Molecular Mechanisms for Stabilizing Biologics in the Solid State

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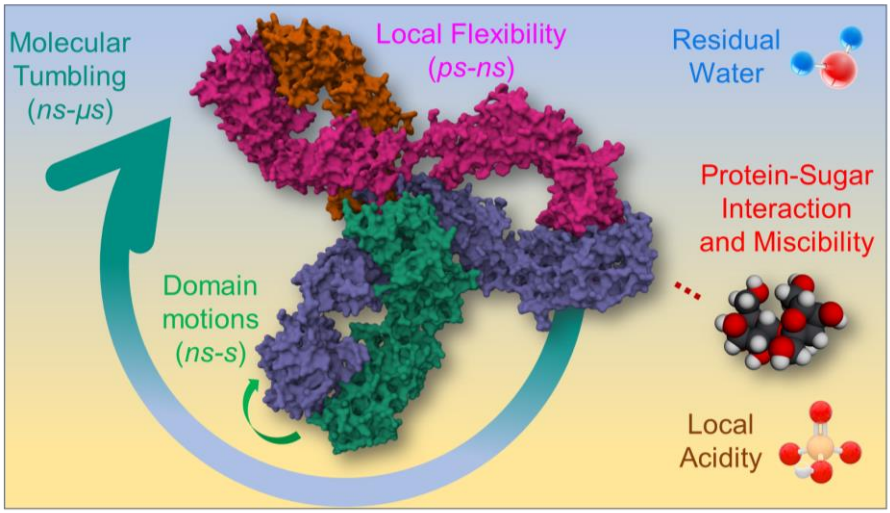
Graphical Abstract



Abstract

Protein drugs exhibit challenges of biophysical and biochemical instability due to their structural complexity and rich dynamics. Solid-state biologics aim to enhance stability by increasing molecular rigidity within the formulation matrix, representing a primary category of drug products alongside sterile liquid formulations. Understanding the molecular mechanisms behind the stabilization and destabilization of protein drugs, influenced by formulation composition and drying processes, provides scientific rationale for drug product design. This review aims to elaborate on the two primary models of water-to-sugar substitution and matrix vitrification, respectively, via thermodynamic and kinetic stabilization. It offers an up-to-date review of experimental investigations into these hypotheses, specifically elucidating protein structure and protein-excipient interactions at the molecular level, molecular dynamics across a broad range of motion regimes, and microscopic attributes such as protein-sugar and protein-salt miscibility and microenvironmental acidity, in relevant liquid, frozen, and solid states, using advanced biophysical techniques for solid-state analysis. Moreover, we discuss how this mechanistic understanding facilitates the investigation and prediction of critical stability behaviors and enables the design of solid biological drug products.

Keywords: Biologics; Lyophilization; Stability; Interaction; Miscibility; Molecular dynamics

Abbreviations

ADC	antibody drug conjugates
AFM	atomic force microscopy
cAMP	cyclic AMP
BSA	Bovine Serum Albumin
CSA	chemical shift anisotropy
cryo-EM	cryogenic electron microscopy
D_2O	deuterium oxide
DAR	drug to antibody ratio
DES	dielectric spectroscopy
DPDH	disodium phosphate hydrogen dodecahydrate
DSC	differential scanning calorimeters
ESD	electrostatic spray drying
FDA	food and drug administration
FTIR	fourier-transform infrared spectroscopy
HDX	hydrogen-deuterium exchange
HDX/MS	hydrogen deuterium exchange mass spectrometry
hGH	human growth hormone
HPSEC	high pressure size exclusion chromatography
HPV	human papillomavirus infection
HSA	human serum albumin
IL-1ra	IL-1 receptor antagonists
IR	infrared spectroscopy
LDH	lactate dehydrogenase
LOVE	liquid-observed vapor exchange
MAS	magic-angle spinning
MD	molecular dynamics
MSD	mean-square displacements
MVD	microwave vacuum drying
NMR	nuclear magnetic resonance
NS	neutron scattering
PBS	phosphate buffered saline
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
ssPL-MS	solid-state photolytic labeling mass spectrometry
SAXS	small angle X-ray scattering
SCF	supercritical fluid
SD	spray drying
SEC	size exclusion chromatography
SEM	scanning electron microscopy
SFD	spray freeze drying
ssNMR	solid state NMR
ssFTIR	solid-state fourier transform infrared spectroscopy

ssHDX	solid state HDX
SXPD	Synchrotron X-ray diffraction
TEM	transmission electron microscopy
TFFD	thin-film freeze-drying
TOF-SIMs	time of flight secondary ion mass spectrometry
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

1. Introduction

Biopharmaceutical products represent a primary category of therapeutics in the modern global pharmaceutical market. There is growing interest in developing peptide, protein, antibody, and vaccine products that have been widely used to treat or prevent a variety of debilitating and life-threatening diseases in immunology, infectious diseases, and oncology. The global biologics market size was valued at USD 419.07 billion in 2023 and is projected to surpass around USD 698.17 billion by 2031.[1] A majority of these products are formulated in the solution and introduced through parenteral administration. 66% of these parenteral protein formulations were manufactured as liquid formulations compared to lyophilized formulations (34%). However, the long-term stability of biologicals in solution often poses challenges in pharmaceutical development. Numerous proteins tend to lose their structural stability in the solutions state, making them prone to undergo changes in their shape or conformation.[2] These alterations can occur due to thermal, mechanical and chemical stresses that proteins experience during purification, processing, shipping, and storage.[3] As such, manufacturing biological products in solid dosage form play a crucial role particularly in enhanced storage stability and ease of transportation.[4, 5]

Specifically, the global lyophilized injectables market was valued at USD 3,365.4 million in 2023 and is projected to reach USD 4,978.3 million by 2030.[6] In biological drug products, both protein-protein and protein-excipient interactions can contribute to stabilizing or de-destabilizing effects. The intermolecular interactions, which occur at a molecular level, can lead to potential problems in maintaining the desired stability of the drug. The drug substances with large molecular size and conformational flexibility have a higher propensity of aggregation which could cause risk in immunogenicity if dosing in vivo. The stability of a protein formulation can often be affected by its active ingredient interacting with the other substances present in the formulation. In solid products, protectant excipients are widely utilized which govern the stability of large molecules, including sugars, polymers, surfactants, etc. Sugars such as disaccharides are often used as stabilizers to retain the native structure of functional biomolecules.[4] Examples include Herceptin and Adcetris, which both include sugar stabilizers such as trehalose or sucrose to increase the storage stability. Surfactants are also commonly used excipients to mitigate the interfacial stress. However, polysorbates can serve as a double-edged sword to induce protein aggregation as a concern of biophysical instability as well as biochemical degradation,[7-10] in addition to the intended function of mitigating the formation of interfacial stress-induced protein particles. Therefore, it is critical to design dry biologic formulations guided by scientific rationales through a mechanistic understanding of the critical attributes, excipient selection, and their impact on protein structure and dynamics.

Besides the scientific design of formulation composition, the development of solid protein formulations requires robust and efficient drying processes. In the past decades, traditional lyophilization technology has been widely utilized.[4] It is known that lyophilization technology is a batch process and time-consuming with very low energy usage efficiency.[11, 12] In recent years, advanced alternative drying methods, e.g., spray drying and lyosphere, are gaining increasing attention in addition to complimentary techniques like microwave drying to improve drying cycle times.[13-16] However, biomolecules can still undergo assorted stress, e.g., temperature, shear stress, and interfacial stress, during drying steps which can induce unfolding, aggregation, and loss of functionality.[17] For example, high temperature in spray drying can induce protein denaturation and rapid removal of water can introduce air-liquid interfacial stress and subsequently result in aggregation. Understanding the impact of formulation process conditions on the protein's structural and dynamic stability can aid in manufacturing solid protein formulation with robust storage stability.

Aside from drying techniques, understanding the specific characteristics that influence the delivery and stability of protein drugs is also crucial in the development of biological products. This knowledge serves as a fundamental basis for comprehending the underlying mechanisms involved in the development process. Analytical tools, specifically those in structural biophysics and dynamics, including Fourier-transform infrared spectroscopy (FTIR), neutron scattering (NS), dielectric spectroscopy (DES), Nuclear Magnetic Resonance (NMR), have been increasingly utilized to characterize protein structure and motion in the solid states. For example, NMR spectroscopy, both solution and solid-state NMR, is a sophisticated technique used in the biophysical analysis of proteins.[18] It has been utilized to study various aspects of protein behavior such as conformational stability, interactions between proteins, complexes formed between proteins and excipients, as well as the arrangement of molecules in insoluble fibrillar aggregates.[18-22] These studies aid the development of rational formulation designs from molecular mechanism understanding. Theories regarding the mechanism by which sugars or polyols stabilize protein both during drying and storage have been proposed and widely discussed in the literature, including vitrification theory, the water replacement theory, and refinements focusing on the local mobility of protein.[23] However, the mechanism of dry-state biologics stabilization is still not completely understood. Hence, we will provide an overview and clarification of up-to-date protein stabilization mechanisms or hypotheses in the solid state. Such mechanistic insights via advanced characterization can enable better insights into the impact of formulation design on protein structure and stability, which ensures the ultimate quality of the protein solid products.

In this review, we will provide a brief description of protein stability considerations in formulation development and an overview of emerging drying techniques and their applications in **Section 2**; protein dynamic and stabilization mechanism of biologics in solid state in **Section 3**, mechanistic factors that impact protein stability in **Section 4**, mechanism driven drug product design from optimizing formulation composition in **Section 5**, and new and evolving modalities and delivery of non-parenteral biologics will be discussed in **Section 6**. Overall, our focus is to elaborate structural mechanisms that underlie protein stability and provide a mechanistic understanding of the protein-excipient stabilization at the molecular level and provide rationale selection of excipients for solid biological formulation. These insights would be critical to address the stability-related issues in dried protein formulation.

2. Protein stability and modern drying technologies

There are many complex molecular and physicochemical factors driving the design of biologic drug products that impact the biophysical and biochemical stability of protein drugs,[18] as shown in **Figure 1A**. These include protein concentration (up to 100 mg/ml and 200 mg/ml and beyond for high and ultrahigh concentrations, respectively, for subcutaneous delivery), a broad range of temperatures, and the engineering processes that accommodate various administration routes and final drug product forms, from sterile liquid to solid state. Thermal, chemical, and mechanical stresses are present throughout the entire development and manufacturing processes.[24-26] Specifically, as shown in **Figure 1B**, a broad range of temperature stresses, from low-temperature storage and freezing stress from lyophilization to thermal stress from the spray drying process, can significantly affect protein aggregation behavior. These stresses alter the thermodynamic and energy state of proteins, resulting in increased aggregation, fibrillization, degradation, oxidation, and other processes that can compromise stability, quality, and bioavailability. Among several formulation strategies, solid biologics via drying processes have shown advantages in preserving protein

stability. However, changes in protein structure and protein-sugar interactions, acidity shifts, and excipient crystallization can occur during the freezing step of lyophilization, subsequently compromising protein stability in the solids.[20, 21, 27] Therefore, the rational selection of appropriate drying processes, excipients and stabilizers, and storage conditions plays a critical role in crafting stable biologic products, which will be further discussed in this review.

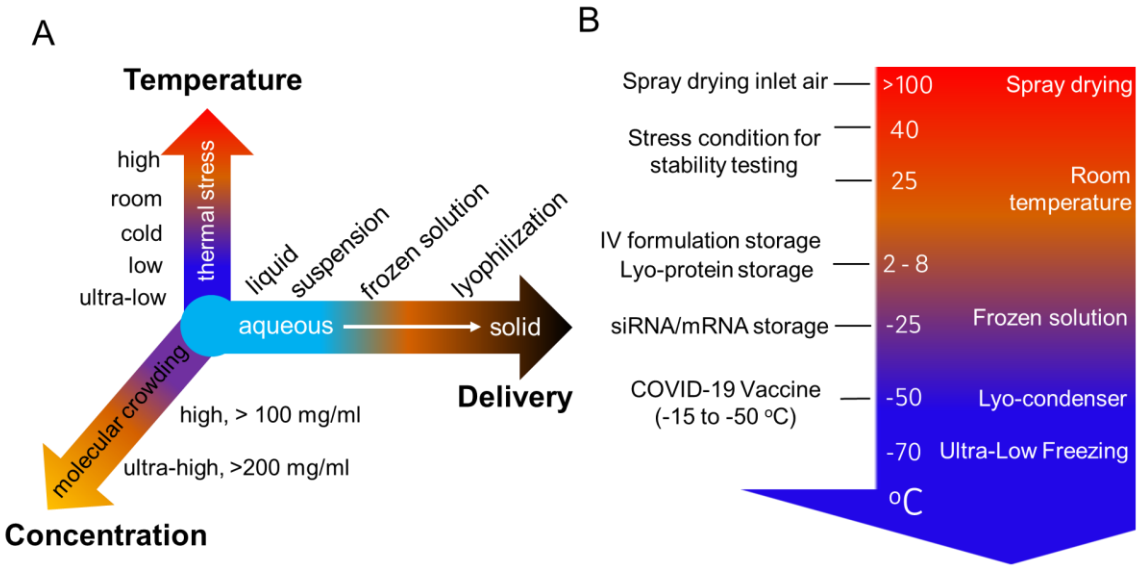


Figure 1. Considerations of biologic stability in formulation development, manufacturing, storage, and shipping. (A) Critical molecular and physicochemical factors and associated thermal stress in formulation design; and (B) Potential thermal stress from a broad range of temperatures during the formulation process and storage. It is worth noting the varied protein concentrations, transitions between liquid, frozen, and solid phases, and the wide range of temperatures, from high to ultra-low, involved in the formulation and manufacturing of solid-state biologics.

