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Review

Inhomogeneous Distribution of Components in Solid Protein Pharmaceuticals: Origins, Consequences, Analysis, and Resolutions

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ABSTRACT

Successful development of stable solid protein formulations usually requires the addition of one or several excipients to achieve optimal stability. In these products, there is a potential risk of an inhomogeneous distribution of the various ingredients, specifically the ratio of protein and stabilizer may vary. Such inhomogeneity can be detrimental for stability but is mostly neglected in literature. In the past, it was challenging to analyze inhomogeneous component distribution, but recent advances in analytical techniques have revealed new options to investigate this phenomenon. This paper aims to review fundamental aspects of the inhomogeneous distribution of components of freeze-dried and spray-dried protein formulations. Four key topics will be presented and discussed, including the sources of component inhomogeneity, its consequences on protein stability, the analytical methods to reveal component inhomogeneity, and possible solutions to prevent or mitigate inhomogeneity.

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Introduction

A major concern in the development of protein pharmaceuticals is to manage their inherent instability. Aqueous formulations often have an insufficient shelf-life, requiring an extensive yet expensive cold chain system that is hardly available in remote areas, especially in developing countries, for preservation and distribution. Transforming protein into the solid form by processes such as freeze-drying or spray-drying can be necessary to achieve sufficient stability and reduce dependence on the cold chain. These processes, however, introduce a variety of stresses that are often detrimental to the protein. To preserve the protein structure both during drying and subsequent storage, the incorporation of stabilizing excipients is of paramount importance.^{1–3} Typical excipients for solid protein pharmaceuticals are di- and polysaccharides, other types of polymers, and amino acids.^{1,4–7} Freeze-drying or spray-drying promotes the formation of a stabilizer matrix in the glassy state, protecting the protein. Other additives like buffers, surfactants, and antioxidants are also often combined to further enhance the stability of solid protein pharmaceuticals.^{8,9}

Although many critical issues regarding protein stability have been studied, little attention has been given to the inhomogeneity of solid protein formulations. Inhomogeneity in the solid state can be defined as an inhomogeneous, unequal distribution or phase separation of components on a molecular scale. Inhomogeneous distribution of components has been previously reported in freeze-dried and spray-dried formulations of food and small molecule drugs and was associated with detrimental effects on product stability and performance.^{10–13} It is highly likely that solid protein pharmaceuticals can experience inhomogeneity as well with consequences on their stability.^{6,14–18}

Currently, only differential scanning calorimetry (DSC) and X-ray diffraction (XRD) are frequently used to identify inhomogeneity in solid formulations, but their sensitivity can be insufficient to monitor inhomogeneity at a smaller scale in solid protein formulations. The recent introduction of techniques with better sensitivity and higher resolution has brought some attention to this problem in the last few years. Tackling product inhomogeneity can further reduce the fraction of deteriorated protein, preventing potential harmful effects after administration.^{19–21} Therefore, we will provide a comprehensive overview of inhomogeneous component distribution in solid protein formulations produced by freeze-drying and spray-drying in this review.

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Principles of Protein Stabilization in the Solid State

To realize the detrimental effects of inhomogeneous distribution of components, especially between proteins and stabilizers, it is necessary to understand the mechanism(s) by which the excipients stabilize the protein. There are 2 major theories explaining protein stabilization in the solid state: the vitrification theory and the water replacement theory.

The vitrification theory describes the kinetic trap of the highly viscous stabilizer matrix in the glassy state that slows down protein molecular mobility, which then leads to a decrease in the degradation processes. To achieve the most effective stabilization, the storage temperature should be below the Kauzmann temperature (which is around half of the melting point or 50°K below the glass transition temperature (T_g)) where the system achieves negligible molecular mobility.^{22,23} Any excipient that can form a glassy matrix, especially with high matrix T_g , has the potential to stabilize proteins during and after freeze-drying and spray-drying. Prime examples of stabilizers that can form amorphous matrix are saccharides such as trehalose, mannose, sucrose, and a handful of amino acids including arginine and polymers (like dextran or serum albumin).

The water replacement theory assumes that the natural protein conformation is driven entropically in the water environment.²⁴ The loss of the protein solvated layer of hydrogen-bonded water after drying can jeopardize the natural conformation.²⁴ Excipients, especially saccharides, that can form hydrogen bonds with proteins can replace the water environment and prevent a loss of the three-dimensional protein structure.²⁴ Apart from hydrogen bonding, interaction at aromatic sites through CH group can also play a (modest) role in this stabilization theory.^{25–28} The concept of thermodynamic stabilization further adds that in the aqueous environment, the equilibrium between protein native and denatured state is prone toward the native state.⁸ Dehydration exposes the protein to a different environment and shifts the equilibrium to forming the denatured state. Stabilizers that form hydrogen bonds with proteins increase the free energy of the denatured state and thus shift the equilibrium back to favoring the native state.^{24,29,30}

It is difficult to attribute the stabilization of protein to just one mechanism, as it is likely that these 2 theories are intertwined and not mutually exclusive.^{8,24,31} Other explanations have also been proposed. The most notable mechanism that emerged recently is the effect of β relaxation. Historically, the slowest motion of the amorphous stabilizer matrix, which is α relaxation, has been used to correlate with protein degradation rate by assuming that it scales with the T_g .³² α relaxation is highly temperature-dependent and can occur over the long period, serving as the basis for the vitrification theory. However, β relaxation, which happens in a much shorter timescale, was found to also have a strong correlation with protein degradation in some protein formulations.^{33–37} Readers are referred to follow the extensive discussion regarding all stabilization theories elsewhere.^{8,38,39}

Mechanisms of protein stabilization in the solid state are complex but primarily depend on the interaction and incorporation of protein into an amorphous matrix of the stabilizer. Therefore, protein molecules must be homogeneously distributed within the stabilizing matrix. Although a protein formulation containing several excipients can demonstrate a single-phase behavior in the aqueous form before drying, redistribution of components during or after freeze-drying or spray-drying can occur, leading to inhomogeneity.¹⁵

Driving Forces of Component Inhomogeneity

Immiscibility Between Components

Many aqueous mixtures display a limited co-solubility behavior and at high concentrations they can separate into different phases.

The limited co-solubility is well known when mixing macromolecules, for example, a system consists of 1 protein and 1 polymer in water (with the earliest example is the gelatin-starch mixture).⁴⁰ A typical phase diagram of a solution of a protein-polysaccharide is given in Figure 1. Clearly, at low concentrations, component ratio is well below the binodal curve and can form a homogeneous solution at a molecular level at any ratio. This observation was first systematically described by the Flory-Huggins theory. Complete miscibility down to the molecular level of 2 macromolecules can only be achieved when

$$\Delta G_m = \Delta H_m - T\Delta S_m < 0^{41}$$

where ΔG_m , ΔH_m , and ΔS_m are Gibbs free energy, enthalpy, and entropy of mixing at temperature T , respectively. The value of $T\Delta S_m$ is always positive because there is an increase in the entropy on mixing. There is evidence that the mixing entropy of macromolecules is several orders of magnitude smaller than that of monomers.^{42,43} Therefore, the value of ΔG_m and thereby the mixing state mostly depend on the enthalpy of mixing, ΔH_m . The attractive or repulsive force between different macromolecules is vital in determining ΔH_m and thus the homogeneity of the mixture.^{42,43} Because proteins are macromolecules, the mixing limitation due to limited co-solubility also applies to protein formulation, especially in the presence of polymers or other proteins.

Before drying, protein and other excipients are often dissolved at low initial concentrations, giving a clear homogeneous aqueous solution. This is nonetheless situational because as the drying process progress, water is separated from the system and the concentration of components increases dramatically, reaching up to 50 times the initial level before solidification.⁴⁴ The significant increase in concentrations during a freeze-drying process appears in the freezing step. During this stage, water continuously crystallizes into ice, gradually pushing the remaining liquid into many small domains with very high concentration, the so-called freeze-concentrated solutions, which is where phase separation due to limited co-solubility can occur.^{15,45} Certain polymer systems such

Figure 1. Typical phase diagram for a protein-polysaccharide system in water. The curve in the diagram represents the binodal or coexistence of the system, in which it is thermodynamically favorable for 2 components to be fully mixed. The region of composition under the binodal curve corresponds to single-phase mixed solutions. The region above the curve illustrates the limited co-solubility of protein and another polymer as they are each concentrated in the different phases. Reproduced from Tolstoguzov.⁴²

as polyethylene glycol (PEG)-dextran, initially below critical concentrations for phase separation, may show a liquid-liquid phase separation during the freezing step.^{15,46} Unlike during freeze-drying, the increase in component concentration in spray-drying appears in the drying step. Figure 2 demonstrates an example of the limited co-solubility issue in spray-drying. The different occurrence of phase separation due to limited co-solubility between freeze-drying and spray-drying plays an essential role in how immiscibility can be prevented or mitigated.

