EFFECTS OF FORMULATION AND MANUFACTURING CONDITIONS ON PROTEIN STRUCTURE AND PHYSICAL STABILITY

by

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This work is dedicated to my mother, Lisa Marie Wilson, and father, Gregory Lynn Wilson, for always being there for me.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

With expanding interest in the use of novel processing methods for biologics, it remains critical to develop formulations capable of stabilizing the conformational state of proteins and ensuring long-term physical stability. Under manufacturing conditions, proteins are exposed to a variety of stresses that can be detrimental to the physical stability of their native structure. In Chapter 1, a review of the effects of physical stresses induced by manufacturing methods will be discussed, with emphasis on their effect on initiating denaturation and aggregation. The common physical stresses discussed will include temperature, surface-induced stresses, pH effects, freezing, dehydration, and pressure. Specific examples of degradation under these stresses will be mentioned, with formulation approaches that can be used to protect against these factors.

Studies in Chapter 2 examined the effects of formulation and manufacturing methods (lyophilization and spray drying) on protein structure and physical stability. Powders containing one of four model proteins (myoglobin, bovine serum albumin, lysozyme, β-lactoglobulin) were formulated with either sucrose, trehalose, or mannitol and dried using lyophilization or spray drying. The powders were characterized using solid-state Fourier transform infrared spectroscopy (ssFTIR), solid-state fluorescence spectroscopy, differential scanning calorimetry (DSC) and solid-state hydrogen/ deuterium exchange mass spectrometry (ssHDX-MS). SsFTIR and fluorescence spectroscopy identified minor structural differences among powders with different excipients and drying methods for some proteins. Using ssHDX-MS, differences were observed among protein formulations containing sucrose or trehalose and mannitol, and/or with varying processing conditions, including proteins like β-lactoglobulin, for which standard characterization techniques showed no differences. Proteins processed by spray drying typically showed greater heterogeneity by ssHDX-MS than those lyophilized; these differences were not

detected by ssFTIR or solid-state fluorescence spectroscopy. The ssHDX-MS metrics were better correlated with protein physical instability measured by size-exclusion chromatography in 90-day stability studies (40°C, 33% RH) than with the results of DSC, ssFTIR, or fluorescence spectroscopy. Thus, ssHDX-MS detected subtle changes in conformation and/or matrix interactions for these proteins that were correlated with storage stability, suggesting that the method can be used to design robust solid-state protein drug products and processing methods more rapidly.

From this work, it was established that population heterogeneity in spray-dried formulations was higher than those that were lyophilized, potentially due to the impact of the air-liquid interface. In order to investigate how excipients can influence the composition of protein at the surface and population heterogeneity, Chapter 3 and 4 focus on spray drying of proteins formulated with sugar-containing excipients and on the effects of surfactant inclusion, respectively. For examining the impact of saccharide-containing formulations, spray-dried formulations of myoglobin or BSA were prepared without excipient or with sucrose, trehlaose, or dextrans. Samples were characterized by ssFTIR, DSC, size exclusion chromatography (SEC), and scanning electron microscopy (SEM). Protein surface coverage was determined by X-ray photoelectron spectroscopy (XPS), while population differences were determined by ssHDX-MS. From these techniques, structural differences were exhibited with the inclusion of different excipients, with dextran formulations indicating perturbation of secondary structure. XPS indicated sucrose and trehalose reduced protein surface concentration better than dextran-containing formulations. Using ssHDX-MS, the amount of deuterium incorporation and populations present were largest in samples processed with dextran. Linear correlation was found between protein surface coverage and ssHDX-MS peak area (R^2=0.8530) for all formulations

with saccharide-containing excipients. This suggests that lower molecular weight species of saccharides tend to enrich the particle surface and reduce protein concentration at the air-liquid interface, resulting in reduced population heterogeneity and improved physical stability, as identified by ssHDX-MS.

In Chapter 4, the impact of surfactants on surface composition heterogeneity and physical stability of spray-dried protein formulations were examined. XPS was applied to determine surface composition and ssHDX-MS was used to measure the population heterogeneity of protein in the formulations. Polysorbate 20 or poloxamer 188 were included in formulations containing no bulking agent, sucrose, or mannitol, and spray-dried. From XPS results, adding surfactants greatly reduced protein concentrations on the particle surface. SsHDX-MS data did not show significant differences in deuterium exchange or protein populations with the inclusion of surfactant, except in the case of mannitol-containing formulations. With both techniques, physical stability could not be correlated to the population heterogeneity due to the difficulty in probing the complex interactions of surfactant at the interface. These results provide insight on the challenges of using ssHDX-MS to examine the complex interactions of proteins and surfactants in the solid state. Further studies are warranted to better understand the impact of surfactants on protein stability in spray-dried formulations.

Lastly, Chapter 5 details a summary of the conclusions formed for the work in this thesis. Suggestions for potential directions for future work are provided based on observations of results and less-explored areas for manufacturing methods and formulation effects on protein physical stability.

CHAPTER 1. EFFECT OF MOLECULAR-SCALE MANUFACTURING STRESSES ON PROTEIN STRUCTURE

1.1 Introduction

With the expanding use of biologics in the pharmaceutical industry, the continued development in understanding how processing affects protein stability and function remains vital for formulation design. Proteins typically exist in nature as large, folded structures held together by different types of interactions, including hydrogen bonds, van der Waal's forces, electrostatic interactions, and hydrophobic interactions.[1] These interactions are critical for the specific functions of a protein molecule, including binding and solubility. While protein molecules have some flexibility that is required for its function, maintaining the native conformational state is a challenge in the development of biologic drug products.[2]

Proteins used in pharmaceutical development can undergo a number of processing steps, both during synthesis of the product, purification, processing, and storage and handling.[3] The number of steps can be impacted by the desired dosage form as well, with different challenges associated with keeping the proteins in an aqueous solution or by drying to a solid state.[2] In industry, the most common approach to produce a solid state product is by lyophilization. This technique utilizes freezing and drying steps in order to remove moisture and achieve a stable product.[4] In recent years the number of drying methods utilized have expanded in the pharmaceutical industry, such as spray drying, spray freeze drying, and foam drying, among other methods that are still being further explored.[5]

Each of these processing methods has varying stresses that can alter the protein's native conformation, with some of the common stresses experienced for lyophilization and spray drying listed in Figure 1.1. For these stresses, a high amount of strain is placed on the protein's

interactions with itself and its environment, which can lead to an energetically unfavorable state, whereby dissociation of the structure occurs.[1] Denaturing stress can impact proteins on different structural levels, such as damage to the secondary, tertiary, or quaternary structure depending on the strength of the stress during processing. In addition, intermediate states may be formed, where the protein has a slightly altered conformation.[6] These intermediates can refold into the native state, or may be further denatured and aggregate.

Lyophilization	Spray Drying
• Freezing - Cold Denaturation - Freeze Concentration - Ice Interface • Solid-Liquid Interface • Pressure • Dehydration	• High Temperature • Air-Liquid Interface • Solid-Liquid Interface • Dehydration

Figure 1.1: Common stresses found during processing with lyophilization and spray drying

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While some individual stresses are capable of conformational denaturation alone, manufacturing exposes the proteins to a number of unique stressors at the same time that can harm or destabilize the drug product. The effect of these stressors is dependent on the rate and strength of exposure to each stress, as well as the formulation parameters used to manufacture a biologic to its final state.[7] In this review, the common stresses that are found to cause physical

denaturation of the protein structure during manufacturing will be presented, as well as formulation strategies that are used to prevent unfolding and aggregation from occurring.

1.2 Temperature

One of the most common pathways for protein denaturation occurs as a result of exposure to temperature changes. The thermodynamics for protein unfolding for a two-state model can be understood as an equilibrium state between the folded native state (N) and the unfolded, denatured state (D).[1] This is represented by the equilibrium equation:

$$K = [D]/[N] \tag{1.1}$$

with K representing the equilibrium constant. Furthermore, the Gibbs free energy between the unfolded and folded state, ΔG_u can be given by the equation:

$$\Delta G_u = -RTlnK = \Delta H_u - T\Delta S_u \tag{1.2}$$

where R is the ideal gas constant, T is the temperature, ΔH_u is the change in enthalpy of unfolding, and ΔS_u is the change in entropy of unfolding.

For globular proteins, the thermodynamic stability is marginal, with the free energy of the folded state only being 5-20 kcal/mol higher than its unfolded state.[8] The activation energy necessary for unfolding to occur is typically in the 25-150 kcal/mole range, which is weaker than covalent or ionic bonds.[7] During exposure to changes in temperature, proteins undergo a change in their free energy that is dependent on its stabilizing and destabilizing forces, which, as previously mentioned, includes hydrogen bonds, van der Waal's forces, electrostatic forces, and hydrophobic interactions. The strengths of these forces are dependent on both global and local interactions of the structure with its environment and within the protein itself. The induction of unfolding can be highly temperature-dependent, and is accompanied with a large increase in the

heat capacity (given by ΔC_p).[9] Since the change in enthalpy and entropy of unfolding are both temperature-dependent, these properties can be understood as:

$$\Delta H(T) = \Delta H_m + \Delta C_p(T - T_m) \tag{1.3}$$

$$\Delta S(T) = \Delta S_m + \Delta C_p \ln(\frac{T}{T_m}) \tag{1.4}$$

where T_m is the midpoint of thermal unfolding on a curve and ΔH_m and ΔS_m are the values of the changes in the enthalpy and entropy of unfolding, respectively, at T_m. These parameters can be inserted into Equation 1.2 to give:

$$\Delta G_u(T) = \Delta H_u(T_m) - T\Delta S_u(T_m) + \Delta C_p[(T - T_m) - Tln\left(\frac{T}{T_m}\right)] \tag{1.5}$$

To better demonstrate the effects of these values in measurable terms (by differential scanning calorimetry), a modified Gibbs-Helmholtz equation can be used to express the free energy of unfolding by its dependence on temperature.[10] This theory is derived from the understanding that as the free energy of unfolding approaches zero, the midpoint of the change in entropy approaches $\Delta H_m/T_m$, which can be given by the expression:

$$\Delta G_u(T) = \Delta H_m\left(1 - \frac{T}{T_m}\right) + \Delta C_p[(T - T_m) - Tln\left(\frac{T}{T_m}\right)] \tag{1.6}$$

With this equation the free energy of unfolding can be plotted as a function of temperature (Figure 1.2). As shown, the kinetics for protein unfolding is shown to be parabolic, with the free energy of unfolding reaching zero at two different temperatures. Beyond these values, the free energy becomes negative, which makes unfolding energetically favorable, and thus could lead to aggregation.

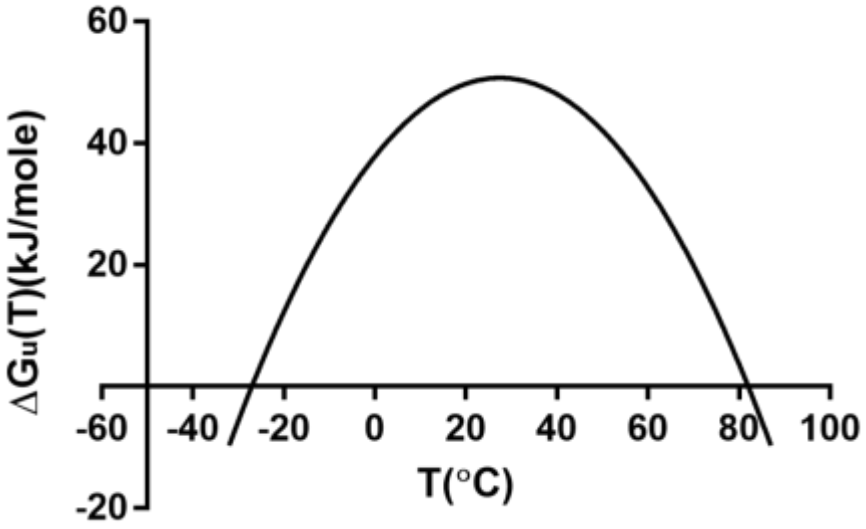

Figure 1.2: A protein stability curve for the Gibb's free energy of unfolding as a function of temperature, given in Equation 1.6. Repurposed from Reference 9.

While a two-state model is used to elaborate on the free energy of unfolding, proteins in nature do not exist in only two specifically folded states. Intermediates with varying levels of partially folded and unfolded protein structures exist and have been identified.[11-13] This is demonstrated for different proteins where unfolding begins to occur before reaching a calculated unfolding temperature, as the intermediates act as nuclei for denaturation and aggregation. At higher temperatures, this leads to a process called high temperature denaturation, while at lower values cold denaturation occurs.

1.2.1 High Temperature Denaturation

For thermally labile compounds, high temperatures used for drying pose a significant risk to their stability, particularly in the case of biologics.[14] Manufacturing approaches for the drying of proteins using high temperature include spray drying[5] and convective oven drying.[15] During

spray drying, a solution containing the biologic formulation is atomized by air into droplets. These particles are briefly exposed to a hot drying gas which remove water and form the dry solid particle to be collected. In a convective oven dryer, solutions or dry samples are exposed to elevated temperatures for a set period of time to reach desired moisture content. These values are significantly higher than the denaturation temperature, or melting point, of most proteins, which usually begin to unfold in a range between 40-80°C.[16]

As indicated in Figure 1.2, at this high temperature unfolding is energetically favorable. As the temperature increases, so does the kinetic energy of the bonds that make up the protein. As a result, increasing heat can lead to the disruption of hydrogen bonds and the interactions of nonpolar amino acids groups, which leads to denaturing.[1] There exist many possible intermediates that can form during unfolding, which can be dependent on the drying rate utilized as well as the excipient composition when exposed to high thermal stresses.[11] For example, above 50°C β-lactoglobulin showed decreasing α-helical and β-sheet content with increasing disordered structure. Above 60°C, aggregation is observed and an increase in intermolecular β-sheet content.[17] For thermally-sensitive proteins, the denatured states tend to form irreversible aggregates, even upon reconstitution, since aggregation occurs quickly upon unfolding from this stress.

For most proteins during spray drying, however; thermal denaturation is not normally observed even at temperatures above 100°C due to the self-cooling effect by solvent evaporation.[18] Due to this self-cooling effect, the temperature will normally not rise about the wet bulb temperature, which is the lowest temperature present on a droplet's surface during evaporation. The wet bulb temperature increases as the drying process continues, but the interior of the particle maintains a temperature 10-15°C lower.[19] Once a critical concentration on the

surface of the droplet has been reached, secondary drying will occur until the particle reaches the dry bulb temperature, which is close to the outlet temperature of the spray-dryer.[18] While this high temperature drying is taking effect, the melting temperature of the formulation is increasing due to dehydration, a stress which will be addressed in a later section.

With all protein formulations, the time of exposure and temperature used play a critical role in the level of denaturation that can be observed. Avoiding long exposure times to high temperature is one such approach, which is why using a lower inlet temperature on a spray-dryer is preferred, since outlet temperature has been well-documented to affect the level of denaturation and aggregation more than inlet temperature.[20-22] Primary structure of the proteins also plays a significant role, as compounds containing a greater amount of hydrophobic amino acid residues tend to have a higher stability against thermal stress than more hydrophilic proteins.[18] The selection of excipients that form strong hydrogen bonding with the protein are also capable of providing protection against high temperature denaturation and improving the overall stability.[23]

1.2.2 Cold Denaturation

Temperature effects on the unfolding of proteins are parabolic, as shown in Equation 1.6. While high temperature effects are well-documented, there also exists the possibility for denaturation at low temperatures for globular proteins, where the free energy for unfolding is similar to that of the higher temperature-induced state.[24] This cold stress can be induced during the freezing step in a manufacturing process like lyophilization, or during normal storage at sub-zero temperatures. Unlike at high temperatures, however; unfolding at lower temperatures is thought to occur by a different mechanism. In cold denaturation, the speculated factor for unfolding is due to changes in the interaction between water and the nonpolar groups of the

protein.[25-27] As the temperature drops below a certain point, it becomes entropically unfavorable for water and the hydrophobic amino acids to not interact in its native state. As a result, the hydrophobic groups become hydrated, which weakens hydrophobic interactions used to stabilize the globular molecule. This change in hydrophobicity results in the collapse of tertiary structure. Experimental evidence has been shown to indicate that globular proteins are capable of possessing a cold denaturation temperature.[28] Two examples are in β-lactoglobulin and apomyoglobin. In the case of β-lactoglobulin, it was demonstrated that the intermediates formed as a result of thermal stresses were different depending on the temperature used, which supports the theory that unfolding at cold temperature occurs by a different mechanism.[12] Similarly, apomyoglobin has been studied by Sabelko *et al.* and demonstrated that stability is related to the protein's hydrophobic core at colder temperatures.[13] In this instance, below the denaturation temperature, the helices forming the core of apomyoglobin become detached, followed by the observation of unfolding.

While high-temperature unfolding is well-reported in literature, cold-induced stresses have not been as prevalent, even though most globular proteins are expected to denature at both high and low temperatures.[29,30] This is due to difficulty in analysis in aqueous solutions below 0°C, where freezing can occur. Freezing can induce a variety of conformational stresses, including cold denaturation, which makes isolation of this particular stress for study difficult. Typically this can be overcome by the use of a denaturant such as urea, although there have been reports of cold denaturation occurring at temperatures above freezing. [31,32]

While this stress is capable of affecting pharmaceutically relevant molecules, such as monoclonal antibodies[33], unfolding by cold denaturation is largely reversible.[30] By increasing the temperature through thawing an aqueous solution or by reconstituting a lyophilized sample, the

aggregation or denaturation that occurred is reversed. In addition, for dried samples the protective effect is increased due to the removal of water, where storage of the product in freezing conditions will likely not result in significant stress. Storage conditions will also determine whether or not denaturation will occur, as for most proteins the temperature of unfolding is below -20°C in an aqueous solution.[28] In terms of formulations, the use of a cryoprotectant, such as sucrose or trehalose[34], will also further improve the stability against this stress factor during processing.

1.3 Surface-Induced Denaturation/Shear

During processing of protein formulations, it is inevitable that solutions will be exposed to some type of foreign interface, whether by filtration, filling, or drying methods.[35] Exposure to these interfaces can result in interactions of the protein by adsorption, which can lead to shearing conditions and unfolding in relation to its environment. This is due to the amphipathic nature of proteins, which leads to high concentrations orienting at the surface because of their polar and nonpolar side chains.[36,37] For this type of denaturation stress, there are two potential hypotheses regarding the underlying principles that lead to unfolding: (1) Surface-induced denaturation due to surface adsorption, aggregation of intermediate states, and/or recycling of unfolded proteins into solution; or (2) Shear-induced denaturation by structural changes of proteins in bulk solution by shear force, aggregation of intermediate states, or solid surface adsorption.[7] In recent studies, it has been demonstrated that shear alone is not a significant enough factor to lead to structural changes for a protein, even at conditions higher than those that would be found in traditional manufacturing processes.[38] For unfolding, a force of 20-150 pN is generally required for proteins, such as a monoclonal antibody. Shear alone is only capable of generating a force of 0.06 pN, far lower than what is required.[39] This indicates that another foreign interface in tandem with shear

may be the critical factor in generating a high enough stress that would lead to denaturation. The three types of these surface-induced stresses found in manufacturing are solid-liquid, air-liquid, and liquid-liquid interfacial stresses.

1.3.1 Solid-Liquid Interfacial Stress

During all phases of processing, protein formulations will be exposed to solid surfaces, either in a vial or device for storage of the drug product[40], membranes during dialysis[41], or the equipment used in the manufacturing process.[42] The determining factor concerning whether the protein will adsorb to a surface depends on the surface interacting with the protein, and the state of the protein's bonding interactions, in particular electrostatic and hydrophobic interactions.[38]

If the protein favorably interacts which the solid surface it is being processed in, the protein can adsorb onto the surface. During this adsorption the protein may be folded or partially unfolded, with a higher probably of adsorption occurring for a partially unfolded protein, as the exposure of the hydrophobic residues increases the number of possible interactions with the surface.[37,43] The initially adsorbed samples act as nuclei at this interface, whereby additional molecules passing through the solid can attach to these sites, increasing the size of the aggregates.

The stability of the structure also plays an important role. As defined by Nakanishi *et al*., there are two classes of proteins based on their stability that determines which types of surfaces will lead to adsorption: "hard" and "soft" proteins.[37] For a hard protein there is a high internal stability, or strong hydrophobic interactions, which help to maintain the protein structure against adsorption. For these types of molecules, adsorption at the solid interface is unlikely on a hydrophilic surface unless there is a strong electrostatic interaction that disrupts the protein structure, or if there is a strong hydrophobic surface used. An example of this is lysozyme, which has a higher amount of aggregation due to adsorption that occurs on a more hydrophobic surface,

polytetrafluoroethylene, than on glass.[44] For soft proteins, a low internal stability, or weaker hydrophobic interactions, is present, which allows for adsorption on any surface where there is a gain in conformational entropy by unfolding and attaching to a solid interface. An example of a soft protein is a monoclonal antibody, which can unfold during adsorption to stainless steel.[45]

The ionic strength of the solution can affect the likelihood of solid-liquid interfacial stress occurring during formulation. Increasing the ionic strength can weaken the repulsion interactions found between the protein and solid during processing, leading to aggregation.[46] In addition, the pH of the solution can also dictate the electrostatic interactions that can occur between a protein and its environment during processing. One example is catalase, which can bind to hydroxyapatite even when protein and surface are both negative due to the high ionic strength of the buffering agent.[47]

There also can be interfacial stress induced by the breaking off of proteins or solid into the solution of the formulation that can lead to additional aggregation.[36] Denatured proteins may desorb from the surface and move back into solution, and serve as additional sites of nucleation where proteins can unfold and aggregate. The equipment used for manufacture can also introduce particulates into solution by leaching or shedding of sub-visible particles from the equipment during frequent, regular usage.[48] These particulates can serve as additional nucleation sites that further increase aggregate formation and denaturation.

To reduce the interfacial stress present from the equipment on affecting the protein, selection of materials that are unfavorable to adsorption for hard proteins is one approach. Controlling the pH and ionic strength to prevent electrostatic attractions is another possibility. For soft proteins, sugars and surfactants can prevent aggregation by interactions that reduce the amount of protein interacting with the solid surface, such as the case of preventing interaction

with the ice interface during lyophilization.[49,50] Reducing the flow rate of the material being processed can also limit the combined effects of shear with solid-liquid interfacial stress that can promote adsorption during processing.[38]

1.3.2 Air-Liquid Interfacial Stress

Of the interfacial stresses, the air-liquid interface is considered the most problematic, as air is present in nearly all types of pharmaceutical processing.[43] One such example of exposure of a protein solution to an air-liquid interface is spray drying[51,52], where air is used to atomize droplets to control particle size during drying. In addition, this stress can be induced upon completion of unit operations, from filling, which introduces an air headspace[53], to agitation during shipping and handling.[54]

During exposure to this air-liquid interface, the amphiphilic nature of the protein can lead to adsorption at the surface, which leads to a higher concentration of protein found at the interface than present in solution.[52] As the protein moves to the surface, there may be exposure of the hydrophobic core due to unfolding in order to equally align at the surface. This unfolding leads to an increased denaturation and protein damage present at the surface, which increases its tendency for other proteins to aggregate as crowding occurs.[55] Similarly, the increase in protein concentration at this interface leads to an increase in the number of interactions of protein molecules by a decrease in free volume, which can cause additional conformational dissociation and alteration of the tertiary structure. As an example, this surface reduction can be caused in manufacturing by increasing the atomizing air flow used for drying. The increase in atomization air flow rate for spray-freeze drying bovine serum albumin led to a decrease in particle size, but an increase in the number of aggregates observed by increased protein interactions at the air-liquid interface.[56]

To reduce the possibility of denaturation and aggregation at the air-liquid interface, several approaches can be taken. Reducing the exposure of protein to air during processing is one approach, whereby decreasing the headspace reduces the area of exposure and thus the potential for surface aggregation.[35] In manufacturing situations where the use of air cannot be avoided (i.e. spray drying), the use of surfactants can alleviate this issue.[57-59] Surfactants are also amphipathic molecules which directly compete with proteins for adsorption at the surface. This in turn decreases the presence of proteins found at the air-liquid interface, reducing the occurrence of denaturation and aggregation. Conversely, avoiding the excipients that increase a protein's free energy of unfolding is also important, such as sucrose, which has been found to increase the aggregation of an immunoglobulin during storage.[60]

1.3.3 Liquid-Liquid Interfacial Stress

In the presence of a two-fluid system, proteins may adsorb to the interface and become denatured. This type of processing stress can often be found during purification[61], encapsulation of protein molecules by emulsion-based methods[62], or in the event of contamination by another foreign liquid.[63] Similar to solid-liquid interfacial stress, protein molecules orient themselves at the interface between solvents to reduce surface tension. In order for the reduction in surface tension to occur, the protein may undergo rearrangement of its native structure so that the exposure of hydrophilic and hydrophobic residues will reduce the free energy found at the surface and stabilize the solution.[64]

There are several factors that affect the extent of denaturation that can occur. The properties of the two fluids can determine the amount of adsorption that results, particularly if there are significant solubility differences for the protein being processed.[65] The difference in solvent area exposed and interfacial tension between solutions in an aqueous-organic solvent

system affects the extent of denaturation, as has been demonstrated for some enzymes.[66] Unlike at a solid-liquid or air-liquid interface, at a fluid interface the protein tends to have greater molecular mobility, due to the interface having a higher mobility.[64] This can lead to increased aggregation in solution due to recycling of denatured proteins back into the bulk.

Similar to the other interfacial stress systems, the use of excipients are capable of reducing the denaturing stress found in these systems. Surfactant has been shown to prevent the adsorption of proteins under this type of stress similar to its activity for other interfacial stresses.[67] Selection of solvents used for methods of processing can prevent unfolding and loss of structure experienced, such as avoiding the use of polyethylene glycol-dextran solutions in lyophilization, which causes phase separation on freezing.[68] Additionally, avoiding contamination by oils, such as silicon oil, can prevent the presence of a fluid-interface forming during processing and handling.[63]

1.4 Dehydration/Moisture Content

Dehydration is a critical step in formation of solid protein formulations, which is accomplished by many of the previously mentioned processing conditions such as lyophilization[69] and spray drying.[70] A critical component during dehydration is to remove water without disrupting the interactions that stabilize the protein structure. Decreasing moisture content has typically been shown to increase the long-term stability of the drug products[71], as long as samples can be stored in appropriately dry conditions. Unintended exposure to moisture prior to rehydration can have deleterious effects on protein efficacy, as components may begin to precipitate and aggregate when exposed to small amounts of water.[72]

Proteins in aqueous solutions exist in a hydrated state, with a fluid hydration shell that envelops the biologic molecule.[73] During the drying process, whether by high temperature drying

or sublimation, the surface area of protein in contact with the hydration shell decreases as a result of water removal. This change reduces the number of water molecules available for interaction with the protein. This leads to increased exposure between protein molecules or with excipients as the solution becomes more concentrated, and thereby increases the occurrence of intermolecular interactions with the components of the formulation that can stabilize the drug product.[1,74]

There exists a point where the removal of water can be detrimental to protein structural stability. As a result of moisture removal, the free energy of the protein will change, and become less stable. To overcome this energy difference and move to a more stable state, the protein can unfold from its native conformation and form intramolecular interactions with itself or aggregate by forming intermolecular interactions with nearby protein molecules.[73] These increased interactions can result in destabilization of the structure, especially in instances where there is not an excipient that replaces the hydrogen bonding that was disrupted by moisture removal.[75]

To prevent aggregation from intermolecular interaction between proteins, the selection of excipient is critical in formulation development. Commonly used excipients include carbohydrates such as sucrose and trehalose[70], as well as sugars like mannitol.[58] These excipients are considered important due to their stabilizing ability of the protein. One widely accepted theory for the use of these compounds is in their ability to serve as a hydrogen bond replacement for water.[76,77] Due to the number of hydrogen molecules and flexibility of these excipients, proteins can form strong hydrogen bonds with them upon depletion of the hydration shell with the excipient preferentially excluded from the surface.[78,79] This leads to increased structural stabilization as the protein does not require unfolding to reach a thermodynamically favorable state.

A significant issue in excipient selection that can occur during drying and storage is on the state of the excipient molecule. In ideal concentrations, the excipients and protein both remain amorphous in a structurally stable state.[21] At excess concentrations of excipient, however, molecules of the excipient can form interactions with each other, leading to nucleation or small crystals. In minute amounts, these crystals may not have any deleterious effects on the formation.[80,81] If, however, these crystals act as nuclei for other excipient molecules, it can potentially remove available hydrogen bonds for the protein by exclusion. This phase separation in turn can lead to increased perturbation of the protein's structure, and lead to denaturation. In addition, preventing moisture exposure prior to rehydration for use is another important aspect to consider, as increased moisture uptake is capable of causing phase separation of the excipients.[82] Crystallization may occur during storage, which will also disrupt the stabilization of the structure. Therefore, it remains important to consider not only the use of excipients, but how to prevent water exposure.