Drying of peptide and protein drugs utilizes a few engineering processes with the goal of the removal of water and stabilizing protein in solid-state. This section will briefly discuss both traditional and emerging drying techniques for producing protein solids. A schematic illustration of drying methods is presented in **Figure 2**. The advantages and limitations of each drying method as well as the stresses that protein may encounter during freezing and drying are summarized in **Table 1**. The choice of drying technique is a decision made with many considerations including drying efficiency and product storage stability. Comprehensive reviews of these techniques can be found in recent articles.[13-16] We would like to briefly introduce their crucial process and our perspective on the advantages and disadvantages of their mechanistic impacts on protein stability discussed in the following section.

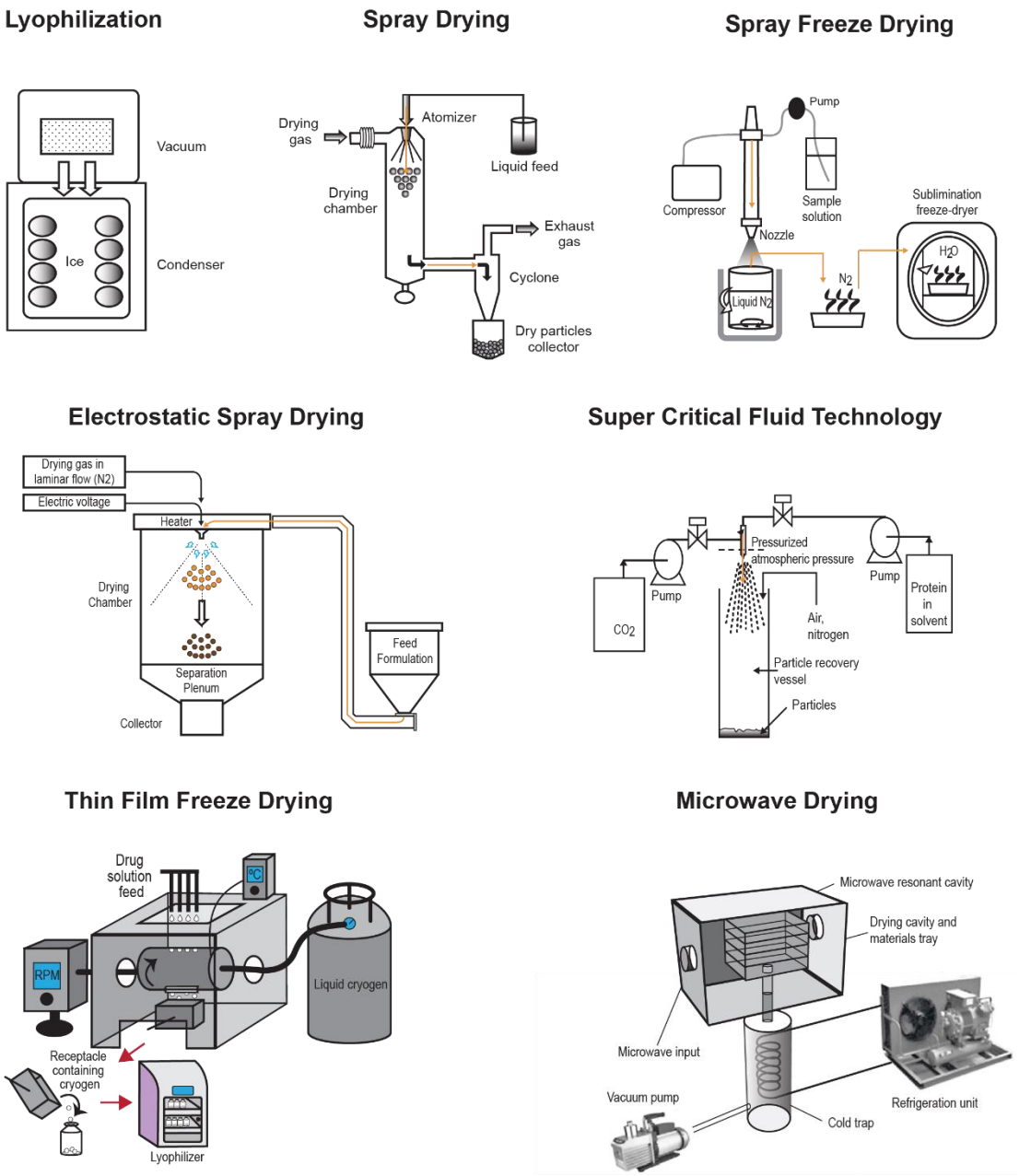


Figure 2. Schematics of various current and emerging drying techniques for solid protein drug products. 13-16, 28-31

Table 1. Drying techniques for solid-state biologics formulation and manufacturing processes.[13-16, 28-31]

Drying Process	Stress	Advantage	Limitations
Lyophilization	– Freezing stress – Excipient Crystallization – Interfacial stress – pH shift	– Low temperature drying operation – Precise dose – Controlled moisture content – Short reconstitution time	– Expensive set up – Long processing time – Batch process

		– Process and knowledge well established	– Not suitable for particle size control
Spray Drying	– Heat stress – Atomization stress – Interfacial stress – Excipient crystallization	– Cost effectiveness – Rapid drying process – Continuous process – Particle engineering with controlled size – Good aerosolization	– Compromised yield – Non-aseptic
Spray Freeze Drying	– Atomization stress – Interfacial stress – Freezing stress	– Rapid freezing – High yield – Particle engineering with controlled size – Suitable for thermally sensitive material	– Time consuming from multiple steps – Costly – Require liquid N_2
Electrostatic Spray Drying	– Thermal stress – Atomization stress – Interfacial stress	– Mild process condition – Particle engineering with controlled size – Continuous process – Good aerosolization	– Lack of industrial benchmark – New regulatory paradigm
Supercritical Fluid Drying	– Atomization stress – Interfacial stress	– Fast process – No surface tension effects – Solvent-free and mild temperature	– Exposure to organic solvent – Unusual set-up – Costly
Lyosphere	– Freezing stress – Excipient crystallization – pH change – Interfacial stress	– Drug substance usage improvement – Compact and faster drying – Supply chain flexibility – Enable combination products – Formulation space flexibility	– Fragile particle – Lack of industrial benchmark – New regulatory paradigm
Microwave Drying	– Thermal stress – pH change	– Rapid dehydration – Formulation homogeneity – Cycle times reduction	– Lack of industrial benchmark – Not compatible with solvent processing
Thin Film Freeze Drying	– Freezing stress – Excipient Crystallization – Interfacial stress – pH shift	– Low temperature drying operation – Controlled moisture content – Advantageous for preparing dry powders of large molecules since it involves	– Lack of industrial benchmark – Requirement of soluble compounds in process solvent

		low shear stress, minimizing any potential detrimental effects	

Lyophilization, known as freeze-drying and illustrated in **Figure 2**, is a process that involves water freezing followed by sublimation for water removal and is widely used for the stabilization of biologics. The lyophilized biologics formulations provide better physical and chemical stability and long-term storage stability.[32, 33] Rational design of freeze-drying process and formulation composition is critical to enable stable lyophilized protein formulation.[5, 34, 35] Freeze drying is a batch process and is time-consuming and energy-intensive. If the process is not well optimized, it can result in not only a longer drying time but also compromised product stability.[32, 36-38] Protein unfolding can also occur due to the stress of freezing and drying, and therefore efficient formulation is needed to inhibit unfolding at each step.[39-42] Aside from process development, the formulation excipient selection, such as sugar excipient, pH and buffer, is also crucial in maintaining the stability of protein during the freeze-drying process, storage, and extended shelf-life.[34]

Compared to traditional lyophilization technology which is a batch process and time-consuming with very low energy usage efficiency, the spray-drying (SD) process involving solution rapidly atomization and evaporation has raised growing interest in manufacturing biologic solids due to its continuous process with a high output rate. It also provides the opportunity for designing inhalation biologic products with suitable powder flowability by controlling the particles' properties through the spray drying process.[41, 43] Exubera as an inhalable insulin product and Raplixa as a mixture of thrombin and fibrinogen are recent approved examples of biological products using SD techniques.[44, 45] However, there is a concern about the stability of spray-dried protein solids because spray drying may result in the displacement of protein on the surface of the droplet due to the extraction of moisture. The removal of water from the center of the droplet to the surface can result in the diffusion of proteins to the edge of the dried particles, exposing them to air-liquid interfacial stresses that induce protein unfolding and loss of functionality. Therefore, understanding the impact of formulation conditions on the distribution of protein and its structure can aid in designing drying conditions to create more homogeneous drug products with robust stability. Considering for nature of SD process, it has its own distinct unique caveats. The product yield depends on the work scale and formulation composition, where yield is typically low. Furthermore, SD proteins are typically administrated through parenteral delivery which needs a sterile product. Ensuring the sterility of protein products during the spray drying process poses a significant challenge in maintaining an aseptic operation. Moreover, achieving extremely low residual water content in the final product may necessitate the use of additional drying methods, which could potentially diminish the time and energy advantages associated with spray drying when contrasted with lyophilization.[46]

Spray freeze drying (SFD) refers to the process of atomized liquid stream into a frozen medium followed by sublimation of the frozen droplet.[47] Such a process provides the potential advantage of minimizing the possibility of phase separation between drug and excipient and therefore results in a more homogeneous distributed drug product.[47] During the SFD process, the formation of porous spherical particles leads to an increase in surface area and subsequently contributes to a reduction in the time needed for sublimation and secondary drying.[48] In addition, the spherical shape of these SFD particles contributes to reasonably acceptable flowability and aerosol performance.[29] However, due to the diversity and

complexity of SFD processes, SFD is still highly experimental and only scaled for laboratory purposes in the pharmaceutical applications, which is mostly used in the food idustry.[49-51]

Electrostatic spray drying (ESD) recently has been developed as an emerging drying method to the traditional spray drying process. ESD utilizes an atomizing nozzle where a charge is applied to the feedstock during the atomization.[9, 52] The use of electrostatic technology can overcome the disadvantage of uncontrolled distribution of drugs inside or on the surface of dried particles. The solvent has the highest ability to polarize and will pick up the most electrons by the applied charge. The carrier, being less conductive than the solvent, will pick up fewer electrons, and the active being least conductive pick up the fewest electrons. As such, the formation of a solvent-rich and a solute-rich core within the droplet can improve the microencapsulation efficiency and facilitate drying at a lower temperature.[53] Application of the ESD has been evaluated in nanoparticles, nanosuspensions, microsphere encapsulations, and also food. Though most ESD applications were experimented with the lab scale, nowadays ESD technologies are commercially available ranging from small lab scale to commercial manufacturing.[9, 52]

Supercritical fluid (SCF) drying is another alternative drying method for protein stabilization. There are two proposed concepts of the SCF process for solid protein products. The first concept is to use supercritical fluid as an anti-solvent for water extraction, which leads to the precipitation of concentrated protein and is followed by additional water extraction through freezing and drying before obtaining final dried particles. The second concept is to use supercritical fluid as a propellant or effervescent agent where the SCF is dissolved at high pressure. SCF has been carried out on only a few food products. Some studies have demonstrated that SCF is capable formulating solid protein particles with good stability and acceptable flow properties.[31, 54-58] One major challenge during the SCF process is the loss of other polar soluble compounds along with water.[59]

Lyospheres are defined as round, discrete, and freeze-dried beads composed of a pharmaceutical active ingredient and excipients.[60] The lyospheres provide the advantage of continuous manufacturing and individual beads can be dried in bulk which can be converted into the final product container with flexibility in dose and potency targeting. Such technology enables dose titration, superior combination products, space-saving bulk storage, and flexible drug product packaging, and overcomes the limitations associated with traditional vial images. In addition, lyospheres technology has the potential to improve the stability and aid custom medicines and vaccines including alternative delivery, and novel adjuvants for patients could be made using this technology.[61] The functionally coated lyospheres using a coating that targets release in the colon can also be used for targeted therapies for oral delivery.[62] Despite the mentioned benefits, lyospheres have been recognized as a vulnerable and delicate product, which can pose significant difficulties in terms of handling, transportation, and packaging.[63]

Microwave vacuum drying (MVD) is a proprietary form of applying a microwave under a vacuum to achieve dehydration at lower temperatures. Drying is faster than lyophilization as heat transfer occurs by radiation (microwaves) instead of conduction. The processing time can be reduced to 6-12 hrs compared to days in lyophilization. This provides an alternative fast-drying technology that enables semi-continuous manufacturing and flexible on-demand manufacturing as well as high-volume products.[60, 64] Härdter *et al.* studied stability of 6 monoclonal antibodies following MVD and lyophilization. The microwave assisted drying showed similar storage stability profile as lyophilization but shorten process.[65] Furthermore, they

demonstrated application of MVD would be particularly beneficial for antibody formulations with low concentrations.[66] However, the application of MVD in the pharmaceutical industry is limited due to a lack of understanding of uniform sublimation and interaction between microwave and biologics and excipients in the formulation.

Thin-film freeze-drying (TFFD) is a rapid freezing method that utilizes a solid surface cooled with cryogenic temperatures to freeze samples.[67] This technique involves dropping small liquid droplets onto the surface, which then spread and freeze within a very short time frame of milliseconds. Subsequently, the frozen thin films are dried through lyophilization, resulting in powders with favorable aerosol characteristics. These powders are characterized by their low density, large specific surface area, and brittle matrix structure.[68] The technique of TFFD has been employed to create dry powders for the pulmonary delivery of small molecule drugs and vaccines.[69-72] Recently, TFFD has been widely utilized for large molecules such as monoclonal antibodies, vaccines, and enzymes, as well as particulates like liposomes and small interfering ribonucleic acid (RNA) encapsulated in solid lipid nanoparticles.[73-79] TFFD is advantageous for preparing dry powders of large molecules since it involves low shear stress, minimizing any potential detrimental effects as compared to spray freeze-drying and spray-drying techniques.[80] The chief limitation is that any drug frozen using this method must be soluble in a solvent that works with this process, such as water and certain organic solvents.

