and efficacy (Carpenter et al., 1997; Fradkin et al., 2009; Manning et al., 1989). To minimize protein degradation and achieve the required shelf-life time, lyophilization (freeze-drying) is often employed in order to incorporate proteins into glassy matrices, wherein the proteins generally exhibit slower degradation kinetics than those observed in liquid formulations (Carpenter et al., 1997; Pikal et al., 1991). Unfortunately, storage of protein products in glassy solids does not guarantee an adequate shelf life: both
physical and chemical degradations may still occur at unacceptable rates. Strategies for design of protein formulations that generally result in adequate storage stability are available, but the detailed mechanisms by which proteins degrade in glassy solids are not well understood (Wang, 2000).

In order to more rationally design formulations to reduce degradation rates in glassy states, it would be desirable to understand the factors that control the degradation processes. It has been suggested that embedding proteins in glassy matrices that are rigid and exhibit slow internal molecular motions can improve protein stability because of coupling between matrix dynamics and protein degradation pathways in glassy matrices (Pikal et al., 2008; Wang et al., 2009a). Although it is not clear what type of matrix or protein dynamics are most directly coupled with protein degradation processes, three physical parameters can be used to provide insight into of various aspects of these dynamics. These include the glass transition temperature ($T_g$) which is related to relatively slow “$\alpha$” relaxations, a structural relaxation time ($\tau$) that describes Johari-Goldstein “$\beta_{JG}$” relaxation processes (Johari and Goldstei.M, 1971; Ngai, 2011), and the mean square displacement ($<u^2>$) of hydrogen atoms, a measure of fast, local “$\beta_{fast}$” dynamics(Cicerone and Douglas, 2012). Here $T_g$ is measured (as usual) by differential scanning calorimetry (DSC), (Pikal et al., 2008) and the difference between the storage temperature and $T_g$ ($T-T_g$) is used to estimate dynamics because alpha relaxation ($\tau_\alpha$) decreases by roughly 10-fold for every 5-10 K change in temperature near $T_g$ (Chang and Pikal, 2009) This qualitative approach to estimating dynamics is
applied to comparisons between samples by making an assumption that $\tau_a$ is on the order of 100 s at $T_g$ for all samples. Based on these assumptions, $T_g$ is frequently used as an indicator of large-scale $\alpha$ motions in glassy matrices, and, for a fixed storage temperature, higher $T_g$ values are associated with slower molecular motions.

The rate of structural relaxation can be estimated through time-dependent changes in enthalpy during physical aging of the glassy materials (Craig et al., 2000; Hancock et al., 1995; Kawakami and Pikal, 2005). Here we used previous reports of enthalpy relaxation that had been measured in formulations of the same compositions using an isothermal microcalorimeter known as a thermal activity monitor (TAM) (Chieng et al., 2013a; Chieng et al., 2013b). $\tau$ (the enthalpy relaxation time) and $\beta$ (the stretching exponent) values were obtained by fitting enthalpy relaxation data with KWW and Modified Stretch Exponential (MSE) equation, which is described by Kawakami et al. (Kawakami and Pikal, 2005). Due to the high variability of $\tau$ and $\beta$ values, the authors reported the more robust and reliable value of the stretched time constant, $\tau^\beta$ (Kawakami and Pikal, 2005). Larger values of $\tau^\beta$ correspond to slower rates of Johari-Goldstein relaxation within the glass (Ngai, 2011).

Local, fast $\beta_{\text{fast}}$ relaxation processes can be measured by neutron scattering (Cicerone and Douglas, 2012). In the neutron scattering experiments, the mean-squared displacement ($<u^2>$) of hydrogen atoms is measured over nanosecond time scales. The reciprocal of the mean square displacement ($<u^2>^{-1}$) has been used to
correlate protein stability with local mobilities in glassy states, and smaller \(<u^2>^{-1}\) values imply a higher local mobility within the glass (Cicerone and Douglas, 2012).

In another (non-exclusive) explanation of the mechanism of protein stabilization in lyophilized formulations, Carpenter et al. proposed that retention of native-like protein structure is a critical factor for storage stability of lyophilized proteins (Carpenter et al., 1998). During both the freezing step and drying stage of lyophilization, proteins are susceptible to structural damage, manifesting as unfolding, loss of higher order structure, and aggregation upon rehydration. Stabilizing excipients are used to inhibit protein unfolding during freezing and drying, which minimizes aggregation observed upon rehydration and increases stability during storage (Allison et al., 1999; Carpenter et al., 1998; Kendrick et al., 1996). Solid-state infrared spectroscopy provides a useful tool to examine the protein secondary structures in dried formulations.

The current study examined formulations of rhGH containing the disaccharides sucrose or trehalose and various amounts of HES, which upon lyophilization yielded glasses with a wide range of glass transition temperatures, \(\tau^6\) values, \(<u^2>^{-1}\) values, and degrees of retention of native secondary structure. Through this study, we illustrate the impact of protein structure and glassy state dynamics (modulated by formulation and storage conditions) on the kinetics of three important types of protein degradation: aggregation, deamidation and oxidation.

3.2 Materials and methods
3.2.1 Materials

rhGH was expressed, refolded and purified from *E. coli* as described previously (Crisman and Randolph). Sucrose and trehalose were purchased from Mallinckrodt Baker (Phillipsburg, NJ).\(^1\) Hydroxyethyl starch (HES; Viastarch) was purchased from Fresenius (Graz, Austria). All other chemicals were of reagent grade or higher. 5 mL glass lyophilization vials (Product Number 68000318) and butyl rubber stoppers (Product Number 19560042) were purchased from West Pharmaceutical Services, Linville, PA.

3.2.2 Composition of Formulations

rhGH was prepared in nine different formulations. Each of the formulations contained 1 mg/ml rhGH, 10 mM Tris, and 5% (wt/v) excipients, at pH 7.4. The 5% excipients were composed of HES and a disaccharide (trehalose or sucrose) in different combinations for each formulation. In formulations one through five, the ratios of HES to trehalose were 5:0, 4:1, 1:1, 1:4 and 0:5, respectively. In formulations six through nine, the ratios of HES to sucrose were 4:1, 1:1, 1:4 and 0:5, respectively. When lyophilized, formulations one through five yielded dry solids with disaccharide

\(^1\) Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.
mass fractions $\Phi$ of 0, 0.20, 0.49, 0.78 and 0.98; $\Phi$ for formulations six through nine were 0.20, 0.49, 0.78 and 0.98.

### 3.2.3 Lyophilization Process

Lyophilization was conducted in a FTS Systems Lyostar lyophilizer. After 1 ml of rhGH formulation was added to each lyophilization vial, the vials were placed on the lyophilizer shelf at room temperature. The shelf and formulations were equilibrated at 10°C for 60 minutes. Samples were then frozen by decreasing the shelf temperature from 10°C to -5°C at a rate of 1°C/min, holding the temperature constant at -5°C for 20 minutes, and then decreasing the temperature from -5°C to -45°C at a rate of 1.3°C/min. Samples were maintained at this temperature for 400 minutes. After freezing, primary drying was initiated by reducing the chamber pressure to 70 mTorr, and by increasing the shelf temperature to -20°C at a rate of 2.5°C/min. The shelf temperature and chamber pressure were held constant at -20°C and 70 mTorr, respectively, for 1400 minutes. Secondary drying was then initiated by increasing the shelf temperature to 33°C at a rate of 0.3°C/min. Samples were held at 33°C and 70 mTorr for two hours before venting with dry nitrogen, and vials were sealed in the chamber.

### 3.2.4 Measurement of Residual Water Content

Residual water contents of the lyophilized samples were measured with Karl-Fisher titration (May et al., 1982). A nitrogen-purged dry box was utilized for sample
handling. Samples were dissolved in methanol. A gas-tight syringe was used to take small aliquots of dissolved samples, which were assayed for water content using a Mettler DL37 KF coulometer (Hightstown, NJ). Samples from triplicate vials were analyzed for each formulation.

### 3.2.5 Measurement of Glass Transition Temperature

Placebo samples of formulations without rhGH were analyzed with a Perkin-Elmer Diamond DSC. The DSC was calibrated with indium for temperature and enthalpy. Samples were weighed and prepared in the nitrogen-purged dry box. To measure the glass transition temperature ($T_g$), samples were heated at a rate of 100°C/min. An initial heating scan was run to 10-20°C above the expected $T_g$, the sample was cooled to 20°C, and $T_g$ was determined from the onset of the thermal transition measured during a second heating scan. For each formulation, samples from triplicate vials were analyzed. The standard deviations of the average of the triplicate measurements are presented.

### 3.2.6 Solid Protein Secondary Structures by Infrared Spectroscopy (IR)

The freeze dried formulation was gently grounded with KBr (about 300 ug rhGH mix with 500 mg KBr) using a mortar and pestle, then transferred into a stainless steel die and pressed into a disc with a vacuum press. Infrared spectra were measured on a Bomem MB-series spectrometer (Montreal, PQ, Canada). The instrument and sample compartment were continuously purged with dry nitrogen.
For each sample, 32 interferograms were collected and averaged using a resolution of 4 cm⁻¹. A seven-point Savitsky-Golay function was implemented to calculate the second derivative spectra (Dong et al., 2006). The second derivative spectra were then later processed by baseline correction and area-normalization to 1 (Kendrick et al., 1996). Because rhGH is a 4-α-helix bundle protein, the prominent negative peak appearing near 1654 cm⁻¹ was assigned to α-helix content in the protein (Devos et al., 1992). After area normalization, the depth of the alpha helix peak height was recorded. Finally the peak width at half height (w₁/₂) was calculated by subtracting the low wavenumber from the high one at half peak height (Chirgadze et al., 1976; Dong et al., 1995). Triplicate samples were used for each formulation lyophilized formulation. The spectrum of native, aqueous rhGH (control) was obtained by placing 10mg/ml protein solution between two CaF₂ windows (Biotools Inc, Jupiter, FL), with a path length of 6 µm.

Correlation coefficients r and areas of spectral overlap were also utilized to quantify the overall structural similarity. After baseline correlation and area normalization, correlation coefficients between the IR spectra for rhGH in glassy, lyophilized samples and that of a reference measured in an aqueous liquid formulation were calculated based on equation 1 as described by Prestrelski et al. (Prestrelski et al., 1993).

\[
 r = \frac{\sum_{i=1}^{n} (X_i Y_i)}{\sqrt{\sum_{i=1}^{n} X_i^2 \sum_{i=1}^{n} Y_i^2}}
\]  

(1)
Here, $X_i$ and $Y_i$ are the intensities of reference spectra and sample spectra at wavenumber $i$.

In order to compute the area of spectral overlap, spectra for glassy, lyophilized samples and that of a reference measured in an aqueous liquid formulation were first baseline-corrected and area-normalized to 1. Then the spectra were overlaid, and the area under the overlapping curves computed by integration (Kendrick et al., 1996).

3.2.7 Circular Dichroism Spectroscopic Determination of rhGH Secondary Structure after Reconstitution of Lyophilized Samples

Circular dichroism (CD) spectra of rhGH in the various liquid formulations prior to lyophilization and after reconstitution of lyophilized samples were recorded with a Chirascan-Plus (Applied Photophysics, UK) circular dichroism spectrometer. Lyophilized rhGH samples were reconstituted with water and diluted to a concentration of 0.25 mg/ml using the appropriate liquid placebo formulation. For each sample, triplicate CD spectra from 200 to 260 nm were obtained and averaged. CD spectra were converted to molar ellipticity by dividing by protein concentration, cell path length and the number of amino acid residues in rhGH, and then the signals were normalized to UV absorbance at 215nm. Triplicate vials were analyzed for each formulation condition.
3.2.8 rhGH Aggregation, Deamidation and Oxidation Rates in Lyophilized Formulations

The stability of rhGH within the dried samples was tested after incubation at temperatures of 313, 323 and 333K over a period of 16 weeks. In addition to protein structural measurements by IR and CD spectrosopies, several analytical methods were implemented at time points of 0 (immediately following lyophilization), 1, 4, 9 and 16 weeks to determine the protein’s rates of physical and chemical degradation. Triplicate vials were analyzed for each formulation at every time point and for every incubation temperature.

Size exclusion chromatography (SEC) was used to determine the amount of rhGH monomer in liquid samples prior to lyophilization and after reconstitution of lyophilized samples with water (Pikal et al., 1991; Riggin et al., 1988). Samples (20 ul) were injected onto a TSK G3000SWxl column (Tosoh Bioscience LLC, Montgomeryville, PA), with a mobile phase of 0.025M ammonium bicarbonate at flow rate of 1 ml/min. Eluting protein was monitored by UV detection at 214 nm, and the peak area under the curve was recorded to quantify the amount of monomer in the rehydrated samples.

Deamidation of rhGH was measured in liquid samples prior to lyophilization and after reconstitution of lyophilized samples with water by anion exchange chromatography (IEC) using a Tosoh TSK SuperQ-5PW column as described previously (Fradkin et al., 2009). Absorbance at 280 nm was recorded for quantifying the amount of native protein in the rehydrated samples.
Reverse phase chromatography (RP) was employed to quantify the extent of rhGH oxidation. A Jupiter C18 column with a isocratic mobile phase of 29% n-propanol and 71% 50mM Tris buffer at pH 7.5 was used in this assay, with UV detection at 220 nm to determine the amount of native protein in the reconstituted sample (Riggin et al., 1987).

The three main degradation pathways (aggregation, deamination and oxidation) of rhGH were characterized. For the results from each chromatographic analysis, a first-order kinetics analysis was used to calculate apparent first order rate constants (k) for protein degradation from the best-fit slopes of lines for plots of ln (P) versus time (weeks), where P is the concentration of monomeric or native protein. Values for each of three replicates recorded at each time point were used to determine values of k at each temperature. Typical R^2 values for the resulting fits were 0.85 or greater.

### 3.3 Results

#### 3.3.1 Lyophilization and Glass Transition Temperature

The lyophilization cycle resulted in elegant cakes for all formulations with no observable shrinkage or cracking of cakes. The water contents for all lyophilized formulations were <1% by mass, at time 0 and after 16 weeks of storage. DSC results showed that the T_g values for the nine formulations after lyophilization covered a wide range (Table 3.1). Samples with increasing concentrations of HES yielded higher
Tg values, and trehalose samples had higher Tg values than did the corresponding sucrose formulations.

Table 3.1: Tg (onset) temperatures for the various lyophilized formulations tested. Note the increasing Tg of the formulations as the concentration of HES is increased. All formulations contained less than 1% water.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Tg (onset) (K) ± SD</th>
<th>Formulation</th>
<th>Tg (onset) (K) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) HES 5%</td>
<td>476±2</td>
<td>(6) HES 4% + Sucrose 1%</td>
<td>431±2</td>
</tr>
<tr>
<td>(2) HES 4% + Trehalose 1%</td>
<td>439±2</td>
<td>(7) HES 2.5% + Sucrose 2.5%</td>
<td>354±4</td>
</tr>
<tr>
<td>(3) HES 2.5% + Trehalose 2.5%</td>
<td>392±3</td>
<td>(8) HES 1% + Sucrose 4%</td>
<td>343±1</td>
</tr>
<tr>
<td>(4) HES 1% + Trehalose 4%</td>
<td>384±2</td>
<td>(9) Sucrose 5%</td>
<td>337±2</td>
</tr>
<tr>
<td>(5) Trehalose 5%</td>
<td>371±2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

During storage at 333 K, samples containing only sucrose as an excipient exhibited partial collapse and significant cake shrinkage. Because the glass transition temperature (337±2K) for the 5% sucrose samples was close to the 333K incubation temperature and it is possible that at least local regions within the sample were not glassy at this temperature, these samples were excluded from further analysis.

3.3.2 Protein Structures Analyzed Immediately after Lyophilization in Glassy Solids

Immediately after lyophilization, dried solid samples of formulations were analyzed with IR spectroscopy to obtain second derivative IR spectra. Figure 3.1-A
shows spectra for formulations containing trehalose, and Figure 3.1-B illustrates spectra for formulations containing sucrose. Prominent in each spectrum was a negative peak at 1654 cm\textsuperscript{-1} which arises from the dominant α-helical secondary structure of rhGH, a four-α-helix bundle protein (Devos et al., 1992). Table 3.2 listed structural similarity of lyophilized protein samples and native aqueous protein, by change of IR α-helical peak width at half height, correlation coefficient r and area of overlap. Formulations containing more sucrose or trehalose showed more structural similarity to native protein and a narrower α-helical signature peak.

Figure 3.1: 2\textsuperscript{nd}-derivative FTIR analysis of dried solid formulations analyzed immediately after lyophilization. A) Formulations containing trehalose; B) Formulations containing sucrose. The negative peak at 1654 cm\textsuperscript{-1} arises from α-helical regions of rhGH. For both sucrose- and trehalose-containing formulations, greater α-helical content is observed with increasing disaccharide:HES ratios, but the peak is deeper and narrower in the sucrose-containing formulations, suggesting a more narrow distribution of α-helical environments. Formulations contained 5% HES (---), 4% HES and 1% disaccharide (-----), 2.5% HES and 2.5% disaccharide (-----), 1% HES and 4% disaccharide (-----), 5% disaccharide (-----).
Table 3.2: Similarity of solid protein structure to native aqueous protein by different methods (change of IR α-helical peak width at half height, correlation coefficient and area of overlap).