Although the Flory-Huggins theory only describes the immiscibility between 2 macromolecules, limited co-solubility can occur in any system regardless of the molecular size. Separation can be found in mixtures of low molecular weight stabilizers (trehalose, sucrose, arginine, or glutamate) and large molecules, which are typical for solid protein formulations. For example, a freeze-dried mixture of trehalose and dextran was found to be phase-separated.⁴⁸ Other freeze-dried mixtures that experience or potentially experience the same issue are sucrose-hydroxyethyl-starch, sucrose with trehalose, polyvinylpyrrolidone (PVP), dextran, or polyvinylpyrrolidone-co-vinyl acetate (PVP-VA) and trehalose-PVP.^{49–51}

Several factors determine whether or not phase separation of components occurs during freeze-drying and spray-drying. First, the ratio between components is an important factor governing immiscibility (Fig. 1) as shown in freeze-drying studies.^{44,52} Thermal history (such as annealing or the freeze-drying or spray-drying condition) affects immiscibility differently through changing component mobility.⁷ Annealing in freeze-drying which raises product temperature above the T_g' (the glass transition temperature of the maximally freeze-concentrated solutions) to produce a good cake structure can introduce sufficient molecular mobility to solutes thereby promoting phase separation.^{53,54} Elevated storage temperature and humidity can compromise the amorphous state of the formulation. In this condition, water can absorb and act as a plasticizer, lowering the T_g of the freeze-drying and spray-drying amorphous formulations. Similarly, the residual moisture as well as its inhomogeneous distribution may lead to the same outcome. The lowering of the T_g can be such that the molecular mobility of components is high enough to allow for phase separation, especially by crystallization of the stabilizer. The nature of components is also a fundamental aspect which dictates the mixing property through preferential or unfavorable interaction between excipients.⁵⁵ Components also have an impact on the time during which the concentrated solution remains in the liquid state to allow for the formation of a colloidal dispersion during freeze-drying and

spray-drying.⁴⁸ Finally, salts and buffers can negatively influence the miscibility of components. Increasing the concentration of certain salts in buffers can lead to salting-out effects and destabilize the system in 2 different ways.⁵⁶ At high concentration, salting-out salts can reduce protein solubility and cause aggregation in the remaining liquid domains.^{55,57} Furthermore, high level of salting-out salts can alter protein-excipient and excipient-excipient interaction leading to phase separation. For example, the lyophilized ovalbumin-PVP system alone exists as a single phase but it is phase-separated when NaCl (at least 50 mM) is present in the aqueous solution prior to freeze-drying. Likewise, the single-phase lysozyme-PVP system is destabilized with the addition of Na₂SO₄ (10 mM) or sodium phosphate buffer (20 mM).⁵⁵ Diethylaminoethyl modified dextran can be phase-separated from non-modified dextran during freezing in the presence of NaCl, NaSCN, sodium phosphate buffer, or Tris-HCl buffer at concentrations as low as 10 mM even though they are miscible in salt and buffer-free environment.⁵⁸ Frozen mixtures of protein (recombinant human albumin, gelatin) or polysaccharide (dextran) and various amino acids (L-arginine, L-arginine hydrochloride, L-arginine monophosphate, sodium L-glutamate) exhibited phase separation when NaCl or buffers were added to the formulation.^{7,45} The effect is concentration-dependent and could be the result of altered hydration states in macromolecules or concealment of the electrostatic effects between them.^{7,55,58}

Adsorption at Various Surfaces for Surface-Active Components

Limited co-solubility is not the only origin of component inhomogeneity. As proteins are generally surface-active, they can be adsorbed at various interfaces formed during the drying process. In the freeze-drying process, an ice-liquid interface exists during the freezing step. This interface is the border between the growing ice crystals and the continuously decreasing remaining solution. The area of this interface is affected by the freeze-drying process, specifically the ice formation rate and the annealing step. More details about process-related effects on the ice surface area will be provided in the later section. During the spray-drying process, on the other hand, a huge air-liquid interface is created during spraying and subsequent drying. The interfacial properties, as well as the adsorption of protein at the air-liquid interface, have been characterized thoroughly, unlike the ice-liquid interface, which severely lacks insights. It is unknown which interface is more harmful to protein. Some studies indicate that the air-liquid interface has a higher affinity of protein adsorption than the ice-liquid interface as

Figure 2. Separation of HPMC (red) and maltodextrin (blue) of individually dried particles of different compositions A1, A2, and A3 captured by confocal Raman microscopy. Reprinted with permission from Nuzzo et al. Copyright 2015 American Chemical Society.⁴⁷

it has been found that up to 65% of protein can be present at the surface of spray-dried particles, while this was only 11% for some freeze-dried products.^{17,18,59} However, surface adsorption seems to be specific for the formulation as well as the drying process. Some formulations have more protein adsorbed at the ice-liquid interface after freeze-drying than the air-liquid interface after spray-drying and vice-versa.⁶⁰

Surface adsorption of proteins is a competitive process and depends greatly on their concentration, the ratio of components, and the presence of competing surface-active molecules. Popular surface-active molecules usually found in protein formulations are Tween 80, Tween 20, and human serum albumin (HSA). Their main role is to stabilize protein by suppressing aggregation, inhibiting protein-protein interaction, and assisting in protein refolding.^{1,61–63} At high concentration, these surface-active molecules can competitively adsorb and replace protein from the interface (such as the case of bovine serum albumin [BSA] replacement from the spray-dried particle surface when the β -lactoglobulin:BSA weight ratio is higher than 1). At lower concentrations, the adsorption is independent of the presence of the other surface-active molecule.^{10,18,64,65} Protein composition at the surface increases when the concentration increases, eventually reaching a level at which the rise in surface protein content is much less pronounced.⁶⁴ Environmental pH can strongly affect protein surface adsorption by altering its conformation and thus surface activity.⁶⁶ Interaction with other excipients can govern the surface adsorption to some degree.^{67,68} In the spray-dried dipalmitoylphosphatidylcholine surfactant (DPPC)-lactose formulation, no noticeable surface adsorption of DPPC was found.^{67,68} Interaction between DPPC and lactose was perhaps the reason behind this observation.^{67,68} Moreover, crystallization of component such as lactose during spray-drying can affect the surface composition of spray-dried particles.^{59,69} On account of the short lifetime of the droplet during spray-drying compared to freeze-drying, the adsorption of protein to the air-water interface can be assumed to be mainly diffusion-limited.⁶⁴ Small proteins with faster diffusion rate can adsorb to the surface before droplet solidification is initiated. In freeze-drying there is more time for other processes to occur such as interaction with the interface, protein structure rearrangement and thermal convection which could play a role in the overall adsorption process.

Another factor that may impact protein surface adsorption is the surface charge. Electric fields are often generated during ice formation of ionic solutions due to preferential inclusion of some ions.⁷⁰ Ion entrapment is thought to be diffusion controlled and depends on their movement speed and the growth rate of the ice interface.⁷¹ The dissociated H^+ and OH^- ions are mainly responsible for this interfacial field.⁷² The electric field potential depends largely on the freezing rate, the concentration, as well as the type of the ionic species present in solution.^{71,73,74} A decrease in the electric potential during freezing of ionic solutions was obtained following an increase in the sucrose concentration.⁷⁵ The electrical field, however, is neutralized after complete ice formation.⁷¹ In spray-drying, the development of the charged surface is the result of particle-particle and particle-equipment interactions.⁷⁶ The basis of electrostatic interaction with protein surface adsorption is complicated and thus is outside the scope of this review.⁷⁷ Nevertheless, its influence on protein surface adsorption during freeze-drying and spray-drying is yet to be researched.

Crystallization of Components

Crystallization of excipients is a well-known event of component separation, especially during freeze-drying of formulations containing buffers. Selective crystallization of one buffering salt

from the buffered protein solution can occur during freezing and dramatically change the pH value. For example, Na_2HPO_4 and K_2HPO_4 crystallize more readily than NaH_2PO_4 and KH_2PO_4 , respectively, because these buffer components have a lower solubility and a higher eutectic temperature.^{1,56} Preferential crystallization of Na_2HPO_4 and K_2HPO_4 can cause a pH drop of 3 units.⁷⁸ In some cases, buffer crystallization can be detected in spray-drying, as in the case of ammonium sulfate in the buffer.⁷⁹ Crystallization of buffers and other excipients during freeze-drying and spray-drying can usually be observed by DSC or XRD analysis of the dried product. However, a recent interesting finding is the crystallization of trehalose during freeze-drying, while it appears to be completely amorphous in the final lyophilized cake. The reason for this is that annealing of the frozen trehalose solution led to the formation of trehalose dihydrate crystals, but during subsequent harsh drying conditions also the water molecules from the trehalose dihydrate crystals were removed by which trehalose turned into the amorphous state.⁸⁰ Therefore, the amorphous state found in DSC and XRD in dried formulations does not guarantee a homogeneous distribution of components. Trehalose crystallization can be facilitated by crystallizing solutes such as mannitol while limited by non-crystallizing components like sucrose or protein.^{52,81} This crystallization behavior of trehalose might be due to the lower solubility, hygroscopicity, and ability to form clusters at higher concentrations compared to other saccharides like sucrose.⁸² Despite the complex crystallization process and transformation between different crystal forms, the dihydrate crystal reported in the study above returns to the amorphous form instead of transforming into the anhydrous crystal.⁸² Whether the subsequent amorphization of trehalose from the dehydrate crystal can restore phase homogeneity and protein interaction is questionable.⁸² Currently, it is unknown to what extent this crystallization event occurs with other excipients and its consequence. Fortunately, only extended annealing time (3 h or more) appears to give rise to trehalose crystallization.

Process-Driven Component Separation

Spray-Drying

There are several mechanisms driving component separation that are rather specific for spray-drying (Fig. 3). During the first

Figure 3. Movements of solvent and chemical components in the droplet caused by spray-drying (— solvent evaporation; — components diffusing away from the surface; — adsorption of surface-active components, triggering the diffusion from the bulk to the sub-surface area; — solvent convection; — solvent boiling when the drying temperature exceeds the boiling point).

stages of the drying process, solvent evaporation at the droplet surface raises the solute concentrations at the air-liquid interface, creating a diffusional flux to the interior of the droplet.⁸³ The diffusion coefficient of different components is unequal, with large molecules like proteins and polymers moving more slowly than small solutes such as salt or low molecular weight saccharides. This is reflected in the Stokes-Einstein equation describing the diffusion coefficient

$$D = k_B T / 6\pi\eta r^{84}$$

where k_B is Boltzmann's constant (1.38×10^{-23} J/K), T is temperature, η is the dynamic viscosity, and r is the spherical particle radius.