3. Mechanisms of protein stabilization

Solid-state proteins are typically formulated with sugars or polyols to enhance stability, with the primary assumption that these carbohydrate molecules contribute to stabilizing the protein structure or rigidity of the molecular dynamics. There are two well recognized hypotheses for how these additives stabilize the dry-state protein during drying and storage. The first hypothesis is the water replacement theory, and the other is glass dynamics or the so-called vitrification theory. The former is a thermodynamic stabilization mechanism while the latter is from the kinetic perspective. A schematic illustration of these theories is presented in **Figure 3**. It is worth noting that although protein stability is often discussed in either one of the theories or the two mechanisms can contribute to protein stabilization simultaneously. In addition, the mechanism of stabilization can be protein specific though the same salt or sugar stabilized is used. Nguyen *et al.* showed that lactate dehydrogenase (LDH) stabilization in the sugar matrix requires vitrification whereas β-galactosidase (β-gal) can partly retain structural stability in the rubbery state. The difference in stabilization may depend on the intermolecular interactions with arginine.[81] In this section, we aim to provide an overview of the water replacement and glass dynamic hypothesis. In addition, we will elaborate on details of the existing experimental evidence of these two dominant theories. Moreover, a few factors that impact protein stability in the dry state will also be discussed.

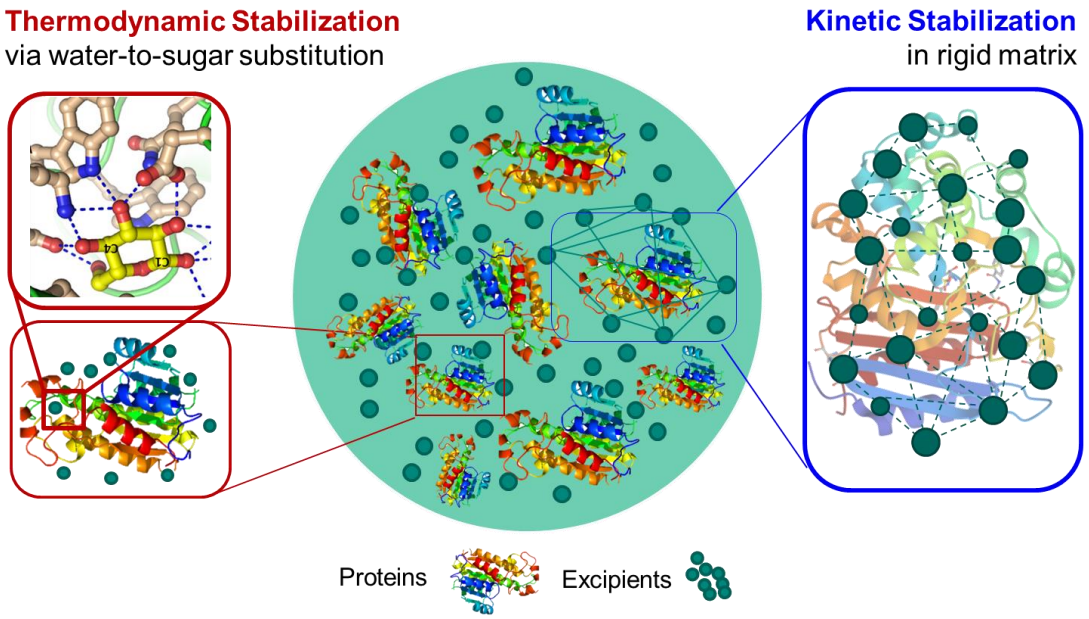


Figure 3. Hypothetic mechanisms stabilizing dry protein through thermodynamic (left) and kinetic (right) stabilization. The models are collectively proposed and investigated in previous studies.[82-84]

3.1 Water replacement theory

From a thermodynamic point of view, protein stability is maintained if the equilibrium between native and unfolded states is more shifted towards the native state. The stabilizer can increase the free energy of protein unfolding and thus drive the equilibrium to a more stable native state.[85] In the solution phase, the stabilization of protein is mainly governed by the preferential interaction mechanism.[86] That is, when the protein is formulated with a stabilizing excipient, the protein prefers to interact with water while the excipient is preferentially excluded from the protein surface. The thermodynamic consequence of such exclusion leads to the protein being preferentially hydrated and the chemical potential of the protein is increased.[32, 85] This induces a thermodynamically unfavorable situation, which in turn stabilizes the native over the unfolded protein state as the increment of chemical potential is higher for the unfolded state than those for the native state and thus increasing the free energy barrier for protein unfolding and denaturation as shown in **Figure 4**.[87] Recently, Arsiccio *et al.* also demonstrated a new thermodynamic model of excluded volume mechanism in protein stabilization which is the creation of a cavity within the solvent and considered as a hard-particle fluid through thermodynamic modeling and experimental data.[88] The formation of hydrogen bonds between water and protein contributes significantly to the protein stability in the aqueous solution.[89] During the lyophilization process, the removal of water leads to the loss of hydrogen bonds between protein and water. Such water removal can result in notable alterations of the protein structure, and therefore protein instability. It has been hypothesized that the hydroxyl groups of the sugars can replace the water in forming hydrogen bonds with protein upon drying processes, conferring thermodynamic stability and providing stability against degradation by replacing the stabilization role with water and stabilizing the native protein conformation.[82] Therefore, the free energy of protein unfolding is increased, thus maintaining the protein stability in the solid state.[90]

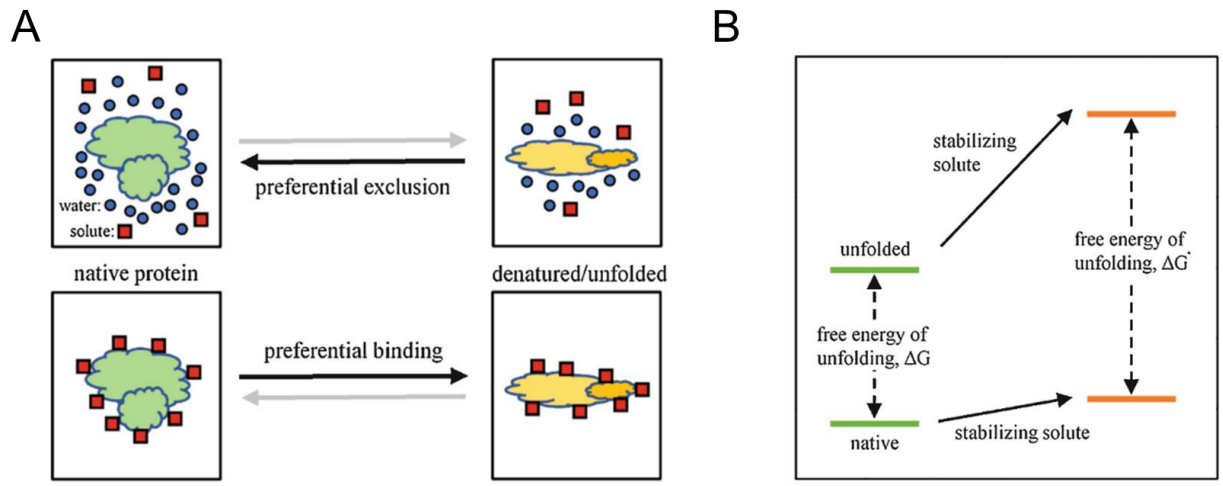


Figure 4. Stabilization of protein in solution state prior to drying by preferential exclusion mechanism. (A) solutes are preferentially excluded from the surface of proteins which stabilizes the native versus the unfolded protein state; and (B) exclusion of solutes from the protein surface increases the free energy barrier for protein denaturation.[87]

A direct measurement of sugar and protein interaction and hydrogen bonding formation is challenging due to the lack of high-resolution structural investigation. This hypothesis was first assessed and proposed by Carpenter and Crowe *et al.*[82] In this original study, FTIR was used to detect the changes in the secondary structure of the protein. The FTIR spectra of freeze-dried lysozyme with and without trehalose were compared to those of fully hydrated lysozyme. It was found that trehalose can prevent the shift of the amide I and II bands of dried lysozyme. Moreover, the presence of trehalose in the formulation results in the appearance of the carboxylate band of lysozyme as shown in **Figure 5A**, a fingerprint band of hydrogen bond, suggesting that trehalose can substitute water to form hydrogen bonds with protein and help stabilize the protein in a near-native state.[82] However, the validation of water replacement theory from the FTIR findings was challenged by Belton and Gil.[91] In their study, they stated that the stabilization of dried lysozyme could be still due to the water-protein hydrogen bonding when the residual water is trapped at the protein-sugar interface upon drying.[91] Nevertheless, Allison and Carpenter *et al.* later reinforced the water replacement theory by showing the evidence that the lysozyme sample with a 4% moisture level had no evidence of an actual peak of carboxylate band and could not give rise to the fingerprint band for sugar-protein hydrogen bond, indicating that the dehydration-induced spectral shifts could not be solely claimed for water trapped at the protein surface. This solidified that the hydrogen bond formed between sugar, not the trapped water, and protein is responsible for the stabilization of solid-state protein.[92] Furthermore, the polymeric sugars like dextran didn't form hydrogen bonds well with proteins as disaccharides with less intensity of the carboxylate band observed. The failure of dextran to inhibit lysozyme unfolding can result from the insufficient hydrogen bond formation between polymeric sugar and protein due to steric hindrance.

The fundamental of water replacement theory is the hydroxyl groups of sugars interact with the surface molecules of the protein via hydrogen bonding. To further detect this mechanistically critical interaction between protein and sugar in the solid state, advanced high-resolution techniques that can identify and quantify site-specific interaction are urgently needed. For this purpose, solid-state NMR has

shown its capability to identify the hydrogen bonds between proteins and excipients. This is often achieved by observing chemical shift changes, intermolecular correlation, or more quantitatively, interatomic distance measurement. For example, in a recent study, chemical shifts of Arg C_ξ peaks were comparable in the mannitol and trehalose formulations as shown in **Figure 5B**, suggesting that they could have interacted with bovine serum albumin (BSA) in similar hydrogen-bonding patterns.[21] In addition, Pielak's group utilized the liquid-observed vapor exchange (LOVE) NMR technique along with the hydrogen-deuterium method to probe the excipient effect on the dry protein structure at the residue level.[93] They found that increased protection of two proteins of the B1 domain of streptococcal protein G and truncated barley chymotrypsin inhibitor 2 when formulated with sugar, especially residues with a greater number of intramolecular H-bonds are better protected, which suggests that sugars retain protein structure in solid state by strengthening intra-protein H-bonds. Whereas, a decreasing protein stability effect was observed when removing three hydroxyl groups (1,2,3-hexanetriol), which indirectly supports the protein stabilization/protection from the water replacement theory.[93]

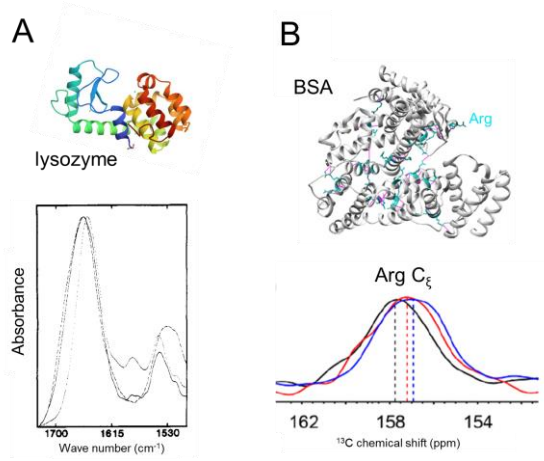


Figure 5. Protein-sugar interaction characterized by FTIR and ssNMR. (A) Amide band region for hydrated lysozyme (dotted line), lysozyme freeze-dried alone (dashed line), and lysozyme freeze-dried in the presence of trehalose (solid line);[82] and (B) Arg C_ξ peaks of spray-dried BSA without any excipient (black), BSA-mannitol (blue), BSA-trehalose (red).[21] X-ray crystallography structure of the BSA monomer (PDB ID:4F5S) highlighting the Arg residues (cyan) and hydrogen bonds with other residues to maintain the secondary and ternary protein structure (magenta).

Overall, all these fundamental experimental studies highlight the critical role of the hydroxyl group in ensuring adequate hydrogen bonding interactions between sugar and protein. Such an interaction requires the binding of sugar to protein in a stoichiometric manner as there might be a limited number of binding sites on the surface of protein molecules that may form hydrogen bonds with stabilization. Therefore, it is expected that a saturation level will be reached if all binding sites are used, and a further increase in sugar-to-protein ratio will not further improve protein stability.[83] Such saturation levels have been observed in several studies. For example, Cleland and Carpenter *et al.* demonstrated that the concentration range of sugars, including sucrose and trehalose, to maximize the native state of the formulations of recombinant humanized monoclonal antibody HER2 is 60-100 mM.[94] Further increase of sugar concentration can only mitigate the rate of protein aggregation slightly. Similarly, a molar ratio of trehalose and recombinant humanized monoclonal antibody anti-IgE (rhuMAbE25) in the range of 300:1 to 500:1 was reported able

to maximize the stability of the spray-dried recombinant antibody.[95] However, if the stability data is replotted in the log scale, a linear correlation was still observed between the stability of rhuMab HER2 and sucrose concentration. Therefore, it is still debatable whether the saturation refers to no additional improvement or is because of the sensitivity of plotting to differentiate the degradation rate.