Another important factor to consider is the drying method utilized. During sublimation in freeze-drying, the initial hydration shell is removed during primary drying by a change in pressure while the solution is frozen.[4] This is followed by a secondary drying in which the remaining bound water found within the folded protein is also removed by using an increase in temperature. This freezing and thawing cycle can impact the bonds of the protein, depending on the remaining moisture content, as the protein may still be interacting with an ice interface that can lead to unfolding, as previously mentioned.[83] For drying by high temperature, a vacuum oven or droplet atomization can be used to dry the particles. With a vacuum oven, monitoring the temperature and pressure is necessary to ensure proteins are not subjected to high temperature conditions or over-drying that can cause structural damage.[15] With atomization for drying, the

removal of water from the droplet can also pull protein molecules to the surface, creating a higher concentration at the surface and increasing exposure to an air-liquid interface that can further lead to loss in conformation.[52]

As stated earlier in this section, to prevent dehydration effects on proteins the use of stabilizing excipients that supplement hydrogen bonding is a common approach.[77] For example, in a spray-dried lysozyme sample, the use of sucrose and trehalose were able to protect the native structure after completion of drying.[70] In addition, prevention of the samples from exposure to excess moisture by proper sealing and storage can prevent phase separation from occurring due to rehydration.

1.5 pH

Although not a directly induced stress, the solution pH is an important factor in determining the interactions of solutes in their environment that should be monitored during processing, as it can be altered by manufacturing steps. The pH dictates the charge state of exposed amino acids, either positive or negative, by protonating or deprotonating an amino acid side chain.[8] This in turn affects the electrostatic interactions of the protein by altering the total charge of the molecule, which can have both local and global effects on the stability of the structure[84] as well as its solubility.[16,85]

At its isoelectric point (pI), the protein has a net neutral charge, and as a result has its lowest solubility at this point.[85] Shifting the pH by either decreasing it (more acidic conditions) or increasing (to more basic conditions) will alter the net charge and increase protein solubility. At higher acidic or basic conditions, destabilization of the structure can result. This is due to the effects of electrostatic interactions that lead to nonspecific repulsions of the amino acids due to increases in charged state.[86] Higher charge results in increased repulsion, which can move amino

acid chains apart, leading to unfolding of the molecule as it moves to a state with lower free energy. In contrast, increasing electrostatic charges may also lead to the formation of salt bridges. In this instance ion pairing, which occurs between amino acid chains of opposite charge, form a strong electrostatic bond that stabilizes the protein conformation.[87]

Electrostatic interactions can also develop between proteins which are capable of stabilizing or unfolding, depending on the pH. Proteins that are highly charged at similar states (either negative or positive) create high repulsive interactions. In a colloidal solution, these repulsions stabilize the proteins and make aggregation energetically unfavorable. However; at pH values where proteins possess both negative and positive charges (typically when closer to the pI); an anisotropic distribution of charges can result, leading to the formation of dipoles for the molecule.[87] These dipoles could then favorably interact with other protein molecules, leading to the formation of aggregates.

There are a number of conformational states that a protein can take depending on the pH utilized. As previously stated, the two-state model of folded and unfolded protein states is not accurate, as there are distinct intermediate states that can be identified for an unstable protein formulation. At extreme pH values, proteins can undergo some conformational changes where they exist in a partially folded state, called the "molten globule" state.[88] In this state, the protein maintains its secondary structure similar to its natively folded state. However; its overall shape tends to be slightly larger, as the hydrophobic core is exposed to the solvent, and the tertiary structure is absent under detectable methods. This is a late-folding state, which while useful for analysis and understanding protein folding, is undesirable in a formulation, as these states can lead to changes in folding of the protein upon processing or exposure to other stresses.

pH of the solution prior to processing has a significant impact on the folded state and other stresses on denaturation.[89] As an example, *Rhizopus niveus* lipase was monitored at several different pH states by Rabbani *et al*.[90] The conformation of this compound had differences in its secondary and tertiary structure depending on state (alkaline, neutral, acidic, or molten globule). Upon exposure to increasing temperature, the lipase underwent denaturation at different temperatures. Other proteins, such as staphylococcal nuclease[91], α-lactalbumin[92], and β-lactamase[93] have also demonstrated pH dependence on affecting secondary and tertiary structure. This illustrates that pH selection is an important consideration for formulation development.

To reduce the effects of pH on denaturation, selection of the appropriate buffer component and pH is critical. Ideally, the solubility should be far enough away from the pI to stabilize the protein without resulting in significant unfolding by repulsive interactions.[85] Concentration also needs to be taken into consideration. At low concentrations, the salts can reduce electrostatic interactions. If concentrations become too high, then buffer components can bind to the protein surface, reducing the stability of the native conformation.[94] Another possibility is preferential exclusion or salting out of the protein in solution. In addition, the processing approach can have an impact on the buffer solubility and affect the pH and ionization strength of the solution, such as the freezing stress before drying[95], which will be discussed in the next section.

1.6 Freezing

During techniques like lyophilization and spray-freeze drying, as their names imply, the temperature of the solutions is decreased until the solvent is frozen before utilizing a drying step. Unlike previously discussed stresses, freezing involves a combination of different factors that can destabilize protein structure, including cold denaturation[4], interfacial stresses[49], phase

separation[81], and changes in the concentration of the protein and its excipients.[96] Freezing of a solution proceeds by super-cooling, ice nucleation, and then crystal growth.[97] Under super-cooling, the liquid state is maintained below the temperature at which ice formation occurs, and then solidifies quickly upon the release of latent heat.[98] As super-cooling leads to formation of ice crystals, the proteins and excipients become excluded from the sites of ice nucleation as the solubility decreases, leading to increased interaction of materials in the matrix as they become concentrated. The ice nuclei begin to increase in size as the lower temperature is maintained until the water in the system has been crystallized.

The rate of cooling dictates the number of ice nuclei formed and the size of the crystals, which is dependent on the formulation design for a protein[83]. Slower rates of cooling lead to less nuclei formation, but larger crystals. Conversely, a high cooling rate leads to the formation of smaller crystals in high amounts. This increase in ice crystals leads to a high surface area by which proteins may adsorb, which can lead to damage and loss of secondary and tertiary structure. For example, a fast-freezing step used for lactate dehydrogenase showed higher structural damage and lower activity recovery than for a slow-freezing rate due to the higher presence of ice interfaces.[83]

The reduction of solubility due to freezing can have effects on the formulation, depending on the solubility of each component at reduced temperatures. Differences in solubility can lead to phase separation, where certain components may crystallize out instead of forming an amorphous matrix.[99] For example, a stabilizing excipient in solution may crystallize at a temperature higher than that of another one, such as raffinose, which can crystallize depending on the lyophilization method used.[100] This reduces the number of interactions present with the excipient, which can expose the protein to cold denaturation stresses or increase the number of

intermolecular interactions between proteins. Typically these issues are avoided by the use of thermally stabilizing excipients which can precipitate out of solution at similar temperatures.[101] Another factor that can be affected by freezing is the ionization and pH strength of the solution. As previously mentioned, pH affects the ionization of the protein, which affects the overall charge and interactions between amino acid chains.[84] The loss of moisture results in a decrease in hydrophobic interactions, and also increases the ionization strength of the solution, and thus the charge state of the protein. In addition, buffer components may crystallize at different temperatures, which can lead to significant changes in pH that lead to denaturation. For example, in a phosphate buffered solution, a dibasic sodium salt will have a significant drop in pH (from 7 to 4) between -0.5°C to -9.9°C, whereas only a minor pH shift can be observed for potassium salts.[95,102]

One approach to control the exposure of proteins to the ice in solution is to control the size of the nuclei formed during the freezing step. In lyophilization, this process is accomplished by controlled ice nucleation, whereby the size of the ice crystals is controlled by a variety of factors.[103] By increasing the size of the ice crystals formed, the surface area exposed to proteins is decreased, resulting in less possible aggregation at these interfacial sites. Conversely, smaller ice crystals may be formed prior to drying to reduce exposure time to cold denaturation by reducing the drying time. The use of surfactants can also be used to reduce the adsorption of proteins at this interface, such as polysorbate 80.[49]

1.7 Pressure

Proteins have elastic properties, which is a necessary attribute to overcome changes in environment[104], as well as critical for function when binding to specific sites for efficacy.[2] At high enough pressures, changes in this elastic effect can alter the volume of the protein in a

defined space in solution, resulting in conformational changes. The change in volume as a result of the change in free energy due to pressure can be given by the equation:

$$\Delta V = d(\Delta G)/dp \tag{1.7}$$

where ΔV is the change in volume, ΔG is the change in Gibbs free energy, and p is the pressure of the system.

The change in volume is a combination of three separate factors: the space occupied by the atoms of the protein, the volume where solvent is excluded (such as the hydrophobic core), and the volume difference that results due to the interaction between the protein and solvent.[105] High pressures will alter each of these factors, which can result in either disassociation of protein molecules or unfolding. The rate of dissociation by denaturation or disassociation due to pressure can be given by the equation:

$$K_d(p) = K_{do}\exp(\frac{p\Delta V}{RT}) \tag{1.8}$$

where K_d is the equilibrium constant for dissociation or denaturation at pressure p, and K_{d0} is the equilibrium constant for structural destabilization at atmospheric pressure.[106]

Weak stabilization interactions in the protein, such as ionic and hydrophobic bonding, maintain conformational stability, which can be broken at higher pressures. For oligomeric proteins, which are primarily connected by electrostatic and hydrophobic interactions, only moderate pressures (0.5-2 kbar) are required for dissociation.[107] Depending on the conformational stability of the protein, unfolding can lead to cavitation, whereby the hydrophobic core is exposed to solvent, which would disrupt the previously stable intra-protein interactions. The formation of these denatured states under high pressures is favorable due to the reduction in volume, as unfolded protein structures have higher compressibility than those in the

folded state.[108] This denaturation can result in the loss of secondary structure, as seen in myoglobin[109], and favor the formation of aggregates.

While high pressure can impact the stability of protein formulations, the pressure conditions required are typically 2-4 kbar.[110] These values are significantly greater than those produced in standard manufacturing processes. However, higher pressures can be used in concentrating proteins in solution and reducing the percentage of aggregates by dissociation. During refolding from non-native aggregates produced during protein synthesis, increasing pressure has been shown to improve the recovery of properly folded states, both with and without chaotropic agents.[111,112] Similarly, at pressures between 1-3 kbar, dissociation of oligomers into monomeric units has been utilized as an effective method of stabilization.[113]

1.8 Conclusions

The development of a drug product often requires exposure to a significant number of stresses. These stresses alone may be minor and largely reversible for many proteins, but in combination can have a detrimental effect on protein structure, leading to the formation of irreversible damage and aggregation. The selection of excipients can be used to reduce the impact these stresses have on the formulation, helping to maintain the intact structure. By understanding the impact these manufacturing stresses have on a protein's conformation, formulation design can be utilized to minimize these effects and achieve stable, efficacious drug products.

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CHAPTER 2. EFFECTS OF DRYING METHOD AND EXCIPIENT ON STRUCTURE AND STABILITY OF PROTEIN SOLIDS USING SOLID-STATE HYDROGEN/DEUTERIUM EXCHANGE MASS SPECTROMETRY (SSHDX-MS)

Adapted with permission from: Wilson NE, Topp EM, and Zhou QT. Effects of Drying Method and Excipient on Structure and Stability of Protein Solids Using Solid-state Hydrogen/Deuterium Exchange Mass Spectrometry (ssHDX-MS). Int J Pharm 567:118470.

2.1 Introduction

The instability of a pharmaceutical protein in solution may necessitate the development of a product in the solid form, which often shows enhanced stability. Factors influencing the stability of solid protein formulations include excipient type and amount, protein concentration, protein:excipient ratio and drying method.[1] For pharmaceutical proteins, the mainstay drying technique is lyophilization. During lyophilization, a solution is frozen and water is removed under vacuum in various stages.[2] Following the freezing stage, frozen water is first removed by sublimation during primary drying. Secondary drying follows, during which additional water bound to the protein is removed to produce the final product. Stresses encountered during lyophilization include cold denaturation, exposure to ice-water interfaces and freeze-concentration.[3] Traditionally, pharmaceutical lyophilization is a time-consuming batch process with low energy efficiency.[4]

Spray drying has attracted increasing interest for manufacturing biopharmaceutical solids because it can be developed into a continuous process with high throughput, and has the capability to achieve satisfactory powder flowability by manipulating particle properties.[5] Examples of spray-dried biological products include Exubera, an inhalable insulin product, and Raplixa, a blend of thrombin and fibrinogen powders produced by aseptic spray drying.[6,7] In

spray drying, a solution is atomized into small droplets which are briefly exposed to a drying gas to produce particles that are collected by a cyclone.[8] Moisture is removed by evaporation, thus resulting in dried particles.[9] Stresses that may affect the structure and stability of the proteins during spray drying are the relatively high temperature and exposure to the air-liquid interface, among others.[10]

Through lyophilization and spray drying, a solid powder containing a biologic product can be formed. Due to the processes and the properties of the proteins, the resulting powders are usually amorphous rather than crystalline.[11] With either method, the removal of moisture can disrupt the higher-order structure of the proteins, leading to exposure of hydrophobic residues that may promote aggregation or increase the risk of chemical degradation.[12] Excipients that stabilize proteins against manufacturing stresses are critical in maintaining protein structural integrity and increasing shelf-life stability. While the effect of excipient has been studied extensively [13-15], relatively little attention has been paid to the effect of processing conditions on protein structure and stability, or to the interactions of formulation and process variables.[16,17]

Common methods for characterizing protein structure such as solid-state Fourier-transform infrared spectroscopy (ssFTIR), circular dichroism (CD) spectroscopy, and fluorescence spectroscopy are capable of identifying global changes in secondary and tertiary structure.[14,18,19] These methods are often unable to detect subtle structural differences of some proteins, either secondary or tertiary, which may impact shelf stability in the long term. Determining protein structure and local environment with greater structural resolution could provide greater insight into the amorphous solid environment experienced by the protein, and guide the rational development of stable formulations. Solid-state hydrogen/deuterium exchange with mass spectrometry (ssHDX-MS) has been demonstrated to be such a method, showing good

correlation between deuterium exchange and physical stability on storage.[20,21] In this technique, a sample is placed into a dessicator containing deuterium oxide (D_2O) at a controlled humidity. In this chamber, exposed hydrogen atoms can be exchanged with deuterium, although due to back exchange only hydrogen atoms on the amide backbone can be monitored by mass spectrometric methods.[22] This provides information on the intra- and intermolecular hydrogen bonding interactions in the formulation matrix that can stabilize protein structure and affect physical stability.

Previously, ssHDX-MS analysis of the effects of processing conditions on monoclonal antibody (mAb) formulations showed different sub-populations of the mAb in spray-dried samples with different deuterium incorporation, suggesting differing protein conformations and/or matrix interactions.[17] However, only one type of protein (mAb) was examined in that work and the aggregation stability was not determined. In the work reported here, four model proteins of myoglobin (16.7 kDa), lysozyme (14.3 kDa), bovine serum albumin (BSA, 66.5 kDa), and β-lactoglobulin (18.4 kDa) were formulated with different excipients and processed by either lyophilization or spray drying. These proteins were selected due to differences in structure and size to examine a broad range of protein characteristics.[11,23] Excipient and formulation selection was based on previous studies with a mAb.[17] The samples were characterized using conventional techniques of ssFTIR, fluorescence spectroscopy, x-ray powder diffraction, and differential scanning calorimetry (DSC), and ssHDX-MS. Stability studies (i.e. protein instability determined by loss of monomeric peak on size exclusion chromatography (SEC)) were performed to identify the effects of processing conditions. The results indicated that conventional techniques did not identify differences between processing methods. By using ssHDX-MS, however, differences in the population of protein species were measured in dried samples produced from different

formulations and drying methods, which showed correlation with storage stability for some of the proteins studied.

2.2 Materials and Methods

2.2.1 Materials

Lysozyme from chicken egg white, bovine serum albumin, myoglobin from equine skeletal muscle, and β-lactoglobulin from bovine milk were purchased from Sigma Aldrich (St. Louis, MO). Protein solutions were dialyzed at 4°C in a 2.5 mM phosphate buffer solution using Slide-A-Lyzer™ dialysis cassettes (Thermo Scientific, Rockford, IL). Solution pH was adjusted to 6.8 using phosphoric acid where necessary. Dialyzed solutions were then diluted to final concentrations of protein and excipient, as indicated in Table A1, for a total solid content of 20 mg/mL. Solutions were then either spray-dried or filled into 2R borosilicate glass vials (0.2 mL per vial) for lyophilization.

2.2.2 Spray drying

Formulations were spray-dried using a Mini Spray Dryer B-290 (Büchi, New Castle, DE). An inlet and outlet temperature of 100 °C and 50-55°C, respectively, with a liquid feed rate of 2 mL/min and an air volumetric flow rate of 600 L/h were used. The collected powders were then distributed into 2R vials (~4mg per vial) and further dried in a lyophilizer for 24 h at 30°C and 100 mTorr to reduce moisture content to values similar to those of lyophilized samples (~2%). The purpose of this additional drying step was to ensure that any potential effects on protein structure and stability are not the consequence of moisture content differences in the dried powders.

2.2.3 Lyophilization

Lyophilization was performed with a Revo® laboratory-scale lyophilizer (MillRock Technology, Kingston, NY). Vials were loaded at a shelf temperature of 25°C, with placebo sucrose solutions (0.2 mL per vial at a concentration of 20 mg/mL) surrounding the samples. The solutions were equilibrated for 5 min, then ramped to 5°C and held isothermally for 15 min. The shelf temperature was then ramped to -5°C and held for 15 min. To induce freezing, the temperature was ramped to -40°C and held isothermally for 60 min. Primary drying was then initiated by ramping the temperature to -35°C and then holding isothermally for 24 h with a chamber pressure of 70 mTorr. For secondary drying, the chamber pressure was maintained, while the temperature was ramped to 25°C and held for 12 hr.

2.2.4 X-ray Powder Diffraction

The crystallinity of lyophilized and spray-dried powders was assessed using a Rigaku SmartLab X-ray diffractometer (Rigaku, The Woodlands, TX) equipped with a Cu Kα X-ray source and Bragg-Brentano geometry. Powders were removed from vials and pulverized onto a glass slide, then loaded onto the slide-holder. Diffraction intensity was measured as a function of 2θ between 5 and 40 degrees. A step size of 0.02° and a scan rate of 35°/min were used.

2.2.5 Solid-State Fourier Transform Infrared Spectroscopy

ssFTIR measurements were conducted in attenuated total reflectance mode using a Nicolet Nexus spectrometer (Thermo Scientific, Waltham, MA) equipped with a Smart iTR accessory. Powders were loaded and compressed against the diamond by a metal anvil. The spectra were collected in the absorbance mode in the range of 800 to 4000 cm^{-1}, with 120 scans and 4 cm^{-1} resolution. Using OPUS 6.5 analysis software (Bruker, Billerica, MA), the results were processed using baseline correction, smoothing, normalization, and second derivatization.

2.2.6 Solid-State Fluorescence Spectroscopy

Intrinsic fluorescence of the solid samples was measured in front surface mode using a Cary-Eclipse spectrofluorometer (Agilent, Santa Clara, CA). The powders (20 mg each) were pulverized, loaded into a sample holder, and pressed against a fused silica plate so that the surface was fully covered with powder. Spectra were collected with a photomultiplier tube of 600, with an incident angle of 25 degrees and a slit width of 5 nm. Excitation occurred at 280 nm, with emission spectra collected between 300-400 nm. The intensities of the spectra as collected were normalized using Prism Software (GraphPad, La Jolla, CA).

2.2.7 Differential Scanning Calorimetry (DSC)

Under nitrogen, 2-4 mg of powder were loaded into hermetic aluminum pans and sealed. Samples were loaded into a Discovery Series DSC 25 differential scanning calorimeter (TA Instruments, New Castle, DE). The sample was analyzed by heating it, starting at -5°C with a ramp rate of 1°C/min to an ending temperature of 180°C. Using the TRIOS software (v4.3.0, TA Instruments, New Castle, DE), the T_g or T_m was determined.

2.2.8 Stability Studies by Size Exclusion Chromatography (SEC)

The stability of the samples was determined by measuring the level of protein aggregates using SEC. Samples were sealed in a desiccator at 40 °C over a saturated solution of magnesium chloride, which generated an environment of 33% relative humidity (RH). At each time point (15, 30, 60, and 90 days), three vials were removed and diluted to protein concentrations of 1 mg/mL. Solutions were centrifuged at 12,000 rpm and 4°C for 10 min to remove insoluble aggregates, and the supernatant was then removed and placed in HPLC vials for analysis. Samples were analyzed on a high performance liquid chromatography system (HPLC, 1200 series, Agilent Technologies, Santa Clara, CA) using isocratic flow of 50 mM sodium phosphate, 100 mM

sodium chloride solution (pH 6.8) over 15 min at a flow rate of 1 mL/min. The column used was a TSKgel® G3000SWXL HPLC Column from Sigma Aldrich (St. Louis, MO). Instability was determined as a percentage of loss of the area under the curve for the monomeric peak of a sample before storage under accelerated conditions, with the exception of BSA, where initial aggregate peaks were also included in determining monomer content.

2.2.9 Solid-State Hydrogen Deuterium Exchange with Mass Spectrometric Analysis (ssHDX-MS)

Vials containing the powder samples were placed in a sealed desiccator at 25°C containing a D_2O solution at 11% RH, which was maintained using a saturated solution of lithium chloride. For each formulation, three vials were removed at various time points (4, 12, 24, 48, and 120 h) following exposure to D_2O vapor. Following removal of each sample, exchange was quenched by rapidly cooling the sample on dry ice. Samples were then stored in a -80°C freezer until analysis. Fully deuterated samples were prepared by dissolving the protein in a solution containing 3 M guanidine hydrochloride, and then placed into a vial containing a 9:1 dilution of D_2O. This solution was then stored at 60°C for 24 hours before being quenched in a 4:1 solution of quench buffer and immediately analyzed.

To determine the extent of deuterium incorporation, samples were reconstituted in 2 mL of a chilled 0.1% formic acid solution (pH 2.5) and 10 µL were injected into a protein microtrap (Michrom Bioresources, Inc., Auburn, CA). Using a high performance liquid chromatography system (1200 series, Agilent Technologies, Santa Clara, CA), the samples were desalted for 1.7 min with 90% water, 10% acetonitrile with 0.1% formic acid, then eluted over 7 min with a gradient of 10% water, 90% acetonitrile with 0.1% formic acid. The columns were housed in a custom-built refrigeration unit[24] maintained at ~0°C to minimize back exchange. The mass spectra of the samples were determined using a 6520 qTOF mass spectrometer (Agilent

Technologies, Santa Clara, CA) in the mass range 200-2000 m/z. Deconvolution of the undeuterated and deuterated samples was used to obtain the masses of the protein, using the MassHunter Workstation Software (version B.04, Agilent Technologies, Santa Clara, CA) to calculate the maximum entropy function. This algorithm converts the mass envelopes of the detected charge states into mass values which correspond to the different species present.

The kinetics of deuterium incorporation was fitted to the mono-exponential model:

$$D(t) = D_{max}(1 - e^{kt}) \quad (2.1)$$

where *D(t)* is the number of deuterons taken up at time *t*, D_{max} is the maximum number of deuterons incorporated, and *k* is the observed rate constant of deuterium incorporation.

2.2.10 Statistical Analysis

The effects of process and excipient on exchange kinetics were compared statistically using Prism Software. For multiple comparisons, a one-way ANOVA followed by Tukey's Test was used.

2.3 Results

2.3.1 Effect of Formulation and Drying Process on Moisture Content and Excipient Crystallinity

All formulations were dried to a moisture content ≤3% (Table A1). For most formulations, the moisture content of the spray-dried samples was slightly less than those that were lyophilized. This can be attributed to the additional drying step at 30°C for the spray-dried samples, which was used to make the moisture content comparable to their lyophilized counterparts.

XRPD measurements for formulations containing sucrose or trehalose were consistent with completely amorphous solids for all processing conditions (Fig. A1). Samples containing

mannitol showed minor peaks on XRPD, indicating the presence of crystalline mannitol. This crystallinity is probably related to the high mannitol content; mannitol greater than 30% (w/w) of the total solid content typically undergoes crystallization.[25]

2.3.2 DSC Analysis

T_g values were determined for sucrose- and trehalose-containing formulations, while T_m was determined for mannitol-containing formulations due to crystallinity. Samples containing sucrose had a lower T_g than other formulations (Table A2), consistent with previous reports.[26] Samples containing trehalose showed process-dependent differences in T_g, with spray-dried samples having a higher T_g than those that were lyophilized. These results may be due to the additional drying step and the lower residual moisture content of the spray-dried samples (Table A1). For samples containing mannitol the T_m was found to be ~159-163°C, which is consistent with crystalline mannitol.[27]

2.3.3 Secondary Structural Analysis by ssFTIR

Amide I region of ssFTIR spectra was collected for each formulation and used to compare protein secondary structure (Fig. 2.1). The FTIR bands for myoglobin were in general agreement with spectra reported previously [11] and prior to processing (Fig. A3), with a band at ~1656 cm^{-1} indicating α-helical structure (Fig. 2.1A). For myoglobin formulations containing trehalose, the bands were similar, with only a slight decrease in intensity for the spray-dried formulation (Fig. 2.1A). In myoglobin samples containing mannitol, a decrease in band intensity and increased broadening were observed relative to lyophilized samples. In sucrose-containing myoglobin formulations, lyophilization showed slight structural perturbation, with a band shift to 1658 cm^{-1}, which still is consistent with α-helix structure.

For the spray-dried formulations of BSA (Fig. 2.1B), FTIR bands were observed at ~1656 cm^{-1}, which corresponds to α-helix structure, as reported previously for BSA.[28] A decrease in band intensity was observed for spray-dried vs. lyophilized formulations, with the exception of the trehalose formulation, for which band intensities were similar. BSA spray-dried with mannitol showed a slight increase in the breadth of the band (Fig. 2.1B), which suggests perturbation of the α-helix.

For β-lactoglobulin (Fig. 2.1C), FTIR bands were observed at ~ 1638 cm^{-1} (β-sheet content), ~1660 cm^{-1} (turns), and ~1690 cm^{-1} (β-sheet content). In all spray-dried formulations of β-lactoglobulin, a band shift to ~1640 cm^{-1} was observed, along with significant broadening, suggesting an increase in structural heterogeneity of the β-sheet. Band intensity at ~1640 cm^{-1} was less in the trehalose- and mannitol-containing formulations for both processing conditions.

For lysozyme (Fig. 2.1D), FTIR bands were observed at ~1625 cm^{-1} (β-sheet), 1646 cm^{-1} (random coil), ~1658 cm^{-1} (α-helix), 1675 cm^{-1} (turns/loops), and 1690 cm^{-1} (turns). No significant differences in peak pattern or intensity were observed among the different formulations and processing conditions.