On account of the slow diffusion, there are more large molecules present near and at the surface than in the interior of the drying droplet when solidification begins, creating a concentration gradient in the final dried particle.^{85–87} Thus, when spray-drying a protein solution with a low molecular weight stabilizer, protein enrichment at the surface cannot only be expected by its surface-active nature but also by its relatively slow diffusion rate which indeed was found in a modeling study for albumin-trehalose combinations.⁸⁶ The diffusional motion away from the surface can be considered as the primary movement, making component distribution predictable simply by knowing the diffusional coefficients of solutes compared to the evaporation rate.^{64,87} Studies have demonstrated that this assumption can predict the separation of components pretty accurate. That is, using mathematical modeling, without taking the contribution of surface activity into account, large molecules such as BSA or pullulan, a polysaccharide, were found to dominate the surface of spray-dried particles compared to smaller molecules like trehalose, with enrichment level of BSA close to the result of analytical techniques.^{18,86,88} The analysis of the spray-dried hydroxypropyl methylcellulose (HPMC)-lactose particles provides experimental evidence that surface enrichment of high molecular weight components, in this case HPMC, without significant surface activity does occur.⁸⁹ Although spray-drying a mixture of a low and a high molecular weight component will result in particles with the large molecule dominating the surface, spray-drying a mixture comprises of 2 large molecules that can have unexpected consequences. Spray-drying of HPMC and maltodextrin led to different surface compositions depending on their ratio in the mixture.⁴⁷ Although HPMC mostly presents at the surface layer due to a higher molecular weight, the reverse with maltodextrin forming the surface layer can be the result of its overwhelming concentration in comparison to HPMC. In another special case, one component with a slow diffusional coefficient may reach the saturation point at the surface, increasing the local viscosity with subsequent solidification earlier than in the rest of the droplet and form a shell.⁸⁷ Generally which component dominates the shell can depend on several chemical characteristics including viscosity and saturation concentration.^{87,90}

During the drying process, the movement of components by other mechanisms is also happening. A rarely investigated process is the internal solvent convection due to temperature and pressure difference in the droplet.⁸⁷ If the drying temperature is above the solvent boiling point, the movement of liquid to escape the droplet is more intense and can affect the diffusion of proteins and other excipients. More extensive details of these phenomena can be found elsewhere.⁸⁵ In addition, the surface adsorption of proteins and other surface-active agents can further create another gradient concentration right below the droplet surface, triggering another diffusional motion of components, also known as Stefan flow.⁹¹ Stefan flow can be the origin of the layer depleted of surface-active components (BSA, poloxamer) below the spray-dried particle surface enriched with these molecules.⁸⁹

Freeze-Drying

The process-driven component separation is also present in freeze-drying. When water begins to crystallize, it pushes the remaining solutes away from the ice-liquid interface.⁹² The excluded solute is transported away from this surface by diffusion and convection and is influenced by differences in interfacial energy as well as the ice crystal lattice structure.^{72,93–95} Development of the concentration gradient made by freeze-drying is relatively complex but seems to primarily depend on diffusion of components following Fickian law, the growth velocity of the ice interface, and potential interfacial interaction of components.⁷² The ice inclusion of some solutes can further contribute to the developing concentration gradient.^{72,92,96–100} Readers can follow the extensive mathematical description given by Tiller.¹⁰¹ This (macroscopic) concentration gradient is demonstrated in one study of trehalose-BSA and trehalose-immunoglobulin G2 formulation. The solute concentration is 2- to 3-fold higher toward the bottom and radial center of the vial freeze-dried in stagnant air freezer.⁹² This is because ice nucleation was initiated by the ice fog technique that started the water crystallization process from the top, pushing other solutes toward the bottom of the vial. A concentration gradient starting from the ice nucleation area in approximate agreement to the diffusion equation is also detected in freeze-drying of different molecules including sucrose, PEG, BSA and polyvinyl alcohol (PVA).⁷² Large molecules like BSA move slower and thus cannot generate a significant gradient profile compared to small molecules like trehalose.^{92,100,102} In some cases, the complex diffusion behavior of some large molecules might not always follow the diffusion law.⁷² In addition, the formation of dendrites can enclose part of the unfrozen solution and prevent components from diffusing away from the surface.^{92,102} Within these confined areas further diffusion might continue to happen and create inhomogeneity in this microenvironment.^{72,92} This seems to be the main difference in concentration gradient developed in freeze-drying compared to spray-drying. The rapidly reducing air-liquid interface of spray-drying droplet traps the slowly diffuse large molecules at the surface but still allows small molecules to equally distribute. The growing ice and dendrites stop or trap slow-moving molecules in the ice structure instead and push the fast-moving solutes away. The concentration gradient is more profound in large freeze-drying tanks of bulk protein solutions where heat and mass transfer are limited by their size compared to small vials used in laboratory context.^{102,103}

Limitations in Solute Evaporation

Given the role of water in the solid state of the glassy matrix, its inhomogeneous distribution can have a strong impact on protein formulation stability. Most residual water in the final product is located around charged and polar residues of proteins.⁴⁹ In freeze-drying, water sublimation comprises 2 separated steps: primary drying and secondary drying. Secondary drying is the most relevant step regarding the residual moisture.¹⁰⁴ The flow of water vapor faces 3 barriers which are the dried-product layers, the semi-stoppered vial, and the chamber resistance.¹⁰⁵ Heterogeneous nucleation of ice can also contribute to difference in water vapor resistance from within the formulation.¹⁰⁶ Several studies tried to simulate water sublimation in freeze-drying and the mean to control it as a fundamental product quality, but still a tool to map moisture distribution is not yet available.^{104,107,108} It is suggested that drying is faster on the top and along the wall of the vial than at the center due to better heat and mass transfer.¹⁰⁹ One study supports this argument by pointing out that the top sections of the freeze-dried cakes of dextran, HSA, and bovine somatotropine have less moisture than the bottom sections.¹⁰⁹ The faster drying rate

along the vial wall is still yet to be understood, although cake shrinkage may help to detach the drying solid from the vial wall and open new interface for facilitated water evaporation.¹⁰⁹

In spray-drying, water evaporation starts at the surface of each droplet. When a shell has formed, a significant decrease in evaporation rate is expected and the drying kinetics keep falling.^{10,11} The temperature in the spray-dryer now greatly influences further drying, especially whether it is below or above the solvent boiling point. Above the boiling point, there are strong convection and potentially bubble formation within the droplet core that can break the particle or create holes to facilitate water evaporation. If no particle breakdown is observed, a moisture gradient may be developed in the drying droplet following Fickian diffusion.¹¹⁰ Spray-dried particles are expected to have a less inhomogeneous moisture distribution compared to freeze-dried cakes as their small size permits better heat and mass transfer. Nevertheless, there is the belief that the moisture gradient may mitigate after 1 day of storage. Apart from theoretical assumptions, no concrete evidence about the phenomenon of water distribution in freeze-drying and spray-drying formulation has been provided.

The Consequences of Different Types of Inhomogeneity on Protein Stability

Destabilization Caused by Protein-Stabilizer Separation

It is clear that for optimum stability, the protein and the stabilizer should homogeneously be mixed with each other on a molecular level. This promotes both hydrogen bonding and strong immobilization by the amorphous matrix described by the 2 main theories of protein stabilization in the solid state. The separation of proteins from the protective agents is detrimental to their stability because it either alters the protective effect of the amorphous matrix or shifts the equilibrium to favor protein denaturation in the protein-rich domains. Separation also results in areas with increased component concentration that may undergo excipient crystallization.¹⁰² Poor homogeneity between the protein and the stabilizer even at the nanometer scale can correlate with decreased stability.^{92,102,111} There is further evidence that inhomogeneity not only affects protein stability during drying but also during the subsequent storage period.^{6,14–18} The same can be said regarding moisture distribution in protein formulations. Inhomogeneity of moisture distribution that brings down the local T_g in the product close to the storage temperature is unfavorable to formulation stability.¹¹² In some formulations that already have low enough moisture content, the effect of inhomogeneous moisture distribution on the glassy matrix of stabilizers may be negligible. Should the distribution of water during storage happen, moisture inhomogeneity may not be a big issue.¹⁰⁹ Still, in case of extreme inhomogeneity, the dried part of the solid may have proteins to suffer over-drying damage while the area with excess water can experience phase separation or component crystallization.

Following the risk of component separation driven by the increase in concentration, it is also noteworthy to discuss the stress of this concentrated solution on the natural conformation of the protein. The increased concentration during freeze-drying and spray-drying is expected to be detrimental to protein stability.⁹² This is due to the high level of any potential reactants, thereby increasing the rate of chemical reactions. Additionally, high protein concentration has the risk of aggregation, especially with hydrophobic proteins having low aqueous solubility.^{16,113–115} Nevertheless, this ignores the self-stabilization effect of some proteins at high concentration. Bevacizumab aggregation decreases with increase in protein concentration.¹¹⁶ The mechanisms are assumed to be either the formation of more stable multimers or the

minimization of surface effects in multiphase systems.¹¹⁷ β -Lactoglobulin self-stabilizes by the volume exclusion effect or the macromolecular crowding effect arising from nonspecific steric repulsion between molecules.^{118,119} Freezing of lactate dehydrogenase (LDH) produces a freeze-concentrated solution of protein but its stability increases due to the self-stabilizing effect of LDH at high concentration.¹²⁰ As for the concern of protein concentration passing the point of aqueous solubility, the increase may not be significant in freeze-drying as large molecules are not likely to develop a strong concentration gradient as discussed above. The risk of aggregation may still be present in spray-drying or freeze-drying of hydrophobic proteins with low aqueous solubility.

Destabilization Caused by Protein Surface Adsorption

In the case of surface adsorption, protein destabilization is not entirely due to the separation from the stabilizer matrix. The adsorption of protein at any interface locates the molecule between 2 environments of different hydrophobicity. To minimize the system-free energy, protein has to adopt a conformation different than the native state such as forming antiparallel β -sheets at the gas phase or moving hydrophobic groups away from the aqueous phase.^{121,122} The larger the specific surface area, the higher the fraction of protein located at these interfaces which results in increasing risk of conformational changes. The conformational change is often just the first stage of further physical degradation during storage such as aggregation leading to complete deactivation.^{6,56,121–125} Moreover, proteins located at surfaces are more prone to chemical degradation pathways such as oxidation and deamidation as demonstrated with freeze-drying formulations of recombinant human growth hormone (rhGH) and keratinocyte growth factor-2.^{6,126}

Surface Denaturation During Freeze-Drying

The low temperature in freezing can inflict major damage to protein structure. The nature of protein cold denaturation is still unclear, with some evidence pointing toward solvophobic interaction, dissociation of subunits in protein quaternary structure, or decreased free energy of unfolding.¹ Several factors affect protein cold denaturation including the pH, protein structure, protein concentration, and excipients.¹¹⁷ For example, on account of their tendency to undergo subunit dissociation, oligomeric proteins like aldolase and LDH are more susceptible to structural alteration in frozen environments than monomeric proteins.¹²⁷