Though it is difficult to directly probe the protein and sugar interaction, vibrational spectroscopic tools, such as FTIR and Raman spectroscopy, were still often utilized to correlate the secondary structural changes with protein stability and assess the sugar stabilization effect in preserving the protein's native state in the dried state.[83, 96, 97] For example, Chang *et al.* have examined the effect of different sugars on the secondary structure of IgG1 antibody.[98] Both trehalose and sucrose can retain a more "native-like" structure compared to sorbitol. Generally, the spectral correlation coefficient, r, represents the degree of similarity of protein secondary structures and a large r value (≤ 1) denotes less structural changes during drying.[83] A number of studies have utilized r to quantitatively assess the stabilization effect of sugars on the protein formulations.[39, 40, 42, 97, 99] High r values were often obtained for the dehydrated proteins with the presence of stabilizers. Meanwhile, more stable protein formulations are accompanied by larger r values, indicating a more "native" protein structure in the dry state. Such a correlation between stability and protein structure similarity provides evidence for the water replacement hypothesis as an indirect measurement.

However, despite evidence in support of the water replacement theory, some experimental findings are contradictory to the theory. It was found that the correlation between protein stability and the preservation of secondary structure is not valid in all circumstances. On the one hand, several studies have reported significant improvements to protein stability while no spectroscopic changes of secondary structure were observed.[83, 98] On the other hand, there are cases where higher protein stability was achieved but with a large perturbation of the protein secondary structure.[100] For example, Costantino *et al.* demonstrated stability enhancement of recombinant human albumin with the presence of dextran as a stabilizer even though a lower degree of retention of secondary structure was observed compared to the formulations without additives.[100] These findings suggest that water replacement theory is not the exclusive mechanism for the stabilization of solid proteins. In other words, thermodynamic consideration is necessary but may not be sufficient for the explanation of protein stability in the dry state. In this regard, a hypothesis that is related to the dynamics of formulations provides an alternative to uncover the stabilizing effect of sugars or polyols on dry proteins and will be discussed in the following section.

3.2 Glass dynamics hypothesis

3.2.1. Molecular motions of solid-state proteins

Proteins possess inherent flexibility, which is determined by their structures and is essential for carrying out their biological functions.[101] The magnitude of movement is related to the specific functional requirements proteins need to fulfill. The regions of plasticity or rigidity within a protein fold, as well as the degree of conformational flexibility during binding and functional processes, primarily depend on electrostatic and hydrophobic properties or their compensatory interactions.[101] Therefore, proteins have inherently enriched molecular dynamics which can be crucial for their functionality and stability. The protein molecules undergo a variety of structural transitions at timescales such as the fast (ps-ns) as well as the intermediate and slow (μs -s) timescale, as illustrated in **Figure 6**. These dynamics display physical motions ranging from global rearrangement and collective domain movement (>μs),[102] loop motion and gating (ns-ms),[103, 104] backbone fluctuation or rotation diffusion/molecular tumbling (ns),[105] side-chain

rotations (ps-ns),[106, 107] and bond vibrations (fs-ps),[108] are relevant to protein functionality,[109] and also stability in solution and solid state after drying.

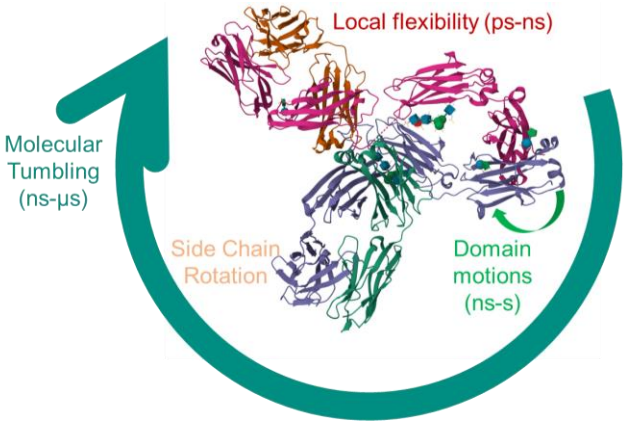


Figure 6. Illustration of molecular dynamics in proteins covering fast and slow motional regime. Protein structure adapted from PDB: 5DK3.[110]

As shown in **Figure 4**, proteins interact with water molecules while the excipient is preferentially excluded from the protein surface, and the formation of hydrogen bonds between water and protein contributes significantly to the protein stability in the aqueous solution. After lyophilization, the removal of water largely slows down the global motion, which makes the local motion a primary component that may modulate the protein dynamics and therefore stability at the temperature below the glass transition temperature (T_g). Therefore, it is crucial to understand how the excipients or drying processes impact the protein dynamic, such as global or local motions, in the solid state and potential correlation with storage stability.

3.2.2. Dynamics that relevant to solid biologic formulations

Protein solids after lyophilization retain as amorphous glassy material which generally demonstrated greater molecular mobility. Two broad classes of dynamics in glasses or polymer sciences have been recognized: primary or global relaxation and secondary or local relaxation. These dynamic terms are also utilized in solid protein dynamic characterization as a refined stabilization mechanism and are typically described as "α-relaxation" and "β relaxation", respectively.

The α-relaxation, also known as "structural relaxation" or "global relaxation", which the vitrification theory is based, represents the slowest dynamic process in glasses on the timescale of seconds to months, which is strongly temperature and viscosity dependent and involves highly cooperative reorientation via translational and rotational movement of whole molecules.[84, 111, 112] These relaxations are crucial for highly coordinated reorientation within a substance. In essence, the vitrification hypothesis suggests that when a system transforms into a glassy state, it becomes considerably more stable due to the significantly restricted molecular mobility in that state in which the protein is immobilized in a rigid and amorphous glassy sugar matrix. As degradation processes rely on molecular mobility, the limited molecular motion in the glassy state leads to a lowered rate of degradation. Consequently, when the system remains below the glass transition temperature, it behaves primarily as a solid, exhibiting a substantially reduced degradation rate.[113]

Aside from slow relaxation, there are local high-frequency dynamics often referred to as the "fast dynamics of glasses". These dynamics involve collective motions on small length scales, occurring on timescales of a nanosecond or less. The fastest β_{fast} relaxation occurs on a timescale of picoseconds and is associated with intra- and inter-molecular collisions. Johari-Goldstein β (β_{JG}, also known as β_{slow}), was mostly discussed and considered as another dynamic index to probe the protein stability in the solid state. Johari and Goldstein made a significant discovery by identifying secondary relaxation in glasses of rigid molecules in 1970.[114] They concluded that certain types of secondary relaxations must involve rearrangements in the orientation and position of one or more molecules within a localized region.[114] Subsequently, such relaxation was named "JG β relaxation" after these authors. The slow β_{JG} relaxation occurs typically on a timescale of microseconds to milliseconds and is associated with small amplitude twisting or crankshaft-like motions, such as the rotational motion of a methyl group or a polymer side chain.[115] It is widely recognized that β_{JG} relaxation serves as a precursor to α-relaxation, which exhibits a slower dynamic as a result of an increase in cooperativity.[116] It has been well held that high-frequency β-relaxations govern the local mobility and are temperature-independent.[117] Although such β relaxation processes may not be the dominant factor at temperatures near or above T_g, their importance is much more emphasized at temperatures below T_g due to their relatively low activation energy.[83] In addition, these relaxations were considered to have a notable effect in limiting the transport of small molecules that may lead to protein aggregation in glasses.[118] That is, by slowing β dynamic processes, diffusive motions of small reactive species such as oxygen and water in glasses can be restricted.[118]

3.2.3. Techniques to characterize the protein motion and correlation with protein stability

Overall, global and local mobility both have respective impacts on the physicochemical stability of protein in a dry state and the strength of impact depends on which length scale of molecular mobility is the determinant factor for protein stability. For example, when the short-time scale of molecular mobility is more dominant, analysis of the amplitude of β relaxation processes is necessary for the design and prediction of protein stability. To further investigate the correlation between protein mobility and stability, there are a few spectroscopic and diffraction techniques that have been extensively utilized to characterize protein dynamics such as slow and fast motion in terms of α-relaxation and β-relaxation including DSC, DES, NS, ssNMR, FTIR, and solid-state hydrogen-deuterium exchange (HDX). The pros and cons of each characterization technique are summarized in **Table 2.** In this session, we will discuss the application of these various techniques in protein dynamic characterization and correlation with protein stability in the solid state.

Table 2. Characterization techniques of protein structure and dynamics in the solid state.

Techniques	Measurements	Primary Applications in Structural and Dynamic Analysis of Solid-state Biologics	Probing Local or Global Molecular Attributes	Non-invasive Analysis
FTIR	C=O, N-H and C-N vibrations	Secondary structure	Global	Yes
ss-HDX	Amide hydrogens exchanged with deuterium	Tertiary structure and dynamics	Local and global	No
DSC	T_g or changes in heat capacity	Structure and dynamics	Global	No

Dielectric spectroscopy	Rotational motions of dipole-bearing groups	Dynamics	Local and global	Yes
Neutron Scattering	Neutron scattering by hydrogen atoms	Dynamics	Local and global	Yes
ssNMR	^{1}H, ^{13}C, ^{15}N, and ^{31}P chemical shifts, relaxation time, CSA, and dipolar coupling	Structure and dynamics	Local and global	Yes

3.2.3.1. Differential scanning calorimeters (DSC)

Literature has suggested that α-relaxation primarily involves segmental relaxations of the main chain, which is typically associated with the material's structural relaxation and is inherently intermolecular.[119] As the temperature approaches the glass transition temperature (T_g), the relaxation time for α-relaxation experiences a significant increase, contributing to the formation of the glassy state at lower temperatures. As such, DSC, which measures the glass transition temperature (T_g) of protein in the solid state, is the most frequently used technique to estimate global mobility in a glassy state.[120] It has been reported that substantial changes in protein stability were observed for temperatures below and above T_g and a common stabilization strategy is to increase the temperature of T_g to slow down the α-relaxation. One empirical way to estimate the degree of α-relaxation dynamics is based on the postulation that $\log\tau_\alpha$ scales with the difference between the storage temperature and T_g (T_g-T) approximately, as suggested by the Williams-Landel-Ferry (WLF) equation. Thus, in general, if a glassy matrix has higher T_g, the slower α dynamics and the protein degradation processes will be suppressed to a larger extent. The correlation between protein stability and (T_g-T) has been demonstrated in a variety of sugars and polyols. For example, in a study by Prestrelski and coworkers, the stability of interleukin-2 was compared in formulations with the presence of different carbohydrates, such as sucrose, trehalose, raffinose, and stachyose.[40] It was found that excipients with higher T_g, e.g., raffinose and stachyose, exhibited higher levels of long-term stabilization effect on interleukin-2. Moreover, Roy and Maloney *et al.* demonstrated a positive correlation between the degradation rate of freeze-dried monoclonal antibody-vinca alkaloid conjugate and (T-T_g) at temperatures 0-30°C above T_g.[121]

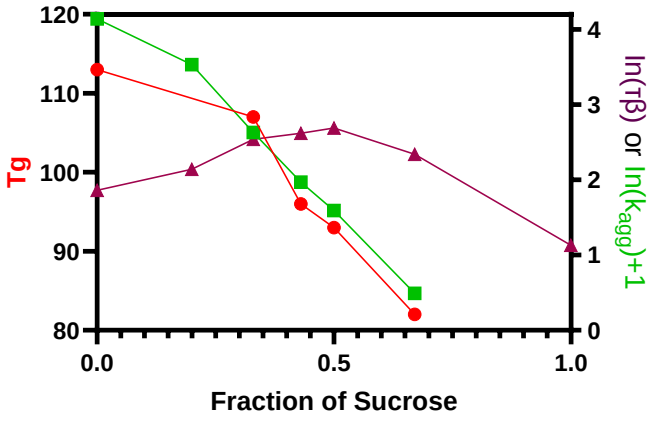


Figure 7. Correlations between Tg, structure relaxation time, and stability in an IgG1 formulation with different weight fraction of sucrose. Plotted from data in Chang *et al*.[98]

Nevertheless, failures of correlating protein stability with T_g were also extensively reported in the literature.[4, 11, 122, 123] The universal correlation between the temperature difference of storage temperature and T_g and protein stability is mostly observed when the system near to or above T_g. When the storage temperature is well below T_g, there is little correlation between protein formulation stability and temperature difference between storage temperature and T_g.[11, 118, 124, 125] In addition, protein formulations of a lower T_g were found more stable than those of a higher T_g, especially when sugar matrices are compared to polymers. For instance, a higher stabilization effect was seen for sucrose and trehalose than maltodextrin and polyvinylpyrrolidone (PVP) on vacuum-dried restriction enzyme *Eco*RI, even though the sugars possess much lower T_g values than polymers.[123] Moreover, Chang *et al*. found that the stability of lyophilized Interleukin-1 Receptor Antagonist formulation was significantly improved although the T_g of the formulations gradually decreased by increasing the concentration of sucrose from 1 to 10%.[11] Similarly, as shown in **Figure 7**, negative correlation between the $T_g - T$ and stability of the IgG1 with different weight ratios of sucrose was observed.[98] In addition, the mobility of IgG1 τ^{β} was not monotonically correlated with the level of sucrose where there is a maximum reached at a 1:1 ratio. These contradictory results indicate that T_g alone is not sufficient to predict protein stability. T_g is a critical temperature of molecular mobility of amorphous materials but may not be a direct predictor for protein mobility.[4] It has been demonstrated that the scaling between $\log\tau_\alpha$ and $(T-T_g)$ can be quite different for various materials, which depends on the fragility, Kauzmann temperature (T_k), and thermal history of the glassy matrix.[84] Moreover, several reports have suggested that T_k, sometimes defined as critical molecular mobility temperature, might be more accurate indicating the molecular mobility in protein formulations.[126]