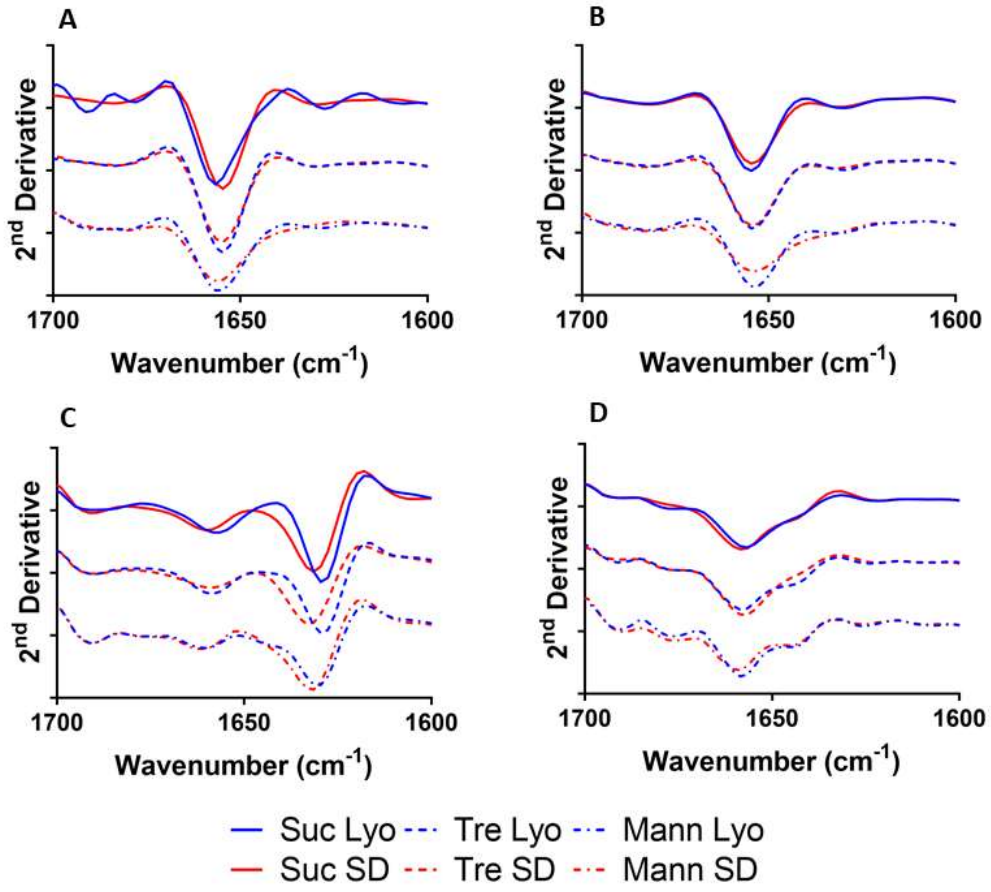

Figure 2.1: Solid-state FTIR spectra of formulated myoglobin (A), BSA (B), β-lactoglobulin (C), or lysozyme (D).

2.3.4 Tertiary Structural Analysis by Intrinsic Fluorescence

Intrinsic fluorescence has been used previously to measure the exposure of tryptophan to its environment[29], which corresponds to changes in tertiary structure. A fully exposed tryptophan residue in the solid state has a fluorescence maximum at ~334 nm. Changes in folding correspond to shifts in the peak. Peak intensity is not considered significant in the solid state because concentration dependence is unlikely to occur.[29]

For all myoglobin formulations, the protein concentration was too low for accurate measurements by the instrument (Fig. 2.2A). For BSA (Fig. 2.2B), the fluorescence peak of all lyophilized samples occurred at 324 nm, while for spray-dried samples there was a blue shift to 320 nm. As the moisture content of the spray-dried samples is slightly less than the lyophilized samples, this may be attributable to hydration differences rather than differences in folding. For β-lactoglobulin (Fig. 2.2C), no significant peak shifts were observed due to formulation or processing conditions.

Lysozyme formulations (Fig. 2.2D) all showed significant differences in peak position that depended on formulation and processing conditions. Both mannitol-containing formulations displayed red shifts (332 nm for lyophilized, 336 nm for spray-dried) relative to other excipients. Sucrose-containing formulations had a peak at 324 nm for lyophilized samples, and at 327 nm for spray-dried. No difference in processing conditions was observed for any protein formulated with trehalose (Fig. 2.2).

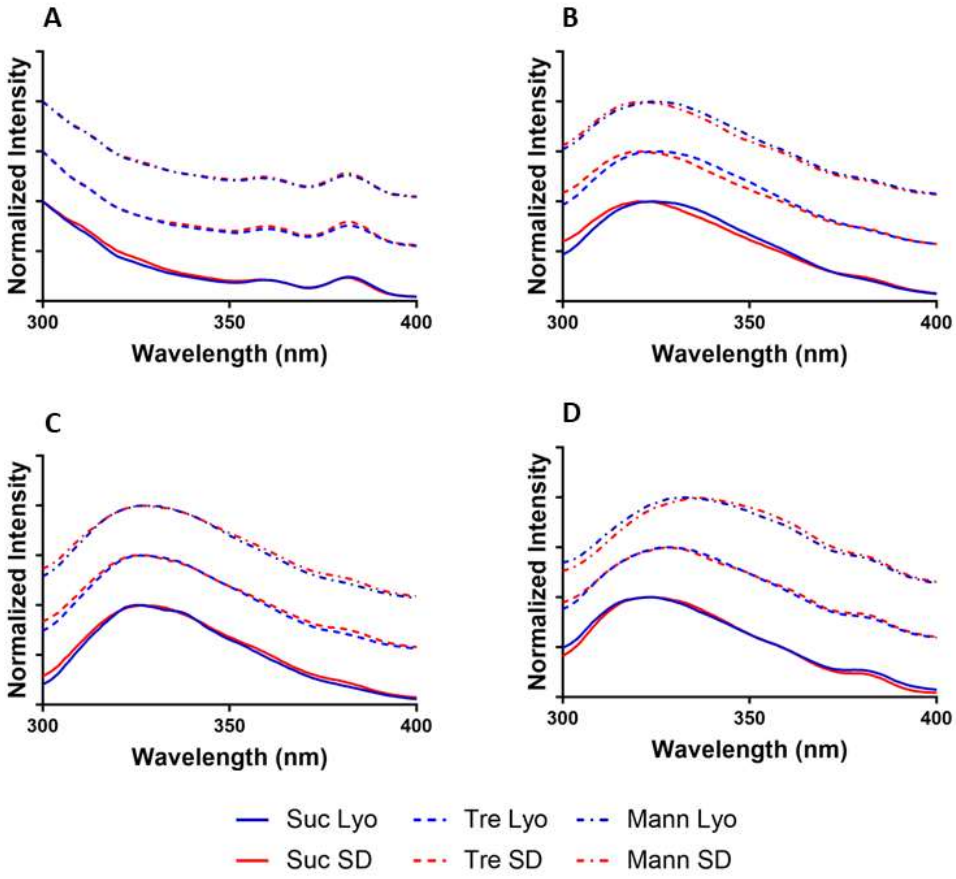

Figure 2.2: Solid-state fluorescence spectroscopy of myoglobin (A), BSA (B), β-lactoglobulin (C), or lysozyme (D).

2.3.5 Protein Conformation and Matrix Interactions Using ssHDX-MS

Protein conformation and matrix interactions between protein and excipients in the solid state were monitored by deuterium incorporation as a function of time. Several factors can affect the rate of exchange, including inter- and intramolecular interactions in the dried matrix, relative humidity, temperature, and the mass transport of D_2O vapor into the sample.[30,31] In this study, the temperature and relative humidity were kept constant.

In a previous study, we showed for a monoclonal antibody that mass transport was complete within 48 h, and that the density of the powder did not affect mass transport.[17] Similarly, as shown for each protein in Figure 2.3, mass transport does not affect the extent of deuterium incorporation, although for the smaller proteins mass transport is completed before the first time point (4 h). This further confirms that density does not significantly affect the rate of deuterium exchange.

The extent of deuteration was used to compare the differences among formulations and drying processes for each protein. In the myoglobin formulations (Fig. 2.3A), 5 days of deuterium exchange showed no significant differences between spray drying and lyophilization for the sucrose and trehalose-containing formulations. Spray-dried myoglobin samples containing mannitol showed slightly greater deuterium uptake than the lyophilized samples. For all other proteins studied, no differences in deuterium uptake as a function of time were observed for formulations produced by different methods. Mannitol-containing formulations had higher deuterium incorporation than those containing sucrose or trehalose, which were comparable. This suggests the differences in deuterium incorporation are primarily dependent on intermolecular interactions between the protein and mannitol or trehalose within the solid, as found in previous studies.[20]

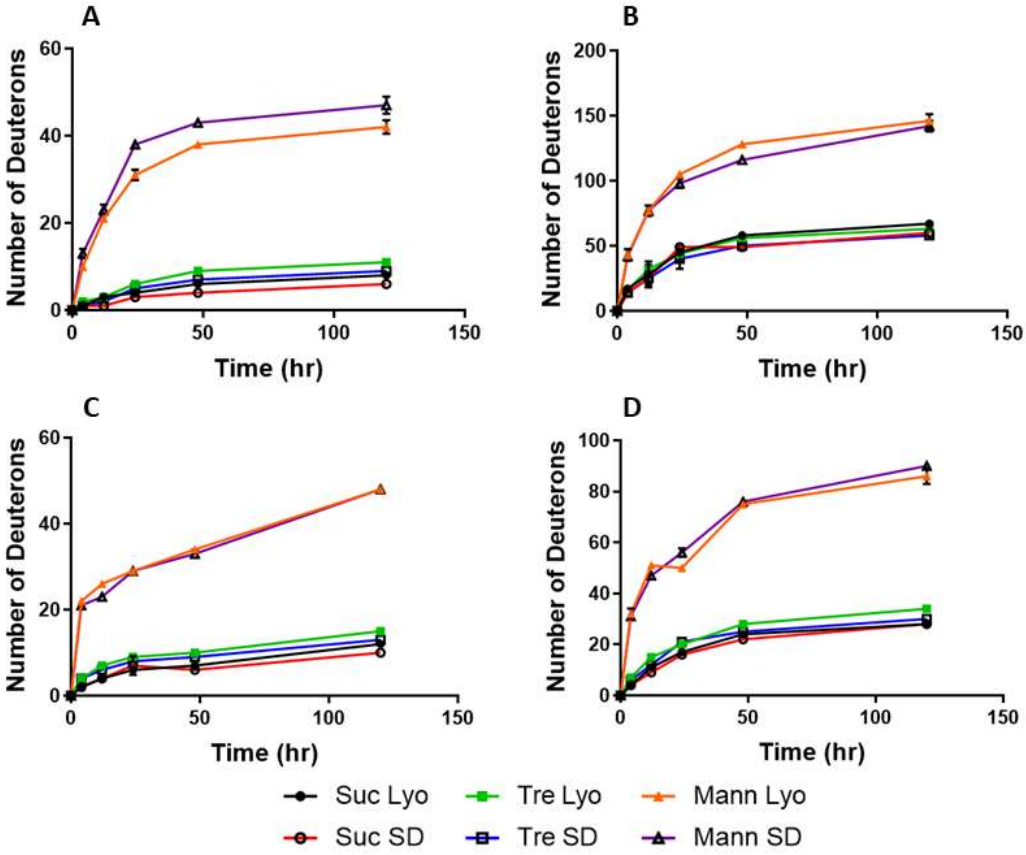

Figure 2.3: Kinetics of hydrogen/deuterium exchange in the solid state for myoglobin (A), BSA (B), β-lactoglobulin (C), and lysozyme (D).

In addition to determining total deuterium incorporation by ssHDX-MS, the deuterated mass spectra were also examined to identify any differences in the shape and width of the mass envelopes. Differences were observed based on both formulation and processing conditions. For all proteins studied, no differences were observed pre-processing and prior to deuterium exchange (Fig. A4). For myoglobin, sucrose and trehalose (Fig. 2.4A and B), formulations showed no differences in their spectra after 5 days of deuteration, and the spectra were slightly broadened relative to that of the undeuterated protein, as expected.[17] In contrast, substantial peak broadening was observed in the spectra of mannitol-containing lyophilized and spray-dried

formulations, as compared to the undeuterated protein (Fig. 2.4C). This peak broadening may be attributed to greater heterogeneity of protein conformations and/or matrix interactions in the solid formulation, which results in a broader range of deuteration states. As mass spectrometry cannot distinguish between intermolecular and intramolecular interactions, population heterogeneity is used to refer all possible differences in inter- and intramolecular hydrogen bonding as identified by ssHDX-MS. The presence of two peaks in the lyophilized mannitol powder is consistent with two distinct populations, one of which is more protected from deuterium exchange (Fig. 2.4C). In contrast, the spray-dried formulation has a broad shoulder on the left side of its peak, suggesting a distribution of protein populations that are not well-resolved. Similar observations were made for all formulations containing lysozyme (Fig. 2.5).The deconvoluted mass envelope for undeuterated BSA indicates the presence of isoforms (Fig. 2.6). During deuterium exchange, peak broadening results in some degree of merging of these species, making it difficult to distinguish the effects of excipients and processing conditions. In sucrose- and trehalose-containing formulations (Fig. 2.6A and B), similar peak broadening was observed for both lyophilized and spray-dried samples. Mannitol-containing samples (Fig. 2.6C) showed greater peak broadening than the sucrose and trehalose formulations.

In β-lactoglobulin, the deconvoluted mass envelopes were consistent with two predominant species and some additional isoforms (Fig. 2.7). An average of the deuterium uptake for each of each of the dominant species was used to calculate deuterium uptake (Fig. 2.3C). Upon deuteration, solid samples containing either sucrose or trehalose (Fig. 2.7A and B) produced similar mass envelopes, with the exception of a shoulder to the right of each main peak for samples produced by spray drying. This suggests the presence of a second population that differs from the main peak in protein conformation and/or matrix interactions for the spray-dried

samples. For β-lactoglobulin samples containing mannitol (Fig. 2.7C), two distinct peaks were observed in lyophilized formulations, again consistent with different populations, although part of this peak splitting may be due to deuteration of the lower molecular weight isoforms. In contrast, the spray-dried formulation has a broad shoulder on the left, suggesting a higher concentration of less deuterated protein in the spray-dried formulation than in the lyophilized formulation.

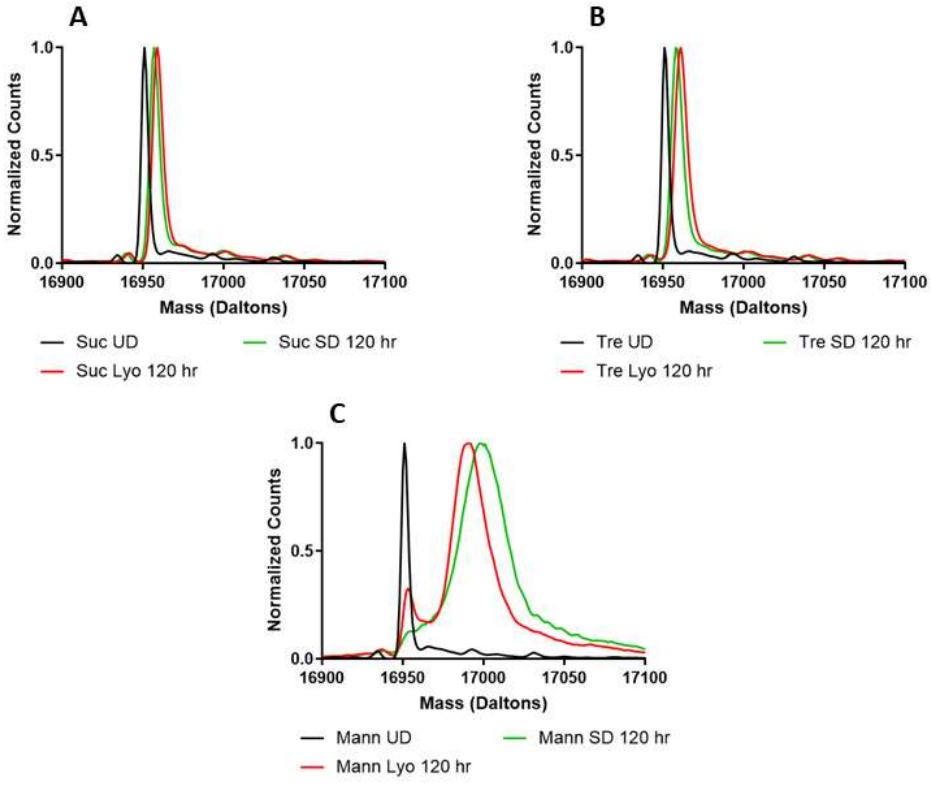

Figure 2.4: Deconvoluted mass spectra of formulations prepared by lyophilization or spray drying with myoglobin and sucrose (A), trehalose (B), or mannitol (C).

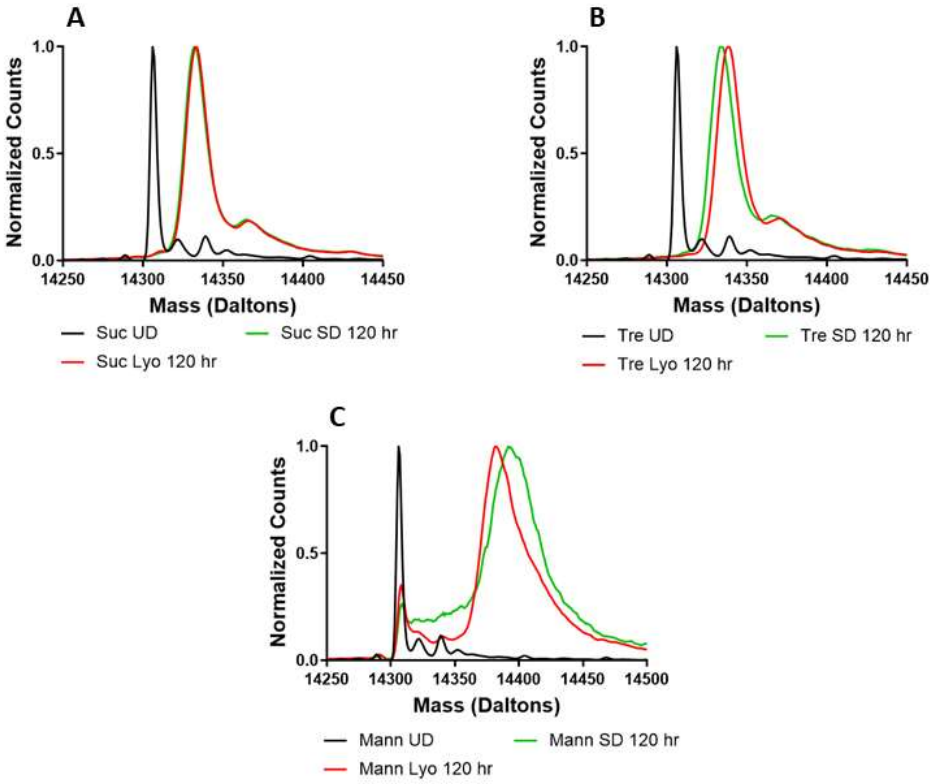

Figure 2.5: Deconvoluted mass spectra of formulations prepared by lyophilization or spray drying with lysozyme and sucrose (A), trehalose (B), or mannitol (C).

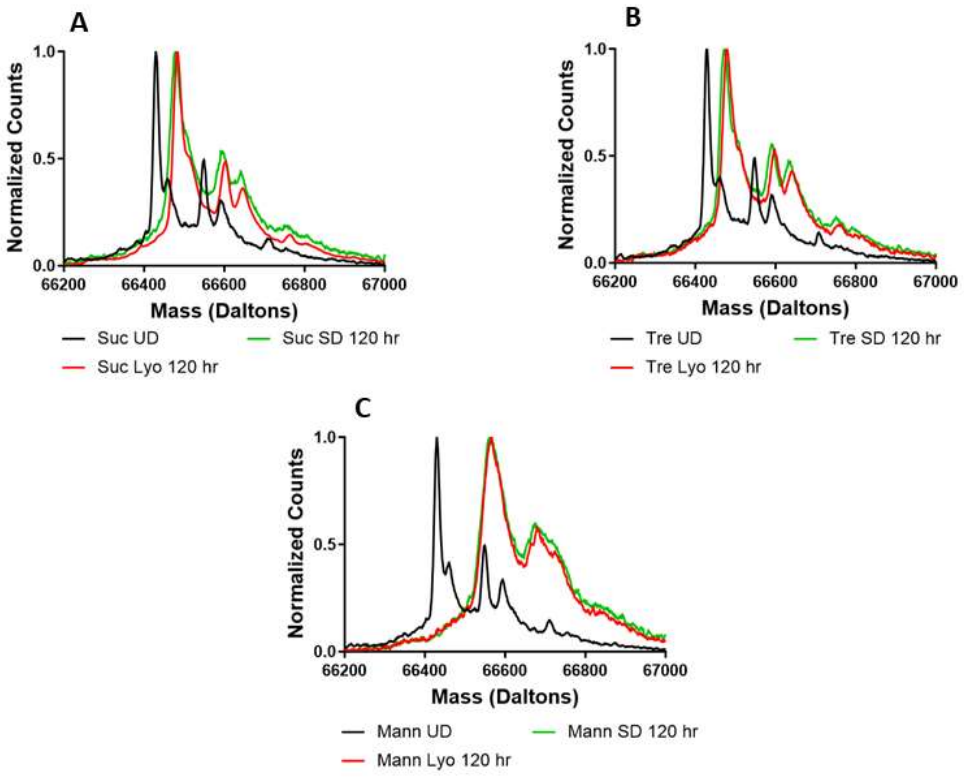

Figure 2.6: Deconvoluted mass spectra of formulations prepared by lyophilization or spray drying with BSA and sucrose (A), trehalose (B), or mannitol (C).

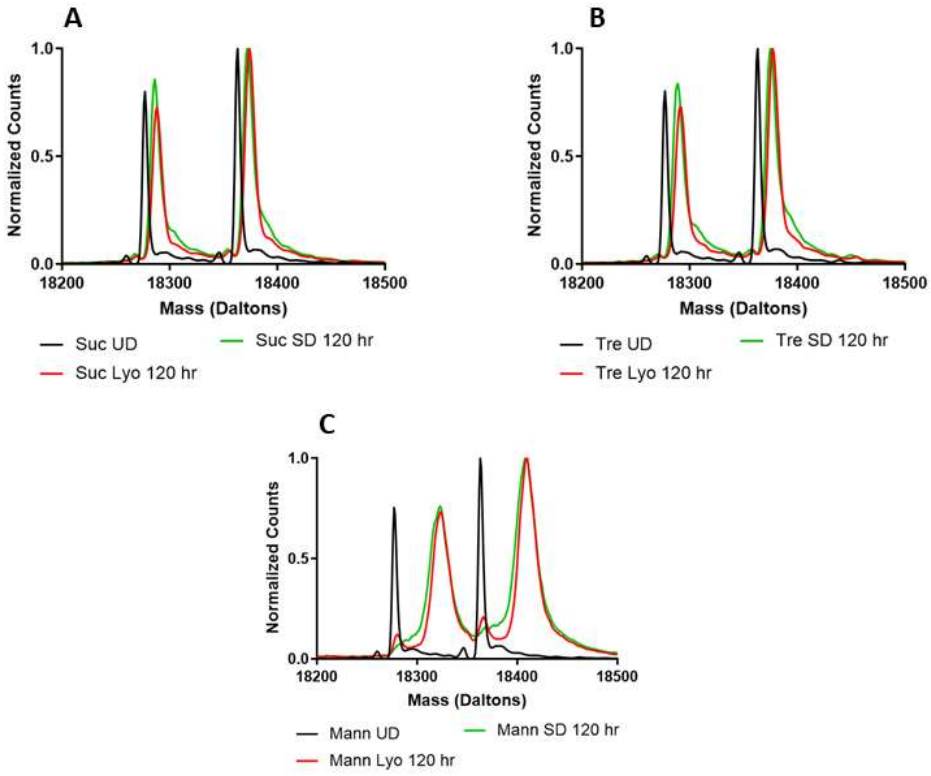

Figure 2.7: Deconvoluted mass spectra of formulations prepared by lyophilization or spray drying with β-lactoglobulin and sucrose (A), trehalose (B), or mannitol (C).

2.3.6 Stability Studies

90-day stability studies were conducted at 40°C and 33% RH to determine the effects of excipients and formulation on protein stability. For most of the studies (Fig. 2.8), the percentage of aggregates was greatest in formulations containing mannitol, with the exception of myoglobin spray-dried with sucrose and all formulations of lysozyme. In the lysozyme formulations, spray-dried samples had greater aggregate content than the lyophilized samples. With BSA, the formulations all contained aggregates prior to drying as BSA normally form dimers at

physiological pH. The samples containing sucrose and trehalose remained consistent in the aggregate content throughout the study.

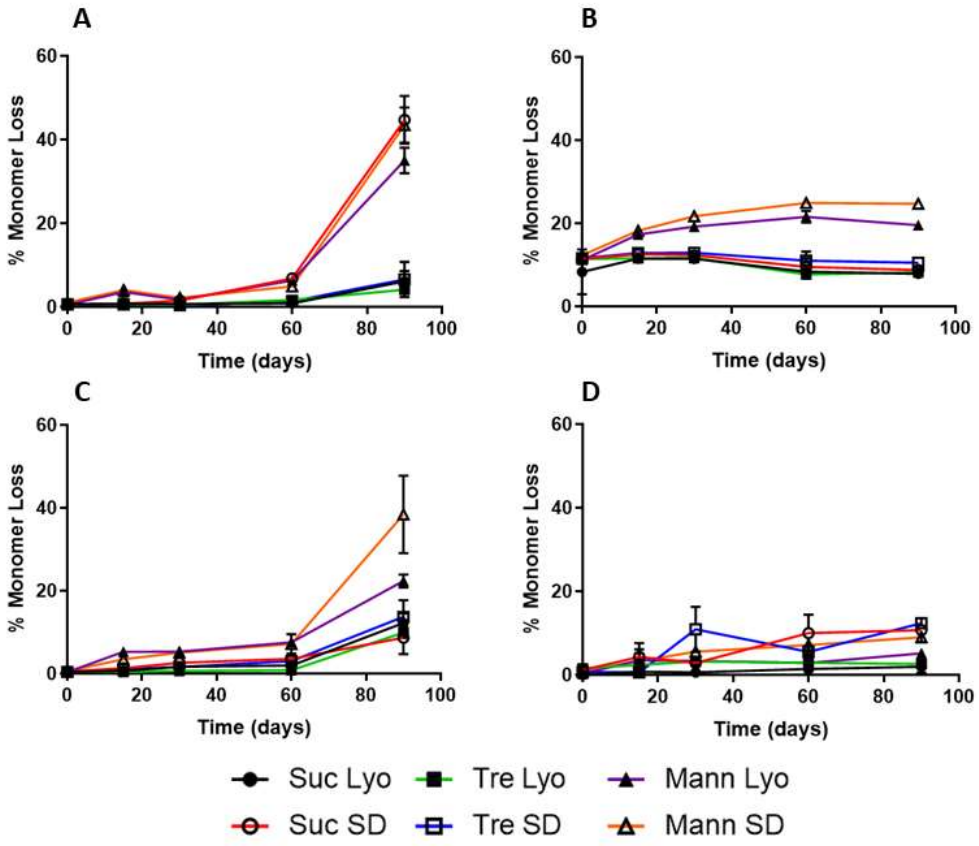

Figure 2.8: Stability studies of formulations containing myoglobin (A), BSA (B), β-lactoglobulin (C), or lysozyme (D).

2.4 Discussion

While the effects of excipients on the stability of lyophilized proteins have been well-studied, relatively few published reports have investigated the effects of different drying methods for various formulations. ssHDX-MS has been shown to be useful in investigating the effects of formulation on protein structure and matrix interactions in solid powders. In the present study,

ssHDX-MS has been used together with orthogonal methods to characterize dried powders of four different model proteins produced using different excipients and drying methods.

The kinetics of deuterium incorporation in ssHDX-MS were fitted to the mono-exponential model (Fig. 2.9). For the proteins studied, with the exception of myoglobin, there were no significant differences in the rate constants (*k*) of deuterium incorporation with changes in formulation or drying method (Fig. 2.9A). This suggests that the rate of deuteration is not affected by the density of the sample, regardless of formulation or processing condition. For myoglobin, there was a statistically significant difference in the rate of deuteration for spray-dried formulations with sucrose or mannitol (Fig. 2.9A). This difference is likely due to the crystallization of mannitol, resulting in more rapid deuteration. With regard to the maximum extent of deuterium exchange (D_{max}, Fig. 2.9B), there were significant differences between formulations containing mannitol and those with sucrose or trehalose for all proteins analyzed. This may also be due to mannitol crystallinity.

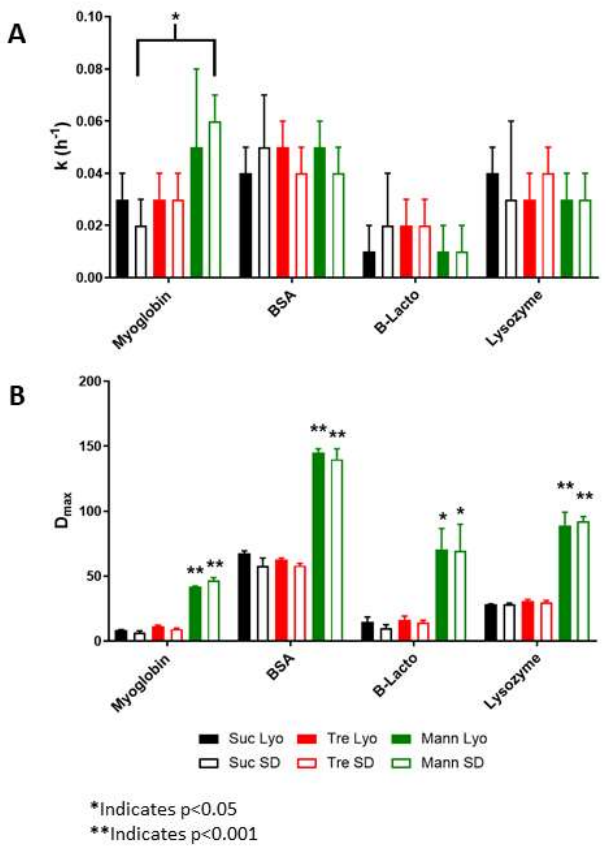

Figure 2.9: Deuterium exchange kinetics for protein formulations fitted to the mono-exponential model in Equation 2.1.