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Similarity of solid protein structure to native aqueous protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Change of IR α-helical peak half width (Δw_{1/2}, cm⁻¹)</td>
</tr>
<tr>
<td>HES 5%</td>
<td>9.44±1.10</td>
</tr>
<tr>
<td>HES 4% + Trehalose 1%</td>
<td>4.93±0.22</td>
</tr>
<tr>
<td>HES 2.5% + Trehalose 2.5%</td>
<td>3.71±0.19</td>
</tr>
<tr>
<td>HES 1% + Trehalose 4%</td>
<td>1.67±0.10</td>
</tr>
<tr>
<td>Trehalose 5%</td>
<td>1.74±0.08</td>
</tr>
<tr>
<td>HES 4% + Sucrose 1%</td>
<td>2.91±0.31</td>
</tr>
<tr>
<td>HES 2.5% + Sucrose 2.5%</td>
<td>2.14±0.23</td>
</tr>
<tr>
<td>HES 1% + Sucrose 4%</td>
<td>1.07±0.10</td>
</tr>
<tr>
<td>Sucrose 5%</td>
<td>0.53±0.02</td>
</tr>
</tbody>
</table>

3.3.3 Protein Structures Analyzed in Liquid State after Lyophilization and Immediate Reconstitution

The secondary structure of the protein was measured by CD spectroscopy at far UV range (200-260 nm) following lyophilization and immediate reconstitution with water. For comparison, the secondary structure of native reference protein prior to lyophilization was also recorded. The spectra for all samples showed a pronounced double minimum around 208 and 222 nm, indicative of α-helix. After reconstitution, the overlaid spectra for all samples were indistinguishable structures from that of
the native rhGH (Figure 3.2). Although there are limits to the resolution in both IR (Jiang et al., 2011) and CD measurements, the CD data suggest that the secondary structural perturbations observed by IR spectroscopic analysis of rhGH in lyophilized, glassy state formulations were reversible upon reconstitution.

Figure 3.2: Protein secondary structure measured by CD spectroscopy. Samples were reconstituted immediately after lyophilization and analyzed. Representative traces for all nine formulations and native protein are overlaid. No significant differences between formulations are evident.

3.3.4 rhGH Stability in Lyophilized Samples

Apparent first-order rate constants for the three main degradation pathways (aggregation, oxidation and deamidation) were determined for each individual lyophilized formulation (Table 3.3). As expected, rhGH aggregated faster at higher incubation temperatures, and both sucrose and trehalose significantly stabilized rhGH against aggregation compared to samples containing only HES. For chemical
degradation, rhGH exhibited increased oxidation and deamidation rates at higher incubation temperatures. Addition of trehalose and sucrose also stabilized the protein against deamidation and oxidation.

Table 3.3: Apparent first-order rate constants for the formation of aggregation, deamidation and oxidation of rhGH in each formulation at different incubation temperatures (313, 323 and 333 K).

<table>
<thead>
<tr>
<th>Formula</th>
<th>Aggregation rate constant (*10^-3 week^-1)</th>
<th>Deamidation rate constant (*10^-3 week^-1)</th>
<th>Oxidation rate constant (*10^-3 week^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>313K</td>
<td>323K</td>
<td>333K</td>
</tr>
<tr>
<td>(1)HES 5%</td>
<td>2.3±0.3</td>
<td>6</td>
<td>6.8±0.53</td>
</tr>
<tr>
<td>(2)HES 4% + Trehalose 1%</td>
<td>1.1±0.1</td>
<td>8</td>
<td>4.9±0.30</td>
</tr>
<tr>
<td>(3)HES 2.5% + Trehalose 2.5%</td>
<td>0.98±0.082</td>
<td>3.1±0.13</td>
<td>6.4±0.34</td>
</tr>
<tr>
<td>(4)HES 1% + Trehalose 4%</td>
<td>0.70±0.064</td>
<td>1.1±0.082</td>
<td>1.3±0.11</td>
</tr>
<tr>
<td>(5)Trehalose 5%</td>
<td>0.50±0.035</td>
<td>1.1±0.11</td>
<td>1.2±0.081</td>
</tr>
<tr>
<td>(6)HES 4% + Sucrose 1%</td>
<td>1.2±0.1</td>
<td>1</td>
<td>5.1±0.41</td>
</tr>
<tr>
<td>(7)HES 2.5% + Sucrose 2.5%</td>
<td>1.0±0.1</td>
<td>0</td>
<td>2.5±0.18</td>
</tr>
</tbody>
</table>
3.3.5 Relationships between Dynamic Relaxation Processes in the Glassy State and Protein Stability

For each degradation pathway the rate was plotted versus glass transition temperature $T_g$, the natural logarithm of the structural relaxation time constant $\ln (\tau^\beta)$, the reciprocal of mean square displacement ($<u^2>^{-1}$) and the change upon lyophilization of the IR $\alpha$-helical peak width at half height, $\Delta w_{1/2}$. Values for $\ln (\tau^\beta)$ and $<u^2>^{-1}$ were taken from previous publications of Pikal et al. (Chieng et al.; Chieng et al.).

Figures 3A-H show the relationship between aggregation rates at temperatures of at 313, 323, 333K and $T_g$, $\ln (\tau^\beta)$, $<u^2>^{-1}$ and $\Delta w_{1/2}$. In all nine formulations, aggregation rates increased with decreasing mass fraction disaccharide in the formulation. This trend is opposite that expected from $T_g$ and $\ln (\tau^\beta)$, measurements, as both of these increased with decreasing disaccharide content. This suggests that alpha relaxation and Johari-Goldstein structural relaxation processes do not directly report on the relevant dynamics. On the other hand, formulations with higher disaccharide content exhibited higher values of $<u^2>^{-1}$, and narrower IR $\alpha$-helical peak widths at half height (reflecting more native-like rhGH structures),
consistent with the idea that fast, local dynamics and secondary structure are relevant to aggregation rates.

Figure 3.3: Correlation of aggregation rates at different temperatures [(○) 313K; (■) 323K; (▲) 333K] with glass transition temperature, relaxation time and IR α-helical peak width at half height. A) Correlation of aggregation rates with glass transition temperature in trehalose formulations. B) Correlation of aggregation rates with glass transition temperature in sucrose formulations. C) Correlation of aggregation rates with relaxation time in trehalose formulations. D) Correlation of aggregation rates with relaxation time in sucrose formulations. E) Correlation of aggregation rates with reciprocal of mean square displacement ($<u^2>^{-1}$) in trehalose formulations. F) Correlation of aggregation rates with reciprocal of mean square displacement ($<u^2>^{-1}$) in sucrose formulations. G) Correlation of aggregation rates with change of IR α-helical peak half width in trehalose formulations. H) Correlation of aggregation rates with change of IR α-helical peak half width in sucrose formulations.
Deamidation rates at temperatures of at 313, 323 and 333K are plotted versus $T_g$, $\ln (\tau^6)$, $<u^2>^{-1}$ and $\Delta w_{1/2}$ in Figures 4A-H. At 323 and 333K, trends found in the deamidation rate constants were consistent with the aggregation results; formulations with higher disaccharide content had slower deamidation. As above, this was contrary to expectation from trends in $T_g$ values and Johari-Goldstein relaxation times $\ln (\tau^6)$, but consistent with trends in secondary structure ($\Delta w_{1/2}$) and local mobility ($<u^2>^{-1}$). At 313K, the rates of deamidation were indistinguishable between formulations.
Figure 3.4: Correlation of deamidation rates at different temperatures \( (\circ) 313K; (■) 323K; (▲) 333K \) with glass transition temperature, relaxation time and IR α-helical peak width at half height. A) Correlation of deamidation rates with glass transition temperature in trehalose formulations. B) Correlation of deamidation rates with glass transition temperature in sucrose formulations C) Correlation of deamidation rates with relaxation time in trehalose formulations; D) Correlation of deamidation rates with relaxation time in sucrose formulations. E) Correlation of deamidation rates with reciprocal of mean square displacement \( (<u^2>^{-1}) \) in trehalose formulations. F) Correlation of deamidation rates with reciprocal of mean square displacement \( (<u^2>^{-1}) \) in sucrose formulations. G) Correlation of deamidation rates with the change of IR α-helical peak half width in trehalose formulations. H) Correlation of deamidation rates with the change of IR α-helical peak half width in sucrose formulations.
For oxidation, all formulations showed faster degradation rates at higher incubation temperatures (Figure 3.5A-H). The trends in oxidation rates at 323 and 333K are similar to those for aggregation and deamidation. Formulations containing more disaccharide had more native-like structure (smaller value of $\Delta w_{1/2}$) and larger value of $<u^2>^{-1}$ (less local mobility) exhibited faster oxidation rates, and these formulations also had higher $T_g$ values and longer relaxation times. This trend was not as pronounced at the incubation temperature of 313K.
Figure 3.5: Correlation of oxidation rates at different temperatures [(○) 313K; (■) 323K; (▲) 333K] with glass transition temperature, relaxation time, and IR α-helical peak width at half height. A) Correlation of oxidation rates with glass transition temperature in trehalose formulations. B) Correlation of oxidation rates with glass transition temperature in sucrose formulations. C) Correlation of oxidation rates with relaxation time in trehalose formulations; D) Correlation of oxidation rates with relaxation time in sucrose formulations. E) Correlation of oxidation rates with reciprocal of mean square displacement ($<u^2>$)$^{-1}$ in trehalose formulations. F) Correlation of oxidation rates with reciprocal of mean square displacement ($<u^2>$)$^{-1}$ in sucrose formulations. G) Correlation of oxidation rates with the change of IR α-helical peak half width in trehalose formulations. H) Correlation of oxidation rates with the change of IR α-helical peak half width in sucrose formulations.
Estimates of the enthalpies of activation ($\Delta H^\ddagger$) and entropies of activation ($\Delta S^\ddagger$) for each of the degradation pathways were computed from Eyring–Polanyi plots of $\ln (k/T)$ vs $(1/T)$ (equation 2a). In addition, Gibbs’ free energies of activation ($\Delta G^\ddagger$) for each degradation pathway were calculated by non-linear fitting of rate constants $k$ and temperature $T$ to equation 2b (Table 3.4).

$$
\ln \frac{k}{T} = -\frac{\Delta H^\ddagger}{RT} + \frac{\Delta S^\ddagger}{h} + \frac{\Delta G^\ddagger}{R}
$$

(2a)
Here, $K_B$ is the Boltzmann constant, $h$ is Planck’s constant and $R$ is the gas constant.

Table 3.4: Enthalpy of activation ($\Delta H^\dagger$) and entropy of activation ($\Delta S^\dagger$) from a fit of data to Equation 2a, and Gibbs’ energy of activation ($\Delta G^\dagger$) from a fit of data to Equation 2b, for aggregation, oxidation and deamidation processes within each formulation. Values of activation parameters are not reported for sucrose-only formulations because formulations may not have been completely glassy at the highest temperature tested (333K).
Values of $\Delta G^\dagger$ were the highest for aggregation (an average across all formulations of 96.2±0.4 kJ/mol) as compared to deamidation and oxidation, which showed essentially equivalent activation free energies (averages 93.0±0.6 kJ/mol and 92.9±0.7 kJ/mol, respectively) (Figure 3.6 and Table 3.4). $\Delta G^\dagger$ values for aggregation increased as the ratio of disaccharide:HES increased; values of $\Delta G^\dagger$ for deamidation and oxidation were relatively insensitive to disaccharide content. Also, the values of $\Delta G^\dagger$ were highest in formulations with the greatest degree of secondary structural retention (Figure 3.6).

Figure 3.6: Gibbs energy of activation $\Delta G^\dagger$ for each degradation pathway: (○) aggregation; (■) deamidation; (▲) oxidation as a function of $\Delta w_{1/2}$, the change of IR helix peak width at half height from that of native rhGH. A) Formulations with HES and trehalose. B) Formulations with HES and sucrose.
3.4 Discussion

Samples with increasing mass fraction of trehalose or sucrose showed slower local mobilities (higher value of reciprocal of mean square displacement, $<u^2>^{-1}$) and concomitantly, lower rates of aggregation, deamidation and oxidation (Figures 3.3-3.5). Thus it is reasonable to expect that small-scale local motions exert some control over reactivity. However, the fact that plots of degradation rate constants vs. $<u^2>^{-1}$ measured at various temperatures did not collapse to a single line suggests that fast local mobility is not the sole controlling factor for reactivity in this system.

Proteins may be unfolded during lyophilization and long term storage (Carpenter et al., 1998). Unfolded protein structures in lyophilized cakes may be particularly prone to aggregation, which we speculate only occurs to any significant extent upon rehydration. More native-like structures were seen in the formulations with high disaccharide:HES ratios. HES is a large polymer with a mean molecular weight of 200 kDa, and HES-containing glasses have relatively high $T_g$ values. However, due to steric constraints, HES has diminished capacity to form hydrogen bonds with proteins during dehydration, which may contribute to the failure of HES to inhibit protein unfolding and loss of $\alpha$-helical structure during drying (Garzon-Rodriguez et al., 2004). On the other hand, trehalose and sucrose, which readily hydrogen bond with proteins during drying, are commonly reported to be effective as stabilizers of native structure during lyophilization.
There was a strong positive correlation of degradation rates, especially those for aggregation, with the IR α-helix peak half width. As rhGH is a helix-rich protein, the α-helix peak width at half height is expected to be sensitive to changes in the local helical environment. In this case, the smaller half width likely indicates a more narrow distribution of α-helical states, as well as more native-like secondary structure content. Hence, degradation rate constants increased with greater degrees of protein structural perturbation in the dried solid.

rhGH in sucrose-containing formulations retained more native-like structure than in trehalose-containing formulations, in contrast with a previous report that found, based on IR analysis, that the structure of rhGH was identical after lyophilization in trehalose- or sucrose-containing formulations (Chang and Pikal, 2009). The explanation for this discrepancy is not clear, although these formulations did not contain HES, and in addition the buffer, protein concentration, and protein:disaccharide ratios were different from those used in the current study.

Recently Cicerone and Douglas suggested that protein secondary structure did not have a significant impact on the degradation rate for a series of proteins in glassy sucrose and trehalose matrices (Cicerone and Douglas, 2012). Their conclusion was based on an analysis of correlation coefficient values (r) (Kendrick et al., 1996) for amide I infrared spectra obtained as a function of disaccharide content. These measurements were interpreted to suggest that, whereas protein structure in disaccharide glasses was a function of disaccharide mass fraction at Φ<0.5, it was invariant at disaccharide mass fractions Φ>0.5. Then, based on roughly constant
slopes of plots of $\ln(k)$ vs. $<u^2>^{-1}$ for all values of $\Phi$, they suggested that the entire second term of Equation 3 must be negligible for all $\Phi$ values, and hence $\ln(k)$ did not depend on protein secondary structure.

$$\frac{d \ln(k)}{d \langle u^2 \rangle^{-1}} = \frac{\partial \ln(k)}{\partial \langle u^2 \rangle^{-1}} + \frac{\partial \ln(k)}{\partial r} \frac{d r}{d \Phi} \frac{d \Phi}{d \langle u^2 \rangle^{-1}} \quad (3)$$

Following the approach of Cicerone and Douglas (Cicerone and Douglas, 2012), we assume that rhGH degradation rates are determined by both local mobility and rhGH structure, and write that:

$$\frac{d \ln(k)}{d \langle u^2 \rangle^{-1}} = \frac{\partial \ln(k)}{\partial \langle u^2 \rangle^{-1}} + \frac{\partial \ln(k)}{\partial w_{1/2}} \frac{d w_{1/2}}{d \Phi} \frac{d \Phi}{d \langle u^2 \rangle^{-1}} \quad (4)$$

Here, we simply replace the use of $r$ as a secondary structure indicator in equation 3 with the amide I peak half width $w_{1/2}$. In contrast to the previous work (Cicerone and Douglas, 2012), we observe significant differences in rhGH structure as a function of disaccharide content, even for $\Phi$ as high as 0.98 (Figure 3.7). Hence the second term on the right hand side of Equation 4 cannot be neglected and we cannot exclude the possibility that protein secondary structure influences reaction rates in the glassy state. Previous publications suggested that local, fast relaxation processes at least in part responsible for protein degradation in lyophilized, sugar-based matrices (Cicerone and Douglas, 2012; Ngai, 2011; Pikal, 2013). However, it is also not surprising that protein structural perturbation might couple with local fast motion to enhance degradation rates, since the structurally perturbed proteins would be starting from a state more prone to degradation (Allison et al., 1999; Allison et al.,
2000; Carpenter et al., 1998; Katayama et al., 2009; Kendrick et al., 1996), and hence require smaller additional local motions to undergo reaction.

Figure 3.7: Change of IR α-helical peak width at half height for protein secondary structure as a function of disaccharide mass fraction Φ in freeze-dried glasses. Open symbols (○) represent trehalose and HES formulations; closed symbols (■) represent sucrose and HES formulations.

One explanation for the apparent discrepancies between the current work and that of Cicerone and Douglas (Cicerone and Douglas, 2012) may be that the analysis of protein secondary structure by the correlation coefficient method may lack sufficient resolution in some cases, especially for proteins whose native structure is high in β-sheet, turn, random structures (Cicerone and Douglas, 2012). The amide I spectral correlation coefficient (r) and amide I area of overlap are commonly used parameters for estimation of the degree of protein secondary structure retention in the dried solid state (Kendrick et al., 1996; Prestrelski et al., 1993). However, previous literature reported that neither method is always sensitive to structural
differences (D'Antonio et al.; Jiang et al., 2011; Wang et al., 2009b); in some cases limits of detection may require as much as 25% of the protein to be unfolded for a perturbation to be detected (Jiang et al., 2011). In contrast, the high α-helix content of rhGH may have allowed more sensitive detection of small structural perturbations.