3.2.3.2. Dielectric spectroscopy (DES)

Proteins consist of a unique sequence of amino acids that are connected by peptide bonds and these repeating units of amino acids (-CO-CR-NH-) possess polar characteristics.[127] In addition, these amino acid side chains can be neutral, polar, or charged, further contributing to their polarizability. Due to their polarizability, they are sensitive to dielectric techniques and present specific dielectric constant in defined conditions.[128] The protein formulation conditions such as plasticizer additives, pH, moisture content, and temperature affect the dielectric properties of the protein, including electronic and atomic polarization, dipolar reorientation, and space-charge interfacial polarization.[129] As such, dielectric spectroscopy (DES) was widely used as a direct approach for relaxation and dynamic and antiplasticization measurement for glassy and small molecules.[128-132]

DES is commonly used to probe protein motions across a wide frequency from 10^{-6} to 10^{10} Hz and temperature ranges.[133] In many cases, the dynamics of proteins are studied in aqueous solutions or different hydration levels or solvent systems. The broad frequency range was used to probe the fast coupled protein-hydration water relaxation and slow relaxation of large-scale domain-like motions of proteins such as lysozyme and myoglobin.[134] More specifically, megahertz (MHz) to terahertz (THz) dielectric spectroscopy probes dynamic processes between 10^{-6} and 10^{-12} s and bulk water relaxation near 20 GHz and protein tumbling near 1−10 MHz can be identified simultaneously.[135] The reorientation motion of protein such as myoglobin as a local β-relaxation was observed in the sub-MHz frequencies.[134-136] In addition, the protein hydration as preferential exclusion and protein-water interaction induced protein dynamic changes were

studied.[137-139] For example, lysozyme is found to surrounded by a tightly bound layer of ~165 water molecules in terms of picosecond dynamic through the dielectric response analysis over the range of 65 to 700 GHz.[137] Furthermore, DES was utilized to explore different orientational polarization mechanisms including relaxation, such as the dynamics of loosely bound, tightly bound, and bulk water and investigate the collective vibration motions of protein and protein-water interaction.[138] However, these techniques pose challenges in lyophilized solids due to the impact of their moisture water content and their porous structure.[84] In one of the very few demonstrations of its use in solid protein formulation, mechanistic understanding of sugar-induced protein stabilization via DES was investigated through protein embedded in a sugars and water system which comprehensive relaxation of sugar, water, solvent, and protein was studied.[140] Large-scale conformational myoglobin motion induced slower protein relaxation was observed due to the relaxation of trehalose below T_g, which was associated with intramolecular rotations of the monosaccharide rings in trehalose.

In some studies, the concept of antiplasticization to independently manipulate the α and β relaxations is employed,[118, 141, 142] thus allowing us to differentiate their respective contributions to the stability of freeze-dried proteins. Antiplasticizers refer to molecular or nanoparticulate substances that lead to the increased rate of α relaxation (resulting in a lower glass transition temperature, T_g), negative deviations from volume additivity upon mixing, and an increase in elastic moduli,[143, 144] while concurrently slow down the higher frequency β relaxation process. This contrasts with regular plasticization, where both the α and β relaxations are accelerated upon the addition of a diluent. Antiplasticization has been observed in several polysaccharide mixtures,[145] particularly enriched by water and glycerol.[146, 147] Multiple research studies have demonstrated the antiplasticization of trehalose when combined with glycerol at different concentrations.[142, 148, 149] DES has been used to study the antiplasticization effect of melt-quenched trehalose-glycerol glasses. The dielectric relaxation times with varying amounts of glycerol were measured covering a broad frequency range between 40Hz to 18GHz. A linear proportionality between the relaxation time of a relatively fast dielectric relaxation process and mass fraction of glycerol up to 0.2 below a certain antiplasticization temperature which is 297K in the study.[142] In addition, a linear relationship between protein degradation rates ($\log\tau_d$) and activation energy (E_a) of the β_{JG} relaxation process was derived in more than 100 antiplasticized sugar glass formulations at 23°C.[118, 142] In contrast, no evident correlation was found between protein degradation and ($T-T_g$), suggesting that faster dynamic processes may be linked directly to the degradation rates. Further, a more quantitative characterization using DES was established and an antiplasticization factor as a ratio of JG relaxation time for the mixture and pure trehalose was used to measure the extent of antiplasticization.[141] This factor is temperature sensitive which is about 1.6 at 293K but less than 1 above 323K.

Though most of these DES studies investigated the correlation between the protein stability and antiplasticization effort, they mainly studied the additive-induced sugar antiparticipation and limited studies focused on the protein itself.[141, 142] There are some limitations of DES in protein dynamic characterization. DES serves as a technique with an extremely wide frequency range, but it is often challenging to interpret the data obtained. Moreover, dielectric measurements cannot offer microscopic insights, and distinguishing between relaxation modes associated with proteins and solvents is not straightforward.[135, 150, 151] Sometimes, the correct assignment of spectra in DES is required but challenging to differentiate the relaxation process of water and protein overlap in hydrated lyophilized solids.[133]

3.2.3.3. Neutron scattering spectroscopy

Neutron scattering (NS) spectroscopy is a nondestructive technique that can be used to study the dynamics of protein in the liquid and solid states. It is sensitive to the position and motions of atoms and can probe spatial and temporal aspects of dynamics, which make it easier to determine what is being observed and the nature of the motion. The protein dynamic in the solid state can be easily impacted by external factors such as excipients, moisture/water content, and temperature. Several studies have connected high-frequency motions with the long-term stability of proteins.[117, 118, 152-159] NS can be used to probe the protein dynamics in solid form and the principles and different types of neutron scattering have been extensively described in previous studies.[160-162] This method involves monitoring the change in neutron scattering intensity by hydrogen atoms in the protein over time to observe protein dynamics. By measuring the mean-square displacements (MSD) ($<u^2>$) of atoms within the protein molecule, the impact of external factors on protein dynamics can be evaluated. NS was extensively utilized to characterize the β_{fast} dynamic process in dry proteins. This technique measures the $<u^2>$ of atoms in glasses, which represents the amplitude of fast motions on the nanosecond scale.

Tsai *et al.* applied *in situ* elastic NS to study the effect of glycerol and hydration on the dynamic processes of lysozyme powder.[159] Glycerol was shown with a stabilization and destabilization effect when the temperature is below and above the dynamical transition temperature (T_d), as evidenced by the changes of $<u^2>$ at different temperature regimes. Cicerone *et al.* provided the first direct evidence correlating β relaxation processes with protein stability.[153] They utilized elastic and inelastic coherent NS to investigate the stability of horseradish peroxidase and yeast alcohol dehydrogenase in the binary glasses of trehalose and glycerol. By adding a small mole fraction of antiplasticizers, i.e., glycerol, the stability of these enzymes was improved with the suppression of the short-time scale dynamics and protein motions even though the τ_α is lowered. Such a relationship between protein stability and β_{fast} relaxation was further validated using inelastic NS measurement. The degradation rates of either chemical or physical degradation processes of 11 proteins correlate linearly with $<u^2>^{-1}$, indicating that β_{fast} relaxation plays an important role in protein stabilization.[118] These observations suggest that protein stability is more directly coupled with high-frequency β relaxation processes, rather than α relaxation. Later, another study leveraged molecular dynamics simulation and NS to evaluate the impact of sugar and water on the protein motion in the lyophilized glassy matrixes.[154] Trehalose was reported to rigidify the lysozyme as evidenced by the inverse relationship between protein flexibility and stability in NS measurements. Interestingly, this study highlighted the importance of hydration levels in the solid state. Lysozyme was found to have a greater tendency to interact with water rather than trehalose in the hydrated matrix. Furthermore, the presence of trehalose significantly slows down the relaxation of hydrogen bonds between lysozyme and water and the formation of hydrogen bonds to fill the void space. Furthermore, some studies have utilized NS to study the impact of buffer composition on the internal protein dynamics in the lyophilized powder. For example, human butyrylcholinesterase lyophilized in three different buffers demonstrated distinct protein internal motions, where salt-free and sample with Tris-HCl sample showed similar small-amplitude motions but sample containing sodium phosphate demonstrated highly reduced mean-square displacements at ambient temperature.[163]

Neutron spectroscopy can yield highly valuable details regarding the microscopic aspects of molecular motions, including atomic motion geometry across various length scales, through the analysis of scattering wave-vector dependencies.[164] Nevertheless, the frequency range accessible through neutron

spectroscopy is limited, primarily enabling the study of protein dynamics on a timescale ranging from picoseconds to nanoseconds.[165] While neutron scattering can provide valuable information in conjunction with vibrational spectroscopy methods, it does have certain drawbacks. Larger sample sizes are required due to the weak neutron sources and low efficient neutron detector.[166] In addition, The highly specific and accurate nondestructive tool of neutron activation analysis makes it expensive and impractical for routine use.[167]

3.2.3.4. Solid-state nuclear magnetic resonance (ssNMR)

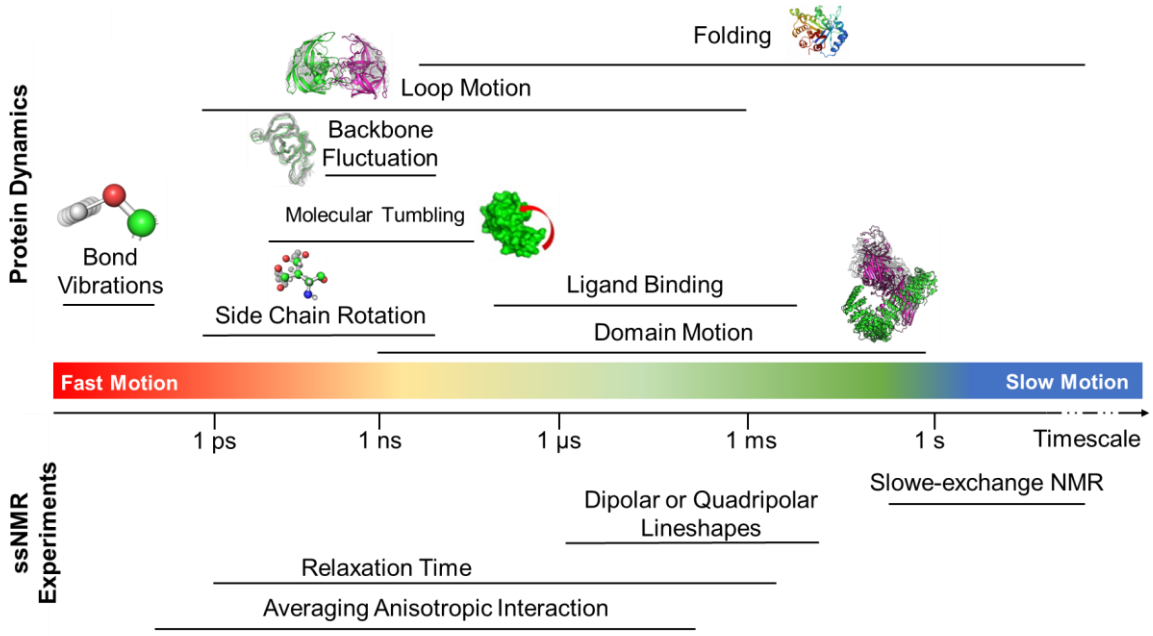


Figure 8. Illustration of molecular dynamics in proteins characterized by ssNMR spectroscopy.[168-170]

NMR spectroscopy plays a significant role in the investigation of protein dynamics since it allows for the determination of dynamics at the level of individual atoms.[171] The movement of a molecule, whether it involves small-scale bond vibrations or large-scale domain motions, causes atoms and bonds to reorient in space. Consequently, this reorientation alters the spatial arrangement of electrons as well as various interatomic distances and orientations. These variations in bond angles, distances, and electronic environments can result in the fluctuations of spin interactions. Chemical shift, chemical shift anisotropy (CSA), relaxation time, and dipolar coupling measurement from NMR are commonly used to probe these dynamic changes. Solid-state NMR (ssNMR) has a natural advantage over solution-state NMR in detecting motions that occur over timescales exceeding tens of nanoseconds, even when there are no isotropic differences in chemical shifts,[172] as shown in **Figure 8**. Especially, magic-angle spinning (MAS) NMR was used to probe motions on the nanosecond-microsecond-millisecond timescales by spin-relaxation experiments.[172-174] It has been widely used for side chain dynamics characterization. For example, the line shape and spin relaxation measurements via ssNMR parameters can probe specific site motion. Side chain motion of dry *streptomyces* subtilisin inhibitor protein power has been observed and quantified by ^{2}H ssNMR.[175] Comparison solution ^{15}N relaxation data and solid-state $^{1}H^{N}$–^{15}N dipolar couplings suggested little or no protein backbone dynamic but evidence of ns-us fast motions in the flexible loops and termini.[176]

In addition, the combination use of R_1, $R_{1\rho}$, and S^2 data for both $^1H\alpha$–$^{13}C\alpha$ and ^1H–^{15}N have also been studied as a powerful tool to characterize three timescales of motion (slow and global motion, intermediate, and fast motion), such as application in backbone dynamics of HET fibrils.[177] Moreover, ssNMR often is used to investigate the motion of secondary structure elements. Such as the loop and helix dynamics in an intramembrane protease were characterized by relaxation dispersion and conformational changes by ssNMR in the liposomes.[178] Transient unfolding of a β-sheet in the excited state of a macroglobulin mutant D76N beta-2 was found by MAS ssNMR probing the intrinsic dynamics of both wildtype and mutant forms of proteins both correlating to its increased aggregation propensity.[179] ssNMR is also particularly powerful for large protein complexes characterization. Zinke *et al.* found that bacteriophage tail tube-bending mediated by highly flexible hinge regions through ^{15}N R_1 and ^{15}N $R_{1\rho}$ relaxation rates and cryo-EM characterization.[180]