With myoglobin, none of the standard methods of analysis were able to detect structural differences. However, drying method related differences were detected using ssHDX-MS when examining the peak areas of the normalized deconvoluted mass spectra as a function of the percentage of deuterium exchanged (Fig. 2.10). In ssHDX-MS, mannitol samples showed two distinct populations that were not identified by conventional methods. Some broadening of the spectra and corresponding increases in peak area are expected over the deuteration time course, since the ensemble of protein molecules displays a distribution of deuteration kinetic behavior. The distinct populations observed for myoglobin cannot be attributed to this phenomenon, which instead may suggest the presence of aggregates or isolated populations trapped within a

crystalline mannitol matrix, either of which will limit exposure to D_2O. Interestingly, there were no differences in deuteration for myoglobin processed with sucrose or trehalose, which differs from previous studies of a monoclonal antibody, where differences in deuterium incorporation for these two excipients were observed.[17]

There was little difference in ssFTIR spectra with either changes in excipient or processing conditions, with the exception of β-lactoglobulin (Fig. 2.1). The shifting and broadening of the ssFTIR peaks for spray-dried β-lactoglobulin suggest an increase in the heterogeneity of protein structural states in the solid sample (Fig. 2.1C). In ssHDX-MS studies of β-lactoglobulin samples, there were no significant differences in the extent of deuterium uptake over time with different drying methods (Fig. 2.3C). However, the deconvoluted mass spectra showed shoulders on the right for sucrose and trehalose formulations (Fig. 2.6A,B) and on the left for mannitol samples (Fig. 2.6C) that were spray-dried, consistent with greater heterogeneity in these samples (Fig. 2.6). In addition, the peak areas of the deconvoluted mass envelopes for spray-dried formulations were consistently greater than corresponding lyophilized samples throughout the deuteration time course (Fig. 2.11), further suggesting population heterogeneity.

Solid-state fluorescence spectra showed process related differences for BSA and β-lactoglobulin (Fig. 2.2B,C). For BSA, these changes in the exposure of the hydrophobic tryptophan may be due to the lower moisture content of the spray-dried samples after the additional drying step. In ssHDX-MS analysis of these samples, spray-dried samples showed higher peak areas of the deconvoluted mass envelopes than lyophilized samples (Fig. 2.11). This suggests that either interactions between the protein and the matrix are weaker and more variable in the spray-dried samples and/or that there is a broader distribution of protein structures. Similar inferences cannot be made for BSA samples due to merging of peaks.

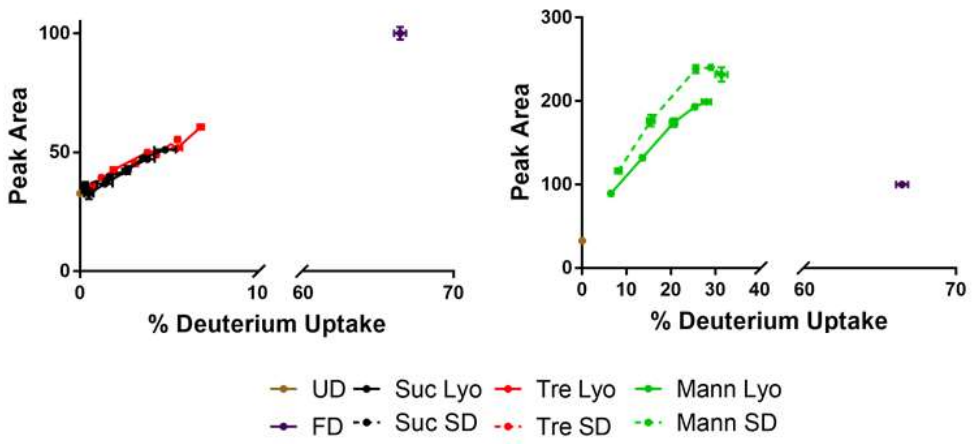

Figure 2.10: Peak areas of the deconvoluted mass envelope as a function of deuterium incorporation for myoglobin. Peak areas are measured as a percentage of the area of the fully deuterated (FD) sample.

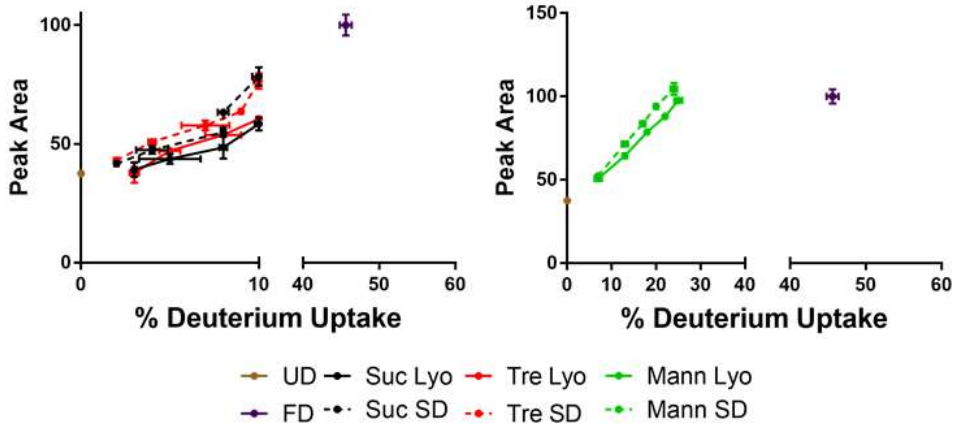

Figure 2.11: Peak areas of the deconvoluted mass envelope as a function of deuterium incorporation for BSA.

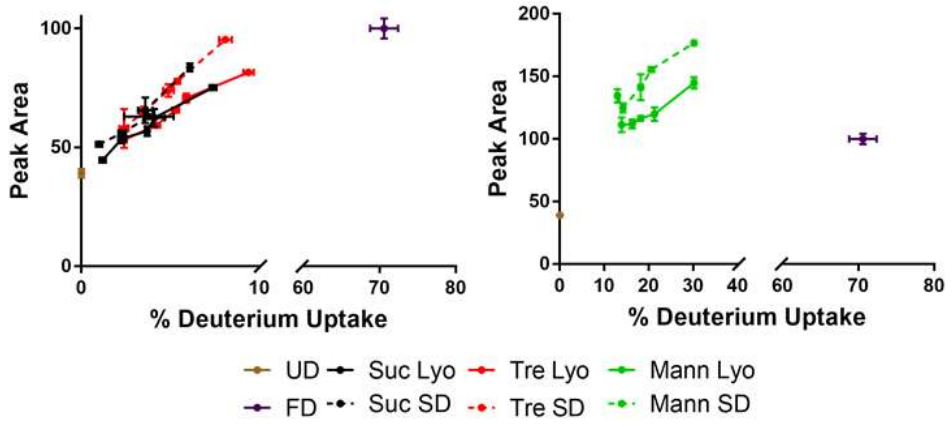

Figure 2.12: Peak areas of the deconvoluted mass envelope as a function of deuterium incorporation for β-lactoglobulin.

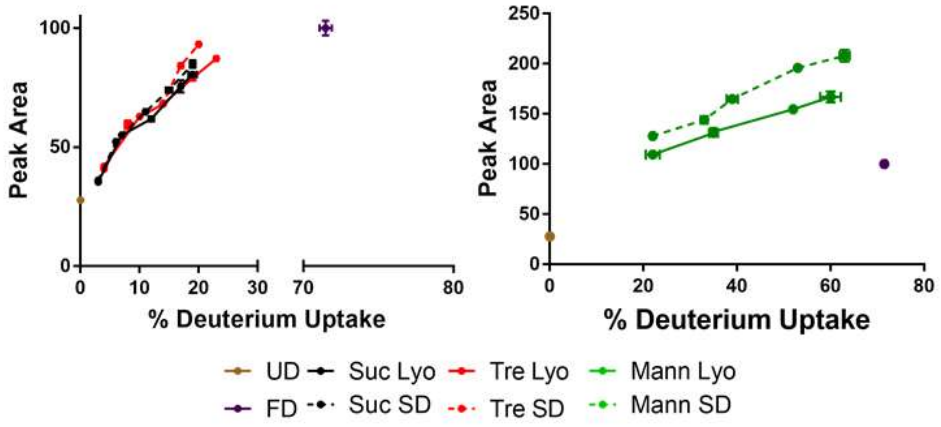

Figure 2.13: Peak areas of the deconvoluted mass envelope as a function of deuterium incorporation for lysozyme.

Conventional methods for analyzing proteins in solid powders were not strongly correlated to stability on storage (Fig. 2.14), a finding consistent with a previous study by our group.[20] ssHDX-MS metrics were more strongly correlated with stability. In previous reports, higher deuterium uptake has been correlated with decreased storage stability,[20,21] suggesting that

weaker intermolecular interactions between protein and excipient or changes in intramolecular interactions in the protein lead to both greater deuterium incorporation and poorer stability. For the proteins studied here, mannitol formulations showed greater deuterium uptake, presumably due to phase separation caused by the crystallization of the excipient (Fig. 2.3). This phase separation reduces the number of possible interactions between protein and excipient, exposing more sites for deuterium exchange to occur. Mannitol samples also had the highest aggregate content on storage, with the exception of spray-dried myoglobin with sucrose (Fig. 2.8), which is likely due to the crystallization of sucrose during storage (Fig. A2). Similar results were obtained when correlating stability with either the deconvoluted peak area or the maximum deuterium incorporation (Fig. 2.14D and E). This suggests that while peak area may be a better indicator of the populations present in the sample, it is not necessarily a better predictor of physical stability on storage.

In the proteins and formulations studied here, population heterogeneity was generally greater in spray-dried samples than in lyophilized samples, as indicated by ssHDX-MS peak area (Figs. 2.4-7, 10-13). This may be related to exposure of protein, preferentially distributed to the air-liquid interface of the droplets, to high shear during atomization. A previous report of structural heterogeneity of a spray-dried monoclonal antibody is in agreement with this finding.[14] Our previous studies have also shown heterogeneity in the surface composition of spray-dried particles.[32,33] Proteins have higher molecular weight and lower rates of diffusion than the small-molecule excipients[34], which may limit redistribution of the protein away from the interface during drying. Further studies characterizing heterogeneity in dried particles and its effects on protein structure and stability are warranted.

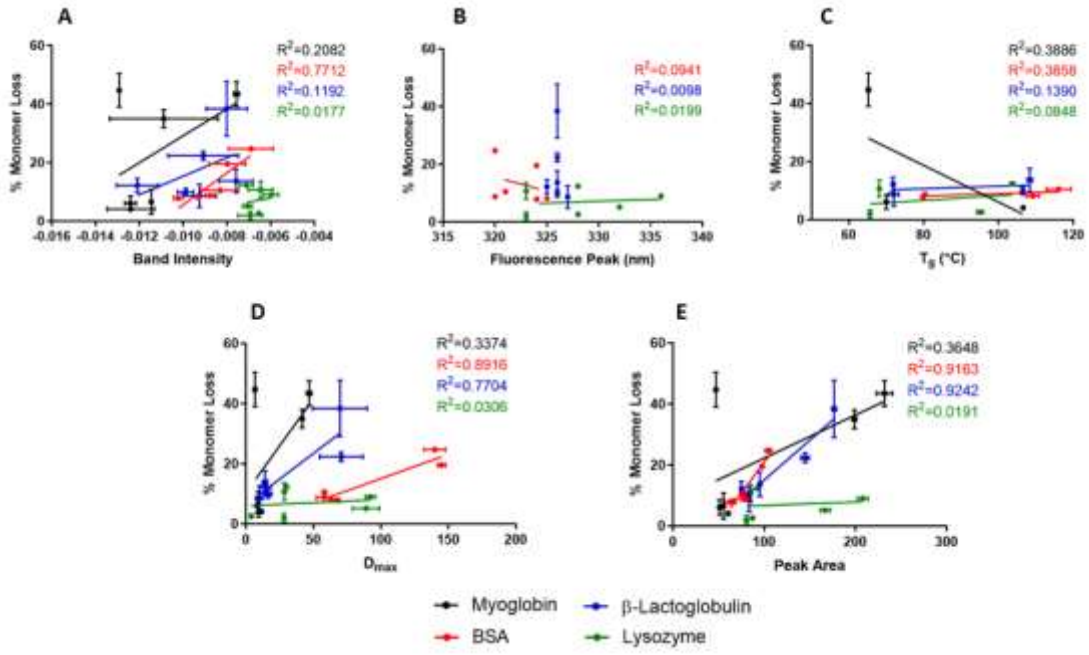

Figure 2.14: Correlation of stability to ssFTIR (A), solid-state fluorescence spectroscopy (B), Tg (C), Dmax (D), and peak area of deuterated samples (E).

2.5 Conclusions

The effects of processing conditions and excipients on protein structure and physical stability were studied by ssHDX-MS and conventional characterization methods of ssFTIR, solid-state fluorescence spectroscopy, and DSC. While the conventional approaches detected some differences between processes and formulations, there was no strong correlation with physical stability. With ssHDX-MS, a greater correlation to physical stability was found, with greater level of instability generally corresponding to higher D_{max} and peak area. In addition, ssHDX-MS was capable of identifying the population heterogeneity within a protein formulation, with increased heterogeneity occurring in spray-dried formulations as compared to the corresponding lyophilized samples. The results demonstrate that ssHDX-MS can be used as a tool not just for predicting physical stability, but also in the identifying differences in processing conditions which could lead to the development of more robust protein formulations.

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CHAPTER 3. SURFACE COMPOSITION AND FORMULATION HETEROGENEITY OF PROTEIN SOLIDS PRODUCED BY SPRAY DRYING

3.1 Introduction

To address stability concerns for protein formulations in solution, additional drying steps are often used to better stabilize proteins in the solid state. The most common drying approach in the pharmaceutical industry is lyophilization, which freezes the solution, followed by primary and secondary drying under reduced pressure to remove water from the protein and produce a solid product.[1] While this technique is well-understood in pharmaceutical product development, it is a time-consuming batch process with low energy-usage efficiency, and may expose sensitive biologics to freezing-related stresses.[2] Consequently, alternative drying techniques have been explored for solid formulation development of pharmaceutical proteins.

Spray drying is a processing method that has been widely used in the food industry[3,4], and has increasingly attracted interest for biopharmaceutical manufacturing.[5] This technique has been successfully used to produce marketed biological products, such as Raplixa and Exubera.[6,7] During spray drying, a solution is fed into a spray nozzle, where liquid is atomized into droplets that are briefly exposed to a drying gas at a set temperature. These droplets are rapidly dried into solid particles by evaporative diffusion of water, and then collected by cyclone. As with lyophilization, proteins are subjected to additional stresses during spray drying, which include high temperature, dehydration, and air-liquid interfacial stresses, among others.[8]

Exposing proteins to these stressors can lead to deleterious conformational changes which affect drug product stability, depending on the extent and length of exposure to each stress. One of the most problematic stresses in spray-drying is exposure of the protein to the air-liquid

interface.[9] The amphiphilic nature of the protein can lead to adsorption at the surface, leading to higher concentrations of protein at the interface than dispersed in solution.[10,11] Protein migration to the surface can cause exposure of the hydrophobic core in order to align at the interface. This unfolding leads to increased denaturation and protein damage, which increases the tendency for aggregation to occur during drying.[10]

To reduce this stress, excipients, such as surfactants, can be used to directly compete with the protein for adsorption at the surface.[12] Less explored are the effects of hydrogen-bonding excipients, such as sugars, and the effects of excipients with increasing molecular weight, on reducing the protein surface composition, as well as their effects on formulation heterogeneity. Previously, solid-state hydrogen/deuterium exchange with mass spectrometry (ssHDX-MS) has been used to demonstrate the effects of drying methods and formulation composition on population heterogeneity[13], which was found to correspond to physical stability.[14] In ssHDX-MS, a sample is stored in a desiccator containing a saturated salt solution in deuterium oxide (D_2O). Exposed hydrogen atoms along the protein amide backbone are exchanged with deuterium, which can be monitored by mass spectrometry. This exposure and protein sensitivity to exchange provides information on the hydrogen bonding interactions in the formulation matrix, which may be related to the conformational stability of the protein and its physical stability.[15]

In the work reported here, the model proteins bovine serum albumin (BSA) and myoglobin were formulated with sugar excipients of low and high molecular weight to examine their impact on particle surface composition and protein conformational heterogeneity, following spray drying. The excipients selected were sucrose, trehalose, dextran of molecular weight 20,000 or 70,000 g/mol (listed as dextran 20K or 70K, respectively) based on their differences in size.[16,17] The samples were characterized regarding secondary structure using solid-state Fourier-

transform infrared spectroscopy (ssFTIR), the levels of formed aggregates by size exclusion chromatography (SEC), and glass transition temperature (T_g) by differential scanning calorimetry (DSC). In addition, surface composition was determined by X-ray photoelectron spectroscopy (XPS), and the population heterogeneity was determined by ssHDX-MS. Using these techniques, a correlation between surface composition and population heterogeneity was determined for the spray-dried proteins studied.

3.2 Materials and Methods

3.2.1 Materials

Bovine serum albumin, myoglobin from equine skeletal muscle, and sucrose (Bioextra, ≥95%, GC) were purchased from Sigma Aldrich (St. Louis, MO). D-(+)-trehalose dehydrate was purchased from Fisher Scientific (Fair Lawn, NJ). Dextrans of molecular weight 20,000 and 70,000 were procured from Afla Aesar (Ward Hill, MA) and Tokyo Chemical Industry Company (Portland, OR), respectively. Protein-containing solutions were dialyzed with a 2.5 mM phosphate buffer solution at 4°C for 24 hours using Slide-A-Lyzer™ dialysis cassettes from Thermo Scientific (Rockford, IL). Phosphoric acid was used when necessary to adjust solution pH to 6.8. Excipient-containing solutions in the same buffer conditions were prepared separately, and then diluted with the dialyzed solutions for a final concentration of 10 mg/mL protein and 10 mg/mL excipient.

3.2.2 Spray drying

Samples were spray-dried using a Büchi Mini Spray Dryer B290 (New Castle, DE) with an inlet temperature of 100°C and outlet of 50-55°C. Solutions were atomized by an air volumetric flow rate of 600 L/h with a liquid feed rate of 2 mL/min. Dried powders were

collected and distributed into 2R borosilicate glass vials (~4 mg per vial) and further dried in a vacuum oven at 30°C and 100 mTorr for 24 hours to reduce moisture content to ~2-3%. Additional drying was performed to reduce the effects that varying moisture may have on protein structure and stability.

3.2.3 Karl Fischer Titration for Moisture Content Analysis

Moisture content was determined by coulometric titration using an 831 KF Coulometer (Metrohm, Riverview, FL). Samples were reconstituted with 1 mL of methanol (anhydrous, septum sealed bottle DriSolv®, Sigma Aldrich, St. Louis, MO). The suspension was then injected into the cell and titrated with Riedel-de Haën Hydranal® Coulomat reagent (Hoechst Celanese Corp., Germany) for 5 min to reach end point for moisture determination. Samples were measured in triplicate.

3.2.4 X-ray Powder Diffraction

A Rigaku Smart Lab X-ray diffractometer (The Woodlands, TX) equipped with a Cu Kα X-ray source and Bragg-Brentano geometry was used to determine the solid form of the dried samples. Powders were pulverized onto a glass slide and loaded onto a slide holder for analysis. The diffraction intensity was measured between 5 and 40 degrees as a function of 2θ with a step size of 0.02° and scan rate of 5°/min.

3.2.5 Solid-state Fourier Transform Infrared Spectroscopy (ssFTIR)

Measurements were conducted with a Thermo Scientific Nicolet Nexus spectrometer (Waltham, MA) in the attenuated total reflectance mode. Samples were loaded onto a Smart iTR™ accessory and compressed against the diamond by a metal anvil. Spectra were collected in absorbance mode with 120 scans in the range of 800 to 4000 cm^{-1} with a 4 cm^{-1} resolution.

Results were processed with OPUS 6.5 analysis software (Bruker, Billerica, MA) using baseline correction, smoothing, normalization, and second derivatization.

3.2.6 Modulated Differential Scanning Calorimetry (mDSC)

Dried powder (2-4 mg) was loaded into hermetic aluminum pans and sealed in a nitrogen-purged glovebox. Samples were loaded into a Discovery Series DSC 25 differential scanning calorimeter (TA Instruments, New Castle, DE), with an empty pan as reference. Powders were heated from 25°C to 225°C with a modulation of ±1°C every 120 s. Glass transition temperatures (T_g) were determined using the TRIOS software (v4.3.0, TA Instruments, New Castle, DE).

3.2.7 Size Exclusion Chromatography (SEC)

The extent of aggregation after spray drying was determined using size exclusion chromatography (SEC). Solutions and dried powders were diluted to protein concentrations of 1 mg/mL and centrifuged for 10 min at 12,000 rpm and 4°C to remove insoluble aggregates. The supernatant was then removed and used for analysis. A high performance liquid chromatography system (HPLC, 1200 series, Agilent Technologies, Santa Clara, CA) was used for analysis with an isocratic flow of 50 mM sodium phosphate, 100 mM sodium chloride solution at pH 6.8 over 15 min at a 1 mL/min flow rate. Eluted samples were detected at 280 nm by a diode array detector. A TSKgel® G3000SWXL HPLC Column (Sigma Aldrich, St. Louis, MO) was used for size exclusion analysis.

3.2.8 X-ray Photoelectron Spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) measurements were performed as described previously.[18] Briefly, the XPS data were collected by an AXIS Ultra DLD spectrometer (Kratos

Analytical Inc., Manchester UK) using monochromatic Al Kα radiation (1486.6 eV). The surfaces of these samples are irradiated with an X-ray beam, which causes core-level electrons to be emitted with a specific kinetic energy unique to each element and chemical bonds with other atoms. The kinetic energy of the photoelectrons is measured by an energy analyzer. This technique has been previously used for protein surface coverage for other compounds and formulations.[19,20]

High resolution and survey XPS spectra were obtained using constant pass energies of 20 and 160 eV, respectively. Data were processed using CasaXPS software. To determine the percentage elemental composition of the formulations, areas of the O 1s, N 1s, and C 1s peaks were calculated following a Shirley background subtraction. Corrections on the corresponding Scofield relative sensitivity factors and inelastic mean free path of the photoelectrons were also applied. The sulfur peaks were not used in these calculations due to its low concentration (<1%) and low relative sensitivity factor. The atomic percentage of nitrogen was used to determine protein surface coverage. Four replicates were measured and the results averaged.

3.2.9 Scanning Electron Microscopy (SEM)

Morphological differences of spray-dried powders were visualized using a Nova NanoSEM 200 system (Fei, Hillsboro, OR). Sample powders were mounted onto a sample holder and sputter-coated with carbon graphite. Particles were then analyzed under vacuum to determine morphological properties.

3.2.10 Solid-State Hydrogen/Deuterium Exchange with Mass Spectrometric Analysis (ssHDX-MS)

Vials containing the dried formulations were stored in a sealed desiccator containing a deuterium oxide (D_2O) solution saturated with lithium chloride to maintain relative humidity at

11% and 25°C. At various time points (4, 12, 24, 48, 120, and 240 h), three vials per formulation were removed and the exchange reaction quenched by rapidly cooling the samples on dry ice. Vials were then stored at -80°C until analysis by mass spectrometry. To produce a fully deuterated control, protein was dissolved in a solution containing 3M guanidine hydrochloride, then aliquoted into a vial containing a 9:1 dilution of D_2O: solution. This solution was then stored for 24 hours at 60° before quenching in a 4:1 solution of quench buffer (chilled 0.1% formic acid solution, pH 2.5) and immediately analyzed.

For mass spectrometric analysis to determine the extent of deuterium incorporation, samples were reconstituted in 2 mL of quench buffer, and 10 µL were injected into a protein microtrap (Michrom Bioresources, Inc., Auburn, CA). Using an HPLC system (1200 series, Agilent Technologies, Santa Clara, CA), samples were desalted in the microtrap column for 1.7 min with 90% water, 10% acetonitrile with 0.1% formic acid, followed by elution over 7 min to a gradient of 10% water, 90% acetonitrile with 0.1% formic acid. To minimize back exchange, columns were housed in a custom-built refrigeration unit with the temperature maintained at 4°C. Mass spectra of samples were determined using a 6520 qTOF mass spectrometer (Agilent Technologies, Santa Clara, CA) in the 200-2000 m/z range. Deconvolution of the samples to determine the mass range of the proteins was performed using the Mass Workstation Software (version B.04, Agilent Technologies, Santa Clara, CA). The maximum entropy function was used for this calculation, which converts the mass envelopes of the detected charge states into values that correspond to the different species present in the formulation.

The kinetics of deuterium incorporation was fitted using the mono-exponential model:

$$D(t) = D_{max}(1 - e^{kt}) \tag{3.1}$$

where $D(t)$ is the number of deuterons exchanged at time t, D_{max} is the maximum number of deuterons that can be incorporated into the sample, and k is the observed rate constant for deuterium incorporation.

3.2.11 Statistical Analysis

The effects of excipient choice on moisture content, surface composition, exchange kinetics, and physical stability were compared statistically using Prism Software (GraphPad, La Jolla, CA). A one-way ANOVA with Tukey's Test was used for multiple comparisons among formulations.

3.3 Results

3.3.1 Moisture Content and Thermal Stability

Formulations all had similar moisture content at the end of vacuum drying (Table 3.1). The amorphous nature of these formulations is indicated by the XRPD patterns (Fig. 3.1); no crystallinity was observed for any system.

For samples where T_g could be determined, sucrose-containing formulations had the lowest Tgs for both myoglobin and BSA, with values between 74-78°C. These values are consistent with those reported for amorphous sucrose.[21] Samples with trehalose also had T_g values consistent with previous reports [21], and were approximately 40°C higher than sucrose-containing systems. Dextran-containing formulations had the highest T_g values for both myoglobin and BSA, and were consistent with values for dextran alone.[22] Differences in glass transition between protein samples can be due to differences in moisture content, which has been demonstrated to impact glass transition.[23]

Table 3.1: Moisture content and Tg for protein formulations. (n=3)

Formulation	Moisture Content (%)	T_g (°C)
Myoglobin Only	2.5 ± 0.7	ND
Myoglobin-Sucrose	2.5 ± 0.6	77.2 ± 2.8
Myoglobin-Trehalose	2.5 ± 0.7	112.9 ± 2.1
Myoglobin-Dextran 20K	3.7 ± 0.6	210.8 ± 0.4
Myoglobin-Dextran 70K	3.0 ± 0.3	219.2 ± 0.5
BSA Only	3.1 ± 0.3	ND
BSA-Sucrose	2.4 ± 0.1	74.7 ± 3.8
BSA-Trehalose	2.5 ± 0.3	105.0 ± 1.3
BSA-Dextran 20K	3.9 ± 0.3	213.3 ± 0.3
BSA-Dextran 70K	2.8 ± 0.1	223.2 ± 0.3

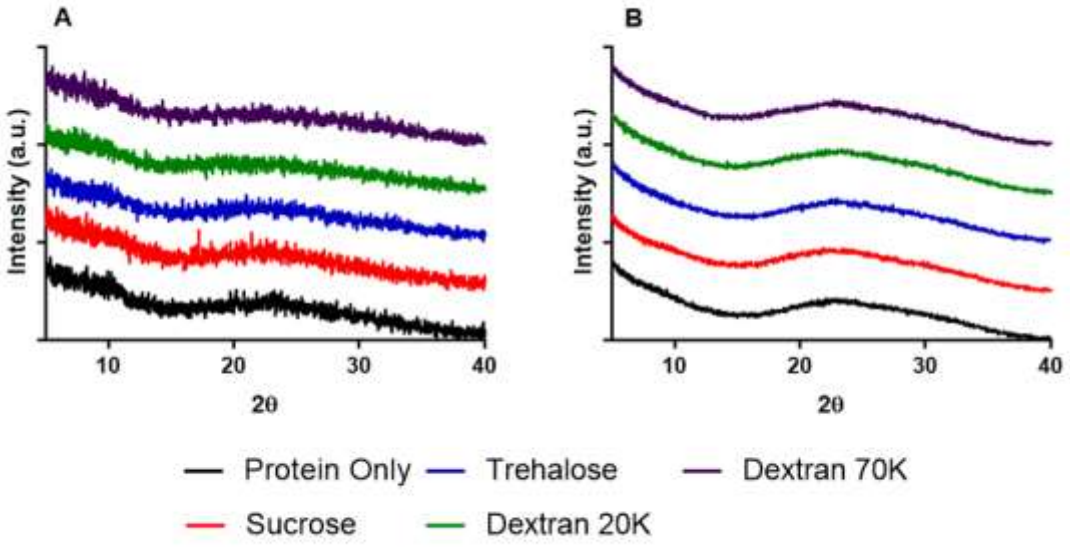

Figure 3.1: X-ray powder diffraction patterns of BSA (A) and myoglobin (B) formulations. Samples were formulated without excipient or with sucrose, trehalose, dextran 20K, or dextran 70K.