Increased retention of native structure for rhGH in the dried solid state correlated with increased free energies of activation (ΔG†) for each of the three degradation modes (Figure 3.6). Presumably, the free energy barriers to structural transitions required for degradation are lower if the initial protein structure is already partially perturbed. Activation entropies were small or negative (Table 3.4), suggesting a transition state that is more ordered than that of a relatively high-entropy ground state. Although somewhat unusual, negative activation entropies have been reported for protein structural transitions in aqueous solutions. For example, activation entropies of -6 to -1kB have been reported for folding of a cold-shock protein (Schuler et al., 2002) and negative activation entropies were reported for lactalbumin aggregation and non-native disulfide formation (Anema, 2001). It is not clear, however, that these results can be directly compared to results for transitions in glassy solids in the (near) absence of water. Previously, it was shown that the excess free entropies of mixing in glassy solid ternary mixtures of ribonuclease, HES and trehalose or sucrose were positive (Katayama et al., 2009). Thus, a possible explanation for the negative activation entropies that we observe is a microscopic demixing of rhGH from the matrix that allows formation of the transition state and concomitantly lowers entropy. In addition, activation entropies
decrease with higher mass fractions of disaccharide, which may imply that transition states of these samples have a more ordered sugar matrix.

The activation free energies for aggregation were the highest of the three modes of degradation (Figure 3.6 and Table 3.4). This may reflect the relatively large activation energy typically required to unfold proteins. For example, Rodriguez-Larrea et al. reported activation energies to unfold lysozyme to be \( \sim 100 \text{ kJ/mol} \) (Rodriguez-Larrea et al., 2006). Compared to the local motions needed to initiate protein oxidation or deamidation in the glassy state (Chang and Pikal, 2009), protein aggregation requires relatively large structural perturbations from the native state. For example, Webb et al. reported that the activation volume change for aggregation of interferon-\( \gamma \) to be an increase corresponding to roughly 20% of the volume change required for complete unfolding (Webb et al., 2001). In contrast, oxidation is likely to occur during storage as a result of diffusion of residual oxygen into the lyophilized cake, where it may react with the exposed oxidation “hot spots”, like Met. The rate limiting step of deamidation (Asn and Gln) in solid state involves formation of a reactive cyclic intermediate (Chang and Pikal, 2009), which requires a smaller, more local degree of motion than that required for formation of the partially unfolded state associated with aggregation.

Finally, the observation that rhGH degradation rates decreased as Johari-Goldstein relaxation rates increased (i.e., with decreasing \( \ln(\tau^0) \), see Figures 3.4cd and 3.5cd) deserves comment. Formulations that exhibited faster Johari-Goldstein relaxation rates also showed improved retention of rhGH native secondary structure
after lyophilization. We speculate that mechanical stresses imposed by the lyophilization process contribute to rhGH unfolding during the lyophilization process. These stresses can cause shrinkage of the glass during drying (Pikal and Shah, 1997; Sadikoglu et al., 1999), and potentially may cause loss of protein secondary structure (Webb et al., 2003). During the lyophilization process, Johari-Goldstein relaxation may act to relieve the buildup of residual stress, thus minimizing unfolding and/or fostering rapid refolding of damaged protein. Samples with faster Johari-Goldstein relaxation would thus be expected to yield protein molecules that are less structurally perturbed and hence less prone to degradation. This interpretation is consistent with results from an earlier study, wherein interferon-γ was lyophilized in formulations containing HES or 1:1: weight ratios of HES:sucrose (Webb et al., 2003). Interferon-γ lyophilized from HES formulations showed more unfolding and greater aggregation than samples lyophilized from 1:1 HES:sucrose, and protein damage was associated with conditions where mechanical stress due to shrinkage was severe enough to cause cracking of the cakes (Webb et al., 2003). In both of these interferon-γ formulations, allowing frozen samples to relax at temperatures above T_g prior to lyophilization eliminated cake cracking and resulted in lower levels of structural perturbation (Webb et al., 2003).

3.5 Conclusion

Rate constants and activation free energies for three degradation processes were strongly correlated with the degree of secondary structural perturbation in
lyophilized rhGH samples. Local mobility measurements made by neutron scattering techniques also showed a quantitative trend of faster degradation in systems with faster local motion, but neither local motion alone nor structure alone were sufficient to determine rates of rhGH degradation. A combination of local mobility measurements and spectroscopic analysis of protein structural retention might be used to predict rates protein degradation in glassy solid matrices. At present, the measurement of secondary structure by IR spectroscopy is more convenient. However, we have made progress on a fluorescence-based metric of fast dynamics that shows promise for use in a freeze-dried format (Cicerone et al., 2011). Finally, we note that although it is likely critical that samples be stored below the glass transition temperature, the glass transition temperature of a lyophilized formulation by itself is a poor predictor of long-term protein stability.

3.6 Acknowledgements

We acknowledge funding from NIH/NIBIB under grant R01 EB006398-01A1.

3.7 References


CHAPTER 4

Storage Stability of Keratinocyte Growth Factor-2 in Lyophilized Formulations: Effects of Formulation Physical Properties and Protein Fraction at the Solid-Air Interface

This chapter has been submitted as: Dilip Devineni, Yemin Xu, et.al. (2014). Storage Stability of Keratinocyte Growth Factor-2 in Lyophilized Formulations: Effects of Formulation Physical Properties and Protein Fraction at the Solid-Air Interface.

4.1 Introduction

A lyophilized formulation is often the choice of dosage form when an aqueous solution formulation of a therapeutic biologic molecule is not sufficiently stable. To achieve its desired shelf life. However, even in lyophilized formulations, proteins are prone to instabilities such as aggregation or chemical degradation. To minimize protein degradation, various excipients are added to the lyophilized formulation (Carpenter et al., 1997; Xu et al., 2013). Key to the development of robust formulations that offer proteins protection against degradation is the formation of a glassy matrix by the added excipients. Commonly used glass-forming excipients include the disaccharides trehalose and sucrose, as well as polymers such as hydroxyethyl starch (HES) (Carpenter et al., 1997).
During storage proteins in lyophilized formulations that contain stabilizing excipients can still degrade, albeit slowly. The factors that control the rates of this degradation are not well understood, and there is still substantial debate about the physical properties of lyophilized formulations that govern rates of degradation. The extent of native structural retention during freeze-drying (Allison et al., 1999; Carpenter et al., 1998; Kendrick et al., 1996b) and the dynamic properties of the glassy matrix (Pikal et al., 2008; Wang et al., 2009b) are most often considered critical to the long-term storage stability of proteins in lyophilized formulations. Other phenomena, such as phase separation (Heller et al., 1996; Heller et al., 1997; Izutsu and Kojima, 2000; Izutsu et al., 1993; Katayama et al., 2009) and interfacial adsorption (Webb et al., 2003; Webb et al., 2002) of protein molecules have been shown to affect protein stability during lyophilization, but their effects on stability during storage have not been investigated. In the current study, we examine the possibility that in addition to the degree of native protein structural retention and glass dynamics properties, the interfacial behavior of proteins in lyophilized solids may be of importance in determining degradation rates.

In the current study as a model protein, we used keratinocyte growth factor-2 (KGF-2), a protein with a beta trefoil structure that is found in numerous growth factors and some human interleukins. The storage stability of KGF-2 was examined in various glassy, lyophilized formulations that contained mixtures of HES and the disaccharides sucrose or trehalose. The rates of aggregation and chemical degradation of KGF-2 were measured, and correlated with measures of native
secondary structure retention and glassy matrix dynamics. In addition, we measured the specific surface areas of various lyophilized disaccharide-HES formulations, and used these data to estimate the amount of KGF-2 present at the glass-air interface. We propose a simple model that accounts for the possibility that the protein molecules adsorbed at interfaces may degrade more rapidly than protein molecules found in the bulk glass.

4.2 Materials and Methods:

4.2.1 Materials

KGF-2 was a generous donation from Human Genome Sciences Inc, Rockville, Maryland. Hydroxyethyl starch / viastarch (HES) was obtained from Fresenius Kabi, Austria, GmbH. Sucrose was purchased from Pfanstiehl laboratories, Waukegan, IL, and trehalose and sodium phosphate was obtained from J.T Baker, PA.

4.2.2 Preparation of KGF-2 freeze-dried formulations

KGF-2 was simultaneously dialyzed and concentrated into 2mM sodium phosphate buffer, pH 6.2 to 10 mg/mL in centrifugal concentrators (Vivaspin®, Millipore) at 10°C. Formulation excipients and their percent weight masses used for preparing formulations of KGF-2 are listed in Table 4.1. Each of the initial aqueous solution formulations of KGF-2 was prepared by weighing excipients into a 50 ml container followed by addition of 5 ml of the dialyzed and concentrated 10mg/mL
concentrated KGF-2 solution. 2 mM sodium phosphate buffer was added to obtain a final volume of 50 ml and a final protein concentration of 1mg/mL. Aliquots of 1 ml solution were pipetted into 5 cc glass vials (13 mm FNT BB LYO). Vial filling took place in a cold room at approximately 2-8°C. The vials were then partially stoppered using double vent Fluorotec rubber stoppers (Daikyo Fluorotec stoppers, West Pharmaceutical, Lititz, PA).

Table 4.1 Formulation excipients and their percent weight masses used for preparing formulations of KGF-2.

<table>
<thead>
<tr>
<th>Formulation#</th>
<th>Formulation additives, % by weight</th>
<th>Buffer used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Trehalose 5%</td>
<td>2mM sodium phosphate, pH 6.2</td>
</tr>
<tr>
<td>2</td>
<td>Trehalose 4%, HES1%</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Trehalose 2.5%, HES2.5%</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Trehalose 1%, HES 4%</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Sucrose 5%</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Sucrose 4%, HES 1%</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Sucrose 2.5%, HES 2.5%</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Sucrose 1%, HES 4%</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>HES 5%</td>
<td></td>
</tr>
</tbody>
</table>

4.2.3 Freeze drying conditions for KGF-2 formulations

The glass vials containing the liquid KGF-2 formulations were loaded onto the shelves of a FTS Durastop® microprocessor-controlled freeze-dryer (SP Industries, Warminster PA) equipped with a Dura-dry MP® condenser unit. The initial shelf
temperature of the freeze dryer was 10°C. Vials were allowed to equilibrate at this temperature for 60 min. The shelf temperature then was reduced to -5°C at 1°C/min and held for 20 minutes, followed by a second ramp to -45°C at 1.3°C/min. After 30 minutes at a shelf temperature of -45°C the chamber was evacuated to a pressure of 70 mTorr, and shelf temperature was increased from -45°C to -20°C over ≈ 10 minutes (2.5°C/min) and held at this temperature for the duration of primary drying (1400min). At the end of primary drying, the shelf temperature was increased from -20°C to +33°C over 3 hours (a ramp rate of ≈ 0.3 °C/min) and held at this temperature for 2 hours for secondary drying. After secondary drying was completed, the chamber was vented with dry nitrogen and the vials were stoppered within the freeze-drying chamber. The vial stoppers were later crimped onto the vials with aluminum seals.

4.2.4 Water content determination in KGF-2 lyophilized formulations

The residual water content in the freeze-dried formulations was determined using a Mettler DL37 coulometric moisture analyzer (Hightstown, NJ) with pyridine free vessel solutions (Photovolt Instruments, Inc., St. Louis Park, MN). Water standards were used to verify the accuracy of the instrument. The vials with freeze-dried samples were placed in a glove box (purged with dry nitrogen gas), and the dried formulations were dissolved in anhydrous dimethylformamide (DMF) (<50 ppm water). Water content in the samples was determined after subtraction of water level in DMF.
4.2.5 Surface Area Measurement

Surface areas of lyophilized formulations were calculated from krypton adsorption isotherms measured using a Quantachrome Autosorb-1 (Boynton Beach, FL). For each formulation, the contents of five vials of lyophilized placebo formulation without protein were inserted into the sample cell, and then the cell with samples was placed under vacuum to remove the moisture.

Krypton adsorption at liquid nitrogen temperature was measured at five points, with helium serving as an inert carrier gas. Brunauer–Emmett–Teller (BET) adsorption theory (Brunauer et al., 1938) was used -- over a pressure range of 0.10-0.30 of the saturation pressure of krypton -- to calculate the specific surface areas.

4.2.6 Storage Stability of KGF-2

The lyophilized KGF-2 formulations were incubated in temperature-controlled incubators at 40, 50 or 60°C. At various time points (0, 1, 4 9 and 16 weeks), samples were removed for the analyses described below.

4.2.7 Infrared Spectroscopic (IR) Analysis of KGF-2 Secondary Structure

IR spectra of KGF-2 in aqueous solution and in freeze-dried formulations were acquired using a BOMEM instrument (ABB Bomem Inc., Quebec, Canada) equipped with GRAMS® software (Galactic Industries Corp., Salem, NH). The IR spectrum of native KGF-2 in aqueous solution was obtained first. An aliquot of 10 mg/mL KGF-2 in 10 mM sodium citrate buffer, pH 6.2, was placed in a variable path length cell with
CaF$_2$ windows (Biotools Inc., Jupiter, FL). Using a path length of 6 µm, a total of 128 interferograms in single-beam transmission mode (resolution of 4 cm$^{-1}$) were collected from 4000 to 1000 cm$^{-1}$ and averaged. After subtraction of water vapor spectra as described by Dong et al (Dong et al., 1990), absorbance data in the amide I region of 1720-1580 were converted to second derivative using a seven point Savitzky-Golay smoothing function. The second derivative spectra were baseline-corrected and their areas normalized to unity. This second derivative spectra was used as an 'aqueous solution control' for evaluation of the lyophilization-induced secondary structure perturbations of KGF-2 in the freeze-dried formulations.

To measure IR spectra for KGF-2 in the lyophilized formulations, the freeze dried KGF-2 sample (approx 200 µg protein) was gently ground with 500 mg of KBr (Thermo scientific, USA) using a mortar and pestle. This mixture was transferred into a stainless steel die (13mm internal diameter) and pressed with a hydraulic press (Carver Model “C”, Wabash, IN) to form a pellet. IR spectra were acquired as described above converted to second derivative spectra. Water vapor spectra were subtracted, and the resulting protein second derivative spectra were baseline corrected and area normalized to unity. The secondary structural changes of KGF-2 in a freeze-dried formulation was assessed using area of the overlap of between a second derivative amide I spectrum for the protein in a lyophilized formulation and that of liquid native protein. (Kendrick et al., 1996a) In addition, spectra were compared by determining the peak width at half height ($W_{1/2}$) for the major second derivative amide I band for KGF-2 at 1647 cm$^{-1}$. For evaluating the potential changes
in secondary structure using the $W_{1/2}$ method, $W_{1/2}$ of the spectrum for the native protein in ‘liquid reference control’, was subtracted from the $W_{1/2}$ of freeze-dried KGF-2 to obtain the relative difference in peak width ($\Delta W_{1/2}$). The values are presented as mean and standard error of duplicate samples of each lyophilized formulation.

4.2.8 Quantitation of KGF-2 Aggregation

Aggregation of KGF-2 in the incubated and rehydrated freeze-dried formulations was quantified using size exclusion-high performance liquid chromatography (SE-HPLC). Triplicate, freeze-dried samples for each formulation, temperature and time point were reconstituted with distilled water, centrifuged at 13500 RPM to pellet potential insoluble aggregates, and the supernatant was collected for analysis. An Agilent 1100 HPLC system equipped with Chemstation™ software was used, together with a TSK Gel G2000SWXL column (30cm×7.8mm i.d., 5µm particle size). The supernatant (40 µL volume) from reconstituted and centrifuged KGF-2 samples was injected into the HPLC system, and the protein was eluted at 0.5ml/min using a mobile phase containing 100mM sodium citrate, 1M sodium chloride, pH 6.2. Eluting protein was monitored by optical absorbance at 280nm. No soluble aggregates were detected by SE-HPLC in this study. Therefore, aggregation was determined directly from the loss of monomeric KGF-2 relative to an un-lyophilized liquid control sample.
4.2.9 Quantitation of KGF-2 Chemical Degradation

Chemical degradation of KGF-2 was monitored by reverse phase HPLC (RP-HPLC). Triplicate, freeze-dried samples for each formulation, temperature and time point were reconstituted with distilled water, centrifuged at 13500 RPM to pellet potential insoluble aggregates, and the supernatant was collected for analysis. An Agilent 1100 HPLC system equipped with Chemstation™ software was used, with a C18 column (2.0 mm x 250 mm, 5 μm 300 Å, YMC, USA). A gradient reverse phase method was used with mobile phase A, 0.1% trifluoroacetic acid (TFA) in water and mobile phase B, 0.07% TFA in acetonitrile. The method consisted of two steps of organic phase gradient at a flow rate of 0.3ml/min. In the first step, a 5% per minute gradient of mobile phase B (B: 5% to 35%) is used. This was followed by protein elution with 0.3% per minute gradient of mobile phase (B: 35% to 42%). Approximately 20 µg of protein were loaded per injection. The total run time was 50 minutes. Absorbance was monitored at 215nm. The percent of chemical degradation of KGF-2 in the sample supernatants was calculated from the peaks areas for native and chemically-altered KGF-2 in the chromatograms with:

\[
\% \text{ degradation} = 1 - \left( \frac{\text{Area of native peak}}{\text{Area of main peak} + \text{Area of degraded species}} \right) \times 100 \quad \text{Equation 1.}
\]

To assess the ability of the method to detect oxidized KGF-2 species, a forced oxidation study of KGF-2 was conducted. KGF-2 was incubated with 0.06% v/v H₂O₂
at 37°C. Samples were collected at 0, 10, 20, 30 and 40 minutes and analyzed by RP-HPLC. The percent chemical degradation increased linearly with incubation time ($R^2 = 0.98$).