Protein cold denaturation can be reversible.^{128,129} Some evidence suggests that irreversible denaturation of proteins during freezing may not be entirely due to cold unfolding.^{120,130–132} Several studies have noticed a link between surface-induced damage and freezing-induced damage. The tendency of surface-induced damage in the presence of a Teflon bead-water interface for 6 different proteins was found to be almost linearly correlated with their tendency to incur freezing-induced damage (Fig. 4).¹³³ The freezing-induced damage may be correlated with the surface of ice crystals formed during freezing. It was hypothesized that protein adsorption at the ice-liquid interface can lead to surface-induced denaturation. Therefore, when the freezing rate increases, the ice crystal size decreases and the total ice surface area can increase as well as the fraction of degraded protein. In several studies, it was confirmed that increased ice surface area created by a high freezing rate is unfavorable for protein stability. This was, for example, demonstrated by freeze-drying of a bovine IgG formulation during which the solution was either rapidly frozen (immersion in liquid nitrogen) or slowly frozen ($2^\circ\text{C}/\text{min}$). Fast freezing resulted in a product with a higher specific surface area, consistent with the higher level of insoluble aggregate formation than slow freezing.¹³⁴

Figure 4. Relationship between freeze denaturation and surface denaturation of 6 different proteins: (1) ciliary neurotropic factor; (2) phosphofructokinase; (3) lactate dehydrogenase; (4) glutamate dehydrogenase; (5) interleukin-1 receptor antagonist; and (6) tumor necrosis factor binding protein. pH shifts and salting-out effect were excluded from the experiment set-up. Reprinted with permission from Chang et al.¹³³

Furthermore, several proteins including tissue plasminogen activator, phosphofructokinase, glutamate dehydrogenase, and LDH, all exhibited increased aggregation in rapid cooling.^{133,135} Slow freezing of LDH and alcohol dehydrogenase solutions (about 1°C/min) led to a higher recovery of activity than fast freezing.¹³⁶ Similarly, when the freezing rate increased from 0.5°C to 50°C/min, more insoluble rhGH was found.¹³⁷ Freezing by liquid nitrogen immersion increased the fraction of aggregated rhGH compared to a freezing rate of 1°C/min.¹²⁶ Liquid nitrogen immersion in freeze-drying of LDH and β -galactosidase showed the lowest recovery of activity.¹³⁸ These interpretations, however, can be limited by the overlapping effect of protein cold denaturation. Lower freezing temperatures in some studies can promote cold denaturation, especially if freezing is carried out by liquid nitrogen immersion. Bhatnagar et al. show that although changing the ice nucleation temperature from -2° to -9.5°C during freezing of an LDH-sucrose system increases the ice surface area, there is no noticeable change in LDH enzymatic activity.^{120,139} As LDH cold denaturation temperature is below -28°C , Bhatnagar et al. experiments carried out above this temperature could exclude the effect of cold denaturation and identify if LDH surface-induced denaturation did happen.¹³² This result contradicts with other findings above that LDH is susceptible to surface-induced denaturation at the ice-liquid interface. It is probable that the approach to control the ice surface area, rather by increasing the cooling rate in other studies or using the controlled ice nucleation method in Bhatnagar et al. study, can result in different adsorption behavior of proteins. Therefore, their results may not be comparable. Further research into this problem is needed.

Surface-induced damage not only occurs during freezing but also affects protein stability during storage, with formulations frozen at high cooling rate displaying faster aggregation (Fig. 5).¹³⁵ It is known that storage stability of solid protein formulation is affected by the retaining natural conformation.^{112,117,140,141} Therefore, the lower storage stability of surface-induced denatured protein above is expected. Protein formulation produced by spray-

Figure 5. Optical density (mean over 340–360 nm range) for reconstituted solutions of lyophilized recombinant tissue-type plasminogen activator products upon storage at 50°C. Vial sizes and cooling rates for preparing the samples are shown in the legend. Straight lines represent the least square linear regression of data. Reprinted with permission from Hsu et al.¹³⁵

drying can be subjected to the same consequence of reduced storage stability.

Although most proteins can adsorb at the ice-liquid interface, the subsequent denaturation after adsorption is highly protein-specific and affected by the drying step.^{16,133,134} Freezing alone is not sufficient to cause lasting structural alteration on some proteins such as recombinant human interferon-gamma (rhIFN-g) and IgG.^{16,94,134} Ice-liquid adsorption and denaturation of rhIFN-g and IgG are reversible after freezing, but the later drying step made this change irreversible.¹⁶ A question that remains unanswered is whether surface-induced damage only affects the single layer of molecules at the surface. In one study, rhIFN-g aggregation was reported to happen only at the monolayer of protein adsorbed at the ice-liquid interface.¹⁷ Future research should shed more light on this matter.

Surface Denaturation During Spray-Drying

Upon spray-drying formulations, the air-liquid interface is the only surface available for protein adsorption and denaturation. Trypsin's loss of activity after spray-drying was partly correlated with protein surface coverage.¹⁸ More protein aggregation was found when small-size particles were produced, as seen in the case of spray-drying rhGH.¹⁴² The air-liquid interfacial area is affected by the atomization step. The higher the atomization air flow rate, the smaller the droplet diameter, leading to a larger surface area which in turn results in increased protein aggregation. Spray-drying of rhGH clearly indicates this trend (Fig. 6).¹⁴³ Nevertheless, the significant increase in protein aggregation from Figure 6 (especially at 800 L/h) can also be the result of shear stress from the atomization air.⁶¹ Upon interpretation of results one must be aware of such potential interfering occurrences on protein aggregation. On the other hand, spray-freeze-drying of BSA using various droplet sizes of the atomized liquid confirmed also that a larger total surface area of the powder increased protein aggregation.¹⁴⁴ Spray-drying of other structures composed of proteins like viruses can also be subjected to surface denaturation.¹⁴⁵

Destabilization Caused by Excipient Crystallization

In the event of inhomogeneity due to the crystallization of stabilizers, their pivotal interaction with protein is lost and so is

Figure 6. Effect of atomization air rate on the formation of insoluble and soluble aggregates in hGH during atomization of 2 mg/mL mannitol-formulated bulk at room temperature. Reprinted with permission from Mumenthaler et al.¹⁴³

protein stabilization effect.^{1,30,146–149} Trehalose crystallization after freeze-drying, even followed by subsequent amorphization, increased the amount of aggregated monoclonal antibody regardless of the amorphous state.^{52,150} After spray-drying or freeze-drying, amorphous stabilizers can crystallize after exposure to high relative humidities and relatively high temperatures.^{147,151} Processing of an amorphous product can also introduce stabilizer crystallization such as in the case of compaction-induced crystallization of trehalose in freeze-dried trehalose-alkaline phosphatase.¹⁵²

Crystallization of buffers yields a distinct effect on protein stability. The pH of the liquid phase can change significantly if one salt in the buffer crystallizes, affecting the protein conformation.^{98,99} Freezing an LDH solution with phosphate buffer had its pH dropped from 7.5 to 4.5 upon crystallization of Na₂HPO₄ and K₂HPO₄, causing protein denaturation.^{153,154} Formation of bovine and human IgG aggregates during freezing was probably the result of a pH shift following sodium phosphate buffer crystallization.¹³⁴ Succinate buffer pH 5 can crystallize as its monosodium salt, reducing environmental pH by 1 to 2 units which was shown to damage rhIFN-γ.¹⁵⁵

Techniques to Analyze Inhomogeneous Component Distribution

The following section describes a variety of techniques that can be used to characterize the homogeneity of component distribution. It includes several conventionally used methods such as electron microscopy and DSC and highlights some techniques with potentially better sensitivity that are yet to be widely utilized. The physical phenomenon behind each technique is complex and thus this section only presents a summary with relevant information regarding the analysis of component inhomogeneity. A brief summary of each technique can be found in Table 1.

Conventional Solid-State Analysis

Electron Microscopy

Electron microscopy is a well-established method that uses a beam of electrons instead of photon light to visualize the sample with a very high resolution. The scanning electron microscope (SEM) and the transmission electron microscope (TEM) are 2 main forms of electron microscopy. In SEM, the microscope detects secondary electrons emitted from excited atoms in the sample, while

in TEM the projection image is generated by analyzing electrons that pass through the sample. Various applications of SEM and TEM for the characterization of solid pharmaceuticals can be found in the literature.¹⁵⁶

Despite being frequently used to characterize sample morphology or structure, images generated by SEM and TEM lack the chemical information needed to understand the distribution of components. Thus, inhomogeneous distribution can only be detected when a distinctive second phase is formed, making its extension from the main solid structure observable (Fig. 7).^{15,187} The phase separation of freeze-dried PEG-dextran presented in Figure 7 is in agreement with the fact that PEG-dextran system can easily separate in the aqueous solution at the same ratio at high concentration.^{14,15}

Because conventional SEM and TEM are very limited in investigating component distribution, they have been modified to provide the lacking chemical information.¹⁵⁶ These modifications include energy-dispersive X-ray spectroscopy and Auger electron spectroscopy, electron energy-loss spectroscopy (EELS), and energy-filtered electron microscopy. Energy-dispersive X-ray spectroscopy and Auger electron spectroscopy are based on the same principle of electron redistribution in the atom-shell after ionization during TEM. The electron transition can result in either an X-ray or Auger electron, which are subsequently detected and analyzed.¹⁵⁶ An EELS instrument detects and analyzes the energy distribution of electrons passing through the sample in scanning transmission electron microscopy. EELS is very sensitive to light elements like carbon, nitrogen, and oxygen. Ricarte et al.¹⁵⁷ used EELS to evaluate the mixing state of phenytoin and HPMC acetate succinate amorphous solid dispersion and found that the resolution of this technique was less than 200 nm. On the other hand, the energy-filtered electron microscopy instrument selects only specific electrons with a certain energy loss to analyze the diffraction pattern.¹⁵⁶ Alternatively, a combination of SEM or TEM with other techniques such as micro-Raman analysis has shown to be a promising approach.¹⁸⁸ Although these methods hold many advantages, they have not been yet applied to analyze inhomogeneity of protein formulation.