3.3.2 ssFTIR Spectroscopy for Secondary Structural Analysis

The amide I region of the ssFTIR spectra (Fig. 3.2) was evaluated to compare the secondary structure of each protein formulation. For BSA (Fig. 3.2A), sucrose- and trehalose-containing formulations had bands at 1653 and 1655 cm^{-1}, respectively, which correspond to α-helical structure and is in general agreement with previously reported spectra.[24] For samples of BSA spray-dried without excipient, there is a band at 1653 cm^{-1}, however there is a reduction in band intensity and increased broadening, which suggests structural perturbation.

For myoglobin formulations (Fig. 3.2B) containing dextran 20K and 70K, bands were detected at 1656 and 1658 cm^{-1}, respectively, with greater band broadening and intensity reduction than sucrose- and trehalose-containing samples, although less than for pure spray-dried BSA. In myoglobin formulations, sucrose- and trehalose-containing samples both had bands at 1653 cm^{-1} corresponding to α-helical structure.[25] For dextran-containing formulations, both showed a band shift to 1654 cm^{-1} and slight broadening relative to formulations containing lower molecular weight excipients. Similar to BSA, this suggests an increase in structural perturbation of the α-helices. For pure spray-dried myoglobin, a broad band at 1651 cm^{-1} was observed, indicating structural perturbation of the predominantly α-helical structure of the protein.

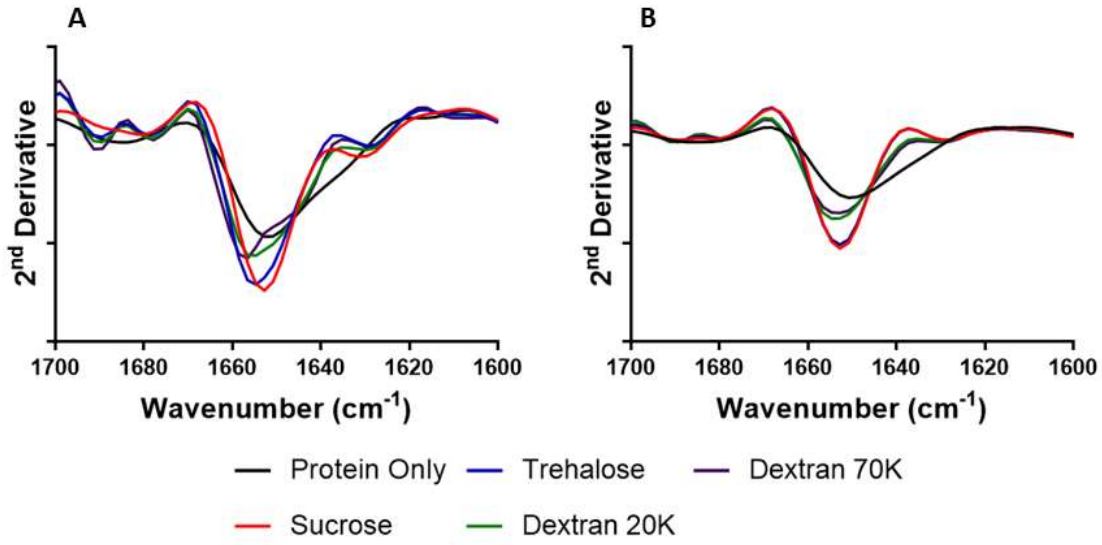

Figure 3.2: Solid-state FTIR spectra of formulated BSA (A) and myoglobin (B).

3.3.3 SEC for Monomer Content Determination Post-Drying

SEC was used to determine the level of aggregates formed after drying (Fig. 3.3). For BSA, the monomer content for all formulations was similar (~83-86%) with the exception of those spray-dried with dextran 70K (~78%). The presence of higher-order aggregates is expected under these buffering conditions, as BSA exists as a mixture of monomers and dimers at pH 6.8.[26] For myoglobin samples, all had similar monomer content (~97%).

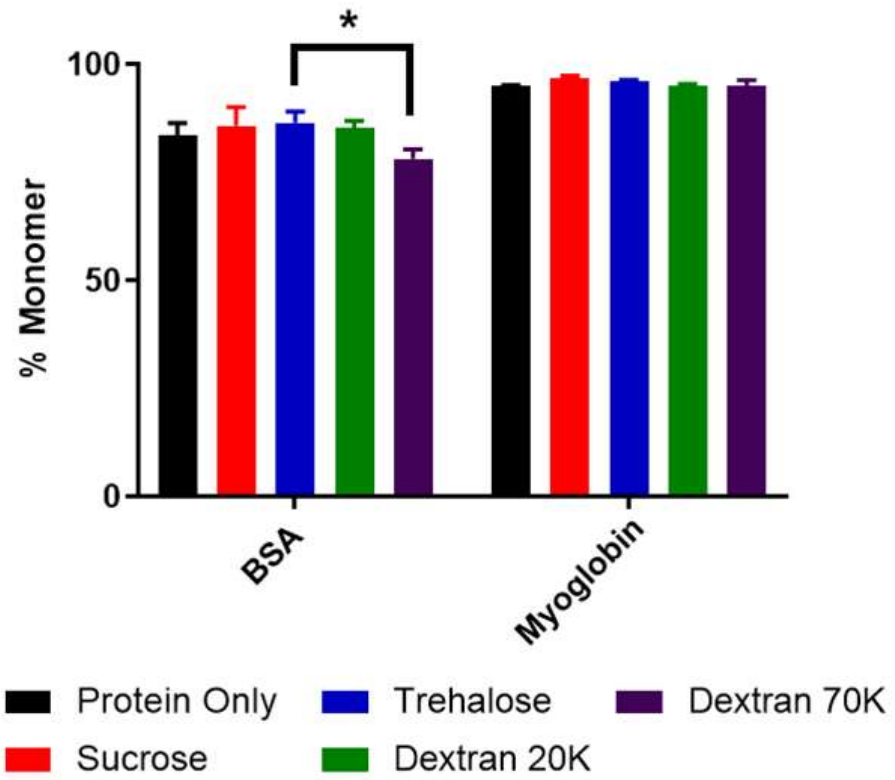

Figure 3.3: Size exclusion chromatography for monomer percentage of BSA and myoglobin formulations. (*Indicates $p<0.05$)

3.3.4 Protein Surface Coverage by XPS

XPS was used to determine the protein composition on the top 10 nm of a particle surface (Fig. 3.4). For excipient-free samples of both BSA and myoglobin, concentrations of nitrogen were in agreement with the bulk composition of each protein (~16% nitrogen, Fig. 3.4A). The addition of excipients to each of the protein formulations resulted in reduced nitrogen composition at the surface, with sucrose and trehalose leading to a significantly greater reduction in surface protein composition as determined from XPS results relative to dextran-containing formulations (Tables B1-4). For the proteins studied, a greater reduction in surface protein concentration was observed in myoglobin as compared to BSA samples.

Due to the absence of nitrogen in the excipients, the amount of nitrogen present at the surface can be used to determine the atomic percentage composed of protein (Fig. 3.4B). Sucrose- and trehalose-containing formulations resulted in the greatest reduction of surface protein, while dextran-containing formulations had the least, regardless of differences in molecular weight. However, the concentration of protein at the surface suggests heterogeneous distribution of molecules in the dried particle, with surface enrichment. The estimated atomic percentage of protein was calculated using the equation:

$$At.\%_{Protein} = \frac{Wt.\%_{Protein} / MWt._{Protein}}{\left(\frac{Wt.\%_{Protein}}{MWt._{Protein}}\right)+\left(\frac{Wt.\%_{Excipient}}{MWt._{Excipient}}\right)} * 100 \quad (3.2)$$

where *At. %* is the atomic percent of the molecule, *Wt. %* is the weight percentage of the molecule in the formulation, and *MWt.* is the molecular weight of the molecule. In a 1:1 w/w ratio of protein:excipient, a homogeneous matrix would yield an estimated surface composition of 0.5, 23.1 and 51.3 atomic percentage protein for sucrose, trehalose, and dextran formulations of BSA, respectively. In myoglobin, expected protein composition values would be 2.0, 54.1, and 80.5% protein for sucrose, trehalose and dextran formulations, respectively. Therefore, there is an excess concentration of protein at the surface, suggesting that the protein is not dispersed homogeneously within the matrix.

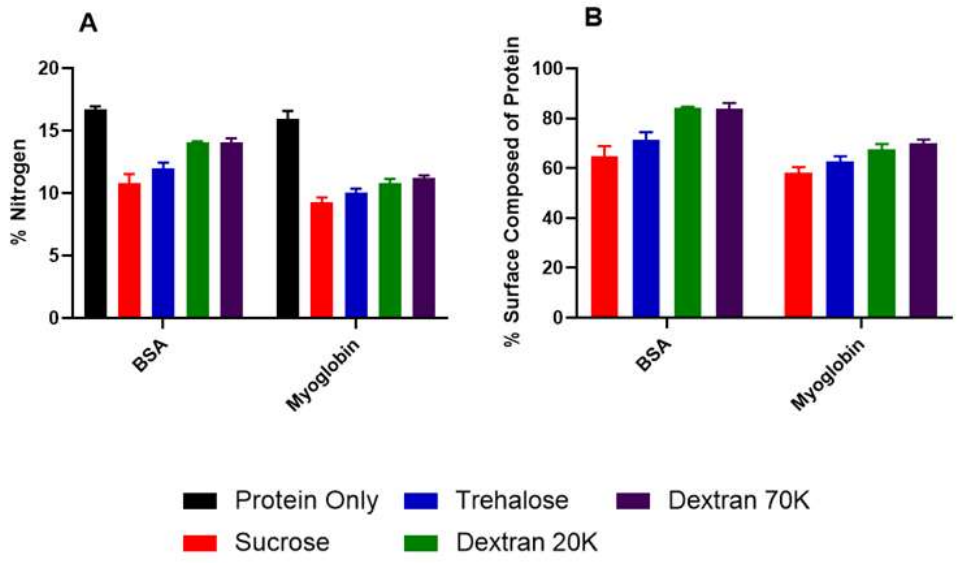

Figure 3.4: Quantification of X-ray photoelectron spectroscopy results for percentage of nitrogen on surface (A) and atomic percentage of protein found on the surface (B).

3.3.5 ssHDX-MS for Protein Conformational Interactions

Matrix interactions in the formulations of dried particles were monitored by measuring deuterium incorporation as a function of time using ssHDX-MS (Fig. 3.5). To control varying factors that can affect the rate and extent of deuterium uptake[15,27], samples were kept at a constant temperature and relative humidity.

For both protein formulations, samples without excipient had the highest amount of deuterium incorporation. This is expected due to lack of hydrogen-bonding interactions provided by the saccharide-based excipients used in other formulations in this study. Dextran-formulated samples with two different molecular weights had similar levels of exchange, with uptake being less than the unprotected samples. The high incorporation in these samples relative to those containing sucrose or trehalose is likely due to the large size and lack of molecular flexibility in

dextran. Rigidity of dextran may prevent intermolecular hydrogen bonding interactions with the protein in specific sites due to conformational differences[17] or phase separation.[28] Samples of sucrose and trehalose had similar levels of deuterium uptake, and were the lowest of the formulations studied.

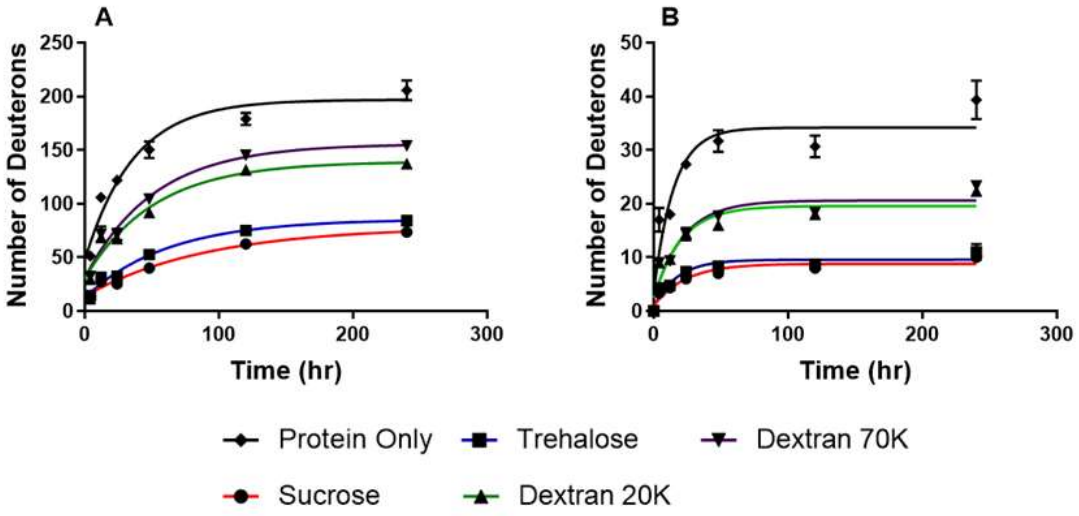

Figure 3.5: Kinetics of hydrogen/deuterium exchange in the solid state for BSA (A) and myoglobin (B).

Deconvoluted mass spectra of the formulations following deuterium exposure over 10 days were examined by ssHDX-MS to identify potential differences in shape and width of mass envelopes. This can provide information about formulation differences that affect the protein populations present. For BSA without excipients (Fig. 3.6A), increased deuterium exposure leads to lower resolution of the isoforms present under mass spectrometric analysis. This can be attributed to variability in the protection of proteins within the matrix, so that each of the isoforms will experience varying degrees of deuteration. Similar results are observed for formulations containing dextran 20K and 70K (Fig. 3.6D and E). For samples dried with sucrose

or trehalose (Fig. 3.6B and C), the deconvoluted mass envelope is better preserved during the process, with some slight broadening with increasing deuterium incorporation.

There are fewer isoforms in myoglobin samples (Fig. 3.7), and a clearer resolution of mass envelopes was achieved in ssHDX-MS. As observed for the excipient-free formulation (Fig. 3.7A), there is a distinct broadening of the mass envelope relative to the undeuterated sample. This suggests a distribution of deuteration and/or conformational states is present in less-protected samples. Similar to BSA, the protein samples processed with sucrose or trehalose (Fig. 3.7B and C) showed lower levels of deuteration and mass envelopes similar to the undeuterated state. Likewise, drying with either of the dextrans (Fig. 3.7D and E) resulted in peak broadening and greater deuterium uptake, although less than the samples without excipient.

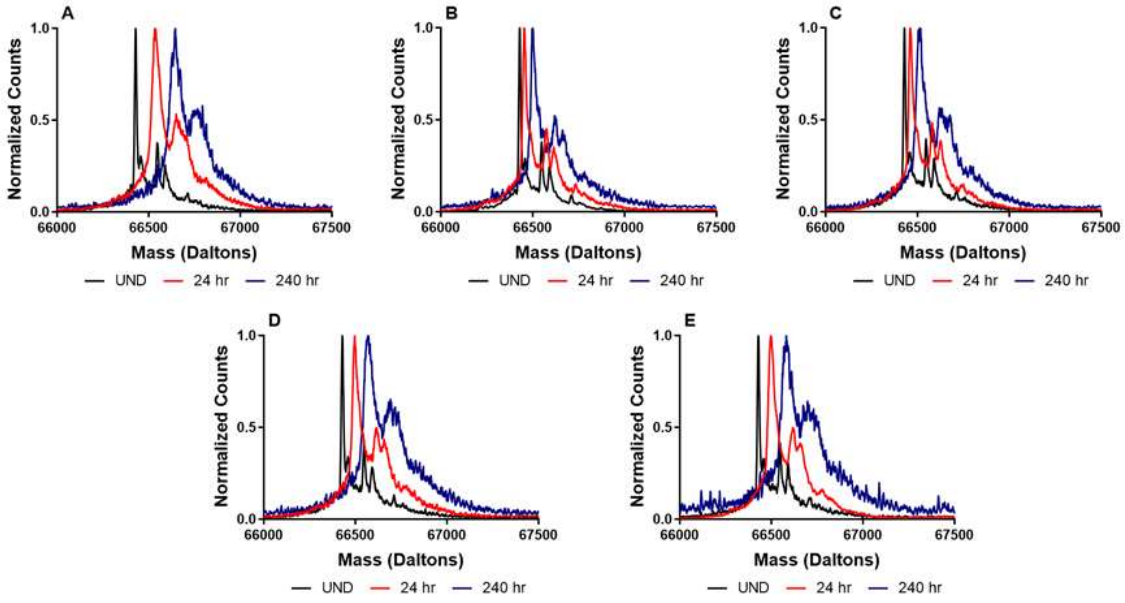

Figure 3.6: Deconvoluted mass spectra of formulations prepared by spray drying BSA without excipient (A) or with sucrose (B), trehalose (C), dextran 20K (D) or dextran 70K (E).

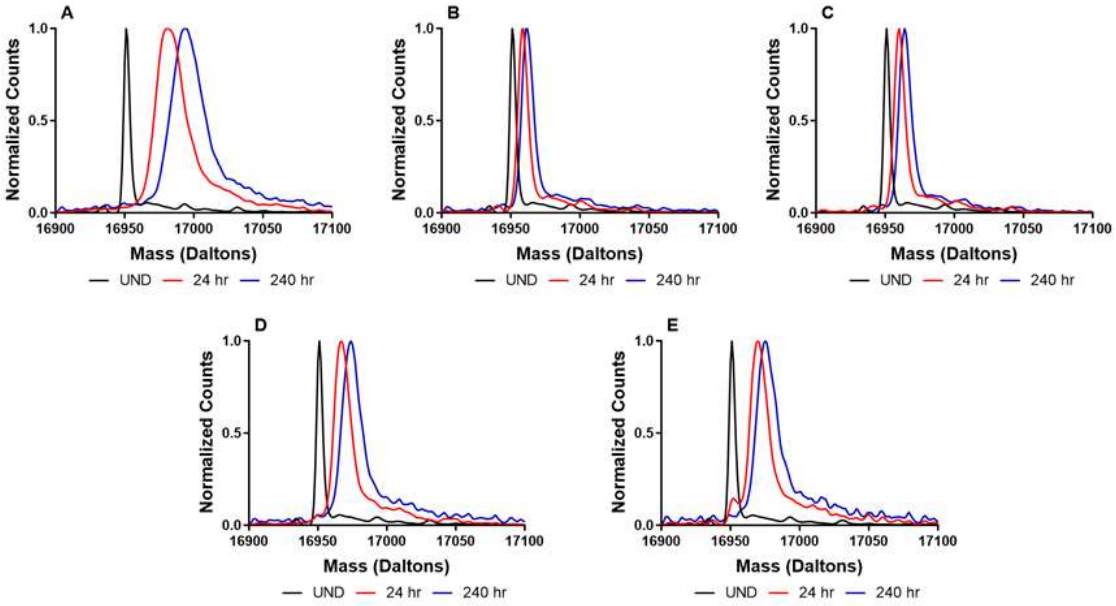

Figure 3.7: Deconvoluted mass spectra of formulations prepared by spray drying myoglobin without excipient (A) or with sucrose (B), trehalose (C), dextran 20K (D) or dextran 70K (E).

To better examine the effects of formulation and spray drying on protein populations, the peak areas of the normalized deconvoluted mass spectra as a percentage of an experimentally fully-deuterated sample were examined as a function of deuterium incorporation (Fig. 3.8). As in previous work, this ratio is thought to reflect differences in the distribution of protein conformational states and matrix interactions in the sample, and may be correlated with long-term physical stability.[13,14] When formulated with an excipient, both BSA (Fig. 3.8A) and myoglobin (Fig. 3.8B) show increasing peak area with increasing deuterium incorporation. At a given level of deuterium incorporation, formulations containing dextran showed greater peak area than those formulated with sucrose or trehalose, consistent with greater population heterogeneity. This suggests that loss of the hydrogen-bonding interactions provided by the

saccharide-based excipients leads to a greater number of conformational states and/or matrix interactions after drying.

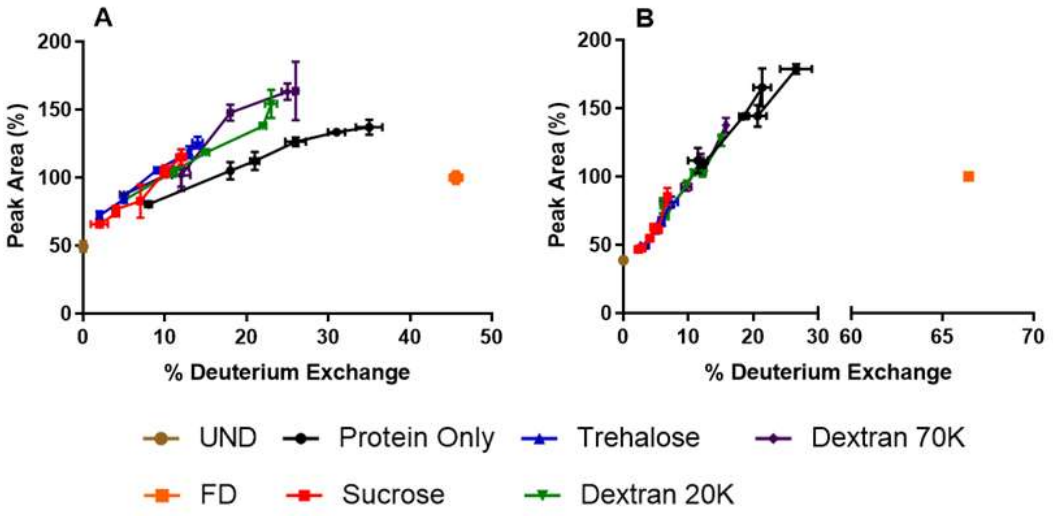

Figure 3.8: Peak areas of the deconvoluted mass envelope as a function of deuterium incorporation for BSA (A) and myoglobin (B). Peak areas are measured as a percentage of the area of the fully deuterated (FD) sample.

3.3.6 SEM for Particle Morphology

The morphology of the spray-dried particles was examined using scanning electron microscopy (Fig. 3.9). Pure spray-dried BSA (Fig. 3.9A) exhibited highly wrinkled particles, which is consistent with literature reports.[29] Particles spray-dried with this protein retain these morphological characteristics despite the addition of excipients.

Differences in particle morphology were observed for myoglobin formulations. Without excipient, spray-dried myoglobin (Fig. 3.9F) exhibits spherical particles with a dimpled surface, which is attributed to buckling due to pressure differences during drying. In sucrose- and

trehalose-containing samples (Fig. 3.9G and H), highly wrinkled particles were formed. For both samples processed with dextran (Fig. 3.9I and J), the particles collapsed with a mushroom-cap shape.

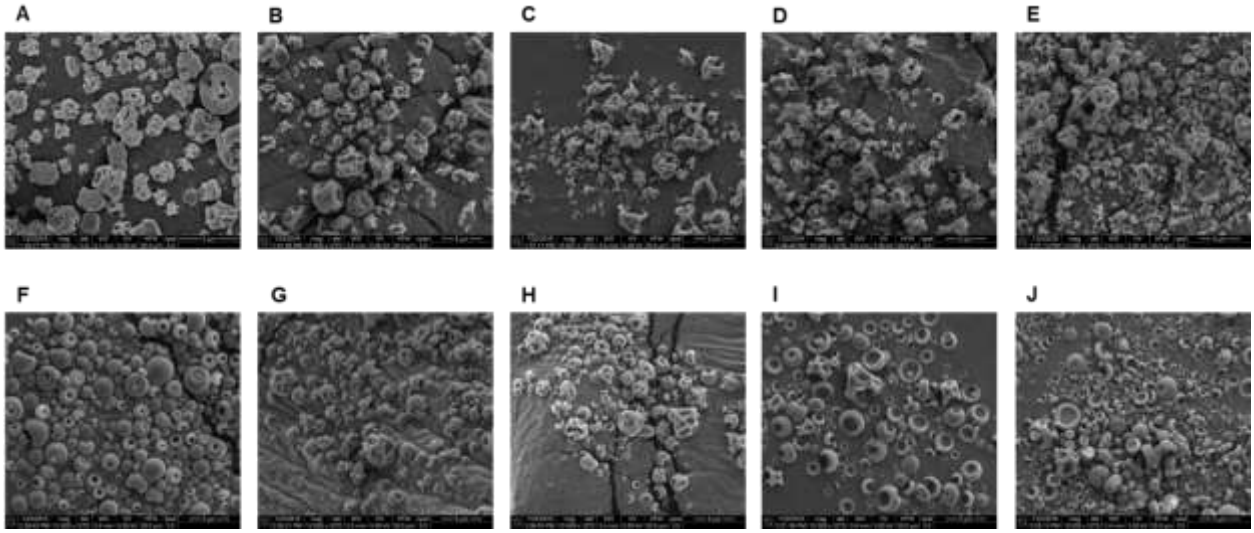

Figure 3.9: Scanning electron microscopy images of spray-dried particles. BSA formulations were processed without excipient (A) or with sucrose (B), trehalose (C), dextran 20K (D) or dextran 70K (E). Samples of myoglobin were formulated without excipient (F) or with sucrose (G), trehalose (H), dextran 20K (I) or dextran 70K (J).

3.4 Discussion

With the growing exploration of alternative drying approaches for biopharmaceuticals, understanding the effects of process and formulation on protein conformation, protein surface coverage, and population heterogeneity are important in developing robust products. Previously, our group has shown that drying method and formulation affect protein populations as measured by ssHDX-MS, which correlates well with protein aggregation on storage.[14] In the present study, ssHDX-MS was used together with XPS and other characterization techniques to explore the

effects of formulation on population heterogeneity in protein formulations processed by spray drying.

The amide I region of ssFTIR spectra (Fig. 3.2) indicated secondary structural differences of protein formulations with and without excipients. For both BSA and myoglobin, the excipient-free formulations showed greater structural disorder than excipient-containing samples, which was expected due to lack of a stabilizing excipient. Inclusion of sucrose or trehalose resulted in better preservation of native structure, which is consistent with previous reports.[24,25] In formulations containing dextran 20K or 70K, some structural perturbations were noted. As these dextran excipients are significantly larger than sucrose or trehalose, they may be less able to interact with the protein to stabilize the secondary structure.[17] In addition, dextran has been shown to undergo phase separation in some protein formulations after lyophilization.[28,30] For this study, only a single glass transition temperature was observed for each formulation (Table 3.1), which suggests a single phase, although there are challenges in identifying multiple phases based on assessment of calorimetric T_gs.

Surface coverage of the proteins was measured by XPS in order to examine the effects of excipients on protein surface distribution following drying. For surface-sensitive proteins, reducing the concentration of protein at the air-liquid interface during drying can lead to reduced aggregation and help retain efficacy.[20] In the present study, higher molecular weight excipients (i.e., dextrans) resulted in greater protein concentrations on the surface when compared to formulations with lower molecular weight excipients (Fig. 3.4). As previously mentioned, this is probably due to the lack of molecular flexibility in the dextrans, which may be incapable of providing site-specific protection of the protein, which can lead to concentration of the protein at the surface and decreased physical stability.