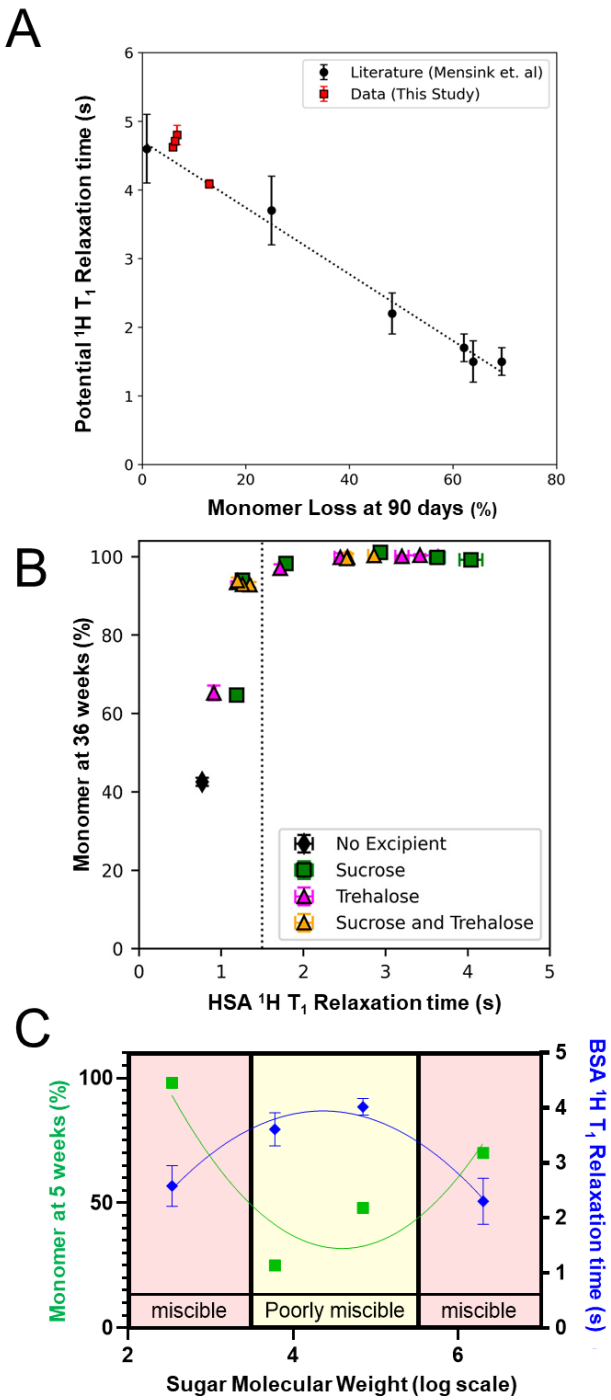


Figure 9. Correlation between ^{1}H relaxation time and protein stability. (A) Correlation between protein aggregation rate (loss of monomer per the square root of time during storage as determined with high pressure size exclusion chromatography (HPSEC)) versus protein ^{1}H T_1 relaxation times for the different formulations;[23] (B) HSA relative monomer percent after storage at 50 °C for 36 weeks as a function of HSA ^{1}H T_1 relaxation time;[181] and (C) An illustrative plot showing the correlation between sugar molecular weight, BSA monomer percentage after 5 weeks of storage at 60°C, and protein ^{1}H T_1 relaxation times across different lyophilized formulations. The figure is plotted from data in Nguyen *et al* [182]

Aside from extensive application in the dynamic characterization of neat solid protein molecules, ssNMR also remains a powerful technique for the investigation of protein conformation and dynamics in lyophilized powders. By utilizing ssNMR relaxation measurements, one can track the mobility of protein side chains, surface groups, excipients, and bound water molecules, correlating these findings to the stability of proteins in freeze-dried solids. The commonly used methods are measurements of the spin-lattice relaxation time in the laboratory (T_1) and rotating frame ($T_{1\rho}$) by ssNMR, where T_1 and $T_{1\rho}$ characterizes the amplitude of the high-frequency (MHz) and low-frequency (kHz) motions, respectively.[183, 184] Many ssNMR studies have described that β-relaxations are more critical to protein stability instead of α relaxations under certain circumstances. [23, 183-189] Relaxation parameters on different time scales can be used to evaluate spin diffusion over different length scales. ^{1}H T_1, typically 1−10 s, corresponds to a spin diffusion over ∼20−100 nm, while ^{1}H $T_{1\rho}$, ranging from 1 to 10 ms, estimates spin diffusion over ∼ a1−20 nm length scale. For instance, Lam *et al.* measured the T_1 values of pure lysozyme, trehalose, lactose, trehalose-lysozyme, and lactose-lysozyme at different hydration levels by ssNMR.[183] The increase of T_1 values induced by both sugars was found well correlated with the enhancement of protein stability. In contrast, the change in aggregation and activity of proteins was attributed to the hydration in the formulations, as reflected by the increased relaxation rates, R_1, or reduced relaxation time, T_1 values, with increasing water contents. Moreover, Yoshioka *et al.* measured the $T_{1\rho}$ for both protein and sugar (trehalose, sucrose, and isomaltose) carbons in freeze-dried lysozyme formulations.[186] It was found that the relaxation time constant (τ_c) and temperature coefficient of β-relaxation were both decreased in the presence of sucrose while increased in the presence of isomaltose, indicating the significant impact of sugars on the β-mobility of the lysozyme. Another study by Yoshioka *et al.* also demonstrated that the degradation rate of lyophilized insulin is determined by β-relaxation rather than global matrix mobility.[187] Compared to the insulin-dextran formulation, the addition of trehalose was shown able to prolong the $T_{1\rho}$ of the insulin carbonyl carbon at 12% relative humidity. Meanwhile, the insulin-trehalose glasses presented a substantially lower degradation rate than the insulin-dextran system at a temperature below the T_g, suggesting β-relaxation is likely the determinant process for protein degradation. Later, Mensink *et al.* and Lay-Fortenbery *et al.* investigated the impact of various sugars on the lyophilized proteins' stability.[23, 190] The relaxation T_1 correlated well with the stability of protein as shown in **Figure 9A**. Recently, Tower *et al.* used ssNMR to predict the stability of lyophilized human serum albumin with the presence of sucrose and trehalose.[181] They explored the correlation of human serum albumin stability as a function of ^{1}H T_1 relaxation time in **Figure 9B**. Interestingly, formulations with relaxation time less than approximately 2s demonstrated lower stability. Most recently, Nguyen *et al.* found that the relaxation time is not always proportionally decrease with increase molecular weight of sugar.[182] BSA with shorter T_1 relaxation time with trehalose and dextran 2000 kDa was found to have better stability as shown in **Figure 9C**, in addition to their good performance in protein-sugar miscibility.

Though ssNMR offers detailed insights into the local movements of specific groups within proteins for examination of protein dynamics, such as methyl groups, it does not provide comprehensive information about larger-scale global motions in existing pharmaceutical applications. In addition, ssNMR relaxation measurement is also sensitive to the moisture or water content in the lyophilized sample which impacts its application in dynamic quantification and corresponding stability correlation.[191] Considering water as the dominant relaxation resource in the lyocake and its significant impact on protein relaxation properties through ^{1}H-^{1}H spin diffusion, interpreting the relaxation values without controlled low moisture levels can

be misleading. Furthermore, there is so-far limited application in high-resolution structural characterization using ssNMR as the orientations of proteins in amorphous solids are considerably more random.[133]

3.2.3.5. Other techniques

Infrared spectroscopy (IR) based on molecular vibration is a well-established experimental technique and its primary application is to characterize the secondary structural of polypeptides and proteins in the solution as well as in the solid state.[192] Most IR structural dynamic studies focused on H-D exchange of proteins on a global scale.[193, 194] Some studies also focus on the exchange rate of secondary structure components.[195] As a successful and one of the very few demonstrations in structural dynamics, Dong *et al.* investigated the exchange rate of cAMP receptor protein secondary structural components and facilitated the interpretation of the ligand-induced conformational and structural dynamics changes.[196]

Hydrogen deuterium exchange mass spectrometry (HDX/MS) has been widely used to study protein structure, interaction, and dynamics. Most HDX/MS studies utilize a continuous labeling strategy where a native protein is exposed to deuterium oxide (D_2O) and the deuterium incorporation is monitored as a function of exposure time. The protein region with open conformations undergoes faster deuterium exchange. Protected or tightly folded regions show slower exchange which is also determined by the dynamic of protein in the solution.[197] This indicates that continuous labeling HDX primarily monitors protein structural dynamic changes, rather than actual structure.[198] However, there is an obvious connection between the two aspects, because tightly folded regions are generally less dynamic than those that are disordered. HDX/MS typically indirectly probe the structural dynamic as a matter of kinetic of deuterium exchange which cannot provide detail of specific motion. As such, HDX was traditionally coupled with NMR analysis to determine protein structure and dynamics at high resolution.[199]

4. Molecular attributes and formulation factors that impacts protein stability

Besides understanding the dynamics-induced protein instability, molecular details also play a critical role in the protein's biophysical and biochemical stability. In this session, we aim to overview the molecular factors including protein structure, miscibility, local microenvironmental pH, packing density, and interfacial stress.

4.1 Protein Structure

Protein structure is highly dynamic and conformational changes can occur as a result of formulation composition or environmental stress. The native structure of a protein and its storage stability are closely correlated.[84, 133] As such, protein drugs are often formulated as lyophilized solids to preserve their native structure and minimize the rate of degradation during storage. Proteins can undergo conformational changes, denaturation, or aggregation which are all examples of protein dynamics, when formulated and stored under unfavorable conditions. These changes can result in loss of biological activity, reduced solubility, increased susceptibility to degradation, and invoke an immune response.[200] The preservation of the solid-state native structure is crucial for maintaining the stability and functionality of the protein during storage.[98] Understanding protein structure and dynamics is challenging and requires advanced biophysical techniques. Solid-state Fourier transform infrared spectroscopy (ssFTIR), solid-state HDX (ssHDX) and ssNMR are used as some common methods for protein structure characterizations.[201]

ssFTIR has been widely used to study the stabilizing effect of excipients on structural changes in lyophilized protein formulation. For example, Allison *et al.* studied the impact of sugar in inhibition of dehydration-induced lysosome unfolding.[92] The degree of structural protection in FTIR amide I infrared spectra correlated well with the ability of sucrose and trehalose to form the extent of hydrogen bonding between sugar and proteins. In another study, the storage stability of IgG1 and human serum albumin (rHSA) study showed a relatively good correlation with the degree of retention of the native structure of protein during dying from the spectra correlation coefficient of FITR spectra.[98] However, the disturbance of secondary structure does not necessarily correlate to the compromised protein stability. According to several studies, there is a negative association between the degree of retention of native protein structure as determined by FTIR and the propensity of protein aggregation for lyophilized human growth hormone,[202] IgG-1,[203] lactate dehydrogenase,[39] recombinant human albumin,[76] and γ-interferon.[204] Due to its low resolution and semi-quantitative characterization tool for protein secondary structure, it has limited predictive power for protein stability or degradation rates in the solid state. Another limitation is that it only characterizes the global protein conformation changes and cannot monitor the local or tertiary structure changes which also impact the protein stability.

HDX has been utilized as a technique to investigate the structure of proteins and solvent accessibility. This is achieved by examining whether a protein retains its native folded conformation in a particular solution formulation.[205] If the components of the formulation fail to stabilize the protein, its conformation may be perturbed, leading to additional aggregation or degradation. By employing HDX, it becomes possible to identify conformational changes, thereby providing insights into the physical stability of the molecule. Understanding protein-excipient interactions is important to optimize protein formulations and identify specific excipients that effectively enable their solid-state stability. ssHDX can be applied as a solid characterization method for protein conformation in the dried formulation by exposing protein solids to the D_2O vapor and followed by mass spectrometry (MS) analysis. In a study of myoglobin, sucrose formulation demonstrated less level of deuterium uptake and correlated well with better storage stability.[201] The correlation between protein stability and the H/D exchange rates has been observed in various proteins, including BSA, lysozyme, and β-lactoglobulin.[21, 201, 206-208] Later on, the extent of hydrogen/deuterium H/D exchange (X_∞) is proposed as a good short-term measurement predictive of long-term stability, indicating the rigidity of structure and dynamics of the protein formulations. Rui *et al.* found that the level of H/D exchange (X_∞) was reduced in the protein that was freeze-dried using a sucrose matrix compared to a trehalose matrix under conditions of 40^{o}C and 11% relative humidity. This finding indicates that the protein retained greater stability in the sucrose formulation, at least under these specific conditions.[209] However, ssHDX might not be capable of detecting differences among various excipients when the presence of excipients does not affect the protein backbone or if excipients interact with the protein through certain mechanisms other than hydrogen bonding.[13, 210]

ssNMR has been widely used to study the structure of insoluble membrane proteins and amyloid fibrils.[211] However, it faces challenges in acquiring high-resolution structural information for amorphous protein solids as they are highly randomly oriented. Nonetheless, ssNMR is still a sensitive spectroscopic tool to provide atomic-resolution structural insights and several studies have evaluated the structure of biological samples including peptides, proteins, and antibodies in the solid-state form.[212] Zhou *et al.* utilized two-dimensional spectra (^{15}N–^{1}H and ^{13}C–^{1}H) and characterized the structure of lyophilized aprotinin and insulin in different physical forms including lyophilized solid, microcrystalline suspension, and fibril

forms.[213] Compared to the relatively simple structure of peptides, there is also growing interest in understanding the higher-order structure of monoclonal antibodies in solid-state form after lyophilization or spray drying. Jacqueline *et al.* demonstrated the feasibility of ssNMR ^{1}H–^{13}C cross-polarization buildup structural fingerprinting on the lyophilized mAbs at natural isotopic abundance using the NIST mAb as a model system.[214]

4.2 Protein-excipient miscibility

As discussed above, the key mechanism of stabilization from the water replacement theory is the hydrogen bonding formation between protein and sugar. As such, good miscibility between protein and sugars at a molecular level is suggested to ensure the protective effects of sugars. Any protein and sugar immiscibility or heterogeneous distribution could result in detrimental effects on product stability due to a lack of sufficient interaction. It has been reported that higher rates of protein aggregation were seen with low miscibility.[23] The sugar selection and drying techniques play a critical role in the final product miscibility. For example, the crystallization of one or more components in the matrix, such as mannitol crystallization, can introduce heterogeneity in freeze-dried formulations. Additionally, heterogeneities can also be introduced by the drying technique, such as the heterogeneously distribution of protein from surface to inner core induced by spray drying process.[206] In such a case, the loss of protein-sugar interaction can lead to significant alterations in protein structure. As such, from a formulation design point of view, the crystallization of sugar needs to be avoided and sugars with a low tendency to crystallize are often preferred.