4.3 Results

4.3.1 Aggregation of KGF-2 after storage and reconstitution of lyophilized formulations

KGF-2 aggregated in all of the lyophilized samples tested, with the extent of aggregation generally increasing with increasing HES content (Figure 4.1). Aggregation was relatively rapid for the first 4 weeks of incubation, and then plateaued for all of the formulations during storage at 40 and 50 °C. During storage at 60°C, formulations with disaccharide alone or 4:1 disaccharide:HES mixtures also showed plateauing. The plateaus observed in disaccharide-containing formulations occurred after 10-30% of the monomer aggregated, with no apparent temperature dependence. During storage at all of the temperatures tested, KGF-2 in the HES-alone formulations exhibited markedly greater rates and extents of aggregation than formulations that contained disaccharides.

Figure 4.1 Percent monomeric KGF-2 in freeze-dried disaccharide-HES formulations as a function of storage time and temperature. Left panel shows percent monomer in 5% trehalose (open circle), 4%trehalose-1% HES (open square), 2.5% trehalose-2.5% HES (open triangle) and 1% trehalose-4% HES (cross) stored and 5% HES (closed circle) at A) 40°C, B) 50°C and C) 60°C. Right panel shows results from
formulations of 5% sucrose (open circle), 4% sucrose-1% HES (open square), 2.5% sucrose-2.5% HES (open triangle) and 1% sucrose-4% HES (cross) and 5% HES (closed circle) stored at D) 40°C, E) 50°C and F) 60°C.
4.3.2 Oxidative chemical degradation of KGF-2 after storage and reconstitution of lyophilized formulations

Oxidative chemical degradation of KGF-2 was measured in the fraction of the protein that did not aggregate (Figure 4.2). Within this fraction, the extent of degradation increased with increasing incubation temperature. And the extent of degradation generally increased with increasing fraction of HES in the formulations. At 40 and 50 °C, the rate of degradation appeared to plateau between 9 and 16 weeks of storage, but at 60°C, plateauing was not apparent. Even for the lowest storage temperature and the most stable formulations (5% sucrose or 5% trehalose), substantial (>20%) chemical degradation was detected. We note that this level, which is likely already unacceptable for a pharmaceutical formulation, may underestimate of total chemical degradation, because it does not include the potentially chemically-degraded molecules in the aggregated fraction.

Figure 4.2. Percent un-oxidized KGF-2 in freeze-dried disaccharide-HES formulations as a function of storage time and temperature. Left panel shows percent un-oxidized in 5% trehalose (open circle), 4%trehalose-1% HES (open square), 2.5% trehalose-2.5% HES (open triangle) and 1% trehalose-4% HES (cross) and 5% HES (closed circle) stored at A) 40°C, B) 50°C and C) 60°C. Right panel shows results from formulations of 5% sucrose (open circle), 4%sucrose-1% HES (open square), 2.5% sucrose-2.5% HES (open triangle) and 1% sucrose-4% HES (cross) and 5% HES (closed circle) stored at D) 40°C, E) 50°C and F) 60°C.
4.3.3 Protein Secondary Structure in Lyophilized Formulations

Infrared spectra of native KGF-2 in aqueous solution and for the protein in freeze-dried formulations (obtained immediately after freeze-drying) are shown in Figure 4.3. The main band at 1647 cm\(^{-1}\) in the second derivative spectrum of KGF-2 is attributed to random coil and \(\beta\)-sheet structure. The bands at ca. 1678 and 1685 cm\(^{-1}\) represent \(\beta\)-turns. (Dong et al., 1990) When spectra for lyophilized samples are compared with the spectrum for native KGF-2 measured in the liquid state prior to lyophilization (dashed line), significant differences are evident, reflecting perturbations of KGF-2 secondary structure. These differences are most clearly observed in the region of 1660-1700 cm\(^{-1}\) and in the region of 1620-1650 cm\(^{-1}\). The degree of structural perturbation is greatest for the formulation containing HES alone (Figure 4.3C), and the degree of perturbation decreases with increasing disaccharide content (Figures 4.3AB). To quantify the magnitude of changes in the infrared spectra relative to the spectrum for the native protein in aqueous solution, we calculated the change in width at half-height for the dominant band in the second derivative spectrum at ca. 1648 cm\(^{-1}\) (Table 4.2). In addition, the % area of overlap between the spectrum for the native protein in aqueous liquid solution and the spectra for the protein in lyophilized formulations was calculated (Table 4.2). In general, increases in spectral half-height widths and decreased areas of overlap were observed with increased HES content. KGF-2 secondary structure in HES without added disaccharides was noticeably more perturbed than in any of the formulations that contained disaccharides.
Figure 4.3. Second derivative FTIR absorbance \( (d^2A/d \theta^2) \) spectra of KGF-2 in disaccharide-HES freeze-dried formulations determined immediately after lyophilization. The dashed trace belongs to the KGF-2 liquid control. Panel A (Sucrose-HES series) and B (Trehalose-HES series) shows increase in perturbation of native secondary structure when freeze-dried with increasing concentrations of HES: 0% HES (blue trace), 1% HES (black trace), 2.5% (red trace), 4% HES (green trace) and 5% HES (magenta trace). Panel C) shows comparison between native secondary structural perturbations of KGF-2 when freeze-dried with ‘HES alone’ (blue trace) and when freeze-dried in ‘buffer alone’ (red trace).
Table 4.2 Lyophilized formulation glass dynamics properties and KGF-2 native secondary structure retention. Tg (°C) values were reported previously by Xu et al. (Xu et al., 2013), and \( \ln \tau^\beta \) (ln hours) and \(<u^2>^{-1}\) values were obtained from Chieng et al. (Chieng et al.; Chieng et al.) ND=not determined.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Tg, °C</th>
<th>( 1/\langle u^2 \rangle ), ( [\AA]^2 )</th>
<th>( \ln \tau^\beta ), ln(hr)</th>
<th>Change in half width for IR band at ca. 1648 cm(^{-1}) (( \Delta W_{1/2} ), cm(^{-1}))</th>
<th>Area of overlap, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>HES 5%</td>
<td>203</td>
<td>2.44</td>
<td>3.8</td>
<td>12.42</td>
<td>78</td>
</tr>
<tr>
<td>HES 4% + Trehalose 1%</td>
<td>166</td>
<td>3.74</td>
<td>3.3</td>
<td>3.73</td>
<td>83</td>
</tr>
<tr>
<td>HES 2.5% + Trehalose 2.5%</td>
<td>119</td>
<td>5.05</td>
<td>3.2</td>
<td>2.51</td>
<td>87</td>
</tr>
<tr>
<td>HES 1% + Trehalose 4%</td>
<td>111</td>
<td>ND</td>
<td>3.1</td>
<td>1.79</td>
<td>87</td>
</tr>
<tr>
<td>Trehalose 5%</td>
<td>98</td>
<td>10.2</td>
<td>3.1</td>
<td>1.62</td>
<td>89</td>
</tr>
<tr>
<td>HES 4% + Sucrose 1%</td>
<td>158</td>
<td>3.43</td>
<td>3.1</td>
<td>3.22</td>
<td>83</td>
</tr>
<tr>
<td>HES 2.5% + Sucrose 2.5%</td>
<td>81</td>
<td>4.42</td>
<td>2.3</td>
<td>0.49</td>
<td>91</td>
</tr>
<tr>
<td>HES 1% + Sucrose 4%</td>
<td>70</td>
<td>5.85</td>
<td>1.9</td>
<td>0.44</td>
<td>90</td>
</tr>
<tr>
<td>Sucrose 5%</td>
<td>64</td>
<td>7.28</td>
<td>1.6</td>
<td>0.03</td>
<td>90</td>
</tr>
</tbody>
</table>

Infrared spectra were also monitored for KGF-2 in the lyophilized samples as a function of storage time and temperature. No significant changes were observed in the spectra after the 16-week storage period (data not shown).

### 4.3.4 Glassy State Physical Properties of Lyophilized Formulations

Glass transition temperatures (Tg) for the lyophilized formulations tested were obtained from a previous publication (Xu et al., 2013) and are shown in Table 4.2. Tg
values were higher for trehalose-containing formulations than in corresponding sucrose-containing formulations. Tg increased with increasing HES content.

Moisture levels in formulations analyzed immediately after lyophilization were all below 1 wt %. Samples tested after storage at 40, 50 or 60°C for up to 16 weeks showed no change in residual moisture levels (data not shown).

Specific surface areas of lyophilized formulations are reported in Table 4.3. Specific surface areas for the lyophilized formulations followed the same order as their Tg values, with specific surface areas increasing with increased HES content, and higher values observed for trehalose-containing formulations compared with sucrose-containing formulations.

Table 4.3. Specific surface areas for lyophilized formulations determined from BET analysis of krypton adsorption, and estimated fraction of KGF-2 on the surface of the lyophilized powders.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Specific Surface Area, m²/g</th>
<th>Estimated Fraction of KGF-2 on Surface, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% HES</td>
<td>2.08±0.01</td>
<td>21.2</td>
</tr>
<tr>
<td>5% trehalose</td>
<td>1.55±0.01</td>
<td>15.8</td>
</tr>
<tr>
<td>5% sucrose</td>
<td>1.17±0.06</td>
<td>11.9</td>
</tr>
<tr>
<td>2.5% HES and 2.5% trehalose</td>
<td>1.93±0.03</td>
<td>19.6</td>
</tr>
<tr>
<td>2.5% HES and 2.5% sucrose</td>
<td>1.63±0.01</td>
<td>16.6</td>
</tr>
<tr>
<td>4% HES and 1% trehalose</td>
<td>2.02±0.06</td>
<td>20.6</td>
</tr>
<tr>
<td>4% HES and 1% sucrose</td>
<td>1.96±0.11</td>
<td>20.0</td>
</tr>
<tr>
<td>1% HES and 4% trehalose</td>
<td>1.66±0.08</td>
<td>16.9</td>
</tr>
</tbody>
</table>
Other glass dynamics parameters (Table 4.2) for the lyophilized formulations were obtained from published values (Chieng et al.; Chieng et al.). These include values for inverse mean square displacement $<u^2>^{-1}$ for hydrogen atoms as measured by neutron scattering, and the relaxation time $\tau^\beta$, which is a parameter that can be measured by isothermal calorimetry (Chieng et al.; Chieng et al.) and that characterizes relaxation processes that correlate with fast Johari-Goldstein motions in the glass (Ngai, 2011).

4.4 Discussion

To analyze the kinetics of KGF-2 degradation in lyophilized formulations, we first attempted to fit the data to two models: a model wherein the degradation was described by simple first-order kinetics, and a model with “square root of time” kinetics (Wang et al., 2009a) $P(t) = P_0 + k\sqrt{t}$, where $P(t)$ is the purity as a function of time, $P_0$ denotes the initial purity of KGF-2 and $k$ is a rate constant. Both models produced poor fits with the experimental data sets (data not shown). Fits of the two models to the data for KGF-2 aggregation were especially poor, as neither model could capture adequately the plateauing of aggregation levels after the first four weeks of storage.

We anticipated that physical and chemical degradation of KGF-2 during storage in lyophilized formulations might be correlated with various parameters that
reflect either: a) relaxation processes in the glass; or b) protein native structural retention. The parameters that we examined included the glass transition temperature Tg of the formulations, which is related to slow, large-scale, “α” relaxation processes in the glass, the relaxation time τ^6 which correlates with faster, Johari-Goldstein motions, and the inverse mean amplitude, <u^2>_1 for hydrogen atoms, which provides an indication of the importance of very fast “β” relaxations. As measures of the degree of perturbation of protein secondary structure, we used the change in peak width at half height (ΔW_{1/2}) for the dominant band at 1648 cm⁻¹ in KGF-2’s second derivative infrared spectrum, and the percent overlap for infrared spectra of KGF-2 in lyophilized formulations compared with those for the native protein in liquid formulations. Plots of the fraction of KGF-2 that was oxidized or aggregated after 16 weeks of storage as a function of each parameter were examined for correlations (Figures 4.4-4.5, Table 4.4). The amount of aggregate formed during storage correlated only moderately (R² values 0.6-0.78, Table 4.4) with increasing Tg, higher levels of rapid motion in the glass phase (smaller values of 1/<u^2>_1) and greater degrees of KGF-2 structural perturbation (either increased ΔW_{1/2} or decreased % spectral overlap). No correlation with aggregation was observed for values of τ^6. For all of the parameters tested, the degree of correlation with the amount of aggregate formed during storage was independent of temperature.
Figure 4.4. Correlation of extent of aggregation of KGF-2 after 16 weeks of storage as a function of glassy state properties and protein secondary structure retention. A) Fraction of KGF-2 aggregated as a function of Tg. B) Fraction of KGF-2 aggregated as a function of the natural logarithm of the characteristic Johari-Goldstein relaxation time ln(τ^β). C) Fraction of KGF-2 aggregated as a function of inverse mean square displacement distance for hydrogen atoms (\(<u^2>^{-1}\)) as determined by neutron scattering. D) Fraction of KGF-2 aggregated as a function of retention of KGF-2 native secondary structure, as reflected in peak width at half height (ΔW_{1/2}) for dominant band in the second derivative infrared spectrum. Diamonds, squares and triangles represent data taken after storage at 40, 50 and 60°C, respectively.
Figure 4.5. Correlation of the fraction of soluble KGF-2 that is oxidized after 16 weeks of storage as a function of glassy state properties and protein secondary structure retention. A) Fraction of KGF-2 oxidized as a function of Tg. B) Fraction of KGF-2 oxidized as a function of the natural logarithm of the characteristic Johari-Goldstein relaxation time \( \ln(\tau^0) \). C) Fraction of KGF-2 oxidized as a function of inverse mean square displacement distance for hydrogen atoms \( \langle u^2 \rangle^{-1} \) as determined by neutron scattering. D) Fraction of KGF-2 oxidized as a function of retention of KGF-2 native secondary structure, as reflected in peak width at half height \( (\Delta W_{1/2}) \) for dominant band in the second derivative infrared spectrum. Diamonds, squares and triangles represent data taken after storage at 40, 50 and 60°C, respectively.
Table 4.4. Correlation coefficients $R^2$ from linear regression plots of the fraction of KGF-2 that aggregated (top) or oxidized (bottom) in disaccharide and disaccharide:HES formulations during 16 weeks of storage, vs. formulation glass transition temperature, increase in infrared peak width, $\ln(\tau^\beta)$, and inverse mean square displacement of hydrogen atoms.

<table>
<thead>
<tr>
<th>Storage Temperature</th>
<th>$R^2$, Fraction KGF-2 Aggregated vs. Tg</th>
<th>$R^2$, Fraction KGF-2 Aggregated vs. $\Delta W_{1/2}$</th>
<th>$R^2$, Fraction KGF-2 Aggregated vs. $\ln(\tau^\beta)$</th>
<th>$R^2$, Fraction KGF-2 Aggregated vs. $&lt;u^2&gt;^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 °C</td>
<td>.78</td>
<td>.70</td>
<td>.34</td>
<td>.72</td>
</tr>
<tr>
<td>50 °C</td>
<td>.72</td>
<td>.62</td>
<td>.24</td>
<td>.74</td>
</tr>
<tr>
<td>60 °C</td>
<td>.77</td>
<td>.69</td>
<td>.28</td>
<td>.65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Storage Temperature</th>
<th>$R^2$, Fraction KGF-2 Oxidized vs. Tg</th>
<th>$R^2$, Fraction KGF-2 Oxidized vs. $\Delta W_{1/2}$</th>
<th>$R^2$, Fraction KGF-2 Oxidized vs. $\ln(\tau^\beta)$</th>
<th>$R^2$, Fraction KGF-2 Oxidized vs. $&lt;u^2&gt;^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 °C</td>
<td>.89</td>
<td>.86</td>
<td>.58</td>
<td>.49</td>
</tr>
<tr>
<td>50 °C</td>
<td>.68</td>
<td>.61</td>
<td>.33</td>
<td>.75</td>
</tr>
<tr>
<td>60 °C</td>
<td>.20</td>
<td>.15</td>
<td>.00</td>
<td>.50</td>
</tr>
</tbody>
</table>

In contrast, at 40° C, the fraction of the protein molecules that were oxidized during 16 weeks storage was well-correlated with Tg and $\Delta W_{1/2}$, but poorly correlated with Johari-Goldstein relaxations ($\tau^\beta$) or fast $\beta$ ($<u^2>^{-1}$) relaxations (Figure 4.4). Correlations for oxidation with Tg and $\Delta W_{1/2}$ decreased as temperature was increased to 50 and 60°C; at 60°C none of the four parameters showed significant correlation.

Overall, the parameter that was most correlated to KGF-2 damage (both aggregation and oxidation) was Tg. Interestingly, however, the correlation was the inverse of that which might intuitively have been expected. Protein aggregation
requires large-scale diffusive motions which slow as Tg increases, yet protein degradation increased as Tg increased. Thus, it is not likely that KGF-2 aggregation occurs at a significant rate during storage within the glassy solid.

If aggregation does not occur in the glassy state, how might the time-dependent increases in aggregate levels be explained? One possible explanation is that small-scale motions, especially the fast β relaxations reflected in measurements of $<\mathbf{u}^2>$, might allow the protein to undergo time-dependent structural changes within the glassy formulations, creating populations with aggregation-prone structures that more rapidly aggregate upon reconstitution. This explanation seems to conflict with the observation that no additional changes in KGF-2 secondary structure could be observed as a function of storage time. However, structural changes in relatively small fractions of the protein population may not be detected by infrared spectroscopy (D’Antonio et al.; Jiang et al., 2011; Wang et al., 2009c).