Differential Scanning Calorimetry

DSC is an analytical technique that is widely applied in pharmaceutical formulation sciences. DSC measures the thermodynamics of heat-induced transitions in solid materials, for instance: crystallization, glass transition, crystal melting, and degradation. Characteristics of thermal transitions like the temperature, shape, and number of transitions are important for sample assessment and provide information on the structural arrangement of components in a solid.^{7,55} The introduction of modulated DSC further improved the sensitivity to record thermal transitions by separating the heat flow that does and does not respond to a changing heating rate also referred to as reversing and non-reversing heat flow, respectively. Because most formulations after freeze-drying and spray-drying are amorphous, the T_g is primarily used to evaluate components miscibility.^{50,55,58} In the ideal situation of homogeneous distribution, only 1 T_g can be found, and it can be estimated by using the Gordon-Taylor equation for 2-component systems:

$$T_g = (w_1 T_{g1} + K w_2 T_{g2}) / (w_1 + K w_2)^{189}$$

where w_1 and w_2 and T_{g1} and T_{g2} are the weight fractions and glass transition temperatures (in Kelvin) of the first and second component, respectively. K is a constant that can be estimated from the densities of the components or the change in heat capacities

Table 1
An Overview of Currently Available Techniques to Analyze Inhomogeneous Component Distribution

Analytical Techniques	Obtained Information	Advantages	Disadvantages	Examples of Analyzed Systems	Modifications
Conventional solid-state analysis					
Electron microscopy	Sample texture phase separation analysis in the presence of a distinctive second phase	High image resolution	Lack of chemical information	<ul style="list-style-type: none"> PEG 3350-dextran 500000¹⁵ 	Addition of chemical information: <ul style="list-style-type: none"> Energy-dispersive X-ray spectroscopy¹⁵⁶ Auger electron spectroscopy¹⁵⁶ Electron energy-loss spectroscopy^{156,157} Energy-filtered electron microscopy¹⁵⁶
Differential scanning calorimetry	Thermal transition properties and phase separation	Widely available equipment Small sample size, rapid analysis	Unable to detect phase separation of strong glass formers and inhomogeneity in case the domain size is below 30 nm	<ul style="list-style-type: none"> Almost every protein formulation produced by freeze-drying and spray-drying 	Improved sensitivity: <ul style="list-style-type: none"> Microthermal¹¹ Nanothermal analysis¹⁵⁸
X-ray diffraction (PDF function)	Crystallinity Amorphous component separation	Widely available equipment	Rather novel technique with limited experience and validation Semi-quantitative technique	<ul style="list-style-type: none"> PVP 30000-dextran 10000⁵⁰ PVP 10,000-dextran 5000 and PVP 29,000-dextran 10,000¹⁵⁹ 	
Solid-state nuclear magnetic resonance	Component-component interaction and miscibility	Detects immiscibility at very small scale of 2–5 nm	Low accessibility. Sophisticated data analysis	<ul style="list-style-type: none"> IgG-saccharide (dextran, inulin, or trehalose)¹¹¹ 	
Surface characterization					
X-ray photoelectron spectroscopy-electron spectroscopy for chemical analysis	Surface elemental composition and bonding	Surface sensitive (below 10 nm penetration depth)	Interference from components with similar atom compositions	<ul style="list-style-type: none"> rhIFN-g-trehalose¹⁷ Trypsin-saccharide (lactose, sucrose, dextrin, mannitol)¹⁸ Whey protein-lactose-soybean oil emulsions⁵⁹ Albumin-DPPC-saccharide (lactose, trehalose, or mannitol)⁶⁷ 	
Time of flight secondary ion mass spectrometry	Surface and close-to-surface chemical information	Highly surface sensitive (1–2 nm penetration depth) High molecular specificity	Complex data analysis Low accessibility	<ul style="list-style-type: none"> rhIFN-g-trehalose-Tween 80¹⁷ 	Surface chemical mapping: <ul style="list-style-type: none"> ToF-SIMS imaging^{160,161}
Surface tension characterization	Dynamic and equilibrium surface tension reduction	Simple and fast testing method	Indirect prediction	<ul style="list-style-type: none"> BSA-trehalose-polymers (HPMC or Poloxamer 188)¹⁶² Trypsin-lactose¹⁸ rhGH-Tween 20¹⁴² 	
Sub-surface imaging using spectroscopic techniques					
Fluorescent spectroscopy (coupled with a confocal microscope)	Visualization of labeled component distribution	Widely available equipment Fast data acquisition Can create a 3-dimensional image of chemical distribution	Require labeling of component, not all chemicals can be labeled Can change the component properties significantly Noise from unremoved fluorescent label and autofluorescence Relatively low depth penetration	<ul style="list-style-type: none"> BSA-trehalose-polymers (HPMC or Poloxamer 188)¹⁶² Protein (BSA or β-lactoglobulin)-dextran 70000⁶⁴ BSA-dextran T70¹⁶³ 	Improved penetration depth: <ul style="list-style-type: none"> Two-photon microscopy, three-photon-microscopy^{164–166}
Infrared spectroscopy (coupled with a confocal microscope)	Components interaction Chemical homogeneity Component distribution	Direct analysis Higher depth penetration	Low image resolution Interference of sample moisture	<ul style="list-style-type: none"> Trehalose-lysozyme¹⁶⁷ 	Improved resolution: <ul style="list-style-type: none"> Confocal IR microscopy^{168,169} Atomic force microscopy coupled IR^{170,171}
Raman spectroscopy (coupled with a confocal microscope)	Visualization of component distribution	Direct analysis A wealth of chemical information No sample	Weak Raman signal, low sensitivity Samples burning in imaging of small pixels Relatively low depth penetration	<ul style="list-style-type: none"> Acacia gum-maltodextrin-sunflower oil¹⁷² Lactose-macromolecules (BSA, HPMC, and poloxamer)⁸⁹ 	Improved resolution: <ul style="list-style-type: none"> Nearfield scanning optical microscopy set-up for Raman microscopy¹⁷⁴

Table 1 (continued)

Analytical Techniques	Obtained Information	Advantages	Disadvantages	Examples of Analyzed Systems	Modifications
		moisture interference		<ul style="list-style-type: none"> • HPMC-maltodextrin⁴⁷ • PVP 10,000-dextran 5000 and PVP 29,000-dextran 10,000¹⁵⁹ • PVP 29000-dextran 10000⁵³ • Lysozyme-saccharide (trehalose or isomaltose) and β-lactoglobulin-saccharide (dextran or trehalose)¹⁷³ 	<ul style="list-style-type: none"> • Raman atomic force microscopy and Raman-SEM^{175–179} • Improved signal intensity and chemical sensitivity: • Resonance Raman scattering^{180,181} • Surface-enhanced Raman scattering¹⁸² • Tip-enhanced Raman scattering^{183,184} • Stimulated Raman scattering¹⁸⁵ • Coherent anti-Stokes Raman scattering¹⁸⁶

during the glass transition.^{190–192} But usually, K is considered as a fitting parameter.

Sample analysis using DSC is rapid and requires only a few milligrams of sample. The presence of more than 1 Tg or a crystallization or melting event indicates the presence of separated phases. Nonetheless, DSC faces certain difficulties in characterizing the inhomogeneity of solid protein formulations. First, thermal transitions of different components (proteins, stabilizers, and other excipients) must occur at substantially different temperatures to accurately identify separated thermal event should immiscibility exist. Strong glass formers like protein and high molecular weight stabilizers usually have broad glass-to-rubber transitions which can overlap with each other in the thermogram.^{50,53,159,173} In addition, the change in heat capacity during the glass-to-rubber transition of amorphous proteins or polymers is generally small making it often undetectable in the DSC thermogram.^{55,173,193} Second, the separation of components must be significant enough (more than 20% of the total content) to be detectable under DSC thermogram.^{55,194} Furthermore, the size of the separated phase must be larger than 30 nm to generate a thermal event distinctive enough in DSC measurement.⁴⁸ When there is a gradient of the ratio of amorphous components present in a sample (e.g., from the surface to the bulk of the sample), there is no strict boundary between different phases and the gradual change of the Tg in the sample cannot be detected by DSC analysis. Even though observing at least 2 separated Tg is difficult, a notable deviation of the primary Tg from the theoretical value calculated in the Gordon-Taylor equation can be taken as a sign of possible inhomogeneity of the protein formulation.^{49,51} Deviations from the predicted Tg can be the result of low excipients interaction.⁵¹ The agreement of practical with theoretical Tg alone is not yet a sign of homogeneous distribution. In freeze-dried ribonuclease A-trehalose-hydroxyethyl-starch formulation, a good agreement between predicted

and practical Tg was found.⁴⁸ It is difficult to conclude that there is no inhomogeneity in this system which consists of 2 macromolecules and a small saccharide given the fact that inhomogeneity is detected in freeze-dried lysozyme-trehalose.¹⁰⁰ Approaches to break the limitation of DSC in detecting separated domains smaller than 30 nm can be found in local thermal analysis such as microthermal and nanothermal analysis.^{11,158}

X-Ray Diffraction With Pair Distribution Function

XRD is primarily used to identify crystals in the sample, often in combination with DSC.^{48,195,196} The crystalline phase creates a light diffraction pattern called the Bragg's peak that is specific to the crystal structure periodic array with a long-range order of atoms. Recently, a combination with computational analysis called pair distribution function (PDF) can extend XRD capability to analyze the component homogeneity of even amorphous protein formulations.⁴⁸ In XRD-PDF, XRD data of pure amorphous components and the formulation are taken and Fourier transformed into PDF profile. If a sample PDF profile matches with the theoretical profile calculated from the summation of the PDF profiles of the pure components, the components are present in separated phases.^{48,50,197} As opposed to a matching profile, a non-matching profile indicates that the local organization differs from the pure components and thus excipients are considered at least partially miscible. XRD-PDF was able to indicate phase separation of a freeze-dried PVP 30-dextran 10 mixture, a separation which was confirmed by modulated DSC analysis.⁵⁰ The precision of XRD-PDF in analyzing inhomogeneity was comparable to confocal Raman microscopy, another method that will be discussed below.¹⁹⁸ Due to the scarcity of literature, it would be interesting to see the full extent of XRD-PDF capability in analyzing the homogeneity of solid protein formulations further

Figure 7. SEM images of freeze-dried cakes showing the emergence of the PEG 3350-dextran 500000 phase separation. Samples contain 4% PEG, 4% dextran, and 10 mg/mL hemoglobin were annealed at -7°C prior to freezing for (a) 0 h, (b) 6 h, and (c) 12 h. Reprinted with permission from Heller et al.¹⁵

evaluated because it can greatly extend the application of the widely available XRD equipment.