All excipient-containing formulations had higher protein surface concentrations than the theoretical concentrations for a 1:1 w/w ratio of protein:excipient (Fig. 3.4B). The higher surface concentration of the biomolecules suggests slow diffusion of proteins during the drying process. This surface enrichment has been demonstrated to depend on the Peclet number, *Pe*, given by the equation:

$$Pe=k/D_s \tag{3.3}$$

where k is the evaporation rate of solvent and D_s is the diffusivity of a component in the solvent system[31], in this instance a droplet. Under spray drying conditions, the rapid evaporation of the solvent and the differences in diffusion rates of the components in the formulation can lead to a heterogeneous distribution of materials in the dried particle. Proteins, which are surface active molecules with high molecular weight, have a propensity to concentrate at the air-liquid interface, producing a surface concentration greater than that in the bulk. This results in a final product with more unprotected protein on the surface exposed to the environment, which can result in reduced long-term stability.

The kinetics of deuterium incorporation by ssHDX-MS was fitted to the mono-exponential model (Equation 3.1) to quantify any differences in the rate of exchange in the various formulations (Table 3.2). For BSA, there were no differences in rate constant, k, for deuterium incorporation among samples. However, BSA formulations containing dextran had higher D_{max} values than those dried with sucrose or trehalose. This suggests that the excipient used had a significant effect on the extent of deuterium uptake that can occur over time. In myoglobin samples, k was also similar across all formulations. As with BSA, D_{max} values for myoglobin formulations were similar for sucrose and trehalose, which were lower than that of the dextran formulations.

Table 3.2: Deuterium exchange kinetics for protein formulations fitted to the mono-exponential model in Equation 1. (n=3)

Formulation	D_{max}	k (h^{-1})
Myo Only	34.2 ± 1.6	0.067 ± 0.015
Myoglobin-Sucrose	8.8 ± 0.4	0.043 ± 0.008
Myoglobin-Trehalose	9.6 ± 0.5	0.058 ± 0.013
Myoglobin-Dextran 20K	19.6 ± 1.0	0.049 ± 0.012
Myoglobin-Dextran 70K	20.6 ± 0.9	0.045 ± 0.009
BSA Only	197 ± 6.5	0.028 ± 0.005
BSA-Sucrose	78.7 ± 5.1	0.011 ± 0.002
BSA-Trehalose	85.7 ± 4.3	0.016 ± 0.002
BSA-Dextran 20K	139.3 ± 2.4	0.019 ± 0.003
BSA-Dextran 70K	155.8 ± 5.1	0.019 ± 0.003

Differences in ssHDX-MS peak areas suggest differences in the heterogeneity of the protein conformation and/or matrix interactions in the samples (Fig. 3.8). As with the structural perturbations observed in ssFTIR, formulations with higher molecular weight excipients showed increased heterogeneity by ssHDX-MS than those with lower molecular weight excipients. In formulations without any excipient, BSA showed less ssHDX-MS peak broadening consistent with a narrower distribution of states than samples spray-dried with excipients, albeit with higher deuterium incorporation. This inconsistency cannot be attributed to aggregation, as the monomer content was the same for samples analyzed by SEC. The more homogeneous distribution of states for the excipient-free formulation may be due to consistent unfolding or partial unfolding of the protein throughout the matrix, or to other types of protein-protein intermolecular

interactions, which may produce similar conformations while not providing increased hydrogen-bonding interactions.

To examine the relationship between protein surface concentration and population heterogeneity, the ssHDX-MS peak area was plotted as a function of the concentration at the interface determined by XPS (Fig. 3.10). Due to the differences in interactions that may be found in protein-only formulations, these samples were treated as outliers and excluded from the correlation. In formulations containing saccharide-based excipients, a strong linear trend exists between surface coverage and peak area, with higher surface concentrations corresponding to increased population heterogeneity. This may be due to the unprotected protein at the surface, which can unfold, leading to loss of conformation and an increase in observed heterogeneity. Interestingly, this correlation was found to be consistent across not only different formulations, but with the two different proteins as well. Together, the results suggest that lower molecular weight excipients capable of hydrogen-bonding to proteins in the solid state, also reduce surface enrichment of proteins, producing a more homogeneous distribution of protein states and better physical stability.

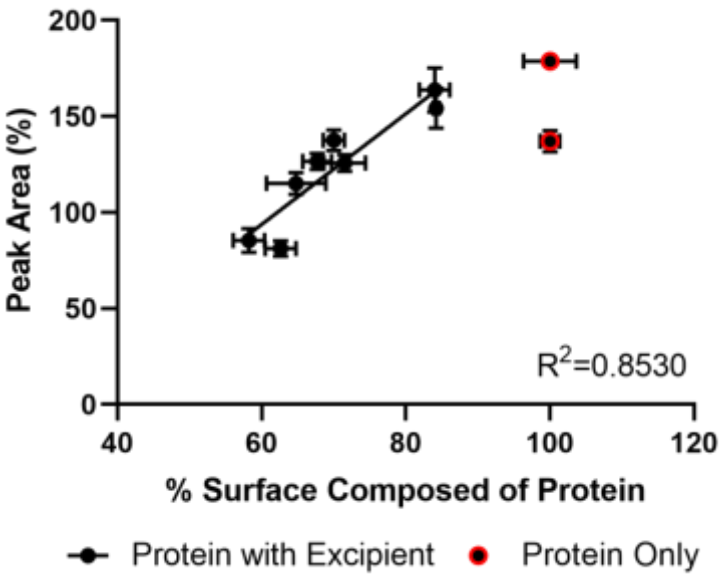

Figure 3.10: Correlation of percentage surface composition of protein to peak area of the deconvoluted mass envelope relative to an experimentally fully-deuterated sample.

3.5 Conclusions

The effects of spray drying and sugar-containing excipients on protein structure, surface coverage, and population heterogeneity were examined by ssFTIR, XPS, and ssHDX-MS. For all techniques, distinct differences were observed in secondary structure, surface coverage, and conformation when processed without excipient and with excipients of different molecular weights. The concentration of protein at the surface of the dried particle showed a linear correlation with protein population heterogeneity (as indicated by ssHDX-MS peak area) when formulated with a sugar-containing excipient. The use of low molecular weight, hydrogen bond-replacing excipients during spray drying thus can reduce both the destabilizing stress at the surface and formulation heterogeneity, leading to improved physical stability and more homogeneous biopharmaceutical products.

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CHAPTER 4. EFFECTS OF SURFACTANTS ON SURFACE COMPOSITION AND PHYSICAL STABILITY OF SPRAY-DRIED PROTEIN FORMULATIONS

4.1 Introduction

In biopharmaceutical development, one processing method with increasing interest to produce solid powders is spray drying. This is attributed to its capabilities for high throughput and to achieve powders with desirable flowability and particle properties[1], as well as usefulness for bulk storage.[2] With this method, a protein formulation in solution is atomized into droplets through a spray nozzle. These droplets are then briefly exposed to a drying gas at a high temperature, which removes moisture by evaporative diffusion.[3] From this, a solid particle is formed, with its morphology dictated by the formulation and processing parameters.[4,5] This material is then collected via cyclone.

One of the most challenging stresses encountered during drying is the presence of the air-liquid interface, which is found not only in spray drying[6], but in other drying approaches such as lyophilization.[7] Due to the amphiphilic nature of proteins, exposure to this interface can lead to surface adsorption, producing concentration differences between the surface and bulk of the solution. Alignment of protein at the surface to reduce interfacial tension can result in exposure of its hydrophobic core[3,8], which could lead to increased denaturation and damage to the protein. This increases the tendency for aggregation to occur, and results in heterogeneous distribution of protein following drying.

In order to reduce the impact of this interfacial stress, formulations containing excipients that directly compete with proteins at the surface are used. One of the most commonly used excipients is surfactants[9,10], which like proteins are also surface active molecules. Similar to

proteins, their amphipathic nature leads to increased concentrations at the air-liquid interface, which in turn reduces protein concentration by directly competing for interfacial placement. This leads to a decrease in aggregation and denaturation, which has been demonstrated for other biologic formulations.[10-12]

Although spray drying typically produces particles with observable microheterogeneity[13], there has been little examination into the impact of excipients on improving the conformational heterogeneity present in protein formulations. Previously, our group has demonstrated that population differences between drying processes can be identified using solid-state hydrogen/deuterium exchange with mass spectrometry.[14,15] By examining the peak area of the deconvoluted mass spectrum, the heterogeneity can be determined relative to other formulations. In addition, it has been found that increased peak area correlates to poorer stability due to the population differences that can impact long-term physical stability.[14] While this technique has been used for comparison between different bulking excipients and drying methods, it has not been applied to excipients with complex interfacial interactions with proteins, such as surfactants.

In the present study, the effects of polyol excipients and the inclusion of surfactants were studied to determine their impacts on the surface composition and physical stability of protein formulations. For this work, myoglobin was formulated without an excipient or with sucrose or mannitol, which are common pharmaceutical excipients. Myoglobin was selected as a model protein, as earlier studies have identified that different excipients can affect the population heterogeneity and physical stability.[14] Surfactants polysorbate 20 or poloxamer 188 were also included or excluded in the formulation prior to drying. Samples were characterized by solid-state Fourier Transform Infrared spectroscopy (ssFTIR) and X-ray powder diffraction (XRPD) for protein structural differences and excipient state, respectively. Glass transition temperature or

melting point was determined where possible. The effect of excipients on protein surface composition was determined using X-ray photoelectron spectroscopy (XPS), and bulk population properties were identified by ssHDX-MS. Physical stability of the formulations were studied under accelerated conditions for comparison to other techniques. These techniques were used to determine the impact on the inclusion of surfactants on formulation heterogeneity, and the viability of ssHDX-MS to correlate these results to population differences and physical stability.

4.2 Materials and Methods

4.2.1 Materials

Myoglobin from equine skeletal muscle, sucrose (Bioextra, ≥95%, GC) and D-mannitol were purchased from Sigma Aldrich (St. Louis, MO). Surfactants poloxamer 188 and tween 20 were procured from Corning (US) and Fisher Scientific (Fair Lawn, NJ) respectively. Protein solutions were purified by dialysis in a 2.5 mM phosphate buffer solution for 24 hours at 4°C using Slide-A-Lyzer™ dialysis cassettes (Thermo Scientific, Rockford, IL). The pH of the buffer solution was adjusted to 6.8 using phosphoric acid when necessary.

For excipient-free formulations, myoglobin was diluted to a concentration of 20 mg/mL. Solutions containing excipient were prepared separately under the same buffering conditions. These solutions were then used to dilute protein solutions for an excipient:protein molar ratio of 400:1 and a total solids content of 20 mg/mL. For surfactant-containing formulations, a separate solution was also prepared and added to the solution for a 0.05% w/v addition of surfactant.

4.2.2 Spray Drying

Formulations were processed by spray drying using a Mini Spray Dryer B-290 (Büchi, New Castle, DE). An inlet temperature of 100°C, an outlet of 50-55°C, liquid feed rate of 2

mL/min, and an air volumetric flow rate of 600 L/h were the processing parameters used. Following drying, samples were collected and distributed into 2R borosilicate glass vials (~4 mg distributed per vial) and further dried in a vacuum oven at 30°C and 100 mTorr for 24 hours.

4.2.3 Moisture Content Analysis by Karl Fischer Titration

Moisture content was determined as described previously.[15] Briefly, an 831 KF Coulometer (Metrohm, Riverview, FL) was used for coulometric titration. Formulations were reconstituted with 1 mL of anhydrous methanol (septum sealed bottle DriSolv®, Sigma Aldrich, St. Louis, MO). Suspension were injected into the cell and titrated with Riedel-de Haën Hydranal® Coulomat reagent (Hoechst Celanese Corp., Germany) until reaching end point for moisture determination (~5 min). Samples were measured in triplicate.

4.2.4 X-ray Powder Diffraction

Potential crystallinity in spray-dried powders was assessed using a Rigaku SmartLab X-ray diffractometer (The Woodlands, TX) equipped with a Cu Kα X-ray source and Bragg-Brentano geometry. Samples were removed form vials and pulverized onto a glass slide, then loaded for analysis. Diffraction intensity was measured between 5 and 40 degrees as a function of 2θ. A scanning rate of 5°/min and a step size of 0.02° were used.

4.2.5 Solid-State Fourier-Transform Infrared Spectroscopy (ssFTIR)

Measurements for ssFTIR were conducted in attenuated reflectance mode using a Nicolet Nexus spectrometer (Thermo Scientific, Waltham, MA). Samples were loaded onto a Smart iTR™ accessory equipped onto the spectrometer, and was compressed against a diamond by a metal anvil. Formulation spectra were collected in the absorbance mode with 120 scans in the range of 800 to 4000 cm^{-1} with 4 cm^{-1} resolution. Results were processed and analyzed with

OPUS 6.5 software (Bruker, Billerica, MA) using baseline correction, smoothing, normalization and second derivatization.

4.2.6 Modulated Differential Scanning Calorimetry (mDSC)

Each sample of dried powder (2-4 mg) was loaded under nitrogen into hermetic aluminum pans and sealed. Samples were loaded into a TA Instruments Discovery Series DSC 25 differential scanning calorimeter (New Castle, DE). An empty pan sealed under nitrogen was used as reference. Powders were heated from 25°C to 180°C at a ramp rate of 1°C/min and a modulation of ±1°C every 120 s. Glass transition temperatures (T_g) or melting temperatures (T_m) were determined using TRIOS software (TA Instruments, New Castle, DE).

4.2.7 X-ray Photoelectron Spectroscopy (XPS)

Measurements to determine surface composition were performed as described previously.[16] Briefly, data was collected by an AXIS Ultra DLD spectrometer (Kratos Analytical Inc., Manchester, UK). Sample surfaces are irradiated with a monochromatic Al Kα radiation source (1486.6 eV), resulting in emission of core-level electrons which possess a specific kinetic energy unique to each element and chemical state, which is measured by an energy analyzer. Constant pass energy of 20 eV and 160 eV were used to obtain high resolution and survey XPS spectra, respectively, and processed using CasaXPS software.

Percentage elemental composition was determined by calculating the O 1s, N 1s, and C1s peaks following Shirley background subtraction and applied corrections on corresponding Scofield relative sensitivity factors and ineleastic mean free path of the photoelectrons. Four replicates were measured and the results averaged. In this study, the sulfur peaks of myoglobin were not used due to concentrations of <1% in the elemental composition and corresponding calculations. Theoretical atomic percentages of protein were estimated using the equation:

$$At.\%_{Protein} = \frac{Wt.\%_{Protein}/\ MWt._{Protein}}{\left(\frac{Wt.\%_{Protein}}{MWt._{Protein}}\right)+\left(\frac{Wt.\%_{Excipient}}{MWt._{Excipient}}\right)} * 100 \quad (4.1)$$

where *At. %* is the atomic percent of the protein, *Wt. %* is the weight percentage of the molecule in the formulation, and *MWt.*is the molecular weight of the molecules.

4.2.8 Stability Studies by Size Exclusion Chromatography (SEC)

Physical stability of samples was determined by monitoring the level of protein aggregations using SEC. Samples were sealed and stored in a 40°C oven. At each time point (15, 30, 60, and 90 days), vials were removed in triplicate for each formulation and reconstituted to a protein concentration of 1 mg/mL. Solutions were centrifuged at 12,000 rpm and 4°C for 10 min to condense insoluble aggregates. Supernatant was then removed and placed into vials for analysis. Samples were analyzed on a 1200 series high performance liquid chromatography (HPLC) system Agilent Technologies, Santa Clara, CA) using an isocratic flow over 15 min at a flow rate of 1 mL/min. The solvent system was composed of a 50 mM sodium phosphate, 100 mM sodium chloride solution of pH 6.8. A TSKgel® G3000SWXL HPLC Column from Sigma Aldrich (St. Louis, MO) was used for size separation. Physical instability was determined by percentage of aggregates formed over time.

4.2.9 Scanning Electron Microscopy (SEM)

SEM was used to examine morphological differences for dried particles. Powders were mounted onto a sample holder and sputter-coated with carbon graphite for 60 s. Samples were visualized using a Nova NanoSEM 200 system (Fei, Hillsboro, OR).

4.2.10 Solid-State Hydrogen/Deuterium Exchange with Mass Spectrometric Analysis (ssHDX-MS)

Deuterium incorporation was examined according to methods used in previous studies.[14,15,17] Briefly, vials containing the spray-dried formulations were stored in a sealed desiccator containing a deuterium oxide (D_2O) solution saturated with lithium chloride (producing a relative humidity of 11%) at 25°C. At time points of 4, 12, 24, 48, 120, and 240 h, three vials for each formulation were removed, capped, and submerged in liquid nitrogen to quench the exchange reaction. Vials were then stored at -80°C until analysis to prevent back-exchange from occurring. For baseline of peak area increase, an experimentally fully deuterated sample was also prepared in solution. For these samples, myoglobin was dissolved in a solution containing 3 M guanidine hydrochloride at pH 6.8, then placed into a vial containing a 9:1 dilution of D_2O under similar buffering conditions. This solution was stored at 60°C for 24 hours, then quenched in a 4:1 solution of quench buffer (0.1% formic acid solution, pH 2.5) and immediately analyzed.

To determine extent of deuterium incorporation, samples were reconstituted in 2 mL of chilled quench buffer, then 10 µL were injected into a protein microtrap (Michrom Bioresources, Inc., Auburn, CA). Using a 1200 series HPLC system, samples were desalted for 1.7 min with 90% water, 10% acetonitrile with 0.1% formic acid in each solvent, then eluted over 7 min to a gradient of 10% water, 90% acetonitrile with 0.1% formic acid. To minimize back-exchange, columns were stored in a custom-built refrigeration unit maintained at 4°C. The mass spectra of spray-dried samples was determined using a 6520 qTOF mass spectrometer (Agilent Technologies, Santa Clara, CA), with deuterated samples being analyzed in the 200 – 2000 m/z mass range. MassHunter Workstation software (Agilent Technologies, Santa Clara, CA) was used to deconvolute the samples and obtain information on the masses of the protein and change

in spectra. Deconvolution was calculated by the maximum entropy function using an algorithm that converts the mass envelopes of detected charged states into mass values.

Kinetics of deuterium incorporation was fitted to a mono-exponential model:

$$D(t) = D_{max}(1 - e^{kt}) \quad (4.2)$$

where *D(t)* is the number of deuterons exchanges at time *t*, D_{max} is the maximum number of deuterons that could be incorporated into the sample, and *k* is the observed rate constant of exchange.

4.2.11 Statistical Analysis

Statistical differences in moisture content, surface composition, deuterium exchange results, and physical stability were compared using Prism Software (GraphPad, La Jolla, CA). A two-way ANOVA with Tukey's test was used for multiple comparisons between formulations with different bulking agents, and with or without surfactant inclusion.

4.3 Results

4.3.1 Moisture Content and Glass Transition Temperature

Formulations were found to have different levels of moisture content (Table 4.1) depending on the bulking agent (p<0.0001). Without sucrose or mannitol, myoglobin samples had slightly higher moisture contents. For samples without a bulking agent glass transition temperature could not be determined. In samples containing sucrose, T_g was between 61-63°C (Table 4.1), consistent with expected values for sucrose[18]. Surfactants did not result in a significant difference in T_g (p>0.05). Likewise; the T_m of mannitol-containing formulations was the same regardless of the inclusion of surfactant, between 159-162°C (p>0.05). For XRPD patterns (Fig. 4.1), those without a bulking agent and formulations with sucrose were found to be

amorphous. Samples formulated with mannitol all exhibited crystallinity (Fig. 1C), which is consistent with previous reports for protein formulations possessing mannitol in high concentrations.[4]

Table 4.1: Moisture content and DSC measurements for myoglobin formulations

Formulation	Moisture Content (%)	T_g or T_m (°C)*
Myoglobin Only	4.4 ± 0.7	N.D.
Myoglobin-PS20	3.4 ± 0.3	N.D.
Myoglobin-Pol188	2.3 ± 0.6	N.D.
Myoglobin-Suc	1.9 ± 0.6	61.8 ± 3.3
Myoglobin-Suc-PS20	2.2 ± 0.7	61.1 ± 1.1
Myoglobin-Suc-Pol188	2.0 ± 0.3	62.7 ± 0.8
Myoglobin-Mann	1.4 ± 0.2	162.2 ± 0.3
Myoglobin-Mann-PS20	1.2 ± 0.5	161.2 ± 0.6
Myoglobin-Mann-Pol188	1.1 ± 0.1	159.9 ± 0.3

*T_g for formulations with sucrose, T_m for formulations containing mannitol. Thermal transitions could not be determined for formulations without bulking agent.

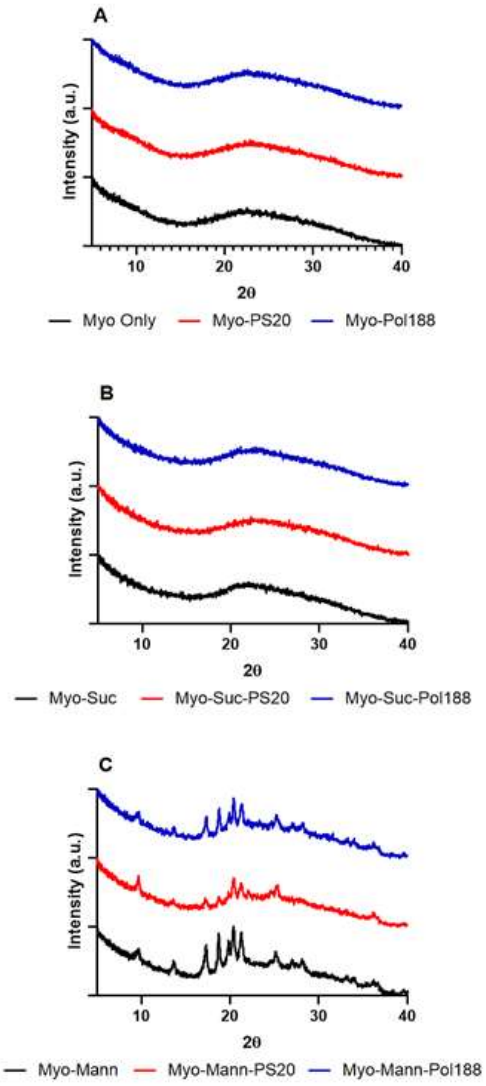

Figure 4.1: X-ray diffraction patterns for spray-dried myoglobin formulations without a bulking agent (A), with sucrose (B), or with mannitol (C).

4.3.2 Secondary Structural Analysis

Secondary structure of each protein formulation was examined using the collected ssFTIR spectra of the amide I region. For myoglobin formulations spray-dried without a bulking agent (Fig. 4.2A), there is a band present at 1652 cm^{-1} corresponding to α-helical structure. The broadness and low intensity of the band relative to other samples suggests structural perturbation,

which is anticipated for proteins processed without a stabilizing excipient. Similar results are observed with the inclusion of polysorbate 20 or poloxamer 188 without any stabilizing excipient.

In formulations containing sucrose (Fig 4.2B), without a surfactant, a band is found at 1655 cm^{-1}, which corresponds to that of myoglobin. In addition, all sucrose-containing formulations were consistent with preservation of the α-helical structure of the protein, which is consistent with literature.[17] For sucrose-containing formulation with surfactants, structural preservation was also observed, although there was a slight band shift to 1657 cm^{-1}.

Formulations containing mannitol showed the greatest degree of structural perturbation (Fig. 4.2C). The primary band at 1658 cm^{-1} corresponded to the α-helix. For mannitol-containing samples without surfactant, broadening of the band was observed, with a small band at 1644 cm^{-1}. This band indicates the presence of disordered α-helix which indicates damage to the secondary structure of myoglobin. In samples of mannitol process with surfactant, an additional band at 1644 cm^{-1} can be observed, which suggests that surfactant may contribute to some preservation of this disordered structure caused by the presence of mannitol.

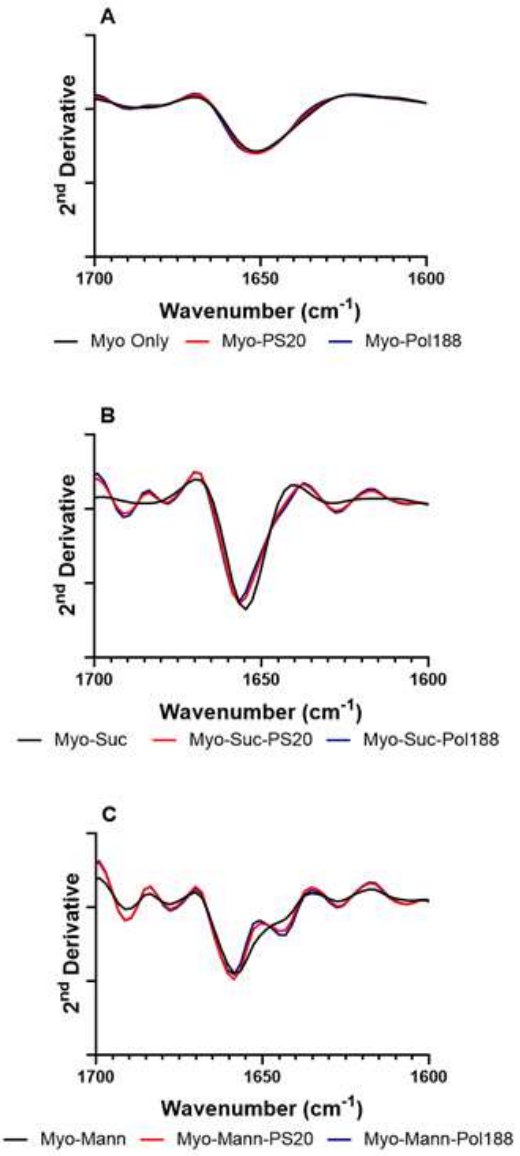

Figure 4.2: Solid-state FTIR spectra of myoglobin formulations dried without a bulking agent (A), with sucrose (B), or with mannitol (C).

4.3.3 Protein Surface Coverage by XPS

XPS was used to determine the composition of the top 10 nm of the surface of the spray-dried particles. Myoglobin processed without excipients was used as the baseline to determine effects of additives on nitrogen (Fig. 4.3A) and atomic percentage of protein surface composition

(Fig. 4.3B). For myoglobin only, the nitrogen concentrations were in agreement with the atomic percentages of the protein, with a value of ~16%. Inclusion of excipients resulted in reduction of nitrogen present at the surface. Without a polyol excipient, surfactant resulted in a significant decrease (p<0.0001), despite being present in low concentrations in the formulation (0.05% w/v). Addition of a bulking agent resulted in a greater reduction in nitrogen content, although this can be attributed to higher concentrations used in the formulation. The presence of both a polyol and surfactant resulted in the highest reduction of nitrogen present at the surface (p<0.0001) for all other formulations as compared to the protein-only formulation).

Due to the lack of nitrogen in the atomic composition of the excipients, the amount of nitrogen on the surface can be directly attributed to the composition of protein (Fig. 4.3B). In formulations with a polyol excipient, the addition of surfactant reduced protein concentrations to ~50% while only composing ~2.4% of the total weight of the sample. Formulations containing sucrose or mannitol reduced the protein surface composition to ~33% and 39%, respectively. Addition of a surfactant to these samples further resulted in a significant reduction in surface protein. For sucrose-containing formulations, a significant difference was observed between the use of polysorbate 20 and poloxamer 188 (p<0.0001), with protein compositions of ~12% and ~6%, respectively. For formulations with mannitol, both surfactants resulted in similar reduction in protein composition at the surface (~16%).

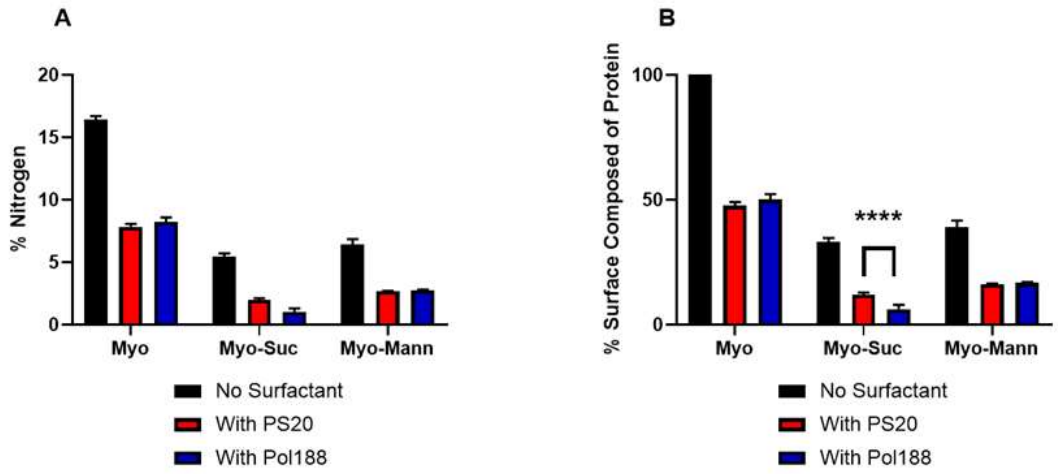

Figure 4.3: Quantification of X-ray photoelectron spectroscopy results for percentage of nitrogen (A) and atomic percentage of protein (B) found on the surface.