The pronounced plateau regions seen in the aggregation data (and in a more muted fashion in the oxidation data) suggest another explanation for the observed degradation kinetics: the possibility that the samples contained multiple environments wherein the protein degrades at different rates. In the freezing step of the lyophilization process, crystals of essentially pure ice are formed, and protein may accumulate at the ice-liquid interface (Twomey et al., 2013). During lyophilization, the ice is removed by sublimation, leaving the accumulated protein at the newly formed glassy solid-air interface (Webb et al., 2002). Protein adsorbed at the surface
may provide a population of molecules that are more susceptible to damage than those in the bulk glass.

Earlier studies showed that recombinant human interferon-γ adsorbs at ice/liquid interfaces during lyophilization (Webb et al., 2002), and bovine serum albumin and trypsin were found to be adsorbed at the surface of lyophilized disaccharide glasses (Millqvist-Fureby et al., 1999). A study on stability of a vaccine lyophilized in carbohydrate glasses found that the rate of degradation increased with the amount of protein present on the surface (Abdul-Fattah et al., 2007b). Of particular interest to the present study, Abdul-Fattah et al. lyophilized rhGH in various carbohydrate formulations and detected rhGH accumulated on the surface of the glassy powders (Abdul-Fattah et al., 2008). In lyophilized sucrose formulations of an antibody, the fraction of the protein found at the glass-air interface increased with decreasing weight ratios of protein:sucrose; 1.0% of the antibody mass in the sample was found at the interface in formulations with a protein:sucrose ratio of 4:1, but this fraction increased from 1.0% to 2.2% and 6.4% as the protein:sucrose ratio decreased to 1:4 and 1:19, respectively (Abdul-Fattah et al., 2007a). The protein:carbohydrate ratio in the current study is 1:50.

Proteins adsorbed at interfaces are often prone to structural damage and aggregation (Bee et al., 2011; Sluzky et al., 1992; Sluzky et al., 1991). For example, lactate dehydrogenase, hemoglobin, Factor XIII and a monoclonal antibody unfolded and aggregated at ice-water interfaces (Kerwin et al., 1998; Kreilgard et al., 1996; Schwegman et al., 2009). Human growth hormone aggregation during freeze-thawing
increased with faster cooling rates, presumably due to the greater amount of ice-water interface present at faster cooling rates. (Eckhardt et al., 1991) Furthermore, for 6 different proteins, the formation of protein particles during freeze-thawing increased with increasing degree of surface denaturation. (Chang et al., 1996)

To explore whether accumulation of KGF-2 on surfaces of lyophilized powders could account for the observed degradation kinetics during storage, we first used BET analysis to measure specific surface areas of lyophilized formulations containing HES, trehalose, sucrose, and 1:1 weight ratio mixtures of HES:sucrose and HES:trehalose. Specific surface areas increased as the Tg of the formulations increased (Table 4.2 and 4.3), a phenomenon that is explained by the slower rates and extents of Ostwald ripening of ice crystals in formulations with higher glass transition temperatures. (Searles et al., 2001) The amount of KGF-2 on the surface was then estimated by multiplying the specific surface area by an estimated a surface coverage of 2 mg/m² (Table 4.3). Estimated maximum ice-water surface coverage for interferon-γ, a similarly-sized protein, were reported as 4.5 mg/m², and protein adsorption to a variety of surfaces such as glass (Bee et al., 2009; Hoehne et al., 2011), silica (Bee et al., 2009; Hoehne et al., 2011), polystyrene (Engel et al., 2002) and cellulose (Bee et al., 2009) has been reported to range from 2-4 mg/m²; thus our estimate of 2mg/m² may yield a conservative estimate of the amount of KGF-2 on the surface of lyophilized samples.
We fit the data for KGF-2 aggregation and oxidation during storage to a simple model that assumes that protein molecules on the surface and in the bulk glass degrade with first order kinetics:

\[
P(t) = P_0 f \exp(-k_{\text{surface}} t) + (1 - f) \exp(-k_{\text{bulk}} t)
\]

Equation 2

where \( P(t) \) is the mass of native protein in the sample as a function of storage time \( t \), \( P_0 \) is the amount of native protein in the sample immediately after lyophilization, \( f \) is the fraction of the protein in the sample found on the surface of the glass, and \( k_{\text{surface}} \) and \( k_{\text{bulk}} \) are apparent first order rate constants for aggregation or oxidation on the surface and in the bulk, respectively of the glassy powders.

For the samples that contained disaccharides or disaccharide:HES mixtures, the best-fit values of \( k_{\text{surface}} \) and \( k_{\text{bulk}} \) for both aggregation and oxidation were temperature-dependent, but were essentially independent of formulation. A global fit to the data for these eight sample types was then used to determine a single value of \( k_{\text{surface}} \) and \( k_{\text{bulk}} \) at each temperature (Table 4.5). Samples containing only HES as an excipient were analyzed separately; values of \( k_{\text{surface}} \) for aggregation in the HES-only formulations were larger than those obtained for the disaccharide-containing formulations, although the rate constants for degradation of KGF-2 on the surface were still much larger than those in the bulk glass (Table 4.5). This is consistent with the observation that the structure of KGF-2 was much more perturbed after lyophilization in HES samples than in any of the samples containing disaccharides,
as evidenced by values of ΔW_{1/2} that ranged from 0.03 to 3.73 cm\(^{-1}\) in formulations containing disaccharide, whereas ΔW_{1/2} in HES samples was 12.42 cm\(^{-1}\) (Table 4.2).

Table 4.5. Apparent first-order rate constants for KGF-2 aggregation and oxidation at the surface and in the bulk glass phase of various lyophilized formulations.

<table>
<thead>
<tr>
<th>Degradation type</th>
<th>Lyophilized Formulation</th>
<th>T=40°C</th>
<th>T=50°C</th>
<th>T=60°C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( k_{\text{surface}} ), wk(^{-1})</td>
<td>( k_{\text{bulk}} ), wk(^{-1})</td>
<td>( k_{\text{surface}} ), wk(^{-1})</td>
<td>( k_{\text{bulk}} ), wk(^{-1})</td>
</tr>
<tr>
<td>Aggregation</td>
<td>disaccharides and disaccharide:HES mixtures</td>
<td>0.07</td>
<td>0.00</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>HES</td>
<td>.21</td>
<td>.01</td>
<td>3.4</td>
</tr>
<tr>
<td>Oxidation</td>
<td>disaccharides and disaccharide:HES mixtures</td>
<td>0.54</td>
<td>0.01</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>HES</td>
<td>0.93</td>
<td>0.03</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Interestingly, for both aggregation and oxidation, the best-fit rate constants suggest that degradation is much more rapid in the population of protein molecules found on the surface as compared to those found in the bulk glass. In fact, \( k_{\text{bulk}} \) for aggregation was essentially zero. For aggregation the small value for \( k_{\text{bulk}} \) is intuitive, because the very high viscosities found in the glassy state should effectively eliminate the large-scale translational motions required for proteins to aggregate. Likewise, because aggregation typically requires protein unfolding, and protein molecules at interfaces are more likely to be conformationally perturbed, the larger values of \( k_{\text{surface}} \) are expected for aggregation. A similar argument can be made to rationalize the values of \( k_{\text{surface}} \) and \( k_{\text{bulk}} \) obtained for oxidation, because protein oxidation
requires diffusion of an oxidizing species (likely O$_2$) to the protein, and rates of oxidation are increased for exposed amino acid residues. Both the diffusion of O$_2$ and the exposure of (normally buried) amino acid residues are expected to be higher in the conformationally-perturbed surface layer, yielding larger values of $k_{\text{surface}}$ as compared $k_{\text{bulk}}$ for oxidation.

Figure 4.6 shows the observed amount of monomeric KGF-2 remaining in each of the nine sample types samples at each of the storage times and temperatures tested, plotted versus the amount predicted based on Equation 2 and the best fit rate constants from Table 4.5. The simple model provided a satisfactory fit to the data, as evidenced by the correlation coefficient $R^2=0.82$.

Figure 4.6. Measured levels of monomeric KGF-2 compared with predictions from the model described by Equation 2 and the associated parameters found in Table 4.5. Data are for all monomer fractions recorded after storage at 1, 4, 9 and 16 weeks and temperatures of 40, 50, and 60 °C, for each of the formulations.
Similar results were obtained from the analysis of KGF-2 oxidation using Equation 2. Figure 4.7 shows the measured fraction of un-oxidized KGF-2 remaining in solution in each of the nine sample types at each of the storage times and temperatures tested, plotted against the fraction predicted from equation 2 and the best fit rate constants from Table 4.5. The simple model provides a reasonable fit to the data (correlation coefficient \( R^2 = 0.84 \)), especially considering the fact that the measured oxidation levels only account for oxidation in the soluble fraction of KGF-2, and likely reflect degradation at multiple oxidation sites.

Figure 4.7. Measured levels of un-oxidized KGF-2 in the soluble fraction compared with predictions from the model described by Equation 2 and the associated parameters found in Table 4.5. Data are for all un-oxidized fractions recorded after storage at 1, 4, 9 and 16 weeks and temperatures of 40, 50, and 60 °C, for each of the formulations.
4.5 Conclusions

Although lyophilized formulations of therapeutic proteins provide effective and relatively stable dosage forms, degradation of the dried protein can occur at rates that are still unacceptable for a pharmaceutical product. Therefore, to optimize stability of proteins in dried formulations, it is important to understand the factors that govern stability during long-term storage. In the current study, we found as expected (Xu et al., 2013) that degradation rates were generally decreased in formulations with greater native state structural retention and with reduced fast $\beta$ relaxations. But these two factors could not account quantitatively for the aggregation and chemical degradation rates of KGF-2 observed. Rather, it appears that a dominant factor governing protein degradation in freeze-dried formulations is the fraction of protein found at the glass solid-air interface. We suggest that future mechanistic studies on lyophilized protein formulations and practical efforts to optimize stability of freeze-dried proteins consider this factor, and develop means by which to reduce surface area of dried formulations and/or minimize protein adsorption at the interface. Such mitigation strategies could include annealing during the freeze-drying process (Searles et al., 2001; Webb et al., 2003) and the inclusion of nonionic surfactants, respectively.
4.6 References


CHAPTER 5
Protein Quantity on the Air-Solid Interface Determines Degradation Rates of Human Growth Hormone in Lyophilized Samples


5.1 Introduction

Lyophilization is widely accepted as an effective method to improve long-term stability of pharmaceuticals, especially therapeutic protein products (Carpenter et al., 1997). In glassy lyophilized solids, both physical and chemical degradation processes are greatly hindered (Carpenter et al., 1997). However, storage of proteins in glassy solid formulations does not always guarantee a desired shelf life (Manning et al., 1989; Wang, 2000). For decades, efforts have been made to choose appropriate formulations and design robust lyophilization cycles to yield stable protein products (Carpenter et al., 1997). It has been widely documented that disaccharides (sucrose and trehalose) are effective stabilizers during lyophilization and storage, and this stabilizing effect has been ascribed to thermodynamic and/or kinetic stabilization mechanisms (Carpenter et al., 1998; Chang and Pikal, 2009; Cicerone and Douglas, 2012). In addition, the lyophilization cycle applied to a given formulation may
determine not only the morphology of the cake and physical properties of the glass, but also the stability of the protein during storage in the dried formulation (Abdul-Fattah et al., 2008; Carpenter et al., 1997; Carpenter et al., 1998; Chang and Pikal, 2009; Randolph, 1997; Xu et al., 2013). For example, the stability of methionyl human growth hormone in dried solid formulations was shown to depend not only on the type of stabilizer included in the formulation, but also on the drying method that was used (Abdul-Fattah et al., 2008). Despite some insights into mechanisms by which excipients provide stability to proteins, developing a formulation that provides adequate protection against protein damage is still a semi-empirical exercise, as is the design of a lyophilization cycle that provides optimal protein stability for a given formulation.

A conventional lyophilization cycle consists of freezing, primary drying and secondary drying steps. The freezing step is of paramount importance (Kasper and Friess, 2011; Searles et al., 2001a). During freezing, most of the water present in the original liquid (about 80% (Kasper and Friess, 2011)) is crystallized into essentially pure ice, which results in a freeze-concentrated solution for the remaining formulation. The pH of the freeze-concentrated liquid may undergo a shift due to preferential precipitation of buffer components, potentially contributing to protein denaturation (Heller et al., 1999; Rathore and Rajan, 2008). In addition, the rates of ice nucleation and crystal growth have large impacts on the ice morphology and ice-liquid interfacial area, and consequently on the final solid products’ specific solid-air
interfacial area formed after drying (Kasper and Friess, 2011; Randolph, 1997; Searles et al., 2001b; Webb et al., 2002).

Several strategies (Kasper and Friess, 2011; Patel et al., 2009) have been developed to manipulate the initial stages of the lyophilization cycle, such as shelf-ramp cooling, annealing, controlled ice nucleation and fast freezing by liquid N\textsubscript{2}. Shelf-ramp cooling is a standard cooling method in commercial freeze-drying, during which shelf temperature of the lyophilizer is decreased in a roughly linear fashion. In this case the maximum cooling rate is limited by the cooling capacity of lyophilizer (Cochran and Nail, 2009; Rambhatla et al., 2004). Shelf-ramp cooling typically results in a high degree of supercooling prior to initiation of freezing, followed by a period characterized by rapid nucleation of a large number of ice crystals (Searles et al., 2001b). Consequently, many small ice crystals are formed. During the primary drying portion of the lyophilization process, ice crystals are removed by sublimation, and the interface between the glass and the voids left behind contribute the specific surface area (SSA) of the resulting glassy solid (Hsu et al., 1995; Webb et al., 2003; Webb et al., 2002). The large number of relatively small ice crystals formed after shelf-ramp freezing in turn yields lyophilized cakes with large surface areas (Kasper and Friess, 2011).

Annealing refers to an additional step that may be added after freezing, during which the sample temperature is maintained between the ice melting temperature and the glass transition temperature of the maximally freeze concentrated solution, \(T_{g}'\) (Searles et al., 2001a; Webb et al., 2003) (or the eutectic melting temperature of
crystalline excipients, if that temperature is greater than $T_g$). Due to both the enhanced mobility of water and the contributions of surface energy to the elevated chemical potential of water in smaller crystals, water is transported from small ice crystals and redeposits onto large ice crystals (Kasper and Friess, 2011). As a result of this Ostwald ripening, larger ice crystals are generated during annealing, which in turn results in lyophilized cakes with reduced specific surface areas (Searles et al., 2001a). For the purposes of this report, this type of annealing process will be termed “pre-drying annealing”.

We note that Ostwald ripening also occurs in lyophilization cycles that use shelf ramp cooling without an annealing step, but to a lesser degree, because the length of time available for ripening (the time during which ice crystals are present and the temperature is above $T_g$) is much shorter in these cycles. To reduce the extent of Ostwald ripening even further, fast freezing methods may be used to limit the time that samples spend at temperatures between the ice melting temperature and $T_g$.

One method of fast freezing is to immerse samples contained in vials into liquid N$_2$ (N$_2$-immersion). Fast freezing also may be achieved by spraying liquid droplets of sample directly into liquid N$_2$ (N$_2$-droplet-freezing) (Bhatnagar et al., 2007; Chang et al., 1996; Heller et al., 1999; Maa et al., 1999; Patapoff and Overcashier, 2002). In the N$_2$ immersion procedure, the relatively low thermal conductivity of the glass vials limits heat transfer, making the effective cooling rate slower than that which can be achieved using the N$_2$-droplet-freezing method (Kasper and Friess, 2011). Compared with the standard shelf ramp cooling both of these two fast freezing methods result
Another type of annealing (herein termed “post-drying annealing”) may be implemented by briefly incubating dry, glassy samples at a high (but sub-$T_g$) temperature at the end of the secondary drying step of the lyophilization cycle. Pikal et al. reported that post-drying annealing could enhance protein stability (Wang et al., 2010). The stability increase was presumably a result of relaxation processes that lead to slower motions in the glassy state (Wang et al., 2010).

The current study examined formulations of rhGH in the presence of three glass forming excipients; sucrose, trehalose and hydroxyethyl starch. These formulations were lyophilized using five different methods, which yielded glassy solids with different SSAs, surface protein contents, glassy state mobilities and degrees of retention of native secondary structure. Because we anticipate that protein molecules located on the surface of lyophilized glassy solids will have significantly faster degradation rates, we hypothesize that the extent of rhGH degradation during storage in various dried solid formulations prepared by different processing methods can largely be ascribed to the resulting levels of rhGH found at the solid-air interface after lyophilization.

5.2 Materials and methods

5.2.1 Materials
rhGH was expressed in *E. coli* and purified as described previously (Crisman and Randolph; Xu et al., 2013). Hydroxyethyl starch (HES; Viastarch) was purchased from Fresenius (Graz, Austria), and sucrose and trehalose were purchased from Mallinckrodt Baker (Phillipsburg, NJ). All other chemicals were purchased as reagent grade or higher. 5ml lyophilization glass vials (Product Number 68000318) and butyl rubber stoppers (Product Number 19560042) were purchased from West Pharmaceutical Services, Linville, PA.