Solid-State Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is a physical phenomenon in which nuclei in a constant magnetic field are perturbed by another weak oscillating magnetic field and produce an electromagnetic signal with a frequency typical for its magnetic field. The NMR spectrum provides a wealth of chemical information comprising chemical mobility, bonding, and interaction between molecules. Specifically, in formulation science, the homogeneity of solid protein pharmaceuticals can be characterized by solid-state NMR (ssNMR). ssNMR is extremely sensitive because it can analyze the mixing state of components at very small domain size (2–5 nm) unlike DSC, which requires the separated domains to be at least 30 nm.^{111,199} Freeze-dried and spray-dried formulations can be characterized by ¹H ssNMR, with spin-lattice relaxation time (¹H T₁) and rotating frame spin-lattice relaxation time (¹H T_{1ρ}) being the main indicators of the mixing state of components. The procedure for protein formulation analysis is similar to that of the widely used drug-polymer solid dispersion mixing state characterization.^{111,199,200} ssNMR has been used to characterize various freeze-dried IgG-saccharide formulations. It was found that at a 2–5 nm scale dextran and IgG display phase separation while trehalose and IgG were miscible.¹¹¹

Surface Characterization

X-ray Photoelectron Spectroscopy-Electron Spectroscopy for Chemical Analysis

With X-ray photoelectron spectroscopy (XPS), also known as electron spectroscopy for chemical analysis, samples are irradiated with X-rays and the ejected electrons are analyzed by measuring their quantity and kinetic energy. XPS allows identification and quantification of the elemental composition and reveals how these elements are bonded (i.e., hybridization of an atom) at the surface of a sample.^{18,87,201,202} Because XPS detects electrons that can escape from the sample, signal coming from the sample surface is much stronger than the signal beneath the surface. This makes XPS a surface-sensitive method and thus suitable to analyze protein surface adsorption. Surface protein is measured by probing nitrogen atoms which are abundantly present in the protein of a formulation. There is a plethora of XPS application examples on surface protein analysis in both freeze-drying and spray-drying formulations.^{17,18,59,67,203} Despite the relative abundance of equipment, XPS is limited to detection of inhomogeneous distribution at the surface level. It is difficult to conclude if the surface enrichment is only a thin coating layer or there is a completely separated phase that extends beneath the surface as the penetration depth of XPS is about 10 nm.⁸⁷ Additionally, there are other excipients containing nitrogen atoms (polymers like PVP, chitosan, another protein such as BSA, amino acids, or buffers like HEPES) that can interfere with protein quantification.^{1,204} In these cases sulfur atoms in methionine and cysteine can be an alternative indicator if they are relatively abundant in the protein structure.^{205,206} The limit of detection might suffer because sulfur is not present in protein as much as nitrogen.^{207–209} In the experience of authors, XPS analysis takes a long time and is not ideal to study surface composition on a large scale.

Time of Flight Secondary Ion Mass Spectrometry

Time of flight secondary ion mass spectrometry (ToF-SIMS) is a form of secondary ion mass spectrometry that uses a focused ion beam to sputter the sample surface in pulses of a few hundred picoseconds to several nanoseconds.²¹⁰ Molecules at the surface are

ionized, ejected, and sometimes fragmented from the original larger structure, which are called secondary ions. Secondary ions are then accelerated in a constant electric field after which they travel through a field-free space before hitting the detector. The time it takes for secondary ions to reach the detector after ejection is measured. The heavier the molecule the slower its ion travels, and as a consequence large structures like proteins and polymers are among the last objects to hit the detector.²¹¹ Similar to XPS, ToF-SIMS is a surface-sensitive chemical analysis technique. However, the molecular specificity is much higher because ToF-SIMS can fragmentize, separate, and analyze the ejected ions.^{211,212} Moreover, the penetration depth of ToF-SIMS is only 1–2 nm, making it much more surface-specific than XPS.²¹³ In addition, ToF-SIMS can be used as a surface chemical imaging method.^{160,161} By scanning a fine-focused ion beam over the surface, like an electron beam in an electron microprobe, mass resolved secondary ion images (chemical maps) can be obtained simultaneously as shown in Figure 8.¹⁶⁰ Further sputtering reveals the chemical information below the sample top surface layer.¹⁶⁰ Despite the high surface sensitivity and chemical specificity, ToF-SIMS can only analyze inhomogeneity at or close to the surface like XPS. Data analysis of complex sample and accessibility of the equipment are major issues hindering the application of ToF-SIMS. The time required to run ToF-SIMS can be significant, but quantitative data and high chemical specificity are an advantage of this method.

Surface Tension Characterization

Unlike XPS and ToF-SIMS, measuring the surface tension at the air-liquid interface is an indirect approach to predict surface inhomogeneity of spray-dried formulations.^{18,162} Surface tension can be measured using a tensiometer, which operates under different concepts like goniometer, Du Noüy ring tensiometer, Wilhelmy plate tensiometer, Du Noüy-Padday method, and bubble pressure tensiometer.^{18,142,162} Among them, the goniometer, or the pendant drop technique, is perhaps the most common method to determine the surface tension. In this method, the characteristics such as diameter and weight of liquid droplets that slowly form out of a capillary are measured. The basic interpretation is the direct relationship between the weight that the droplet starts to fall out of the capillary and the liquid surface tension. More sophisticated interpretation with droplet diameter can be carried out using the Young-Laplace equation recorded by a camera and analyzed using

Figure 8. ToF-SIMS image of a microsphere surface 149 μm in diameter generated from the diagnostic anions identified within the ToF-SIMS spectra. The overlay shows poly(lactic-co-glycolic) acid (green), PVA (blue), and lysozyme (red). Reprinted with permission from Rafati et al.¹⁶⁰

special software. The surface chemical composition is predicted by knowing both the surface tension reduction dynamics (an indication of adsorption kinetics) and equilibrium state due to the adsorption of proteins and other surface-active molecules. If a component strongly reduces the surface tension, the particle surface will likely be enriched with it after spray-drying. In case one component reduces the surface tension faster than the other, it potentially adsorbs at the surface at a greater portion. Moreover, if the gradual addition of another excipient can continuously lower the surface tension of the protein solution, the protein is likely being progressively replaced from the surface by the second agent. Several publications describe how the surface tension can predict the presence of protein at the solid surface of spray-dried formulations depending on the presence of other excipients.^{18,142,162} Thanks to its simplicity, surface tension measurement is suitable for preliminary testing in formulation development.

Sub-surface Imaging Using Spectroscopic Techniques

Fluorescent Spectroscopy

Fluorescent microscopy has been the core of biomedical imaging for a long time and has been applied to characterize the distribution of components in solid protein products. When a fluorescent detector is coupled to a confocal microscope, chemical distribution can be observed not only on the surface sample but also from within the sample through optical sectioning. By stacking the images of different depth levels, a three-dimensional view can be obtained. Before visualization, components need to be labeled with fluorescent agents. For example, BSA incorporated in spray-dried particles can be observed by confocal fluorescent microscopy after labeling with fluorescein isothiocyanate.¹⁶² N-(1-pyrenemethyl)iodoacetamide labeling, an oxygen-sensitive fluorescent agent, can allow the specific analysis of labeled molecule on the sample surface.⁶⁴ Under an oxygen-free environment, all labeled molecules are illuminated, but subsequent oxygen exposure will quench the fluorescent signal of only surface molecules. Subtracting the original signal with the quenched signal can determine the amount of component at the surface quantitatively.^{64,163} The competitive surface adsorption behavior between BSA and β -lactoglobulin after spray-drying was revealed using this method.¹⁶³ One drawback of fluorescent labeling is that it can modify the hydrophobicity of the labeled component, which affects the interfacial affinity and surface adsorption. In some extreme cases, labeled protein even precipitates due to the introduction of hydrophobic surfaces and potential hydrophobic interactions.^{214,215} A more hydrophilic fluorophore can be selected to circumvent these situations. Furthermore, the sample can exhibit background fluorescence due to unbound fluorophores or autofluorescence of other components in the formulation. It is important that after labeling the protein, the free fluorescent agent should be removed. Another disadvantage of fluorescent spectroscopy is to visualize the distribution of all components, all of them need to be labeled with different fluorophores, but not all molecules may possess suitable chemical groups for covalent linkage with the fluorophores.

One factor that may affect the visualization of the sub-surface chemical distribution is the penetration depth of the microscopic system. The penetration depth depends on the ability of excitation laser to reach the focus unscattered and the emitted fluorescence to reach the detector.¹⁶⁴ The excitation laser may pass through small spray-dried particles (10–20 μm), but deeper visualization of thick samples or formulation with translucent materials can be difficult.¹⁶⁵ Two-photon microscopy can bypass this restriction by using photons coming from 2 independent long-wavelength high-power laser beams in pulses of femtosecond to nanosecond to focus and simultaneously light up the fluorophore at the designated depth

spot.^{164–166} The longer wavelength significantly improves the penetration depth, reaching up to 1 mm depth in some studies.^{164,216} The low energy of each laser prevents fluorescence excitation on the traveling path at the focus point, further reducing the background signal.¹⁶⁵ Even in samples with high density it can reach up to 140 μm for sufficient depth profiling of component distribution.¹⁶⁵ Similarly, 3 or more photons can be combined to cause fluorescence excitation in imaging. A compromise when employing 2-photon microscopy is a small reduction in image resolution, but it can be negligible with optimal laser beam setup.^{166,217}

IR Spectroscopy

IR spectroscopy is commonly used to study the mixing state of drug and polymer in amorphous solid dispersions. IR spectroscopy detects the IR light wavenumber at which the vibration of a functional group with a dipole moment is excited. Component miscibility is characterized by analyzing the interactions between different components in a sample that appear in the IR spectra, for example, hydrogen bonds between protein and sugar.^{218,219} Thus, conventional IR is a bulk analysis rather than a technique to map chemical components in the sample for which IR imaging is capable of. IR imaging was first demonstrated in a physical mixture of trehalose and lysozyme using near-IR imaging, indeed showing phase separation as expected.¹⁶⁷ The spatial resolution in this work was quite poor (10–40 μm) which was due to the not very sophisticated equipment available at that time.^{167,220} Nowadays, however, there are several IR microscopy systems that work in the IR and near-IR region capable of providing better spatial resolution to map component distribution.^{221,222} An advantage of long-wavelength light in IR is that a higher penetration can be achieved, reaching up to 2–3 mm depending on the wavelength.^{223–225} Unfortunately, relatively long wavelengths often result in low spatial resolution of around a few μm .^{226,227} Several approaches are under development to improve the resolution of IR microscopy, which include the use of a confocal setup using sCMOS detector with a more effective photomultiplier tube and near-field optical microscope.^{168,169} Combination of IR with atomic force microscopy is a recently introduced approach that can visualize component inhomogeneity at a nanometer resolution.^{170,171} Because near-IR spectroscopy can be used to monitor the moisture content in freeze-dry cake, IR microscopy may be used to visualize water distribution in protein formulation.^{106,228}

Raman Spectroscopy

Raman spectra are recorded differently than both IR and fluorescent spectroscopy where either light adsorption, emission, or reflection is measured. Raman spectroscopy explores the inelastic scattering of light (Raman²²⁹ shift) where incident photons lose or gain energy after interaction with the molecule, which is termed Stokes and anti-Stokes scattering, respectively. Raman spectra are presented as the difference in energy (in wavenumbers) between the incident and scattered light. Anti-Stokes scattering is by far a less common phenomenon than Stokes scattering and thus the majority of Raman spectroscopic equipment employs Stokes scattering to measure the Raman spectra.