Although the importance of miscibility in ensuring protein stability is recognized, there are limited studies to evaluate the impact of excipients and processes in the inhomogeneity of solid protein formulations. This is mostly due to the lack of a high-resolution technique that can identify and quantify the mixing of protein and excipient. Common miscibility/homogeneity characterization techniques like DSC and X-ray diffraction (XRD) can identify inhomogeneity in the solid formulation, but their sensitivity can be insufficient to monitor inhomogeneity at a smaller scale in the solid protein formulations. The traditional high-resolution surface imaging techniques such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM) can achieve the nanoscale resolution often necessary for miscibility evaluations. However, these techniques are not able to identify the chemical compositions of different phases. With more advanced x-ray photoelectron spectroscopy (XPS) and time of flight secondary ion mass spectrometry (TOF-SIMs) surface characterization, which enables the chemical composition characterization of the excipient atoms with nitrogen might interfere with protein quantification.[215] The typical sub-surface imaging using spectroscopic techniques such as fluorescent spectroscopy, IR, and Raman spectroscopy, provides component distribution, but low image resolution and low depth penetration.

ssNMR has been implemented as an advanced and high-resolution tool to assess the molecular mixing of bulk powder samples at sub-100-nm resolution in amorphous solid dispersions (ASDs).[216] Going beyond the applications of small molecule formulations, ssNMR also offers high resolution to identify phase separation in glassy protein formulations.[23] The ^{1}H ssNMR spin-lattice relaxation measurements were typically used to evaluate the miscibility of protein-sugar lyophilizates, illustrated by the three scenarios in **Figure 10**. NMR relaxation values in the laboratory (T_1) and rotating frame ($T_{1\rho}$) represent the averaged properties of multiple nearby nuclei due to homonuclear ^{1}H spin diffusion. Relaxation parameters on different time scales can be used to evaluate spin diffusion over different length scales. ^{1}H T_1 is typically

ranging from 1-10s, corresponding to a spin diffusion over ~ 20-200nm, while ^{1}H $T_{1\rho}$ is from 1-10ms, estimates spin diffusion over ~ 1-20 nm length scale. The miscibility between sugar and protein can be identified on a 1-10 and a 20-200 nm length scale respectively through the relaxation time comparison. It was applied to understand the impact of the molecular weight of sugar on the miscibility and corresponding storage stability of IgG after lyophilization. Smaller sugars like trehalose provide better miscibility with IgG on both small and large scales, whereas the largest protein dextran 70 kDa performed worst and phase separation was observed on both scales. The miscibility behavior correlates with protein storage stability. Smaller sugar trehalose demonstrated better miscibility as a lower tendency of aggregation during storage and vice versa for larger sugars.[23] Further, Chen *et al*. have recently utilized ssNMR to investigate the impact of protein-excipient interaction and miscibility on the physical stability of spray-dried protein formulation.[21] The data suggest substantial immiscibility and phase separation between BSA and Leucine which leads to a poor stabilization effect. In contrast, trehalose showed the best storage stability in spray-dried BSA solids as trehalose remains amorphous and is miscible at both 1-20 and 20-200 nm scale. Buffer salt is the most common excipient used in the protein formulation to retain the appropriate pH and buffering capacity. Mutukuri *et al.* examined the effect of buffer salts on the physical stability of BSA from both spray-dried and lyophilized formulations.[208] ssNMR effectively identified the presence of phase separation between the succinic salt and the BSA in the lyophilized formulation. This phase separation results in a loss of long-term stability. Similarly, Tower *et al.* utilized ssNMR to measure homogeneity on 20–50 and 1–3 nm domains and mobility related to β-relaxation of HSA with trehalose or sucrose lyophilized solid.[181] The trehalose crystallization at higher trehalose to HSA ratio resulted in sample inhomogeneity by differences in the ^{1}H T_1 and ^{1}H $T_{1\rho}$ relaxation time values. In addition, a ^{1}H T_1 relaxation time shorter than 1.5 s indicates an unstable sample under the given stability conditions and ssNMR acquisition parameters.

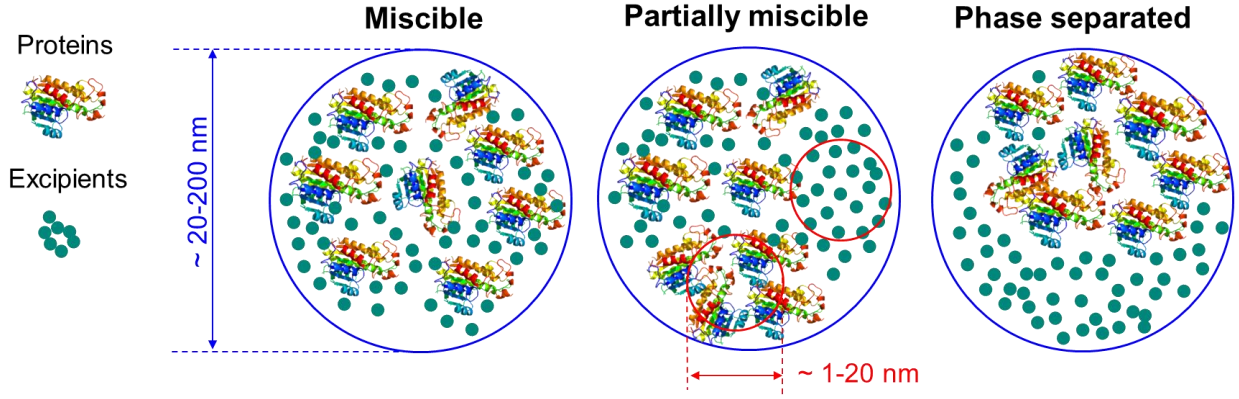


Figure 10. ssNMR quantification of protein-sugar miscibility in dry biologic. The molecular miscibility in all three scenarios can be evaluated at the domain size of approximately 20-200 nm and 1-20 nm, by comparing the ^{1}H spin-lattice relaxation time in the laboratory (T_1) and rotation frames ($T_{1\rho}$) of protein and excipients, respectively.[21, 181, 208]

4.3 Protein-excipient interaction

As discussed in **Section 3.1**, from a thermodynamic perspective, the hydroxyl groups of sugar molecules form sufficient hydrogen bonds with protein substituting the hydrogen bonds that were previously formed between water and protein to preserve the native conformation of proteins. Straight

probing the sugar-water replacement and sugar interaction with protein is challenging in analytical characterization. FTIR was utilized to probe changes in the protein secondary structure as an indirect way to investigate the excipient stabilization effect on protein structure after drying.[82, 98, 195] Carpenter and Crowe demonstrated that sugars can hinder changes in the amide II band through FTIR, which indicates alternations in protein secondary structure and hydrogen after drying as the first evidence of water replacement theory.[82] However, quantitatively characterizing the relationship between alterations in FTIR spectra and the extent of protein structure disruption can be challenging, particularly when there are only slight changes in peak positions. More advanced solid-state characterization technique, such as ssNMR, was further applied to understand the role of protein-excipient interaction in the physical stability of protein after drying. The changes in chemical shifts of NMR spectra can indicate the intermolecular interactions between proteins and excipients.[217] In a recent study of BSA with trehalose and mannitol,[21] found that arginine side chain -NH and -NH_2 are largely involved in hydrogen bonding as H donors to maintain the helical structure and tertiary folding of BSA. The signal assigned to the side chain C_ξ of arginine in the mannitol and trehalose was shifted from that of BSA without any excipient, indicating the disruption of intramolecular hydrogen bonds in BSA and possible formation of new intermolecular bonds with sugar molecules. (**Figure 4B)**

4.4 Microenvironmental acidity

Buffer salt is typically used to maintain the pH of the formulation to control the stability of proteins. A commonly encountered issue is the pH shift during the lyophilization process that can occur due to the undesired crystallization of a buffer component, resulting in loss of protein activities.[218-223] The buffer ionization constant, *pK*a and crystallization of buffer components determine the pH changes in the freezing cycle.[224] Phosphate, histidine, acetate, citrate, succinate, glycine, and tris are the common buffers.[225] Phosphate and succinate are well-known examples of a buffer that can crystallize.[226, 227] On the other hand, buffers like acetate and tris buffer are generally considered non-crystallizing under a representative lyophilization cycle.[228] Aside from the buffer type, researchers have demonstrated that the formulation and freezing condition including sample size and cooling rate can impact the buffer crystallization behavior, such as selective crystallization of disodium hydrogen phosphate.[229] The acidity of lyophilized materials can be evaluated using the Hammett acidity function, which measures the activity of H^+ ions in the solid.[230] This function utilizes an acid-sensitive dye to determine the state of acidity. Hammett acidity has been proven to accurately predict the rates of acid-catalyzed chemical reactions in the solid materials and a correlation between stability and measured acidity in the solid state was established from several studies.[230-235] As a result, it has been proposed as a valuable tool for characterizing and ranking the stability of formulations containing acid-sensitive molecules in dry formats.[236]

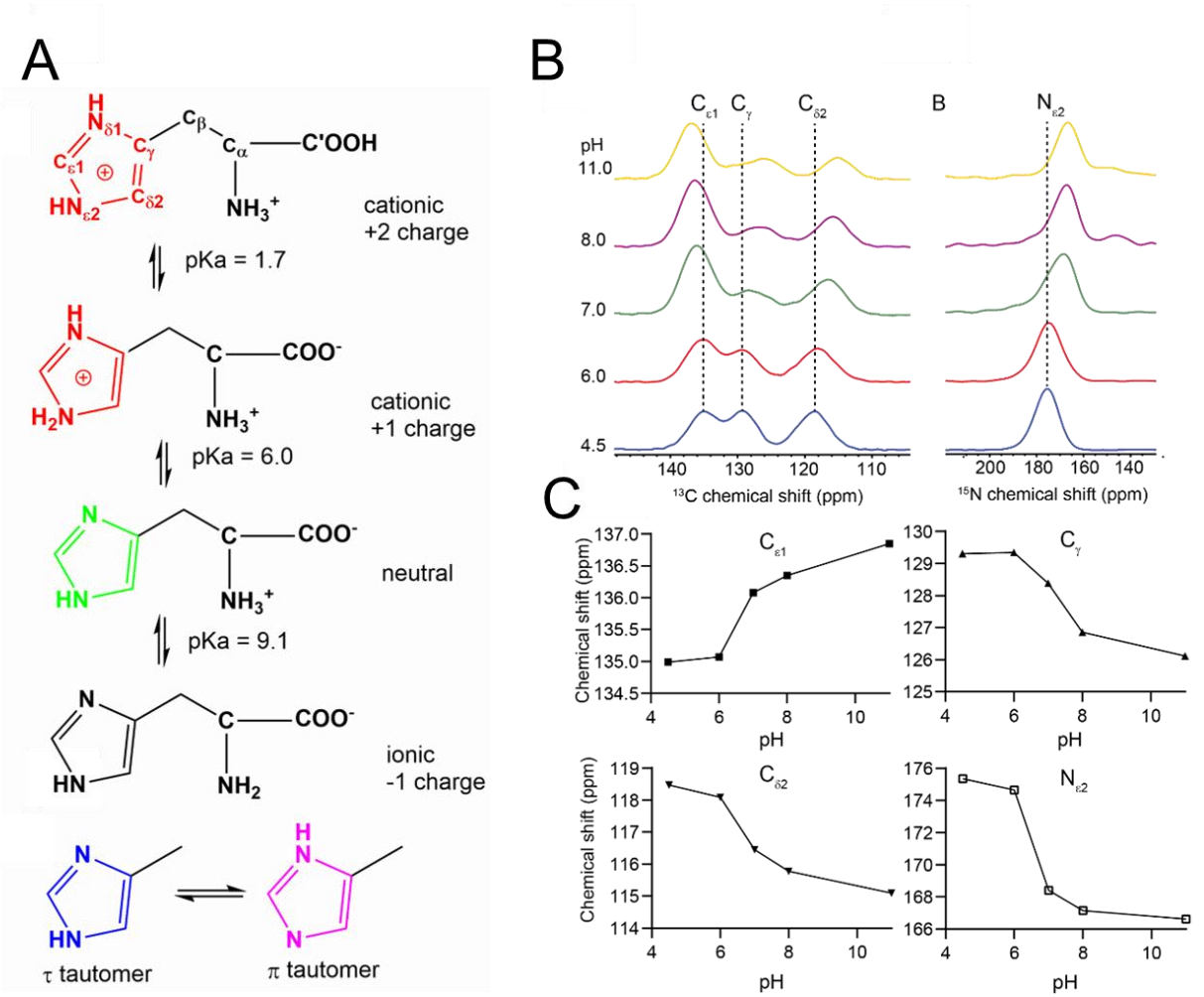


Figure 11. Microenvironmental acidy probed by ssNMR.[20] (A) Protonation states of histidine and tautomerization of its imidazole ring; (B) 1D ^{13}C and ^{15}N spectra of histidine in the lyophilized powder at pH 4.5 to 11.0; and (C) Correlation between C_{ε}, C_{γ}, $C_{\delta 2}$ and $N_{\varepsilon 2}$ chemical shifts of amorphous histidine and pH values at which the formulations were prepared.