5.2.2 Formulation and lyophilization cycle design

rhGH was formulated at a concentration of 1 mg/ml in one of three formulations. In addition to rhGH, each formulation contained 2 mM sodium phosphate at a pH of 7.4, as well as 5% (wt/v) of HES, trehalose or sucrose. Lyophilization was performed using a FTS Lyostar I system. An aliquot (1 ml) of each rhGH formulation was pipetted into vials, and lyophilized with one of five different lyophilization cycles, denoted as standard lyophilization, pre-drying annealing lyophilization, post-drying annealing lyophilization, N₂-immersion lyophilization and N₂-droplet-freezing lyophilization.

In the standard lyophilization cycle, sample vials were loaded onto the shelf, which was at room temperature. The shelf temperature was reduced to 10 °C, and samples were equilibrated at this temperature for 1 hour. Shelf temperature was then decreased to -5 °C at 1 °C min⁻¹, kept at -5 °C for 20 minutes, and then decreased to -45 °C at 1.3 °C min⁻¹. Samples were kept frozen at -45 °C for 400 minutes. Primary
drying was then initiated and performed at a shelf temperature of -20 °C and a chamber pressure of 70 mTorr for 1400 minutes. Secondary drying was then started by increasing the shelf temperature to 33 °C at a rate of 0.3 °C min⁻¹. Samples were held at 33 °C and 70 mTorr for four hours. Finally, vials were sealed in the chamber under dry nitrogen.

For the pre-drying annealing lyophilization cycle, an additional annealing step was added to the standard cycle. After samples were kept frozen at -45 °C for 400 minutes, shelf temperature was increased to -5 °C over 30 minutes. Then shelf temperature was kept at -5 °C for 6 hours before cooling to -45 °C. The shelf temperature was kept at -45 °C for 6 hours, and then the primary and secondary drying steps followed the same protocol as in the standard lyophilization cycle.

Post-drying annealing was performed using the same protocol as standard lyophilization cycle, except that after the standard secondary drying step, shelf temperature was increased up to 50 °C at a rate of 0.3 °C min⁻¹, and held at 50 °C for 6 hours before ending the cycle.

Liquid N₂-immersion lyophilization was carried out by immersing the glass vial containing 1ml formulation into a liquid N₂ bath for 2 minutes and then putting the vials onto the lyophilizer shelf, which was pre-cooled to -45 °C, for 400 minutes. The rest of primary and secondary drying steps were the same as standard lyophilization cycle.

In the liquid N₂-droplet-freezing lyophilization cycle, samples were slowly pipetted into the glass vials, which were filled with liquid N₂. The sample vials were
quickly moved onto the lyophilizer shelf, which was pre-cooled shelf at -45 °C, and held at this temperature for 400 minutes. The rest of the primary and secondary drying cycle followed the same protocol as in the standard lyophilization cycle.

5.2.3 Measurement of residual water content

Residual water contents of the lyophilized samples were analyzed using the Karl Fischer method (May et al., 1982). Triplicate samples were prepared in a dry nitrogen-purged box and measured using a Mettler DL37 KF coulometer (Hightstown, NJ), as described previously (Xu et al., 2013).

5.2.4 Glass transition temperature measurement by differential scanning calorimetry (DSC)

The glass transition temperatures of the maximally freeze-concentrated formulation (T_g') and the lyophilized formulations (T_g) were measured with a Perkin-Elmer Diamond DSC. For T_g' measurement, aqueous solutions (20ul) in aluminum pans were cooled from room temperature to -60 °C at 10 °C min⁻¹. After equilibration at -60 °C min⁻¹ for 5 minutes, samples were heated to -5 °C at 5 °C/min, and kept at -5 °C for 30 minutes before samples were recooled to -60 °C again at 10 °C/min. The second heating scan was up to 10 °C at 5 °C/min. In order to eliminate any thermal history effects, T_g' was determined from the onset of thermal transition measured during the second heating scan. T_g measurement of the lyophilization products
followed the same protocol as described previously (Xu et al., 2013). At least triplicate samples were used to determine the $T_g$ and $T_g'$.  

5.2.5 Protein secondary structure by infrared (IR) spectroscopy

In a dry box lyophilized samples (around 0.3 mg rhGH) were mixed with 0.5 g KBr using a mortar and pestle. After being transferred into a stainless steel die, samples were pressed into a disc using a vacuum press. IR spectra were collected on a Bomem MB-series spectrometer (Montreal, PQ, Canada). The spectra of the dried samples and of native aqueous rhGH were obtained as described previously (Xu et al., 2013). Data were processed to obtain second derivative IR spectra according to a previous publication (Xu et al., 2013). Finally, for the major negative band associated with the $\alpha$-helix content of rhGH the peak width at half height ($w_{1/2}$) was computed by subtracting the low wavenumber from the high one at the half peak height (Xu et al., 2013).

5.2.6 Circular dichroism (CD) spectroscopic measurement of rhGH secondary structure after reconstitution

CD spectra of rhGH in aqueous solution before lyophilization and after reconstitution of lyophilized samples were obtained with a Chirascan-Plus (Applied Photophysics, UK) CD spectrometer. For each sample, the CD spectrum was plotted from 200 to 260 nm by averaging spectra from triplicate measurements (Xu et al., 2013).
5.2.7 Surface area measurement

A Quantachrome Autosorb-1 (Boynton Beach, FL) was employed to measure
the SSA’s of lyophilized formulations, using five-point krypton adsorption isotherms.
For each formulation, samples from five vials of placebo formulation (no protein) were
placed into the cell. The cell with samples was held under vacuum for at least 5 hours
at room temperature to remove the moisture prior to initiation of the surface area
measurement. Triplicate samples were measured to determine the surface area for
each sample.

5.2.8 Electron spectroscopy for chemical analysis  (ESCA)

To prevent from moisture uptake by dried formulations, all sample handling
and preparation were performed in a glove-bag purged with dry air (RH<5%).
Samples of lyophilized powders were deposited using double-sided adhesive tape onto
a sample holder (45°) that was covered with a copper tape. The sample holder was
transferred to the analysis chamber for ESCA measurements. ESCA was performed
with a scanning auger multi probe PHI spectrometer (Model 25-120) equipped with
monochromatic Al Kα source (pass energy 100 eV). The C (1s) photoelectron line at
284.6 eV was used as an internal standard for the correction of the charging effect in
all samples. The vacuum was maintained at ~10⁻⁸ Torr or lower. Spectra were
collected by AugerScan (version 3.22) and analyzed using CasaXPS software (version
2.3.12). At least triplicate samples were used to determine elemental composition for each formulation.

5.2.9 **Calculation of the mass of rhGH on the surface of lyophilized formulations**

ESCA was used to measure elemental compositions of the surface layer of lyophilized powders. ESCA is sensitive to elemental composition in approximately the outermost 100 Å of the lyophilized powders (Webb et al., 2002), and thus was used to measure the mass fraction of rhGH on the surface. The mass of final solid \( m_f \) in each vial after lyophilization is about 52 mg, which is essentially the sum of all the components: rhGH (1mg), sugar (50mg), phosphate buffer (~0.5mg) and residual water (less than 1% of total mass). rhGH contains carbon (C), oxygen (O), nitrogen (N), hydrogen (H) and sulfur (S), and based on its primary sequence, its atomic composition is \( C_{995}H_{1541}N_{263}O_{301}S_8 \). According to the manufacturer, ESCA is not capable of detecting H or any element whose surface concentration is less than 0.1%. In the lyophilized solids which contain protein, sugar and buffer, the overall S content is diluted down to less than 0.1%, thus all ESCA results reflect only the surface elemental composition of N, C and O. Moreover, because neither sugar nor buffer contain N, the N peak is indicative of rhGH molecules in the outmost 100 Å, i.e. protein on the surface of the dried formulation. The calculated theoretical overall N content of rhGH on a sulfur- and H-free basis is 18.0%. To calculate the mass of protein molecules on the surface, we assume that the surface layer thickness probed
by ESCA (l) is equal to the 100 Å (Webb et al., 2002). Furthermore, following the
analysis presented in earlier studies (Abdul-Fattah et al., 2008; Webb et al., 2002),
we assumed that the density of the solid fraction within the cake (ρ) is constant across
the cake, with a value of roughly 1.1g cm\(^{-3}\) (Abdul-Fattah et al., 2008). Under these
assumptions, the mass fraction of rhGH in the surface layer is:

\[
\frac{m_{\text{surface protein}}}{m_t} = \frac{SSA \times l \times \rho \times N\%}{18.0\%}
\]

(1)

where \(m_t\) (52mg) is total lyophilizate mass per vial, SSA is each formulation specific
surface area per gram cake (m\(^2\)/g), \(l\) (100 Å) is the surface thickness, and \(\rho\) (1.1g cm\(^{-3}\))
is the density of the solid within the cake. N\% is the surface N percentage measured
by ESCA.

5.2.10 rhGH storage stability study

Lyophilized samples were incubated for 16 weeks at 323 K. At each time point
(immediately after lyophilization, and after 1, 4, 9 and 16 weeks), after rehydration
of samples with water rhGH aggregation, deamidation and oxidation levels were
measured by size exclusion chromatography (SEC), ion exchange chromatography
(IEC) and reverse phase chromatography (RP), respectively (Xu et al., 2013). At least
triplicate measurements were carried out to determine the quantities of remaining of
monomer/native protein at each time point.

5.2.11 Error analysis
Throughout the manuscript, when error bars are presented, they represent the experimental mean ± standard deviation, based on n≥3.

5.3 Results

5.3.1 Design of the pre-drying annealing step

A requirement for pre-drying annealing is that the sample temperature be maintained below the freezing point but above the $T_g'$ (or the eutectic melting temperature if there are any crystalline components). $T_g'$ values determined from DSC experiments were -15.5±0.3, -37.2±0.2, -38.9±0.2 °C for 5% HES, 5% trehalose and 5% sucrose solutions, respectively. In addition, DSC results showed that ice melt onset temperature of these formulations was -2.5±0.5 °C. Based on these results and previous successful annealing protocols (Searles et al., 2001a), a shelf temperature of -5 °C was selected for the annealing temperature for all three formulations.

5.3.2 Cake structures, water content and glass transition temperatures of lyophilized formulations

Formulations prepared by all five lyophilization cycles resulted in visually elegant cake structures (Xu et al., 2013). The water contents for all lyophilized samples prepared by all of the different cycles were less than 1% wt/wt. The DSC measurements for all three formulations prepared by five different cycles showed single transitions at their respective $T_g$'s. $T_g$’s for the three formulations did not show
large variations as a function of the lyophilization cycle parameters. $T_g$'s were 203±3, 98±2 and 64±2 °C for HES, trehalose and sucrose formulations, respectively (table 5.1) (Chieng et al., 2013a; Chieng et al., 2013b).

Table 5.1. $T_g$ (onset) temperatures measured by DSC, previously reported ln ($\tau^\alpha$, h) measured by thermal activity monitor (Chieng et al., 2013b) and previously reported $<u^2>-1$ measured by neutron scattering (Chieng et al., 2013a) for lyophilized formulations (without protein) prepared using the standard freeze-drying cycle.

<table>
<thead>
<tr>
<th></th>
<th>$T_g$ (onset) (°C) ± SD</th>
<th>ln($\tau^\alpha$, h) (at 323K)</th>
<th>$&lt;u^2&gt;-1$ (Å²)±SD (at 323K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% HES</td>
<td>203±3</td>
<td>3.4</td>
<td>2.25±0.04</td>
</tr>
<tr>
<td>5% Trehalose</td>
<td>98±2</td>
<td>2.0</td>
<td>9.10±0.50</td>
</tr>
<tr>
<td>5% Sucrose</td>
<td>64±2</td>
<td>0.2</td>
<td>6.72±0.17</td>
</tr>
</tbody>
</table>

5.3.3 Protein structure in lyophilized formulations

Immediately after lyophilization, samples were analyzed with IR spectroscopy to obtain second derivative spectra. The dominant negative band at 1654 cm⁻¹ corresponds to the $\alpha$-helical structure of rhGH (Xu et al., 2013). Figure 5.1 shows spectra for rhGH lyophilized in the presence of 5% HES (Figure 5.1A), 5% trehalose (Figure 5.1B) and 5% sucrose (Figure 5.1C), for each of the different lyophilization cycles tested. In general, based on the half-height width and the depth of the negative of the $\alpha$-helix band near 1654 cm⁻¹ measured by IR, rhGH in sucrose and trehalose formulations exhibited greater native-like secondary structural content than rhGH lyophilized in HES formulations; sucrose formulations dried by all cycles led to the
most native-like rhGH secondary structure. Most interesting, lyophilization cycles utilizing either pre-drying annealing or post-drying annealing yielded lyophilized formulations with more native-like rhGH secondary structure than those produced using the standard lyophilization cycle. In contrast, fast freezing methods (N₂-immersion and N₂-droplet-freezing lyophilization) resulted in less native-like structures, as shown in Figure 5.1.

Figure 5.1. 2nd-derivative IR spectroscopic analysis of freeze-dried formulations analyzed immediately after lyophilization. (A) 5% HES formulation, (B) 5% trehalose formulation, (C) 5% sucrose formulation. Formulations prepared by pre-drying annealing lyophilization (red), post-drying annealing (orange), standard lyophilization (purple), N₂-immersion (green), N₂-droplet-freezing (blue), aqueous native control (black).
5.3.4 Protein structure in lyophilized and reconstituted formulations

After lyophilization, samples were immediately reconstituted with water, and the secondary structure of rhGH was measured by far-UV (200-260 nm) CD spectroscopy (Figure 5.2). The spectra for the protein from all the formulations prepared by each of the different lyophilization methods were indistinguishable from that of the aqueous native control protein. The CD data suggested that the secondary structural perturbations observed by IR in lyophilized samples were largely reversible upon reconstitution.

Figure 5.2. Protein secondary structures measured by CD spectroscopy. Samples prepared by different lyophilization cycles were reconstituted immediately after lyophilization, and there were no significant variations in the CD spectra between different samples.
5.3.5 Specific surface area, surface N percentage and amount of protein on the surface

SSA’s of the lyophilized formulations were measured using BET krypton adsorption (Table 5.2). The N\textsubscript{2}-immersion and N\textsubscript{2}-droplet-freezing lyophilized samples had much larger SSAs than the respective samples lyophilized using the standard cycle. Post-drying annealing had minimal impact on the SSAs of the lyophilized formulations we studied. On the other hand, pre-drying annealing reduced SSAs up to 2-fold compared to standard lyophilized samples. Furthermore, regardless of which lyophilization method was used, higher SSAs were found for the HES formulation compared to those for the disaccharide formulations.

Table 5.2. Specific surface areas of lyophilized formulations, atom percent N in the outmost 100 Å layer of lyophilized formulations and the mass fraction of protein in that layer.

<table>
<thead>
<tr>
<th></th>
<th>SSA (m\textsuperscript{2}/g)</th>
<th>N%</th>
<th>Surface rhGH Fraction, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard lyophilization</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% HES</td>
<td>2.3±0.2</td>
<td>1.4±0.2</td>
<td>10.2</td>
</tr>
<tr>
<td>5% trehalose</td>
<td>1.6±0.1</td>
<td>0.6±0.1</td>
<td>3.1</td>
</tr>
<tr>
<td>5% sucrose</td>
<td>1.2±0.1</td>
<td>0.7±0.1</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>Pre-drying annealing</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% HES</td>
<td>1.8±0.1</td>
<td>1.4±0.1</td>
<td>8.0</td>
</tr>
<tr>
<td>5% trehalose</td>
<td>0.8±0.1</td>
<td>0.6±0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>5% sucrose</td>
<td>0.8±0.1</td>
<td>0.7±0.1</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>Post-drying annealing</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5% HES</td>
<td>2.3±0.2</td>
<td>1.2±0.2</td>
<td>8.8</td>
</tr>
<tr>
<td>5% trehalose</td>
<td>1.3±0.1</td>
<td>0.4±0</td>
<td>1.7</td>
</tr>
<tr>
<td>5% sucrose</td>
<td>1.2±0.0</td>
<td>0.5±0.1</td>
<td>1.9</td>
</tr>
<tr>
<td>5% HES</td>
<td>6.0±0.5</td>
<td>1.5±0.2</td>
<td>28.6</td>
</tr>
</tbody>
</table>
ESCA measurements were first performed on a lyophilized control sample containing a 2:1 weight ratio of rhGH to phosphate buffer salts, without additional excipients. The control measurements yielded 12.6 wt% N (on a sulfur- and H-free basis), which compared well with the expected theoretical value of 12 wt% N. ESCA measurements of the %N on the surface of the lyophilized cakes containing glass-forming excipients are reported in Table 5.2. If the rhGH were homogeneously distributed throughout the dried solids, a %N value of 0.35% would be expected. However, in all samples tested, the %N values measured in the outermost surface layer probed by ESCA were significantly higher, indicating the presence of surface excesses of rhGH. Compared to results for the standard lyophilization cycle, the two fast freezing methods (N₂-immersion and N₂-droplet-freezing) both showed slightly higher N% in the outmost 100 Å of the dried formulations. On the other hand, post-drying annealing samples had low N%, and pre-drying annealing samples had N% values equivalent to those for standard lyophilized samples. For all processing methods, samples formulated in HES had higher levels of surface N% than did sucrose or trehalose formulations. Finally, the total amount of rhGH in the outmost 100 Å layer was calculated using the equation (1) (Table 5.2). Regardless of the type
of excipients used, samples prepared by two rapid freezing, liquid N\textsubscript{2} treatment methods showed the highest amounts of protein on their surfaces, as a result of both larger SSAs and higher surface N percentages. Likewise, regardless of the processing method that was used, the amounts of rhGH on the surface of lyophilized HES formulations were higher than those for formulations prepared from sucrose or trehalose solutions.