Just like fluorescence and IR spectroscopy, Raman spectroscopy can also be combined with an optical microscope to enable chemical mapping of a sample. Confocal Raman microscopy is suitable for direct analysis of solid protein formulations without any sample modification. As with other spectroscopic imaging methods, Raman microscopy obtains the spectrum of each pixel from the sample and deconvolutes it to visualize the chemical distribution in the field of view. A distinct separation in a spray-dried maltodextrin-acacia gum-sunflower oil formulation

Figure 9. Internal mapping by confocal Raman microscopy of spray-dried particles produced from the same emulsion consist of acacia gum (red), maltodextrin (blue), and sunflower oil (yellow) under different condition. More details can be found in the original article. Reprinted with permission from Munoz-Ibanez et al.¹⁷²

demonstrated the capability of confocal Raman microscopy in chemical mapping of complex samples (Fig. 9).¹⁷² Thanks to its high resolution and chemical information, confocal Raman microscopy has been applied extensively in the development of many pharmaceuticals, in particular, to investigate component heterogeneity in tablets.^{53,230–232} There are several examples of applications on macromolecule formulations. Phase separation in freeze-dried PVP-dextran mixtures was detected by confocal Raman microscope which corresponded with DSC data.^{159,198} Freeze-dried samples of lysozyme-saccharide (trehalose or isomaltose) and β -lactoglobulin-saccharide (dextran or trehalose) were found to be phases separated using confocal Raman microscopy.¹⁷³ Binary systems consisting of lactose and either BSA, HPMC, or triblock copolymer poloxamer obtained by single droplet drying showed separate domains enriched with macromolecules under Raman microscopy.⁸⁹ Similarly, confocal Raman microscopy images of HPMC-maltodextrin mixtures dried by film drying, single-particle drying, and spray-drying showed distinctive domains specific to the ratio of 2 components.⁴⁷

Because water is a weak Raman scatterer, sample moisture is unlikely to interfere with spectrum acquisition in Raman microscopy.¹⁵⁹ Image acquisition can be optimized to significantly reduce the measurement time of one sample from 2.5 to 0.5 h.¹⁷³ However, due to the rare nature of inelastic scattering, Raman transitions are often weak and signal emitted from components with low concentration or being encapsulated in samples with low optical transparency are difficult to detect using Raman microscopy.^{160,233,234} Inhomogeneity in small particles produced by spray-drying formulations may be hard to characterize due to the resolution limit of the confocal microscope, and focusing the laser beam on very small pixel can cause the sample burning.^{235,236} Therefore, prior beam adjustment can be vital to image acquisition, including laser intensity and exposure time. In advanced microscopes equipped with interchangeable lasers and diffraction gratings, the excitation wavelength is also adjustable. Because the scattering magnitude is inversely proportional to the laser wavelength of the fourth power, Raman signal intensity is much higher when using short wavelength lasers and the spatial resolution is also improved. However, shorter wavelength lasers can induce autofluorescence (or background noise) and disturb the Raman signal.²³⁷ In addition, as with other light-based techniques, depth penetration can be an issue in Raman imaging, especially in non-transparent or translucent samples. Depending on the laser source, imaging can reach up to 60 μm when using longer wavelength (1528 nm) while being limited to 30 μm in case of shorter wavelength (816.7 nm).²³⁸

To overcome the spatial resolution limit, several modified Raman setups have been explored. Introducing the aperture-based setup of Nearfield Scanning Optical Microscopy to Raman can improve the lateral resolution to below 100 nm.¹⁷⁴ By using different methods and correlating the data, such as Raman-atomic force microscopy and Raman-SEM, a more complete understanding of inhomogeneity can be obtained.^{175–179} These combined systems offer a high spatial resolution in addition to the chemical information of Raman spectroscopy. To tackle the sensitivity issue, other forms of Raman spectroscopy can be employed like surface-enhanced Raman, resonance Raman, tip-enhanced Raman (TERS), stimulated Raman (SRS), and Coherent anti-Stokes Raman scattering (CARS). They enhance the Raman signal taking advantage of Raman scattering being intensified in the presence of nearby metal (surface-enhanced Raman, TERS) or when the exciting laser beams match with the vibrational energy transitions of the molecule (SRS, CARS, resonance Raman).^{180–186,239,240} Among these techniques, SRS seems to be the most promising that is still being further improved. The physical principles behind these techniques are complex and thus beyond the scope of this review, but readers can follow a good description and discussion of Opilik et al. and Wei and Min.^{241,242} Despite tremendous advances, these novel yet promising techniques still suffer specific problems such as the scanning tip instability in TERS, the Raman band broadening in resonance Raman, or the background signal in CARS.^{181,183,184} However, the biggest hindrance would be their availability because most of them are only found in specialized labs, limiting their application in analyzing the inhomogeneity of solid protein formulations.

Opinions on the Detection of Inhomogeneity in Protein Formulations

A mixture of 2 components can appear miscible using analytical techniques that measure large domains (DSC, electron microscope) while showing phase separation with techniques that can analyze smaller domains (ssNMR, spectroscopic imaging such as confocal Raman microscopy and possibly fluorescent or IR microscopy).¹¹¹ This is especially important in amorphous formulations comprising of strong glass formers (proteins, polymers) with difficult to detect or overlapping thermal transitions or when the phase borders are unclear (typically the concentration gradient in spray-dried particles). In many instances, inhomogeneity was identified with confocal Raman microscopy while DSC analysis failed to detect component separation.^{159,173,198} Given the complexity of the phase behavior of solid protein formulation, it is often necessary to combine different analytic techniques to

characterize sample inhomogeneity. With a relatively quick analysis and the wealth of chemical information, Raman microscopy seems to be the most capable technique available. Further characterization can be carried out with other techniques to gather quantitative or complementary data. Many techniques introduced previously are still yet to be utilized to study the homogeneity of solid protein pharmaceuticals. Their current major obstacles are the equipment cost, the requirement of expert users, and the low throughput which should all be addressed before widespread application.²⁸

Possible Solutions to Alleviate Component Inhomogeneity and Its Effect on Protein Stability

Mitigating the Surface Adsorption of Proteins

Protein surface adsorption and denaturation is a common process that appears during both freeze-drying and spray-drying. Some formulations can be more vulnerable than others. Those that contain surface-sensitive proteins carry a major risk of surface denaturation. Furthermore, production processes that generate a huge surface area are at risk for surface denaturation. For example, equipment like spray-driers typically produce very small particles (below 5 μm in diameter) that have a large total surface area and their products are likely to suffer a higher risk of surface-induced denaturation of proteins.^{88,243–245} Similarly, freeze-drying processes that use liquid nitrogen immersion can create a large ice-liquid interface and incur significant surface denaturation. Modifying the protein structure with genetic (directed evolution, introduction of an extra disulfide bridge) or chemical alteration can create more stable proteins or modify its surface affinity. One example of such approaches is the improved stability of PEGylated hemoglobin, but it can be time-consuming and negatively affect the protein natural function.^{246–248} To overcome protein surface adsorption and denaturation, 2 approaches are available.

Replacing Proteins at the Surface by Other Surface-Active Agents

The addition of surface-active agents can competitively adsorb and exclude the protein from the air-liquid and ice-liquid interfaces, preventing surface-induced denaturation. Regardless of the chemical structure and the drying process, most surface-active excipients seem to be capable of this task at very low concentration (0.02%–0.12%) even though they cannot completely remove the protein from the surface at these concentrations.^{17,65,120,133,134,142,143,203,249,250} Polysorbate 80 (Tween 80) and polysorbate 20 (Tween 20) are 2 widely used surfactants in marketed protein pharmaceuticals and have been studied extensively for their ability to inhibit protein surface adsorption.^{1,251,252} Surface replacement of protein by Tween 80 in freeze-dried rhIFN- γ formulation was confirmed using ToF-SIMS, corresponding to reduced rhIFN- γ aggregation in formulation with Tween 80 compared to Tween 80-free formulation.¹⁷

Alternatives to conventional surfactants are surface-active polymers as well as other proteins. Poloxamer 188 at 1% completely excluded the BSA from the spray-dried particle surface.¹⁶² HSA was successfully used to protect several cytokines (interleukin-1 α (IL-1 α), IL-1 β , IL-3, and macrophage colony-stimulating factor) after freeze-drying.²⁵³ The presence of 0.05% BSA increased the activity recovery of LDH from as low as 30% during freeze-thawing to 80% after freeze-drying.¹⁵³ Many protein products on the market such as Betaseron®, Kogenate®, and Recombinate™ contain or used to contain human albumin probably to mitigate surface adsorption of the therapeutic protein.¹

Because interfacial adsorption is a competitive process, the amount of surface-active agents may need to scale with the protein

content in the formulation. However, there is a limit to how surfactant can prevent surface adsorption and denaturation of proteins. In one study of freeze-drying IgG, increasing the amount of Tween 80 from 0.02% to 0.1% did not further reduce the aggregation.¹³⁴ Care should be taken to select the proper surfactants as well as their concentration because surfactants can induce protein unfolding. Anionic and cationic surfactants can denature protein by forming the protein-surfactant complex.¹⁸ Polysorbate can undergo auto-oxidation, hydrolysis of the fatty acid ester bond, increase aggregation, or contain peroxide contaminants, all of which are potentially detrimental to protein stability depending on their concentration.^{203,251,253–255} Additionally, surfactants can act as a plasticizer, reducing the T $_g$ of the amorphous matrix that stabilizes the protein.¹⁴⁵ Other pathways via which surfactant can interact with protein leading to unfolding was described by Otzen.²⁵⁶