4.3.4 ssHDX-MS for Conformation Interactions

Hydrogen-bonding interactions in the formulation matrix were studied by ssHDX-MS (Fig. 4.4), which can provide information on the conformational differences in the formulation, as well as serve as a predictor of physical stability.[14,19] For formulations processed without myoglobin, the inclusion of surfactant did not significantly affect deuterium uptake ($p>0.05$). Mannitol-containing samples had similar levels of uptake to the unprotected formulations, with samples containing poloxamer 188 exhibiting the highest deuterium incorporation ($p<0.0001$). For samples containing sucrose, there was significantly lower uptake compared to other formulations ($p<0.0001$). The inclusion of surfactant in these samples had no observable effect on exchange over time.

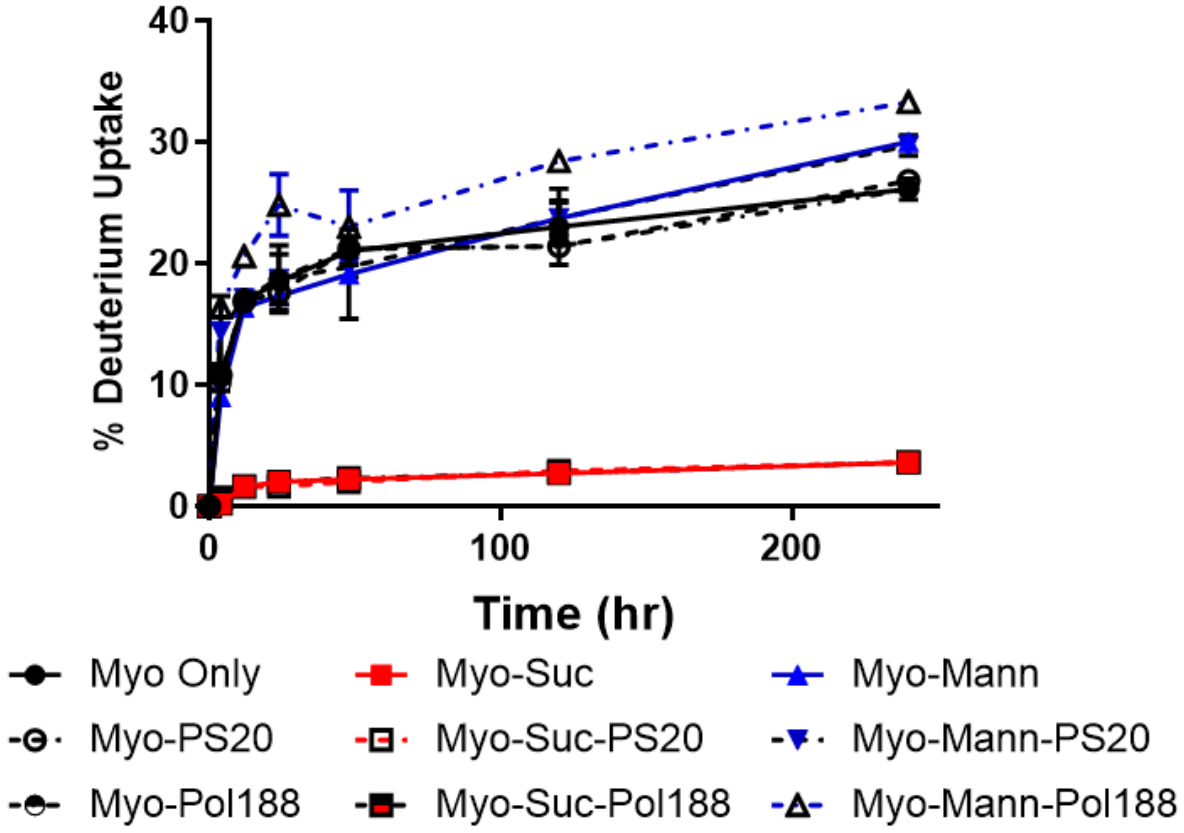

Figure 4.4: Kinetics for deuterium exchange of myoglobin formulations observed by ssHDX-MS. (n=3)

To examine the effects of formulation on spray-dried protein populations, the peak area of the normalized deconvoluted mass spectrum was calculated as a percentage of experimentally fully-deuterated samples (Fig. 4.5). These values were then plotted as a function of deuterium incorporation. As shown in other work, protein population differences can be attributed to conformational or molecular interactions variances.[14,15] In this study, formulations processed with mannitol exhibited the highest amount of species present, regardless of the addition of surfactant. Formulations with sucrose exhibited the lowest degree of population heterogeneity.

For all formulations under study, increasing deuterium uptake typically showed increased population heterogeneity, which is expected due to increasing differences in exchangeability for deuterium. In the formulations without a bulking agent (Fig. 4.5B), protein formulations without a surfactant did not exhibit a significantly lower heterogeneity than those with polysorbate 20 or poloxamer 188 ($p>0.05$). Surfactant-containing samples had similar levels of heterogeneity. In formulations with sucrose (Fig. 4.5C), a similar trend was observed where samples with sucrose had the lowest heterogeneity, but no significant difference with the exclusion or inclusion of surfactant ($p>0.05$). In mannitol-containing formulations (Fig. 4.5D), those containing polysorbate 20 or poloxamer 188 possessed a higher amount of heterogeneous species than myoglobin processed with only mannitol ($p<0.01$). Of those, samples with poloxamer 188 had the highest heterogeneity.

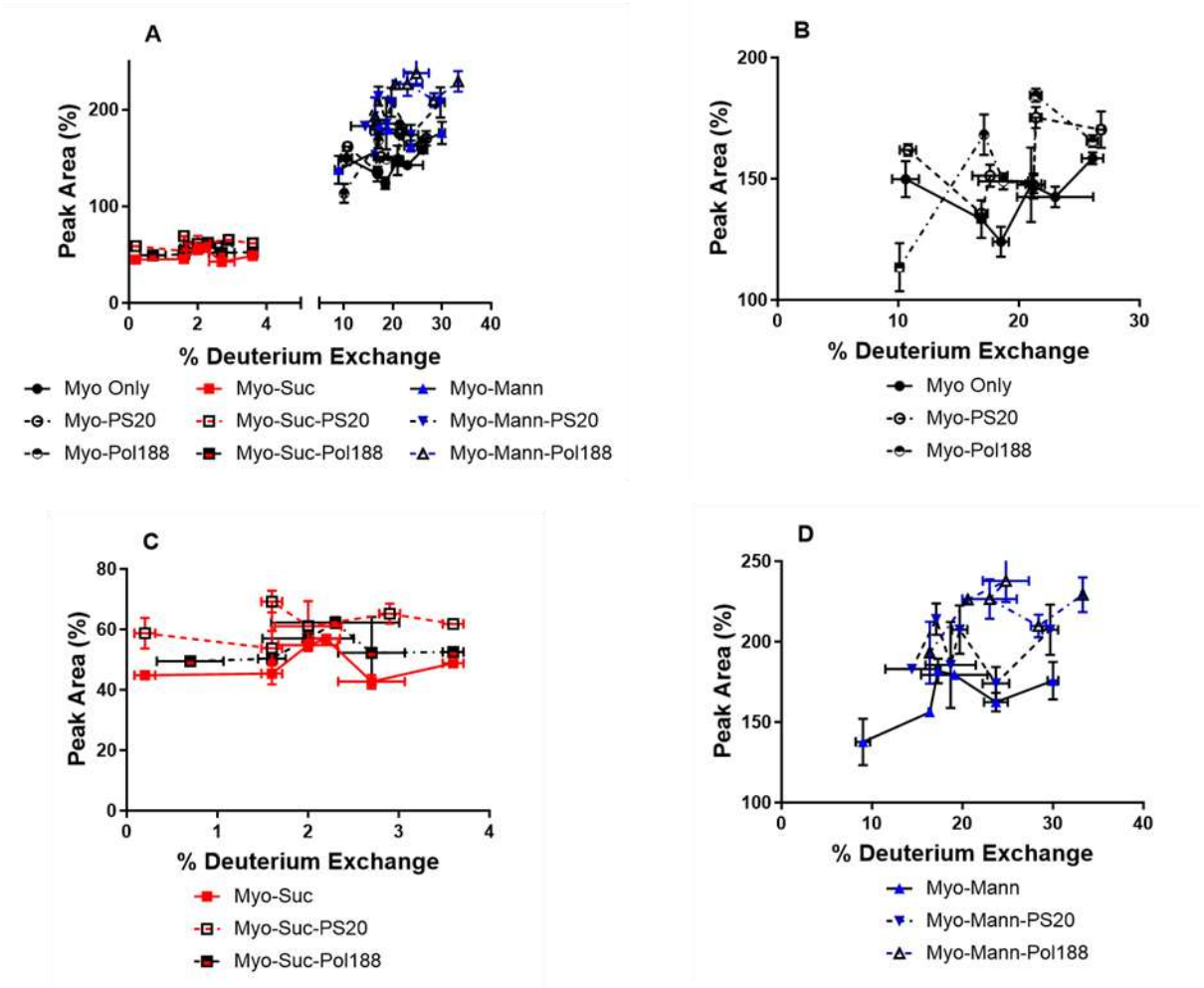

Figure 4.5: Peak areas of the deconvoluted mass envelope as a function of deuterium exchange for all myoglobin formulations (A), and samples formulated without a bulking agent (B), with sucrose (C), or with mannitol (D). Peak areas were calculated as a percentage of the area of an experimentally fully deuterated sample.

4.3.5 SEM for Particle Morphology

Samples without a polyol excipient had a dimpled morphology (Fig. 6). In myoglobin samples dried with sucrose and without surfactant, there was a presence of particles with dimpled and wrinkled morphologies. The inclusion of a surfactant resulted in spherical particles without the dimpled shape.

Samples processed with mannitol possessed distinct differences compared to other samples. For all samples, agglomeration was observed, with no discernible morphology. The presence of crystallinity could be observed on the surface, which is expected due to the high concentration of mannitol present.

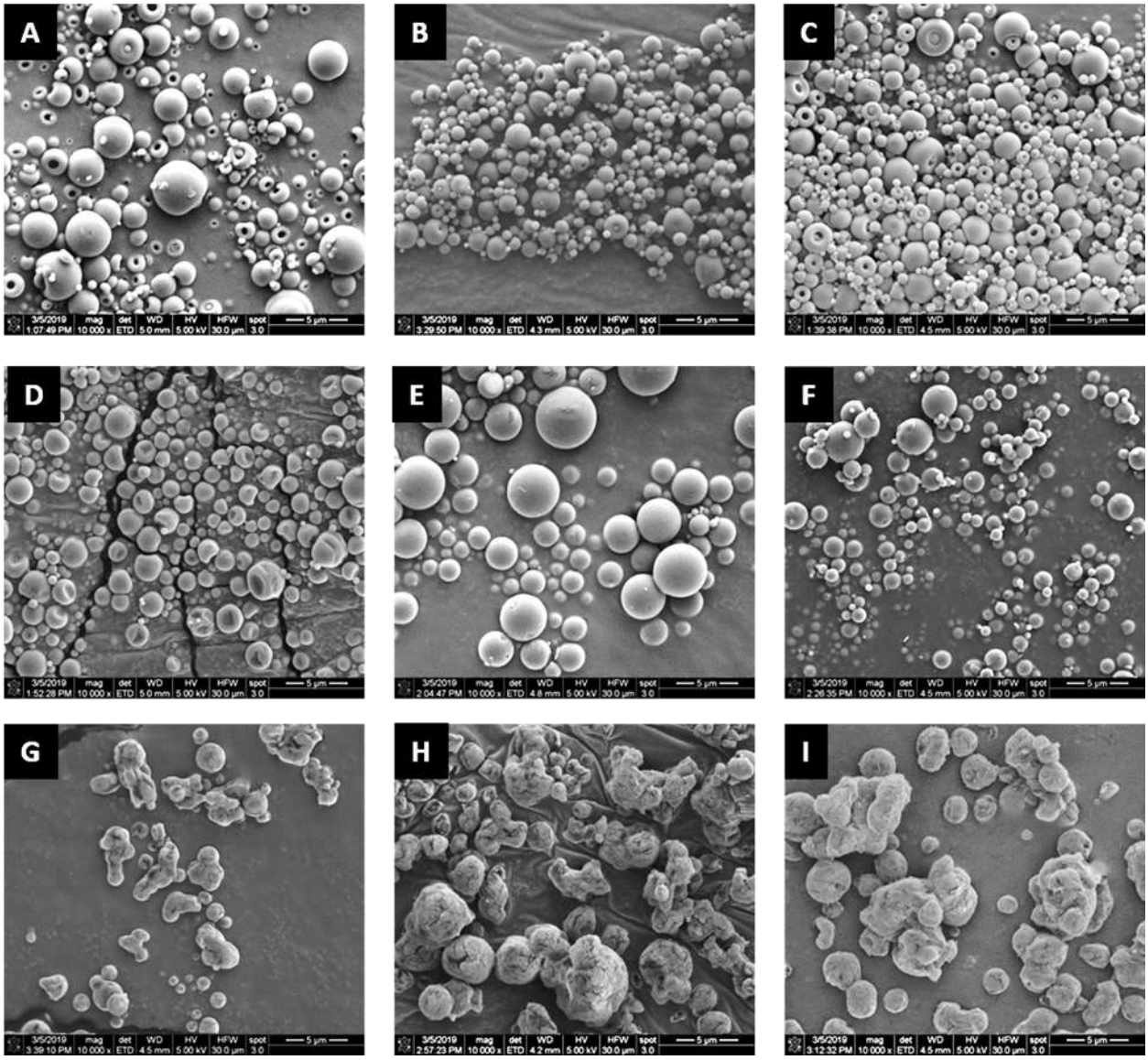

Figure 4.6: SEM images of particles morphology for spray-dried samples formulated without bulking agent (A), with polysorbate 20 (B), or with poloxamer 188 (C). Samples containing sucrose were dried without surfactant (D), with polysorbate 20 (E), or with poloxamer 188 (F). Samples containing mannitol were processed without surfactant (G), with polysorbate 20 (H), or with poloxamer 188 (I).

4.3.6 Stability Studies

Accelerated stability studies were conducted for 90 days at 40°C to examine the effects of excipients on the stability of spray-dried myoglobin formulations (Fig. 4.7). Samples without a bulking agent but containing polysorbate 20 or poloxamer 188 (Fig. 4.7A) showed a slight reduction in monomeric content compared with those without surfactants ($p<0.05$). The most stable formulation was myoglobin formulated with sucrose but without surfactants (Fig. 4.7B), which maintained the highest monomeric content throughout the accelerated study. With the addition of surfactant, significant differences in aggregation were observed ($p<0.0001$) for the sucrose-containing protein formulations. Sucrose formulations containing polysorbate 20 had the highest observed decrease in protein monomer. Mannitol-containing formulations had similar levels of aggregation with and without surfactant (Fig. 4.7C), with formulations containing polysorbate 20 having the higher amount of aggregation ($p<0.05$).

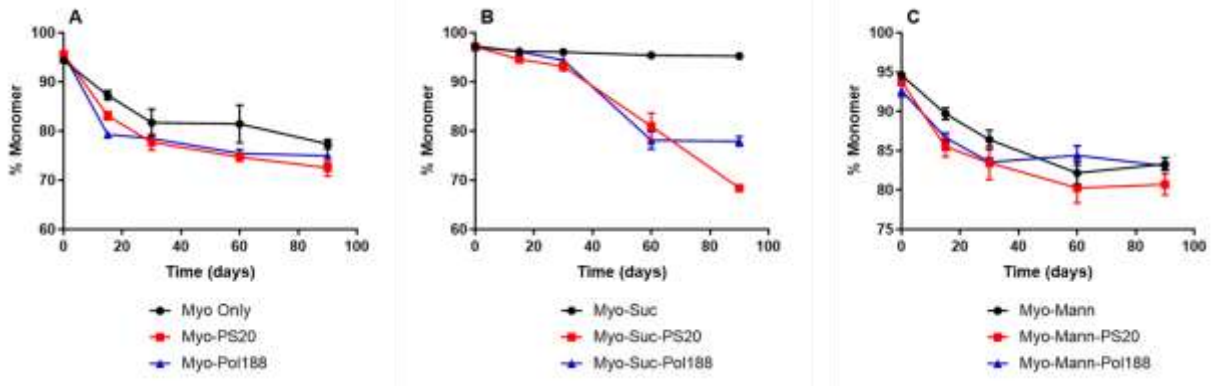

Figure 4.7: Accelerated stability studies at 40°C for myoglobin formulations without a bulking agent (A), with sucrose (B), or with mannitol (C).

4.4 Discussion

While surfactants are an important excipient utilized in the biopharmaceutical industry to reduce the presence of protein at the air-liquid interface, very little work has been done to explore its impact on formulation heterogeneity. In addition, ssHDX-MS has not been examined as a physical-stability predictive tool on the effects of surfactants in a solid protein formulation. Previously, our group has demonstrated that both formulation and processing effects can have an impact on the observed conformational heterogeneity, which correlated well to loss in monomer content.[14] In the present study, ssHDX-MS and other characterization techniques were used to identify the effect of the inclusion of surfactant in spray-dried formulations with different bulking agents.

In the ssFTIR spectra (Fig. 4.2), secondary structural differences were identified between formulations with different excipients. Myoglobin formulations without a bulking agent exhibited disorder of the α-helical structure after spray drying. This is expected due to the lack of a stabilizing excipient that can sufficiently replace the hydrogen bonds lost by the removal of water. The inclusion of surfactant did not show an impact on the secondary structure likely due to their low concentration in the formulation. Formulations containing sucrose preserved the native structure of the protein, which has been previously observed for studies utilizing this excipient with myoglobin.[17,20] This is due to the capability of the excipient to serve as hydrogen bond-replacement molecules. Similarly, surfactant did not have an observable impact on the secondary structure. In mannitol-containing formulations, structural perturbation was observed, with indications of disordered α-helices. Crystallization of mannitol during spray drying led to damage of protein structure, resulting in the observed disorder. At the concentrations under study, mannitol has a tendency to crystallize, which leads to less interaction of the excipient with proteins. This reduced excipient interaction can result in poor physical stability. Surfactants in

this study displayed a more pronounced presence of the disordered helix, which may suggest surfactant could impact protein secondary structure in the solid state for structurally unstable proteins.

XPS provided information on the surface coverage of protein on particles after spray drying (Fig. 4.3). In formulations without a surfactant, excluding the protein-only formulation, there was an excess of protein present at the interface, suggesting the excipients of sucrose and mannitol were not capable of producing a homogeneous distribution in the dried particles. This is likely due to the inability of these excipients to directly compete for adsorption of the interface of the solution prior to the drying step. Following drying, there is protein enrichment on the surface of a dried particle, as the rate of evaporation is faster than the diffusion of solutes.[21] With the addition of surfactant, there is a significant reduction of protein on the surface. While less protein at the interface has been demonstrated to improve stability for a shear-sensitive biomolecule[22], this reduction of protein on particle surface is also indicative of an inhomogeneous distribution. This can be observed by estimating the theoretical atomic percentage of protein at the surface for a homogeneously distributed particle. As calculated using Equation 4.1, the expected atomic percentages of protein on the surface should be ~74% for no bulking agent and with polysorbate 20, ~95% with no bulking agent and with poloxamer 188, and <1% for all other formulations. This indicates that despite an improvement in the homogeneity of protein in the particle, the formulation remains largely heterogeneous.

The deuterium uptake results of ssHDX-MS were fitted to the mono-exponential model indicated in Figure 4.8. For the maximum extent of deuterium incorporation, there is a significant difference between formulations without a bulking agent or with mannitol, and those containing sucrose (p<0.0001). Higher D_{max} values for protein-only formulations or those with

only the addition of surfactant are due to the lack of a water-replacing excipient that can account for the lost hydrogen-bonding interactions post-drying. The additions of low polysorbate 20 or poloxamer 188 are not capable of accounting for the lost intermolecular interactions, due to the low concentration of excipient and the surface activity of the molecules that favors interfacial interactions. This can also be observed in the denaturation of secondary structure following spray drying, where surfactant inclusion does not result in preservation of the α-helical structure of myoglobin. In mannitol-containing formulations, the increased amount of deuterium uptake and high D_{max} values is likely due to the crystallization of mannitol. Crystallization of the excipients reduces the amount of molecules available for hydrogen-bond stabilization for proteins, and allows for greater exposure for deuterium exchange. For the rate constant, *k,* a similar trend can be observed, although for mannitol-containing samples, the inclusion of surfactant resulted in a slight increase in *k* values ($p<0.05$). This may suggest that surfactants contribute a destabilizing effect on the protein in the presence of another destabilizing excipient that leads to an increased rate in deuterium uptake, although no difference could be identified in the D_{max} compared to mannitol-only formulations.

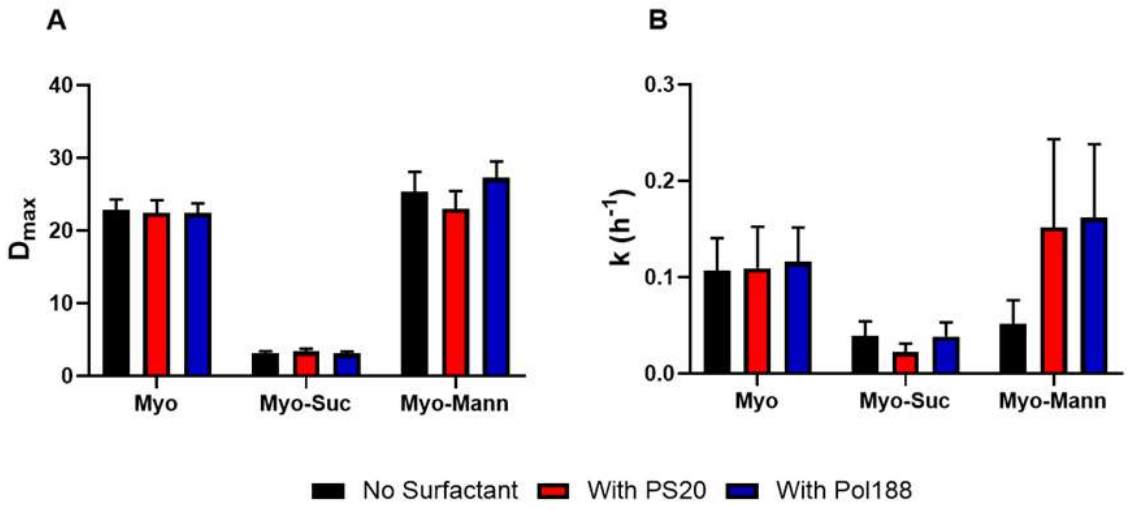

Figure 4.8: Deuterium exchange kinetics for ssHDX-MS results fitted to the mono-exponential model in Equation 4.2.

In this study, the impact of a bulking agent on protein heterogeneity can be identified and quantified by XPS and the peak area of the ssHDX-MS results. The addition of sucrose, a hydrogen bond-donating excipient, interacts with the proteins and provides protection from exchange and reduces the heterogeneity. In contrast, a destabilizing excipient like mannitol, which crystallizes, will have some reduction of protein on surface as measured by XPS, but does increase instability when measured by ssHDX-MS. With the inclusion or exclusion of surfactant, however, it is difficult to determine significant differences by ssHDX-MS with the exception of mannitol-containing formulations. One possibility for this is the limitation of ssHDX-MS as a tool for bulk analysis. While ssHDX-MS provides information on the exposure of protein for the entire formulation, it is not possible to determine deuterium exchange for the surface only. This may mask the contributions of excipient for protection from deuterium exchange when examining peak area due to low concentration at the interface. Another possibility could be due to the interaction of surfactant with proteins. For some biomolecules, it has been demonstrated that surfactants can reduce protein surface enrichment by forming interactions with the protein, such as hydrogen bonding of polysorbate 20 to bovine serum albumin.[23] However, for other molecules, such as monoclonal antibodies[24], these interactions are very weak, making it difficult to measure the interfacial behavior of non-ionic surfactants. In addition, other studies have indicated no protein concentration in solution is reduced at the interface by steric hindrance.[25] The complexity of surfactant interactions at the interface likely may not be due to hydrogen-bonding contributions in the solid-state, which cannot be identified by ssHDX-MS.

Formulations that do exhibit statistically significant differences in heterogeneity are those possessing mannitol. For these samples, surfactant inclusion in an already unstable formulation

contributes to greater heterogeneity. This suggests that the addition of surfactant to less suitable formulations would contribute to greater heterogeneity, and therefore worse physical stability. For formulations that do not contain a bulking agent, other stabilizing contributions from myoglobin (such as electrostatic interactions, for example) may result in more consistent heterogeneity in samples, despite surfactant inclusion. In those containing sucrose, the molar ratio used has been determined to be suitable for a stabilizing protein formulations,[26] which likely results in the negligible contribution of surfactant observed by ssHDX-MS.

In the accelerated stability studies, the inclusion of surfactants in most formulations was found to result in a slight increase in aggregation after 90 days; those spray-dried with sucrose exhibited the most significant decrease in physical stability. This difference is likely due to the crystallization of sucrose due to exposure at high temperatures and humidity (Fig. C1), which has previously been shown to occur under accelerated storage conditions.[14,27] Another possibility is due to oxidation or hydrolysis reactions with surfactants, which could result in chemical degradation of the formulations.[28,29] While there is no pH change in the samples upon reconstitution that would indicate chemical degradation of the surfactants (Fig. C2), there is a possibility that the buffering capacity may be sufficient for protecting the formulation from an observable shift. These minor aggregation differences due to chemical degradation could not be identified by ssHDX-MS or XPS.

4.5 Conclusions

The impact of surfactant inclusion in formulation containing different bulking agents was examined. Using XPS, significant differences in atomic protein percentage at the particle surface were found with different formulations. With ssHDX-MS, differences in protein populations could be identified between formulations with and without surfactant only in the case of

mannitol, an unstable formulation due to crystallization of the bulking agent. For both techniques, no definitive correlation could be made between the composition heterogeneity and physical stability, due to the limitations in measuring the complex interactions of surfactant and protein at the air-liquid interface.

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CHAPTER 5. CONCLUSIONS AND RECOMMENDATIONS

In this work, the effects of different processing methods and excipients were examined in relation to the conformational state of proteins, the heterogeneity of the populations present, and their physical stability. Different characterization techniques, such as ssHDX-MS and XPS, were used to characterize these differences and establish a correlation, if any existed, to physical stability as determined by SEC. With these techniques, significant correlations were found to occur with the heterogeneity of protein populations, as well as identifying the capabilities of existing methods for protein screening when using excipients that may form interactions besides hydrogen bonding.

In the first study, processing and excipient effects on protein structure were analyzed in order to determine what correlation, if any, existed between these techniques and physical studies. Samples were studied using ssFTIR, solid-state fluorescence spectroscopy, DSC, and ssHDX-MS. For the conventional analytical techniques, no strong correlation could be determined due to the challenge in using a bulk analytical method to identify the microheterogeneous states that can exist in protein formulations. With ssHDX-MS, a greater correlation was found with physical stability corresponding to higher D_{max} and peak area values. This is likely due to the capability of the analytical technique to better identify the population heterogeneity. These results demonstrated that spray-dried formulations exhibited higher heterogeneity compared to lyophilized samples. This study showed that ssHDX-MS can be used not only as a tool for predicting physical stability, but also for identifying population differences in proteins due to drying method or excipients used, which could improve development of more robust biologic products.

For the second study, protein formulations spray-dried with different sugar-containing excipients were prepared to examine their effect on protein structure, surface coverage, and population heterogeneity. Samples were analyzed by ssFTIR, XPS, DSC, and ssHDX-MS. With these techniques, distinct differences were observed for proteins when processed with or without excipients of differing molecular weights. For structure, the addition of dextrans or the exclusion of an excipient showed a loss of α-helices. When comparing protein surface coverage by XPS and heterogeneity by the peak area of ssHDX-MS results, a linear correlation was observed for formulations with a sugar-containing excipient. Low molecular weight, hydrogen bond-replacing excipients during spray drying could reduce the destabilizing stress at the air-liquid interface and the population heterogeneity. This could lead to improved physical stability and homogeneity in spray-dried biopharmaceuticals.