5.3.6 Lyophilization impact on rhGH monomer levels

Prior to lyophilization, SEC analysis of rhGH showed that the protein was >99.9\% monomeric. Upon reconstitution of samples immediately after lyophilization, about 2-3\% monomer loss (compared to control samples) was observed in all formulations prepared by the two faster freezing methods (N\textsubscript{2}-immersion and N\textsubscript{2}-droplet-freezing lyophilization cycles) (Figure 5.3). On the other hand, no significant differences were observed in levels of deamidation or oxidation before and after lyophilization process, regardless of which formulation or lyophilization cycle was used (data not shown).

Figure 5.3. Percentage of rhGH monomer detected by SEC analysis after lyophilization with various cycles and immediate reconstitution. (■) standard lyophilization; (▲) pre-drying annealing; (◆) post-drying annealing; (□) N\textsubscript{2}-immersion; (▲▲) N\textsubscript{2}-droplet-freezing. Relatively higher monomer loss was seen in the samples prepared by N\textsubscript{2}-immersion and N\textsubscript{2}-droplet-freezing lyophilization methods.
5.3.7 Storage stability of rhGH lyophilized samples

The percentages of remaining monomeric rhGH, unoxidized rhGH and non-deamidated rhGH were determined at each time point throughout the 16-week incubation study (Figure 5.4). The rate of loss of native protein due to formation of degradation products decreased with increasing time. Some formulations showed plateaus by the end of 16-week incubation study. It is clear that among all three formulations, the highest levels of damage were seen in the samples prepared by fast freezing methods. In contrast, samples treated with annealing (pre-drying or post-drying annealing) showed least damage after the 16-week incubation period. In addition, both deamidation and oxidation were faster than aggregation, and
disaccharide formulations (sucrose and trehalose) exhibited slower degradation kinetics for rhGH than HES formulations did.

Figure 5.4. Storage stability as a function of incubation time at 323 K for rhGH in formulations lyophilized using various cycle parameters. Panels: A) fraction monomeric rhGH remaining after storage in glassy HES; B) fraction rhGH not deamidated after storage in glassy HES; C) fraction rhGH that is not oxidized after storage in glassy HES; D) fraction monomeric rhGH remaining after storage in glassy trehalose; E) fraction rhGH not deamidated after storage in glassy trehalose; F) fraction rhGH that is not oxidized after storage in glassy trehalose; G) fraction monomeric rhGH remaining after storage in glassy sucrose; H) fraction rhGH not deamidated after storage in glassy sucrose; I) fraction rhGH that is not oxidized after storage in glassy sucrose. For all panels, lyophilization cycle conditions are represented by ♦: standard lyophilization, ●: pre-drying annealing, ■: post-drying annealing, ▲: N₂-immersion, ▼: N₂-droplet-freezing. Panel A-C represents HES formulation, Panel D-F represents trehalose formulation, and panel G-I represents sucrose formulation. Connected lines are predicted values from two parameter (k_s and k_b) first-order kinetics model for each individual formulation, using surface protein quantities determined from ESCA and SSA measurements.
5.4 Discussion

We hypothesized that the dominant factor that determines the rate of protein degradation observed during storage of lyophilized formulations is the amount of protein found at the solid-air interface after lyophilization. By employing different glass-forming stabilizers and lyophilization methods, samples with a wide range of masses of protein on solid-air interface were generated as a result of modulating both the SSA of the glass and concentration of the protein in the surface layer.

One major factor contributing to differences in the SSAs observed with the various lyophilization processes is Ostwald ripening of ice crystals, which only occurs
to a significant extent between the time when freezing is initiated and the time when the samples cool down to the glass transition temperature for the maximally freeze-concentrated solution \(T_g\). Samples prepared by the pre-drying annealing method (wherein the temperature was maintained between the freezing temperature and \(T_g\)) had the smallest SSA values of all the methods tested because the time available for Ostwald ripening of ice crystals was longest in that method. In contrast, the fast-freezing methods (liquid N\(_2\) immersion or droplet-freezing) cause the solution temperature to rapidly reach \(T_g\), thus allowing little time for Ostwald ripening and yielding correspondingly high SSA values. Finally, we observed that the SSA values for the formulation processed with the post-drying annealing cycle were equivalent to those for the standard cycle. This can be explained because the freezing portion of the lyophilization cycle was identical for the standard and post-drying annealing methods. Hence, the times available for Ostwald ripening for the two methods were equal, resulting in equal ice crystal size distributions and equivalent SSA values. Also, as long as post-drying annealing is executed well below the system \(T_g\), no change in SSA would be expected during this process.

The N\% (surface N percentage) of the various lyophilized formulations determined by ESCA is indicative of protein fraction on the surface, because no other formulation component contains N. For a given formulation, N\% was similar for samples prepared by pre-drying annealing, post-drying annealing and standard lyophilization methods, consistent with previous studies on methionyl rhGH (Abdul-Fattah et al., 2008). Samples prepared by the two liquid N\(_2\)-treated lyophilization
protocols resulted in higher N% (Table 5.2). In addition, the HES formulations had the highest protein fraction on the surface regardless of which lyophilization method was used, again consistent with the earlier study on methionyl rhGH, which demonstrated that surface concentrations of protein were highest in formulations containing polymeric excipients (Abdul-Fattah et al., 2008). A possible explanation for the higher masses of protein found on the surface of lyophilized HES formulations is that, during the freezing step of the lyophilization cycles, the greater viscosity of HES formulations hindered the diffusion of the protein away from growing ice crystal surfaces. The explanation for the high N% found on the surface of formulations prepared with liquid N₂ immersion or droplet-freezing may be similar. The rapid ice formation in the process and short time between initiation of freezing and sample glassification at T₉’ limited the time available for protein to diffuse away from growing ice surfaces.

Webb et al. reported that lyophilized formulations of recombinant human interferon-γ with higher SSAs also had higher rates of protein aggregation (Webb et al., 2003). It was also noted, based on electron spectroscopy for chemical analysis (ESCA) (Abdul-Fattah et al., 2008) measurements, that the surfaces of the lyophilized solids were enriched in protein. This enrichment was likely due to a combination protein adsorption at ice-water interfaces, and limited ability of large molecules such as proteins to diffuse away from the freezing front causing them to be trapped at the surface (Kasper and Friess, 2011). Consistent with previous reports (Adler et al., 2000; Millqvist-Fureby et al., 1999; Webb et al., 2002), we observed substantial
enrichment of protein on the solid-air surface of lyophilized powders (Table 5.2). Proteins found on the solid-air interfaces of glassy lyophilized solids certainly experience an environment that is dramatically different from that inside the glass (Zhu et al., 2011). Effective glass transition temperatures are expected to be lower in the interfacial region than in the bulk (Liu et al., 2013; Zuo et al., 2013), allowing greater mobility for any protein molecules found at the surface of glassy lyophilized powders.

rhGH structures in dried solids prepared using both pre-drying annealing and post-drying annealing were more native-like than in those prepared using the standard lyophilization cycle, whereas rhGH structures were most perturbed in samples that had been rapidly frozen using liquid N₂. Webb et al. proposed that annealing could serve to alleviate residual stress and reduce the excess free volume of the glass, thus improving protein structures (Webb et al., 2003). However, another explanation could be that protein molecules on the solid-air surface are more prone to structural perturbation. Hence, those samples with smaller quantities of protein at the solid-air surface (i.e., pre-drying annealed samples) should show more retention of native structure, whereas samples with larger surface protein quantities (e.g., those prepared with liquid-N₂ freezing) should show more perturbed structures. This is indeed the case, as can be seen in Figure 5.5, where formulations exhibiting a larger fraction of rhGH at the interface had less native-like structure as measured by IR, i.e. larger values of Δw₁/₂ (Xu et al., 2013).
Figure 5.5. Change of IR α-helical peak half width measured in lyophilized solids compared to that of native rhGH in aqueous solution ($\Delta w_{1/2}$), plotted against the fraction of the total protein found on surface. For some data points, error bars are smaller than the symbols.

In addition, previously we showed that decreased activation free energies ($\Delta G^\dagger$) for aggregation, deamidation and oxidation of rhGH correlated with increasing degrees of protein structural perturbation in lyophilized formulations (Xu et al., 2013). In turn, rhGH degradation (by aggregation, oxidation, and deamidation) was faster in samples wherein the rhGH structure was more perturbed (Xu et al., 2013). A similar result was seen in this study. As shown in Figure 5.6, the fraction of aggregated protein after 16 weeks incubation at 323 K increases with the degree of rhGH structural perturbation, as reflected in the change after lyophilization of the IR α-helical peak width at half height, $\Delta w_{1/2}$. 
Figure 5.6. Correlation of the percent of rhGH found as aggregates measured after 16 weeks incubation at 323K with the change compared to native rhGH in aqueous solution of the IR α-helical peak half width in the lyophilized solid formulations (Δw_{1/2}). For some data points, error bars are smaller than the symbols.

Kinetic model for rhGH degradation in lyophilized samples

Because populations of protein molecules found at the solid-air interface of lyophilized samples are likely to experience a significantly different environment from those molecules found in the bulk, we analyzed the aggregation, deamidation and oxidation kinetics for rhGH by assuming simple first-order dependence of degradation kinetics, both in the surface layer and in the bulk. Thus, for each of the degradation reactions, we write:

\[ P_{st}(t) = P_{st0}e^{-k_{st}t} \]  \hfill (2)

\[ P_{st}(t) = P_{bt0}e^{-k_{bt}t} \]  \hfill (3)

\[ P_{tot}(t) = P_{st}(t) + P_{bt}(t) \]  \hfill (4)
Where $P_{st}$ and $P_{bt}$ are the amount of native protein on the surface and in the bulk, respectively, at a certain time $t$, $P_{so}$ and $P_{bo}$ are the initial amount of native protein on the solid surface and in the bulk, respectively, and $k_{s,i}$ and $k_{b,i}$ are the apparent first order degradation rate constant for protein on the surface and in bulk, for each of the $i$ reactions (aggregation, deamidation, and oxidation). The sum of $P_{st}$ and $P_{bt}$ is equal to $P_{tot}$, the amount of remaining native protein at any given time point, which can be measured by size exclusion, ion exchange and reverse phase chromatographic analysis of the samples after reconstitution. For each degradation pathway and each formulation, using initial amounts of protein on the surface and in the bulk determined from the ESCA and SSA measurements for $P_{so}$ and $P_{bo}$, the two parameters $k_{s,i}$ and $k_{b,i}$ were fit to the data from the 16-week incubation study (Table 5.3), using the evolutionary solving method in the Microsoft Excel® solver package, with constraints of convergence as $10^{-8}$, mutation rate as 0.9 and maximum time as 120 seconds. Convergence to the reported optimal values was obtained from multiple initial guess values. Predicted kinetics based upon $k_{s,i}$ and $k_{b,i}$ determined for each formulation are plotted in Figure 5.4. In general, predicted values from individual two parameter ($k_{s,i}$ and $k_{b,i}$) first-order kinetics model fit well to the experimental data.

As expected, values of the surface-layer rate constants $k_{s,i}$ were much higher than the bulk glass rate constants $k_{b,i}$. For aggregation, the rate constant in the bulk $k_{s,agg}$ was negligible, as expected due to the high viscosity in the glassy state that limits diffusive transport of large molecules such as rhGH and also restricts the
relatively large scale motions required for the protein unfolding that is generally associated with aggregation. Oxidation and deamidation reactions, both of which require smaller degrees of molecular motion than does aggregation (Chang and Pikal, 2009) showed rate constants in the bulk glass that were roughly two orders of magnitude smaller than those observed for rhGH in the surface layer.

Table 5.3. Apparent first-order rate constants for rhGH degradation during incubation at 323 K in the surface layer ($k_{s,i}$) and in the glassy bulk solid portion ($k_{b,i}$) for each formulation and lyophilization cycle. The subscript $i$ refers to the type of degradation: aggregation ($i$=agg), deamidation ($i$=d) or oxidation ($i$=ox). Mean absolute percent deviation for these fits are all below 8%.

<table>
<thead>
<tr>
<th>Route of Degradation</th>
<th>Formulations</th>
<th>Apparent First Order Rate Constants (% per week)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
<td>Pre-annealing</td>
</tr>
<tr>
<td></td>
<td>$k_{s,a}$</td>
<td>$k_{b,a}$</td>
</tr>
<tr>
<td>Aggregation</td>
<td>5% HES</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>5% trehalose</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td>5% sucrose</td>
<td>6.6</td>
</tr>
<tr>
<td>Deamidation</td>
<td>5% HES</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>5% trehalose</td>
<td>32.2</td>
</tr>
<tr>
<td></td>
<td>5% sucrose</td>
<td>30.6</td>
</tr>
</tbody>
</table>
A striking result of the analysis is that the fitted rate constants do not depend on the composition of the formulations or on the lyophilization cycle that was used to generate the samples. Although parameters that reflect molecular motions within the glassy formulations such as the relaxation time $\tau^6$ determined from thermal activity monitor measurements (Chieng et al., 2013b) and the inverse mean square displacement of hydrogen atoms $<u^2>^{-1}$ (see Table 5.1) are different for the three formulations we examine here, the rate constants for the three reactions are insensitive to these glassy-state relaxation properties. In fact, the respective surface and bulk rate constants for the various formulations and lyophilization cycle parameters are so similar that the data for each type of rhGH degradation during incubation at 323K could be fit using Equations 2-4 by two global formulation- and

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1We note that the relaxation properties were measured in protein-free samples and may not reflect motions within the protein molecules themselves.
lyophilization-cycle independent, apparent first-order rate constants. Table 5.4 lists the three sets of global rate constants $k_{sg,i}$ and $k_{bg,i}$, where the subscript $i$ refers to the degradation pathway (aggregation, deamidation or oxidation).

Table 5.4. Global first-order degradation rate constants at 323 K for each degradation pathway (aggregation, deamidation and oxidation) for rhGH on the surface and within the bulk solid of all the formulations.

<table>
<thead>
<tr>
<th>Degradation Route</th>
<th>$k_{sg,i}$ (% per week)</th>
<th>$k_{bg,i}$ (% per week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$i =$ Aggregation</td>
<td>11.6</td>
<td>0 (&lt;10^{-3})</td>
</tr>
<tr>
<td>$i =$ Deamidation</td>
<td>35.7</td>
<td>0.5</td>
</tr>
<tr>
<td>$i =$ Oxidation</td>
<td>90.8</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Figure 5.7 shows the experimentally-determined fractions of the rhGH in the various lyophilized formulations that were damaged by aggregation, oxidation, and deamidation after 16 weeks storage at 323K, plotted against values predicted from Equations 2-4, measured values of the quantity of rhGH in the surface layer and the fitted rate constants presented in Table 5.4. The simple model correlates the data very well, with a regression line for the plot of actual vs. predicted extent of degradation yielding a slope of 1.07±0.02 and a correlation coefficient $R^2=0.98$.

Figure 5.7. Comparison of the predicted fraction protein damaged after 16 weeks incubation at 323K with experimental values measured by liquid chromatography methods (error bars on experimental values are presented in figure 5.4 and omitted here for clarity). The predicted fraction was computed using pairs of global degradation rate constants $k_{sg,i}$ and $k_{bg,i}$. The degradation routes are denoted ●: $i =$ aggregation, ■: $i =$ deamidation, ▲: $i =$ oxidation. The linear regression line has a slope of 1.07±0.02, $R^2=0.98$.  
Proteins lyophilized in glassy formulations often show degradation as a function of storage time (Chang and Pikal, 2009; Xu et al., 2013). This observation is perplexing, especially for protein aggregation, because the high viscosities ($>10^{13}$ Poise (Angell, 1995)) found in the glassy state should preclude the relatively large-scale diffusive motions required for proteins to aggregate. One possible explanation to this conundrum is that during storage, protein molecules in glassy solids might accumulate small conformational changes that prime them to become increasingly aggregation-competent upon reconstitution (Chang and Pikal, 2009; Cicerone and Douglas, 2012). However, no evidence showing the accumulation of this type of aggregate-competent species during storage has been presented (Kaminski et al., 2012), perhaps in part due to the relatively low sensitivity of available optical spectroscopies to examine protein structure in dry solids (Jiang et al., 2011).
Aggregation of proteins after lyophilization and reconstitution is often correlated with the extent of loss of native protein structure that occurs during the lyophilization process, with those formulations and conditions that yield the greatest loss of native structure resulting in the most aggregation upon reconstitution (Xu et al., 2013). Although numerous studies (Carrotta et al., 2001; Chi et al., 2003; Fink, 1998; Fink et al., 1994; Gomez-Orellana et al., 1998; Grillo et al., 2001; Khurana et al., 2001; Kim and Yu, 1996; King et al., 1996; Speed et al., 1997) have demonstrated the role of unfolded or partially unfolded protein molecules in fostering aggregation, the acute loss of structure during lyophilization does not completely explain why aggregation levels increases during storage.