Reducing the Surface Area by Changing the Freeze-Drying and Spray-Drying Process

Rather than preventing the adsorption of protein to the surface, reducing the air-liquid and ice-liquid interface is an attractive alternative that can be achieved by manipulating the freeze-drying and spray-drying process conditions. Because the ice-liquid interface directly correlates with the cooling rate in freeze-drying, the ice surface can be reduced by avoiding fast freezing and adopting a relatively slow cooling rate or adding an annealing step for surface-sensitive protein.^{47,86–91,95} This approach increases the pore size in the final product that in turn prevents the formation of air bubbles during reconstitution and the potential associated air-liquid interface adsorption and denaturation protein.¹⁶ It was demonstrated that rhIFN- γ was much less aggregated after reconstitution when an annealing step was introduced in the freeze-drying process.^{16,17} However, cooling rate manipulation is possible only if the risk of phase separation from components is minimal.¹⁵⁴ Additionally, too slow cooling rate can be detrimental to protein stability because the increase in component concentration of freeze-concentrated solution can facilitate chemical degradation, especially oxidation.²⁵⁷ A different strategy to control ice surface area is to control the nucleation process, especially the temperature when water starts to crystallize. This is carried out by actively initiating ice nucleation using external stimuli like pressure or ice fog. Samples nucleated at higher temperatures show a lower specific surface area than samples treated at lower temperatures.^{119,137,258} Nevertheless, it is worth noticing that reducing the ice surface area can prolong the secondary drying step, reduce water evaporation rate, and possibly cause moisture retention in the center bottom of the cake.¹⁰⁴ In spray-drying, options to reduce the formation of large interfaces are limited because it requires to increase the droplet diameter. The droplet diameter, however, depends largely on the available equipment, making this approach rather restricted.

Preventing Concentration-Driven and Process-Driven Component Separation

Avoiding Immiscibility, Crystallinity, and Salting-out Salts

The immiscibility problem finds its origin in the incompatibility of components, and thus requires careful selection of excipient as well as their concentration to avoid or mitigate this issue. The ratio between components is a crucial factor while the initial concentration has almost no impact on phase separation after freezing.^{44,52} Increasing the ratio of maltodextrin to HPMC changes the separation pattern of the spray-dried particles, particularly from the HPMC-rich to the maltodextrin-rich surface.⁴⁷ Furthermore, avoiding pH values in the solution that lead to component immiscibility by altering the interaction of protein and other components is fundamental.^{259,260} Easily crystallizing salts and

buffers should not be used, while crystallization of stabilizers can be prevented by using adequate storage condition (low temperature and relative humidity), addition of excipients that increase the Tg like Pullulan, or altering their ratio with protein.^{1,147}

Although the presence of salts and buffers can be detrimental to component homogeneity, the effect is not always negative and it seems to closely follow the Hofmeister lyotropic series.^{261,262} Especially anions, but also cations, can strongly affect the solubility and immiscibility of components such as protein and polymer in a fixed order.²⁶² The basis behind the salting-out effect is the strong interaction of salt and water, reducing proteins and macromolecules solvate layer and thereby their solubility.²⁶³ Salting-in salt affects the solubility and immiscibility of proteins and polymer through interactions with functional groups including non-localized attractive dispersion force binding and localized binding, which in turn improves solubility and prevents component-component interaction or immiscibility.²⁶³ As a result, salting-in salts like NaSCN, KSCN, KI, and KBr that prevent phase separation can be incorporated in the formulation, while salting-out salts like Na₂SO₄, Na₂HPO₄, and KH₂PO₄ should be avoided or included at low concentration only.^{15,261}

Arresting Component Separation by Rapid Solidification

When optimizing the formulation is not possible for practical reasons, changing the freeze-drying or spray-drying process to promote rapid solidification and arrest any possible component separation is a viable option. In freeze-drying, reducing the lifetime of the freeze-concentrated solution is fundamental. This lifetime depends on the cooling rate as well as the annealing step.¹⁵ Rapid freezing by liquid nitrogen immersion was enough to halt phase separation in the hemoglobin-PEG-dextran formulation and reduce protein damage.¹⁸⁷ BSA-trehalose-buffer and IgG2-trehalose-buffer solutions frozen using circulating liquid coolant at -20°C show less freeze concentration and inhomogeneity in the cakes compared to stagnant air-freezer which provides a slower cooling rate.⁹² Freeze-drying of IgG2 at high cooling rate ($-20^{\circ}\text{C}/\text{min}$) leads to insignificant gradient concentration profile and the product has better stability during storage.¹⁰² But, as rapid freezing promotes ice-liquid surface denaturation of protein (on account of the larger liquid-ice surface), this approach is useful only if the protein is less prone to interfacial denaturation. Additionally, this approach is not practical in industrial manufacturing, and usually freeze-dryers have a limit to how fast it can cool the protein solutions. Moreover, fast freezing with low target temperature can lead to cold denaturation. There is also a limit to the fast freezing approach, whereas water crystallization is exothermic and may raise the solution temperature up to -2°C .⁵³ The time required for the vial to reach Tg' again can be long enough for phase separation to occur because industrial freezers do not have a fast enough cooling rate.⁵³ Practical approaches to promote complete ice formation are minimizing fill depth and controlled ice nucleation which have been shown to reduce phase separation to some extent.^{53,159} Together with a relatively high cooling rate, controlled ice nucleation was demonstrated to prevent phase separation.⁵³ Two popular approaches to control ice nucleation are the ice-fog and the depressurization techniques. Further benefits of controlled ice nucleation allow the management of interval homogeneity, ice surface area, and the drying rate.²⁵⁸ More details about controlled ice nucleation can be found in the review of Geidobler and Winter.²⁵⁸ A question that remains is whether different controlled ice nucleation methods, even if they follow the same principle such as the ice fog technique, can lead to comparable products.²⁶⁴

In spray-drying, shortening the droplet's drying and solidification time can provide a similar benefit. This can be carried out by either increasing the concentration of feeding solution or the drying rate

through temperature or flow rate increases. Increasing the formulation's solid content can significantly lower the solvent content and thus the solidification rate, but in turn, it can increase the solution viscosity.^{47,90} If the viscous solution is difficult to handle, it is preferable to increase the drying kinetics instead by raising the drying temperature or air circulation or by decreasing the droplet size given the protein is still stable at high heat.²⁶⁵ However, this approach does not provide any considerable change in product homogeneity, especially protein enrichment at the surface, probably because the spray-drying process is already short for any meaningful alteration.^{47,90,265} Nevertheless, there is an observable difference in one study of spray-drying 2 polymer solutions with higher solid content. Sub-surface mapping reveals that the area below the surface exhibits a better distribution pattern and a blurry phase border of 2 polymers, HPMC and maltodextrin.⁸⁹ The effect of this approach may need more verification. At the moment, inhomogeneity in spray-drying should be tackled in the formulation composition rather than altering the spray-drying process.

Management of Moisture Distribution

Controlling inhomogeneous moisture distribution is still a rarely touched area of protein formulation. The ideas suggested here are merely based on the authors' opinion. In freeze-drying, controlling the moisture distribution is difficult and thus the practical approach so far would be to limit the amount of residual water. Theoretically, this is carried out by changing the water desorption rate and drying time.²⁶⁶ When residual moisture is limited sometimes down to 1% in some formulations, inhomogeneous distribution of water can have insignificant impact on protein stability, although the damage of over-drying should also be considered.¹⁰³ Inhomogeneous moisture distribution in spray-drying, especially at the core of the particles, might be prevented by allowing sufficient time for water to effectively escape the droplet. Formation of particle shell that limits the water movement should be avoided or delayed. Hopefully, future research may pick up this issue and study the control of moisture distribution in freeze-drying and spray-drying of protein formulations.

Outlook and Perspectives

The instability of protein in solid pharmaceuticals remains a major concern, and studies have shown that inhomogeneous component distribution can contribute to protein degradation substantially. Inhomogeneity can arise naturally due to the physicochemical properties of components, or separation can be process-driven. Investigation of different types of component distribution is possible with a diverse range of analytical methods. Some techniques can provide extra information regarding protein-excipient and excipient-excipient interactions like the ssNMR or IR. In general, it requires the application of different analytical techniques to reveal the full extent of inhomogeneity in the sample and understand its origins. Depending on the type of inhomogeneity, the available equipment, and the protein characteristics, different approach to mitigate instability can be considered for an individual formulation. Whether surface adsorption or phase separation in the amorphous form contributes more to protein destabilization is difficult to judge and hard to predict. This should be evaluated on a case-by-case basis. Perhaps in protein-rich formulations, the fraction of protein adsorbed to the surface is relatively small and degradation will depend on the sub-surface area, where component inhomogeneity can arise.⁶⁰ In contrast, surface proteins will comprise a major fraction of total protein amount in stabilizer-rich formulations and denaturation due to surface adsorption is, therefore, more important.⁶⁰ Nevertheless, if the protein is

insensitive to surface denaturation, the sub-surface inhomogeneity may play a major role in instability. Despite many advances, several key aspects are still unknown. Protein instability in the different separated domains typically found in spray-drying formulations has not been reported so far, although in our opinion it can be difficult to isolate denaturation in these small areas from the bulk. The properties of the ice-liquid interface, such as its hydrophobicity, protein adsorption affinity, and tendency to facilitate surface denaturation, remain unclear. Inhomogeneous moisture distribution and its effect on protein stability are yet to be investigated thoroughly. Does the moisture inhomogeneity exist as a strong concentration gradient and how to characterize the moisture distribution are potential topics of further investigation. The differences in diffusion rate of components leading to inhomogeneity during spray-drying also occur during freeze-drying. However, to our knowledge, no studies have been published in which this phenomenon is extensively studied. The slowing effect of low temperature on component diffusion during freezing may need to be taken into account. Finally, although recently developed analytical techniques do meet the requirements to analyze inhomogeneity in solid protein pharmaceuticals, most of them still have problems with resolution and availability. In the techniques section of this paper, we present several methods that are promising but yet to be studied or applied. Initiatives exploring them in the characterization of homogeneity in solid protein formulations may be a next step forward in resolving homogeneity related problems in solid state protein formulations.

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