In the third study, the effects of surfactant inclusion in spray-dried protein formulations on surface composition, population heterogeneity, and physical stability were investigated. Surface composition was examined by XPS and ssHDX-MS was used to determine the protein formulation heterogeneity. Surfactants used in this study were polysorbate 20 and poloxamer 188, and were included or excluded in formulations containing no bulking agent, sucrose, or mannitol that were spray-dried. For XPS, surfactants greatly reduced protein concentrations on the particle surface, although the atomic percentage at the surface still indicated formulation heterogeneity. Data for ssHDX-MS did not indicate significant differences in deuterium exchange or protein populations between formulations with and without surfactant, except in mannitol-containing samples. This may be due to limitations of ssHDX-MS to measure complex interactions of protein and surfactant at the air-liquid interface. From these results, no correlation could be

identified to the physical stability of myoglobin. Further studies are warranted to better understand the impact of surfactants in protein stability for spray-dried formulations.

For future studies, several recommendations can be made based on the results of the current work. In the study of processing methods in Chapter 2, lyophilization and spray drying were examined as drying approaches for protein formulations. Alternative drying approaches, such as spray freeze drying, foam drying, and others could also be studied to determine if heterogeneity as determined by ssHDX-MS can be further correlated to physical stability. In addition, for this study, the same molar ratio was applied for all protein formulations to allow for comparison between multiple proteins possessing different characteristics. This work also indicated that for all formulations, spray-dried samples had greater heterogeneity than those that were lyophilized. Examining different molar ratios of excipient with proteins may provide a better understanding of concentration effects on population heterogeneity, for both different excipients and drying methods.

In examining excipient effects in Chapter 3, only sugar-containing excipients of sucrose, trehalose, and different molecular weight dextrans were examined to understand their impact on protein surface composition and population heterogeneity. From this work, a positive linear trend was established between the surface percentage of protein and the observed population heterogeneity by ssHDX-MS. Examination of other types of excipients, such as those that do not contribute hydrogen bonding interactions, may provide additional information on their impact on surface composition and protein heterogeneity.

For the study on surfactant effects in Chapter 4, no discernible correlation could be established between the surface composition, population heterogeneity, and the physical stability of myoglobin when including or excluding a surfactant in the formulation. From the results, the

formulation without excipient had similar monomer content as those with excipients, suggesting myoglobin may be less sensitive to the air-liquid interface than other proteins that have been studied. Examining surfactant effects with a more interface-sensitive protein may provide different results to better understand the effects of surfactant inclusion. In addition, this work indicated a potential knowledge gap in understanding the interactions of surfactant and protein in the solid state. A study examining different proteins and surfactants may provide information on the capabilities of XPS and ssHDX-MS on identifying the complex interactions that occur between protein and surfactant. Other solid-state characterization techniques, such as solid-state fluorescence spectroscopy, may also identify the effects of surfactant inclusion on protein conformation.

APPENDIX A. SUPPORTING INFORMATION FOR CHAPTER 2

Table A.1: Formulation composition and moisture content.

Protein	Excipient	Protein:Excipient Ratio (w/w)*	Moisture Content Lyo (%)	Moisture Content SD (%)
Myoglobin	Sucrose	1:9	1.7 ± 0.2	1.0 ± 0.5
	Trehalose		1.3 ± 0.1	1.0 ± 0.3
	Mannitol		1.1 ± 0.2	0.8 ± 0.1
β-lactoglobulin	Sucrose	1:9	1.5 ± 0.7	1.6 ± 0.1
	Trehalose		1.6 ± 0.3	1.4 ± 0.5
	Mannitol		1.5 ± 0.4	0.8 ± 0.2
Bovine Serum Albumin	Sucrose	3:2	1.7 ± 0.3	1.2 ± 0.5
	Trehalose		1.5 ± 0.7	1.6 ± 0.1
	Mannitol		1.5 ± 0.1	1.1 ± 0.5
Lysozyme	Sucrose	1:10	2.4 ± 0.5	1.4 ± 0.6
	Trehalose		2.8 ± 0.7	2.1 ± 0.8
	Mannitol		1.9 ± 0.3	1.2 ± 0.2

*Different protein:excipient ratios correlate to the same molar ratio. Total solid content is 20 mg/mL.

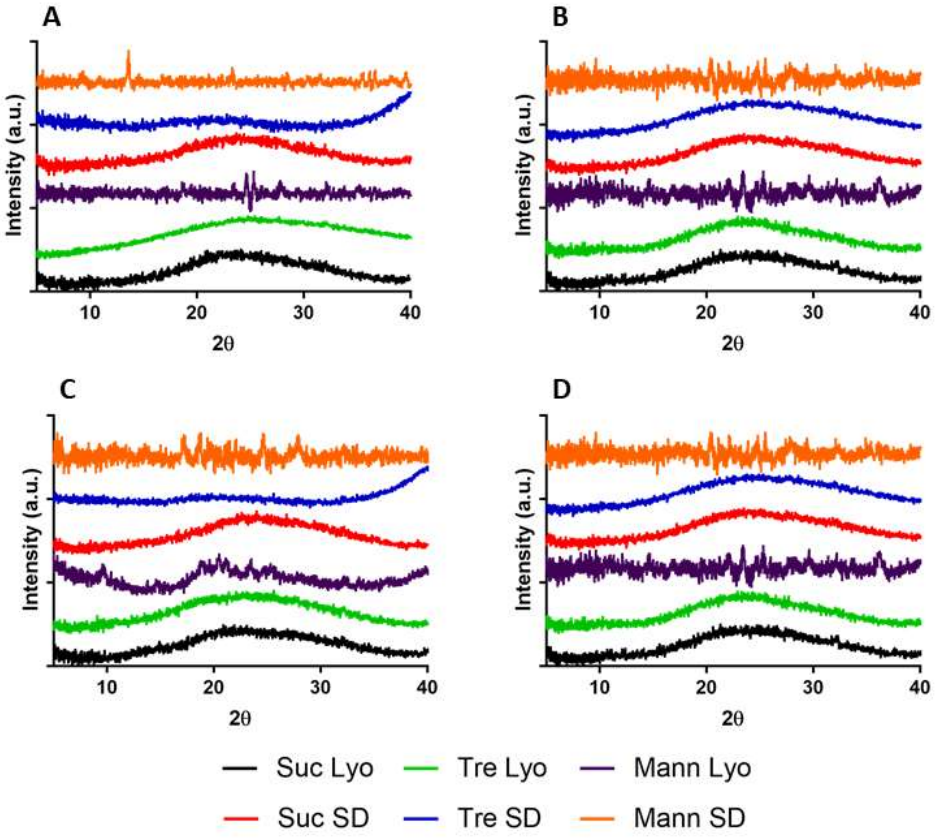

Figure A.1: X-ray powder diffraction powders of myoglobin (A), BSA (B), β-lactoglobulin (C), or lysozyme (D). Samples were formulated with either sucrose (Suc), trehalose (Tre) or mannitol (Mann) and processed by either lyophilization (Lyo) or spray drying (SD).

Table A.2: T_g analysis for formulations produced by spray drying for lyophilization (mean ± SD).

Sample	**T_g or T_m Lyo (°C)***	**T_g or T_m SD (°C)***
Lysozyme/Suc	65.6 ± 0.3	68.1 ± 0.1
Lysozyme/Tre	95.1 ± 0.9	103.7 ± 0.6
Lysozyme/Mann	160.6 ± 0.1	163.4 ± 0.1
BSA/Suc	79.8 ± 0.4	80.4 ± 0.1
BSA/Tre	109.2 ± 1.7	116.1 ± 3.3
BSA/Mann	159.5 ± 0.4	161.8 ± 0.2
Myoglobin/Suc	70.0 ± 0.1	65.2 ± 0.5
Myoglobin/Tre	106.6 ± 0.1	105.9 ± 0.7
Myoglobin/Mann	160.1 ± 0.1	160.9 ±0.4
β-Lactoglobulin/Suc	71.8 ± 0.3	72.0 ± 1.3
β-Lactoglobulin/Tre	106.5 ± 0.2	108.4 ± 0.7
β-Lactoglobulin/Mann	160.4 ± 0.4	163.1 ± 0.1

*T_g for sucrose- and trehalose-containing formulations. T_m for mannitol-containing formulations.

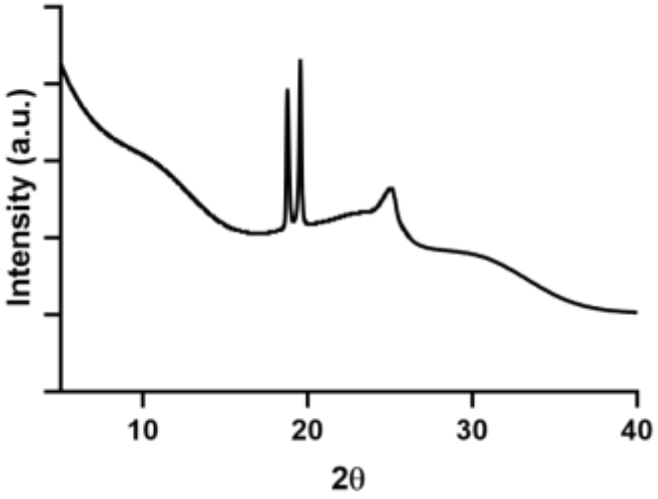

Figure A.2: X-ray powder diffraction powders of myoglobin spray-dried with sucrose after 90 days of storage.

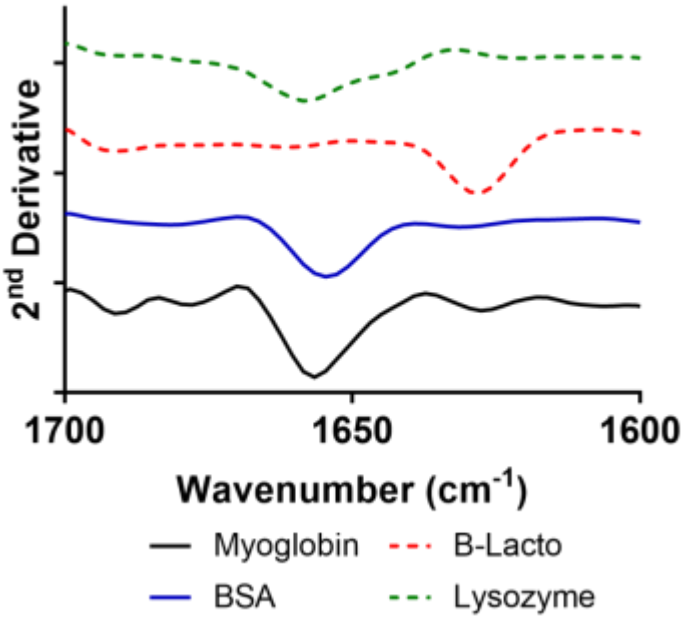

Figure A.3: Solid-state FTIR spectra of proteins prior to formulation and processing.

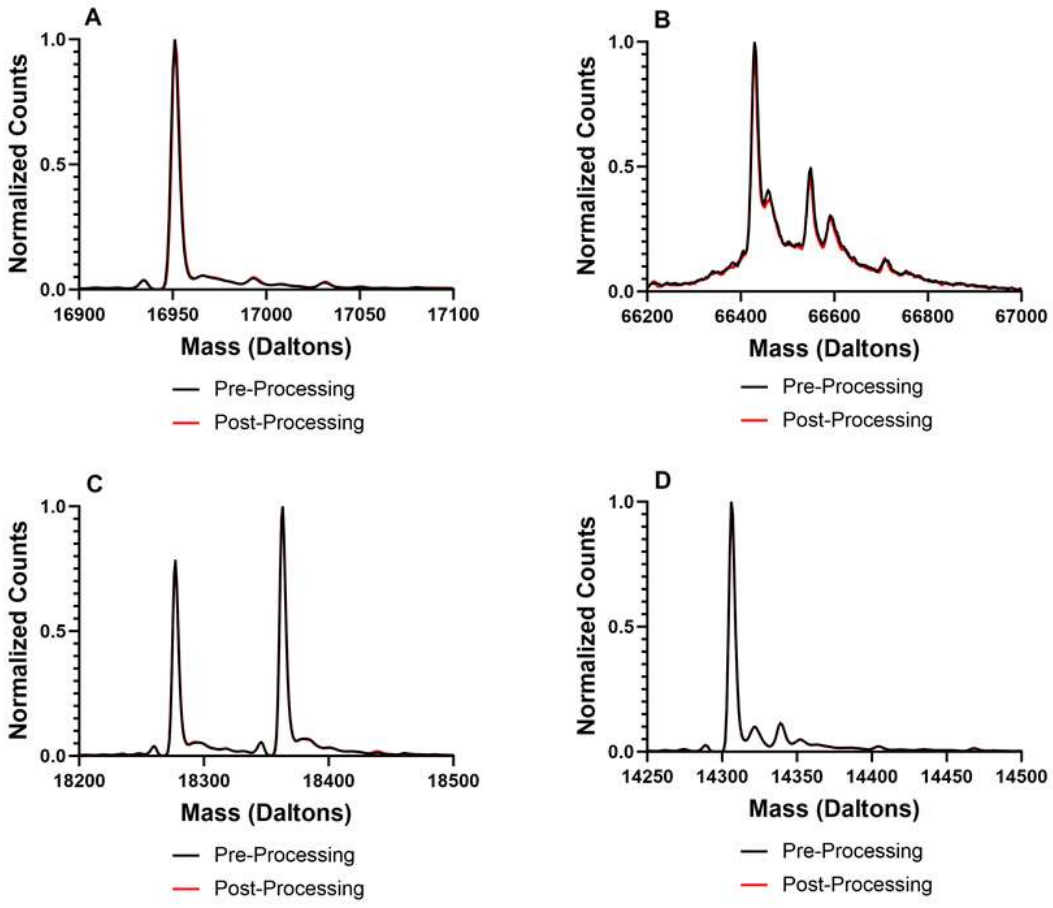

Figure A.4: Deconvoluted mass spectra of proteins pre- and post-processing for myoglobin (A), BSA (B), β-lactoglobulin (C), and lysozyme (D).

APPENDIX B. SUPPORTING INFORMATION FOR CHAPTER 3

Table B.1: Tukey's Test for percentage nitrogen on surface for BSA formulations.

Formulations Compared	**Adjusted P Value**
Protein Only vs. Sucrose	<0.0001
Protein Only vs. Trehalose	<0.0001
Protein Only vs. Dextran 20K	0.0001
Protein Only vs. Dextran 70K	0.0001
Sucrose vs. Trehalose	0.0501
Sucrose vs. Dextran 20K	<0.0001
Sucrose vs. Dextran 70K	<0.0001
Trehalose vs. Dextran 20K	0.0008
Trehalose vs. Dextran 70K	0.0009
Dextran 20K vs. Dextran 70K	>0.9999

Table B.2: Tukey's Test for percentage nitrogen on surface for myoglobin formulations.

Formulations Compared	**Adjusted P Value**
Protein Only vs. Sucrose	<0.0001
Protein Only vs. Trehalose	<0.0001
Protein Only vs. Dextran 20K	<0.0001
Protein Only vs. Dextran 70K	<0.0001
Sucrose vs. Trehalose	0.2415
Sucrose vs. Dextran 20K	0.0049
Sucrose vs. Dextran 70K	0.0010
Trehalose vs. Dextran 20K	0.1485
Trehalose vs. Dextran 70K	0.0244
Dextran 20K vs. Dextran 70K	0.7636

Table B.3: Tukey's Test for percentage protein on surface for BSA formulations.

Formulations Compared	**Adjusted P Value**
Protein Only vs. Sucrose	<0.0001
Protein Only vs. Trehalose	<0.0001
Protein Only vs. Dextran 20K	<0.0001
Protein Only vs. Dextran 70K	<0.0001
Sucrose vs. Trehalose	0.0428
Sucrose vs. Dextran 20K	<0.0001
Sucrose vs. Dextran 70K	<0.0001
Trehalose vs. Dextran 20K	0.0006
Trehalose vs. Dextran 70K	0.0007
Dextran 20K vs. Dextran 70K	>0.9999

Table B.4: Tukey's Test for percentage protein on surface for myoglobin formulations.

Formulations Compared	**Adjusted P Value**
Protein Only vs. Sucrose	<0.0001
Protein Only vs. Trehalose	<0.0001
Protein Only vs. Dextran 20K	<0.0001
Protein Only vs. Dextran 70K	<0.0001
Sucrose vs. Trehalose	0.0710
Sucrose vs. Dextran 20K	0.0005
Sucrose vs. Dextran 70K	<0.0001
Trehalose vs. Dextran 20K	0.0345
Trehalose vs. Dextran 70K	0.0031
Dextran 20K vs. Dextran 70K	0.5221

APPENDIX C. SUPPORTING INFORMATION FOR CHAPTER 4

Table C.5: Two-way ANOVA and Tukey's Test for atomic percentage protein on surface.

Source of Variation	**P Value**
Interaction	<0.0001
Row Factor	<0.0001
Column Factor	<0.0001
Formulations Compared	**Adjusted P Value**
Myo Only vs. Myo-PS20	<0.0001
Myo Ony vs. Myo-Pol188	<0.0001
Myo-PS20 vs. Myo-Pol188	0.1427
Myo-Suc vs. Myo-Suc-PS20	<0.0001
Myo-Suc vs. Myo-Suc-Pol188	<0.0001
Myo-Suc-PS20 vs. Myo-Suc-Pol188	<0.0001
Myo-Mann vs. Myo-Mann-PS20	<0.0001
Myo-Mann vs. Myo-Mann-Pol188	<0.0001
Myo-Mann-PS20 vs. Myo-Mann-Pol188	0.9183

Table C.6: Two-way ANOVA and Tukey's Test for moisture content.

Source of Variation	**P Value**
Interaction	0.0123
Row Factor	0.0133
Column Factor	<0.0001
Formulations Compared	**Adjusted P Value**
Myo Only vs. Myo-PS20	0.0567
Myo Ony vs. Myo-Pol188	0.0002
Myo-PS20 vs. Myo-Pol188	0.0344
Myo-Suc vs. Myo-Suc-PS20	0.7395
Myo-Suc vs. Myo-Suc-Pol188	0.9665
Myo-Suc-PS20 vs. Myo-Suc-Pol188	0.8733
Myo-Mann vs. Myo-Mann-PS20	0.8733
Myo-Mann vs. Myo-Mann-Pol188	0.7395
Myo-Mann-PS20 vs. Myo-Mann-Pol188	0.9665

Table C.7: Two-way ANOVA and Tukey's Test for ssHDX-MS sesults.

Source of Variation	**P Value**
Interaction	<0.0001
Row Factor	0.0003
Column Factor	<0.0001
Formulations Compared	**Adjusted P Value**
Myo Only vs. Myo-PS20	0.2257
Myo Ony vs. Myo-Pol188	>0.9999
Myo-PS20 vs. Myo-Pol188	0.2257
Myo-Suc vs. Myo-Suc-PS20	>0.9999
Myo-Suc vs. Myo-Suc-Pol188	>0.9999
Myo-Suc-PS20 vs. Myo-Suc-Pol188	>0.9999
Myo-Mann vs. Myo-Mann-PS20	0.7454
Myo-Mann vs. Myo-Mann-Pol188	<0.0001
Myo-Mann-PS20 vs. Myo-Mann-Pol188	<0.0001

Table C.8: Two-way ANOVA and Tukey's Test for ssHDX-MS peak area results.

Source of Variation	**P Value**
Interaction	0.0003
Row Factor	<0.0001
Column Factor	<0.0001
Formulations Compared	**Adjusted P Value**
Myo Only vs. Myo-PS20	0.1894
Myo Ony vs. Myo-Pol188	0.5321
Myo-PS20 vs. Myo-Pol188	0.7456
Myo-Suc vs. Myo-Suc-PS20	0.1388
Myo-Suc vs. Myo-Suc-Pol188	0.8305
Myo-Suc-PS20 vs. Myo-Suc-Pol188	0.3496
Myo-Mann vs. Myo-Mann-PS20	0.0003
Myo-Mann vs. Myo-Mann-Pol188	<0.0001
Myo-Mann-PS20 vs. Myo-Mann-Pol188	0.0093

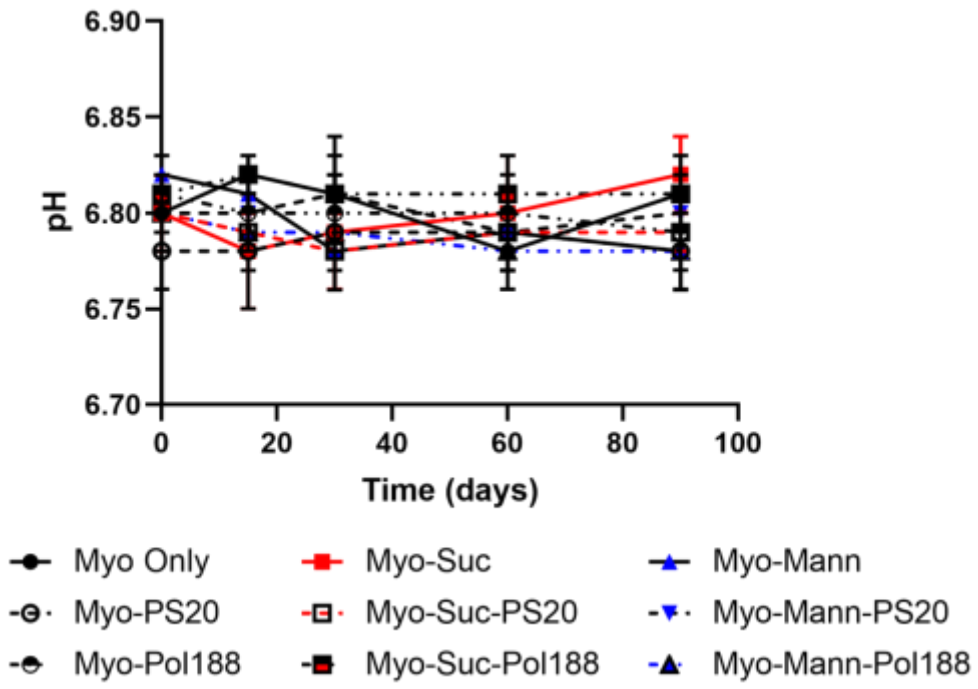

Figure C.5: pH profile for formulations under 90-day accelerated stability study following reconstitution.

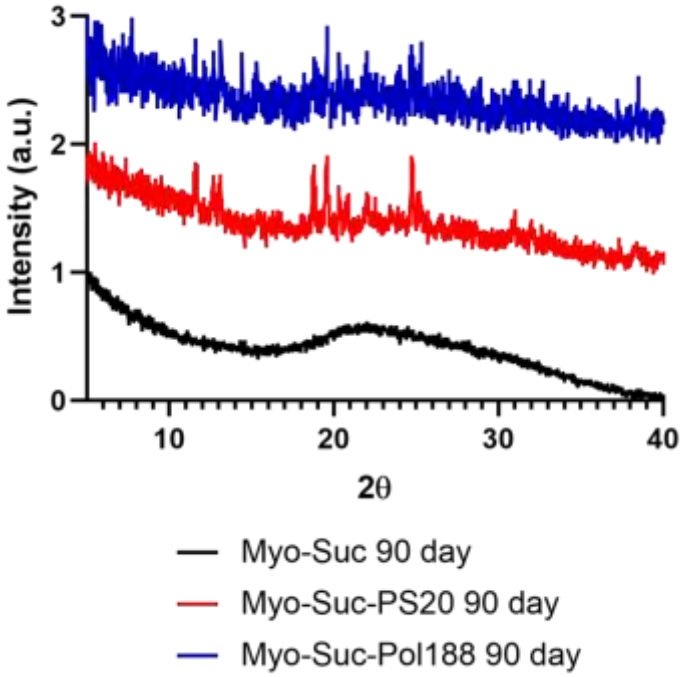

Figure C.6: XRPD results of sucrose-containing formulations following storage at 40°C for 90 days.

VITA

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EDUCATION

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- Process protein formulations through lyophilization and spray drying
- Characterize protein formulations based on secondary and tertiary structure, aggregation, and hydrogen-deuterium exchange

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- Prepared porous polymer particles from pharmaceutical excipients for heterogeneous crystallization
- Designed polymeric films for controlling protein crystallization
- Conducted dissolution studies to monitor drug release for regulatory compliance

Pharmaceutical Sciences Co-op Formulation Scientist, *Merck, Kenilworth, NJ*
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- Designed parenteral formulation approach for controlled release of peptides
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- Analyzed effects of excipients on peptide stability in solution

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- Produced paclitaxel nanocrystals with fluorescent markers for *in vivo* analysis in animal models
- Cultured hepatic cancer cells for *in vitro* analysis of fluorescent markers in paclitaxel

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- Extracted mitochondria from cells using differential centrifugation techniques
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- Performed activity assays spectrophotometrically to determine toxicity effects on ATP production

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Lyophilization, spray drying, spray freeze-drying, hydrogen/deuterium exchange analysis, mass spectrometry, HPLC method development, FTIR, fluorescence spectroscopy, crystallization, X-ray diffraction, differential scanning calorimetry, thermogravimetric analysis, Karl Fischer moisture content analysis, scanning electron microscopy, dissolution

PUBLICATIONS

Wilson NE, Mutukuri TT, Zemlyanov DY, Topp EM, and Zhou QT. Effects of Surfactants on Surface Composition and Physical Stability of Spray-dried Protein Formulations. *J Pharm Sci*. In Preparation.

Wilson NE, Mutukuri TT, Zemlyanov DY, Taylor LS, Topp EM, and Zhou QT. Surface Composition and Formulation Heterogeneity of Protein Solids Produced by Spray Drying. *Pharmaceutical Research.* Submitted August 2019.

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PRESENTATIONS

Wilson, NE. Effects of Processing Method and Excipient on Protein Structure and Stability Using Solid-State Hydrogen/Deuterium Exchange Mass Spectrometry. *Gordon Research Seminar: Preclinical Form and Formulation for Drug Discovery.* 2019. Waterville Valley, NH.

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Wilson NE, Topp EM, and Zhou QT. Process and Formulation Effects on Protein Structure and Stability Using Solid State Hydrogen/Deuterium Exchange Mass Spectrometry (ssHDX-MS). *American Association of Pharmaceutical Scientists PharmSci 360.* 2018. Washington, DC.

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Wilson, NE, Topp EM, and Zhou QT. Impact of Spray-drying and Formulation Design on Protein Stability and Heterogeneity. *Center for Pharmaceutical Processing Research Meeting.* 2018. Storrs, CT.

Wilson, NE, Zhou QT, and Topp EM. Effects of Lyophilization and Spray-drying on Protein Structure. *Garnet E. Peck Symposium* 2018. West Lafayette, IN.

Wilson, NE and Topp EM. Effects of Formulation and Manufacturing Methods on Protein Structure. *International Society of Lyophilization - Freeze Drying Midwest Chapter Conference.* 2017. Chicago, IL.

Wilson, NE, Karaki A, and Chadwick K. Heterogeneous Crystallization as a Technology for Engineering an Excipient/Crystal Drug Dosage Form. *Gordon Crystal Engineering Conference.*2016. Stowe, VT.

Wilson, NE. Heterogeneous Crystallization in Microporous Polymers for Optimizing the Manufacture of Regulatory Compliant Dosage Forms. *Pharmaceutics Graduate Student Research Meeting*. 2016. Kansas City, MO.

Wilson, NE, Karaki A, and Chadwick K. Advanced Manufacturing by Heterogeneous Crystallization in Microporous Particles. *Pharmaceutics Graduate Student Research Meeting*. 2015. Lexington, KY.

Wilson, NE, Ling J, and Chadwick K. Reduced Manufacturing Steps Through Crystallization on Polymeric Excipients. *Garnet E. Peck Symposium* 2015. West Lafayette, IN.

TEACHING EXPERIENCE

Graduate Assistant, *Purdue University*
January 2015 - Present

- Develop and supervise research projects for undergraduate students
- Instruct on instrumentation and standard operating procedures

Teaching Assistant, *Purdue University*
Advanced Biopharmaceutics
January - May 2015

- Evaluated students' understanding of pharmaceutical dosage form components and transport mechanisms

Dosage Forms
August - December 2014

- Instructed students on the different types of pharmaceutical dosage types and related physicochemical properties
- Proctored and evaluated examinations and coursework of 180 students

AWARDS, HONORS and FELLOWSHIPS

- Graduate Assistance in Areas of National Need (GAANN) Fellowship, **2016-2019**. Purdue University.
- College of Pharmacy Research Recognition Award, **2014**. Purdue University.

PROFESSIONAL MEMBERSHIPS

- Reviewer, AAPS PharmSciTech — September 2018 - Present
- Treasurer, AAPS Purdue University Student Chapter — May 2016 - May 2017
- Member, AAPS — September 2014 - Present