In current work, by separating the degradation kinetics between surface and bulk of the solid, we find an alternative explanation for protein aggregation during storage. The majority of the degradation occurs in the population of protein molecules found on the solid surface. Adsorption of proteins to interfaces frequently results in conformational perturbations, gelation, and aggregation (e.g. (Bantchev and Schwartz, 2003; Bee et al., 2009; Bee et al., 2011; Britt et al., 2012; Dickinson, 1999; Kraegel et al., 2008; Vessely et al., 2005)). Furthermore, in thin films of polymethylmethacrylate (Reiter et al., 2005) and polystyrene (Gabriele et al., 2006) formed by drying of spin-coated layers, residual stress remaining in the surface layer causes slow relaxation motions and conformational rearrangement of polymer chains. It is plausible that the correlation that we observe between the fraction of the rhGH molecules found in the surface layer of lyophilized powders and the loss of native
protein secondary structure (see Figure 5.5) is due to a similar effect, wherein protein molecules in the glass-ice surface layer experience residual stress upon drying, causing the observed unfolding. Furthermore, the glass transition temperature near the surface of the glasses varies from that of the bulk. For example, glass transition temperatures found in a surface layer approximately 1000Å thick in polymethylmethacrylate (PMMA) films differ from those of the bulk (Inoue et al., 2013). Yu et al. reported that diffusion on the surface of glasses was at least $10^6$ times faster than bulk diffusion, and glass surface diffusion can cause surface evolution at nm to μm scale (Sun et al., 2012; Zhu et al., 2011), which is a length scale that would be expected to be long enough for (conformationally-perturbed) protein molecules to collide with each other in the surface glass layer, causing aggregation.

5.5 Conclusion

Pre-drying annealing and post-drying annealing both result more native-like rhGH structure after lyophilization. Fast freezing lyophilization cycle is detrimental for rhGH not only during lyophilization process, but also during storage. The amount of protein on the solid-air interface should be a key factor to consider for formulation and lyophilization design, as protein on the surface degrades much faster than that in the bulk. Finally, due to the substantial mobility differences between proteins on the glass surface and in the bulk, calculating both surface and bulk degradation kinetics is recommended in order to rationally design protein formulation and lyophilization process.
5.6 Acknowledgements

We acknowledge funding from NIH/NIBIB under grant R01 EB006398-01A1.

5.7 References


Xu, Y., Carpenter, J.F., Cicerone, M.T., and Randolph, T.W. (2013). Contributions of local mobility and degree of retention of native secondary structure to the stability of


6.1 Conclusions

Proteins are becoming the most important class of pharmaceuticals. However, their marginal stability poses a huge challenge in their development. Since many proteins do not have acceptable shelf life in the aqueous state, a solid-state form is preferred. Lyophilization is widely used to produce solid form protein samples. In using a dried sample, one expects that the mobility which is linked to protein degradation will be greatly hindered, thus suppressing protein degradation reaction. Unfortunately, storage of protein in a dried form does not always guarantee an adequate shelf life (Carpenter et al., 1997; Chang and Pikal, 2009). Both physical and chemical degradation can occur at unacceptable rates. The detailed mechanisms that dictate stability in the dried, glassy solid state are not well understood.

In this work, we are aiming to improve the current understanding about mechanisms of protein stability in lyophilized samples. To achieve this goal, we started from researching the prevailing proposed factors that govern the protein stability in glassy solid. These are protein structure, glass dynamics, phase separation and protein adsorption onto the interfaces (Carpenter et al., 1997; Chang and Pikal, 2009; Randolph, 1997; Webb et al., 2002). There is ample evidence in the literatures shown that each of these four factors has been successful to explain certain observations. However, most of them are only qualitative, because of lack of well-controlled and well-characterized glassy formulations. In this thesis, first, we
examined formulations of rhGH combined with the disaccharides sucrose or trehalose and various amounts of hydroxyethyl starch (HES), which upon lyophilization yielded glasses with a wide range of retention of native protein structure and glass transition temperature, structural relaxation time and the local mobility. We also analyzed samples for various types of protein degradation after storage at temperatures of 313, 323 and 333 K for periods of up to 16 weeks. After a comprehensive analysis, we found that protein degradation rates were higher in formulations with higher glass transition temperatures and slower global dynamic motions. Formulations in which the protein retained more native-like structure immediately following lyophilization exhibited better stability, and these formulations also showed larger free energy barriers to structural perturbations. Protein structure and local mobility in the glass were strongly correlated with rate constants for aggregation, deamidation and oxidation. Secondly, we did a similar study with another model protein, KGF-2. Although, the degradation rates were generally decreased in formulations with greater native state structural retention and with reduced fast β relaxations. But these two factors could not account quantitatively for the aggregation and chemical degradation rates of KGF-2 observed. Rather, it appears that a dominant factor governing protein degradation in freeze-dried formulations is the fraction of protein found at the solid-air interface. In order to quantitatively analyze the impact of protein adsorption onto interface, we lyophilized rhGH with various glass-forming stabilizers, and different lyophilization methods which were incorporated various freezing and annealing steps. The amount of rhGH on the surface of lyophilized
powders were determined from SSA and ESCA analysis. The proposed surface-bulk separate degradation kinetic model suggested that the differences in the extent of rhGH degradation during storage in the dried state between different formulations and processing methods could largely be ascribed to the associated levels of rhGH at the solid–air interface after lyophilization.

6.2 Future recommendations

Certainly, more research is necessary in order to fully understand the intricacies of mechanisms for protein degradation in glassy, lyophilized state. Based on our finding, we have several recommendations for future practice on lyophilization of protein therapeutics.

1) Protein structure in the glassy samples measured by IR showed a quantitative trend to determine rates of rhGH degradation.

2) It is clear that the glass transition temperature of a lyophilized formulation by itself is a poor predictor of long-term protein stability. Although it is likely critical that samples should be stored below the glass transition temperature, otherwise the glass state will be compromised.

3) The protein degradation upon lyophilization can be viewed as results of two underlying competitions: damage and recovery. The lyophilization process itself is well known to produce various stresses onto labile protein molecules (see the main thesis). Therefore, as expected, protein will be damaged to different extents depending on which excipient used to prepare the
formulation. The degree of retention of the native structure after lyophilization is an indicator of the degree of stresses proteins suffered through the lyophilization. On the other hand, it could also be as a result of different recovery capability which glass matrices could exercise to relieve the buildup of residual stress, thus minimizing unfolding and/or fostering rapid refolding of damaged protein. In our study, for an example, in the formulations with same ratio of disaccharide to HES, but different disaccharides (sucrose or trehalose), we consistently saw that sucrose formulation results better structure than that of trehalose. One reason could be the different residual mechanical stress buildup during lyophilization. However, an alternative explanation could be that Johari–Goldstein relaxation may act to relieve the buildup of residual stress; samples with faster Johari–Goldstein relaxation would be expected to yield protein molecules that are less structurally perturbed and hence less prone to degradation.

4) Annealing, as we showed, is an effective method to reduce the surface area of final lyophilizates’ glass-air interface, thus improving the protein stability. However, as we mentioned in the thesis, caution needs to be taken, when 1) high concentration protein formulation is used, as there is a danger that phase separation could occur. In such case, formulation strategy needs to be adjusted accordingly. 2) Annealing is also needed to be well evaluated if the formulation tends to shift pH upon freezing, as annealing will further accentuate pH shifts
and the protein will be exposed to unfavorable pH values for an extended period of time.

5) Our final conclusion pointed out that the amount of protein on the solid–air interface should be a key factor to consider for formulation and lyophilization design, as protein on the surface degrades much faster than that in the bulk. Then how could we control the amount of proteins on the surface? Other than annealing and controlled nucleation strategies we elaborated in this thesis, adding non-ionic surfactant is another way to minimize protein adsorption at the interface. Actually it is most popular way that pharmaceutical industry is using to minimize the protein adsorption on to interfaces, for both aqueous and lyophilized products (Chou et al., 2005; Kerwin, 2008; Stoner et al., 2006). The reason why we did not carry out experiments with addition of surfactant is that we want to make straightforward conclusions with minimal number of variables, such that we could avoid the potential surfactant protein interaction puzzle. Although surfactant is widely used, several wise considerations are still required for solid state protein formulation. 1) We know that proteins are tend to accumulate on the interface, but this accumulation is probably an outcome of at least two effects. One is protein adsorption onto the ice-water interface during freezing step (Webb et al., 2002), which adding surfactant seems to be able to solve the problem. However, the other one is limited ability of large molecules such as proteins to diffuse away from the freezing front causing them to be trapped at the surface (Kasper and Friess, 2011). In this case,
adding surfactant is likely not the right solution. 2) Surfactants are notorious for undergoing auto-oxidation, cleavage at the ethylene oxide subunits and hydrolysis of the fatty acid ester bond (Kerwin, 2008). The by-products of all these reactions could adversely influence the stability of a biopharmaceutical product.

6) Another reason which has impeded our understanding of glassy sample is the limited available techniques, which allow us to look at the protein information without reconstitution. In our work, as well as other literatures, most protein structural information were obtained by solid IR, even though, we know that IR is good for secondary structure information interpretation, but not informative for higher order structure. It is possible that protein molecules lose tertiary structure, but still maintain most of the secondary structure. In that case, information obtained by IR would not be enough to explain the protein degradation behavior. Progress has been made, for example, steady-state tryptophan fluorescence spectroscopy was used to probe the changes of protein tertiary structure in solid state (Ramachander et al., 2008). We are excited to see more powerful techniques to expand our knowledge.

7) Finally, in terms of protein therapeutics development, patient safety is THE most important thing! Recently, protein particles, especially micron and sub-micron protein particles have been found to be capable of promoting immune-responses. There is certainly a need to look into the protein particle formation after reconstitution of lyophilized products.
6.3 References


APPENDIX

A

rhGH fermentation, purification, and characterization

(by Amber Haynes Fradkin and Yemin Xu)

Rosetta DE3 cells containing the rhGH expression plasmids are inoculated overnight in one of the 250ml flask with LB and 50 mg/mL chloramphenicol and ampicillin. At the meantime, autoclaved solution needs to be prepared for fermentation. Each 1L of the fermentation solution contains: Yeast extract (40g), NaCl (10g), Glycerol (25ml), chloramphenicol and ampicillin (50mg), MES (19.52g), at final pH of 6.5.

In the following morning, fermentation is performed in a 4 liter Biostat B (B. Braun Biotech) equipped with pH, temperature, dissolved oxygen, and antifoam control systems with optimized PID values. It is suggested to add autoclaved fermentation media first, then start the control system to adjust parameter to the optimal condition (T=37 °C, CO₂=30% of saturation level, pH=6.5, agitation=200rpm), where pH is maintained by addition of either 1M sodium hydroxide or 1M sulfuric acid. After control panel shows all the parameters are satisfied, the overnight culture is added into the fermenter system from the top inlet. Caution needs to be taken to minimize the potential contamination. At the same time, 5% Antifoam 204 (Sigma) is added to control the excess foam. In general, after 3-4 hours (depending on the
initial overnight culture cell density), the cell optical density (OD) in the fermenter should reach about 10. At that stage, cell density measurement is carried out every 15 minutes until the OD reaches ~15, and at that time, isopropyl β-D-1-thiogalactopyranoside (IPTG) is added into system to a concentration of 0.75 mM. Growth is continuing for another 3-4 hours until cell density reaches the plateau. Cells are harvested by centrifugation at 4000g for 15 minutes and the cell pellets can be stored frozen at -20 °C.

The rhGH cell lysis is carried out by a GEA Niro Soavi Panda Plus. Cell pellets (10% w/v) are added to a solution of 10 mM Tris, 1 mM EDTA at pH 7.5, and mixed to a uniform consistency at 4 °C. Then mixtures pass through the Panda Plus, operating at 1000 bar. rhGH protein is extracted as inclusion bodies after 3 continuous runs through Panda machine. The protein-rich inclusion bodies are centrifuged at 5000g for 40 minutes and stored at -80 °C.

Purification of rhGH from inclusion body is using two-step FPLC. First, rhGH inclusion bodies are suspended in 100 mL of 8M urea, 20 mM Tris, 20 mM cysteine (pH 8.0) at a protein concentration of 1 mg/mL. After one hour of mixing at room temperature, inclusion bodies are broken down and soluble. The solution is diluted to a final volume of 1000 mL in a buffer containing of 20 mM Tris, 15% glycerol, 1M urea, 2.5 mM cysteine (pH 8.0) at a protein concentration of 0.125 mg/mL and held at 4 °C overnight to allow protein slowly fold back to correct conformation. The refolded protein mixture is centrifuged and the supernatant is loaded onto a 50 mL Toyopearl® Super Q 650M preparative column (Tosoh Bioscience, Stuttgart,
Germany). The column is equilibrated in 20 mM Tris (pH 8.0) prior to loading of protein mixture. The rhGH was recovered and fractionated by elution with a 10-column volume linear gradient from 0-500 mM NaCl in 40 mM Tris and 0.4 M urea (pH 8.0). Fractions are analyzed using non-reducing SDS-PAGE. The fractions with monomeric rhGH are pooled together in to a beaker. The pooled samples are added NaCl for a final salt concentration of 2 M. The salt-adjusted rhGH solution is then loaded onto a 75 mL Phenyl Sepharose™ High Performance (GE Healthcare, Piscataway, NJ, USA) column, which is conditioned with 20 mM sodium phosphate, 2M NaCl (pH 7.4) before load of protein. The rhGH is recovered and fractionated by elution with 8-column volume linear gradient from 2-0M NaCl in 20 mM sodium phosphate (pH 7.4). Fractions are analyzed using non-reducing SDS-PAGE and fractions enriched with monomeric rhGH only, with no noticeable dimer, are pooled and stored at 4 °C. For long term storage, these purified protein needs to be further concentrated by spin concentrator, for ease of aliquot and storage at -80 °C.

We use several techniques to characterize rhGH samples. In addition to non-reducing SDS page, we have used UV spectroscopy to measure the protein concentration, SEC to quantify the protein aggregation, IEC to quantify the deamidated species, RP to measure the oxidized products, CD and IR to look at protein secondary structure, micro-flow imaging and flowcam® to test the protein particle formation, etc. The typical chromatography graphs from HPLC (SEC, IEC and RP) are included here (figure A-1, A-2, A-3), and each method could be found in Chapter 3 method section.
Figure A: chromatographic graph for rhGH using SEC (A-1), IEC (A-2) and RP (A-3) methods. Each methods detail could be found in Chapter 3 method section.
B

Solid FTIR protocol
(by Alexander von Allmen and Yemin Xu)

First, we need to turn on FTIR machine and purge with dry nitrogen for 30 minutes, to remove water moisture residual in the system. After that, we need to collect a background scan, which is essentially the spectra of the air and residue background moisture.

To prepare solid IR sample, we first use a clean scapula and obtain the desired amount of solid for the given formulation. The actual mass of sample used needs to be recorded for later on normalization process. Sample is placed into the grinder with 1 packet of KBr, and grinded for three minutes making sure the sample turns from a crystal sugar consistency to a powdered sugar consistency (Different Mixing times have been tested; this amount of time produces the best pellet). In addition, we need to clean FTIR Evacuable Pellet Press with DI water and dry with Kimwipes and dry air. Then, we assemble FTIR Evacuable Pellet Press (Figure B) without anvils or piston, and place one anvil inside with the mirror side facing up, and put the sample into the FTIR Evacuable Pellet Press. Furthermore, we use a Kimwipe if the sample is sticking to the grinder, then place the second anvil with the mirror side facing down into the sample. After that, we gently add piston (Do put too much force onto piton). Then we push rubber ring down to seal the top of the FTIR Evacuable Pellet Press, and place FTIR Evacuable Pellet Press into FTIR infrared Hydraulic Press. We
attach the vacuum hoses to the FTIR Evacuable Pellet Press, turn the vacuum on, and use FTIR infrared Hydraulic Press until the FTIR Evacuable Pellet Press is at 10 tons of pressure. After waiting for 10 minutes (different amounts of vacuum time have been tested 10 minutes is the minimum time to receive a transparent pellet), we release the Hydraulic Press, take out the FTIR Evacuable Pellet Press and remove the base. After flipping the Evacuable Pellet Press upside down and place it in the Hydraulic Press, we place a one inch nut on top of the Evacuable Pellet Press, and use Hydraulic Press until you hear a loud pop. After that, the sample can be removed from the Pellet Press.

Figure B. Assemble of FTIR Evacuable Pellet Press.
After sample is prepared, we place the pellet into the Solid FTIR Cell, and place Solid FTIR Cell into the FTIR Machine and push the two black cylinders together so that the samples is stabilized to the position, then start the data collection. The data analysis is performed by the IR program on the same computer.

Trouble Shooting

If the pellet is cloudy, it is not being ground well enough or the vacuum time is too short

Solution: Increase grinding time and/or increase vacuum time.

If pressure is decreasing quickly for the Hydraulic Press and not staying at 10 tons

Solution: Release pressure take off vacuum tube and pressurize to 10 tons then add vacuum tube to Pellet Press.
C

Raman spectroscopy protocol

(by Christopher Carpenter, Jason Stewart and Yemin Xu)

The Raman machine we use here is manufactured by Bio Tools. This machine is equipped a laser with 100mW at wavelength of 785nm. It has two modes: one for liquid samples, and the other for solid sample. We will describe the protocols for both measurements.

For liquid sample measurement, we fill a Quartz cuvette (10mm path length) with 1ml sample. After turning on the machine and waiting for 15 minutes to allow the laser to warm up, we pull the rod on the right side to the “out position”, which is for the liquid measurement. After inserting cuvette with sample into the sample holder on the top and closing the cover, data can be collected by SnRI peak software.

For the solid sample, first, the rod need to be push in for the solid mode. In addition to opening SnRI peak software, we also need to use Toupview software to focus the laser, by taking real time focusing image (picture), and adjusting the x, y and z nobs. The typical steps will be: 1) focus nothing, but just air, 2) move up the sample stage towards microscope and take images. When sample is in the focus region, the image should be distance from air scan, 3) slowly adjust “z” nob to make the fine focus, until the signal is strong enough, 4) then data can be collected by using appropriate number of average and integration time, to make a smooth curve with a good signal to noise ratio.