DSC, SEM, and low-temperature X-ray powder diffractometry provided evidence of the crystallization of disodium hydrogen phosphate. The pH shift as a consequence of the crystallization of phosphate buffer was detected during the freezing step. The pH values of a frozen solution can be determined using low-temperature pH electrodes operating at sub-zero temperatures, by adding a universal pH indicator solution, or by synchrotron radiation.[237] Studies have shown that the extent of pH shift from phosphate buffer is concentration dependent, where an initial buffer at a pH 7.4 drops to pH 5.2 with a lower concentration of phosphate buffer at 8mM in contrast to pH 4 with a higher concentration of 50 and 100mM buffers.[238] However, even when protein was formulated with a very low concentration of phosphate buffer at 1mM, the crystallization of disodium hydrogen phosphate as a dodecahydrate was still detected by high sensitivity synchrotron radiation technique.[237] Furthermore, when proteins are formulated in the lyophilized state, they exhibit something known as "pH memory", which refers to lyophilized proteins retaining the characteristics and behavior related to pH that they had prior to being freeze-dried. It is crucial

to understand that the impact of pH shift will vary among different proteins and may not elicit the same effect universally. Though IgG2 and bovine IgG were not sensitive to the pH shift, the impact on β-galactosidase and lactate dehydrogenase protein can be detrimental.[221, 222] Furthermore, the protein itself or another excipient can prohibit the buffer-induced pH shift.[239] Many methods have been attempted to measure the pH of protein formulation in the solid state including Hammett acidity function. An indicator dye-sorption method is widely used to measure the solid surface acidity based on the ionization state of the indicator dye molecule.[240] However, discrepancies have been observed comparing the dye-sorption method and slurry pH method depending on the compound and indicator.[241] As such, it is critical to develop a reliable and non-invasive technique to examine the microenvironmental acidity which serves as an indicator of stability and establishes the correlation with the stability of solid biological products after water removal.

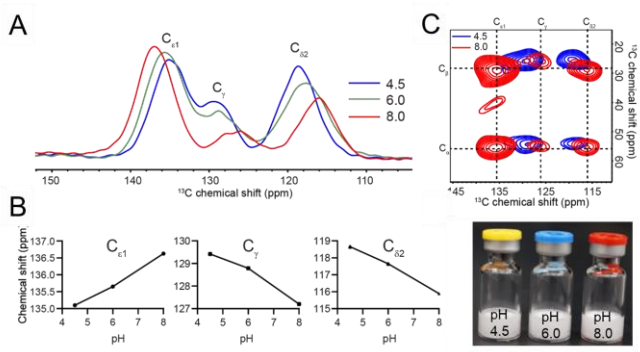


Figure 12. Microenvironmental acidity of lyophilized formulations of HPV probed by chemical shift changes of histidine in the solid state.[20] (A) Chemical shift changes of histidine in HPV vaccine formulations lyophilized at different pH conditions (pH 4.5, 6.0 and 8.0) observed in 1D ^{13}C ssNMR spectra; (B) 2D ^{13}C spectra of HPV vaccine formulations; and (C) Correlations between ^{13}C and ^{15}N chemical shifts and pH values of the starting solution.

Recently, an approach based on solid-state NMR was developed to noninvasively investigate the microenvironmental acidity in the lyophilized matrix and the first reported use of histidine as an indicator for measuring pH changes in the solid protein formulation by ssNMR. Histidine is commonly used in buffer and has unique acid-base properties making it a suitable choice of a pH indicator. Histidine contains four potential sites for protonation: the carboxylate end, two nitrogen atoms of the imidazole ring, and the amine terminal. The sequential deprotonation of these sites, including the carboxylic acid group, imidazolium cation, and amine nitrogen, occurs at specific pKa values of 1.9, 6.1, and 9.1, respectively and it can regulate shifts of pH as protonated τ tautomer and the $N_{\delta1}$-protonated π tautomer in **Figure 11A**. Protonation states of histidine lyophilized at a range of pH values from 4.5 to 11.0 were identified from full ^{13}C and ^{15}N resonance assignments in one-dimensional and two-dimensional NMR experiments in **Figure 11B**. The results demonstrated a pH-dependence of histidine chemical shift in the amorphous state in **Figure 11C**. This study, it was first explored the solid-state pH by ssNMR in the lyophilized human papillomavirus infection (HPV) vaccine formulations and observed microenvironmental pH shift using histidine as an indicator. The chemical shift perturbations shown from pH 4.5 to 8.0 in 1D and 2D spectra in HPV formulation in **Figure 12A** and **12B** were similar to those of histidine formulation alone, indicating that acidity is not affected by the HPV in the lyophilized formulation at the given conditions. C_{ε}, C_{γ}, $C_{\delta2}$ and $N_{\varepsilon2}$ chemical shifts in **Figure 12C** also agree well with the trend of chemical shift and pH established in **Figure 11C**. This ssNMR pH measurement approach was further applied to understand the crystallization of

disodium phosphate hydrogen dodecahydrate-induced phase transition and the pH of the freeze concentration using histidine as a molecular probe and based on the chemical shifts of atoms of interest.[242] It is worth noting that the methodology using histidine as a molecular probe to identify microenvironmental pH has also been utilized in quantifying acidity in pharmaceutical frozen solutions.

Recently, the homogeneity of salt distribution in solid biologics has been evaluated by protein-buffer miscibility using ssNMR. Mutukuri *et al.* found the ^{13}C-detected ^{1}H $T_{1\rho}$ values were significantly different for BSA, trehalose, and succinic acid in lyophilized formulations by ssNMR, as shown in **Figure 13**.[208] This finding offers compelling proof of variability within a domain scale smaller than tens of nanometers. Besides the relaxation measurement, the distribution of chemical shifts implies variations in microenvironments acidity. It has been demonstrated that alterations in acidity, such as in frozen solutions or lyophilized protein formulations, can detrimentally affect stability.[219, 227] The distribution of microenvironmental acidity, as evidenced by the observed protein and excipient immiscibility, strongly aligns with the physical stability of BSA formulations. This is evident by the highest degree of monomer loss detected through SEC analysis in lyophilized BSA with succinate buffer.

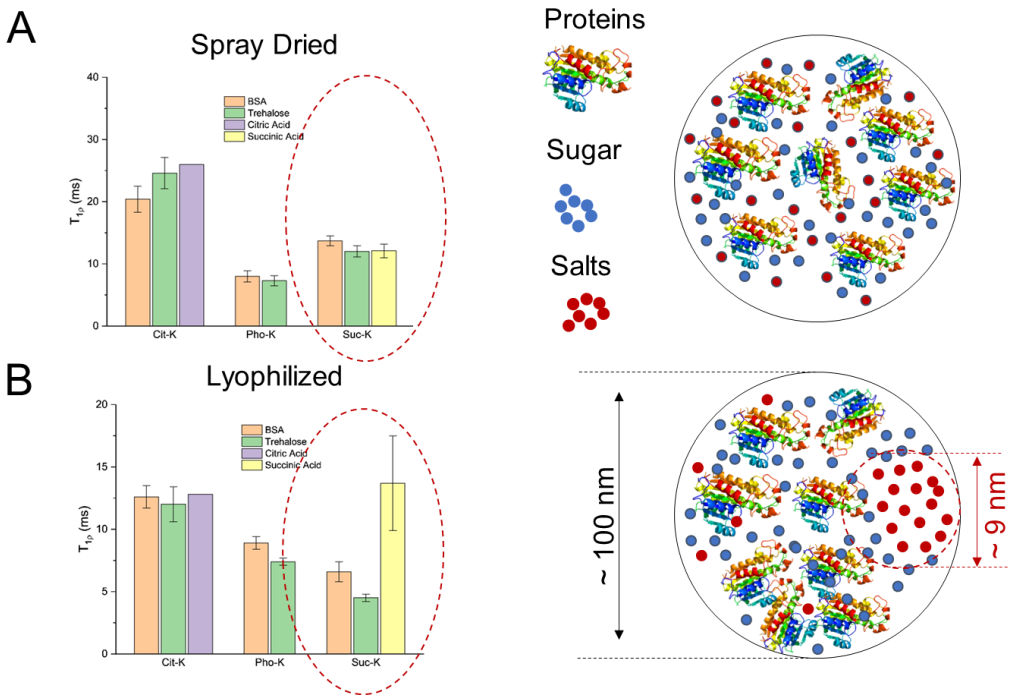


Figure 13. Evaluation of microenvironmental acidity through quantifying the miscibility of protein, sugar, and salt.^{13}C-detected ^{1}H $T_{1\rho}$ of BSA, trehalose, and buffer salt in spray-dried (A) and lyophilized (B) BSA formulations with potassium counterion at T0, and the corresponding scenarios of molecular mixing. Tre: trehalose; Pho: phosphate buffer; Suc: succinate buffer; Cit: citrate buffer; K: potassium.[208] It is worth noting the phase-separated domain of succinic acid in the lyophilized formulation in (B).

4.5 Surface area and interfacial stress

Protein stability is known to be sensitive to interfacial stresses. While processing protein formulations, it is unavoidable that solutions will come into contact with an external interface of some kind.

This exposure can occur through filtration, filling, or drying techniques.[243] Biopharmaceutical products such as spray-dried powders and lyophilized cakes are usually along with high surface areas. Surface regions tend to have increased quantities of surface-active species, such as proteins or surfactants. Protein aggregation at interfaces frequently involves alterations in conformation, as proteins adjust their higher-order structure in reaction to interfacial pressures such as hydrophobicity, charge, and mechanical stress.

During the freezing step in the lyophilization, protein molecules tend to adsorb and accumulate at the interface between ice and water, leading to denaturation and unfolding of proteins. As the protein solution undergoes freezing, the process of ice nucleation and crystal growth begins, creating an ice-water interface. The presence of this interface significantly impacts protein stability during freezing, as protein molecules interact and adsorb at the ice-water interface. Consequently, the ice formation can disrupt the interaction between water and the protein's surface, resulting in the loss of stabilizing hydration interactions between water and the protein components. It has been reported by Abdul-Fattah *et al.* that higher protein stability was seen with lower fractions of protein on the surface comparing different drying processes including freeze drying, spray drying, and foam drying associated with levels of sucrose in the formulation.[244] Similarly, a study of using human growth hormone (hGH), the stability of freeze-dried hGH was found in a good correlation with the surface protein quantity.[97] These experimental results all suggest that the relative fraction of protein on the surface over the bulk needs to be considered as well. Therefore, it is anticipated that the protein at the surface is less stable. Thus, the quantity of protein on the surface will impact the overall stability of the protein.

As discussed in **Section 2**, spray drying attracted many interests as an alternative drying technology for biologicals with the advantages of high throughput, and the ability to obtain desired flowability and aerosolization. However, there is a stability concern with spray-dried protein solids associated with drying stresses such as high temperature, atomization stresses, and air-liquid interfacial stress. Such effects on the stability of the formulation are dependent on the process parameter, protein type, and formulation. Costantino *et al.* discovered a direct correlation between the surface area of spray-dried BSA powder and the loss of monomers.[50] Similarly, a recent study examining the heterogeneity and particle surface composition of spray-dried BSA has indicated that proteins exposed at the surface of particles exhibit a higher degree of variability.[206] A correlation of the percentage surface composition of protein to the peak area of the deconvoluted mass envelope relative to an experimentally fully-deuterated sample which as an indicator for protein population heterogeneity was established.

5. Mechanism-driven drug product design from optimizing formulation composition

5.1 Rationale choice of sugar as protectant

5.1.1 Sugar size and molecular flexibility

As described in **Section 3.1**, it is critical to ensure adequate contact between sugar and proteins to restrict the protein motion. As a good stabilizer for protein stabilization, sufficient hydrogen bonds between protein and sugar, adequate mobility suppression, and sufficiently high T_g during the drying process is the prerequisites. The size and molecular flexibility of sugars determine their ability to stabilize proteins. As such, disaccharides such as trehalose and sucrose are typically considered the best choices for stabilizing disaccharides for therapeutic proteins. Practically, trehalose with good interaction with protein and reduced local mobility is commonly considered an ideal stabilizer.[117] On the other hand, large molecule weight sugar such as dextran is not selected to use as a sugar stabilizer, especially when used alone. The main drawback

is the limited flexibility of the molecular chains which limit their ability to interact with protein and form adequate hydrogen bonding due to their large size. As such, the steric hindrance limits the adequate interaction/hydrogen bonding between protein and sugars and compromises the effect of slowing the protein dynamic in the solid state. The capability of vitrification at the surface of the protein is also compromised.[92] Likely, study has shown that molecularly flexible oligosaccharide inulin 4 KDa provided better stabilization in four different proteins than the similarly sized but molecular rigid oligosaccharide dextran 6 KDa.[49] Recently, Nguyen *et al.* found an interesting correlation between sugar molecular weight and miscibility and stability. They hypothesized that the protein-sugar miscibility is not a function of the absolute size of the sugar, but instead is a function of the relative size of the sugar to the protein.[182] The order of miscibility was found to be trehalose > dextran 2000 kDa > dextran 6 kDa and dextran 70 kDa shown from **Figure 14A** and **Figure 14B**. BSA molecules were dispersed within the polymer chains of dextran 2000 kDa, and hence have a lower tendency to be in contact with other BSA molecules. The proposed model of sugar and protein miscibility was shown in **Figure 14C**.

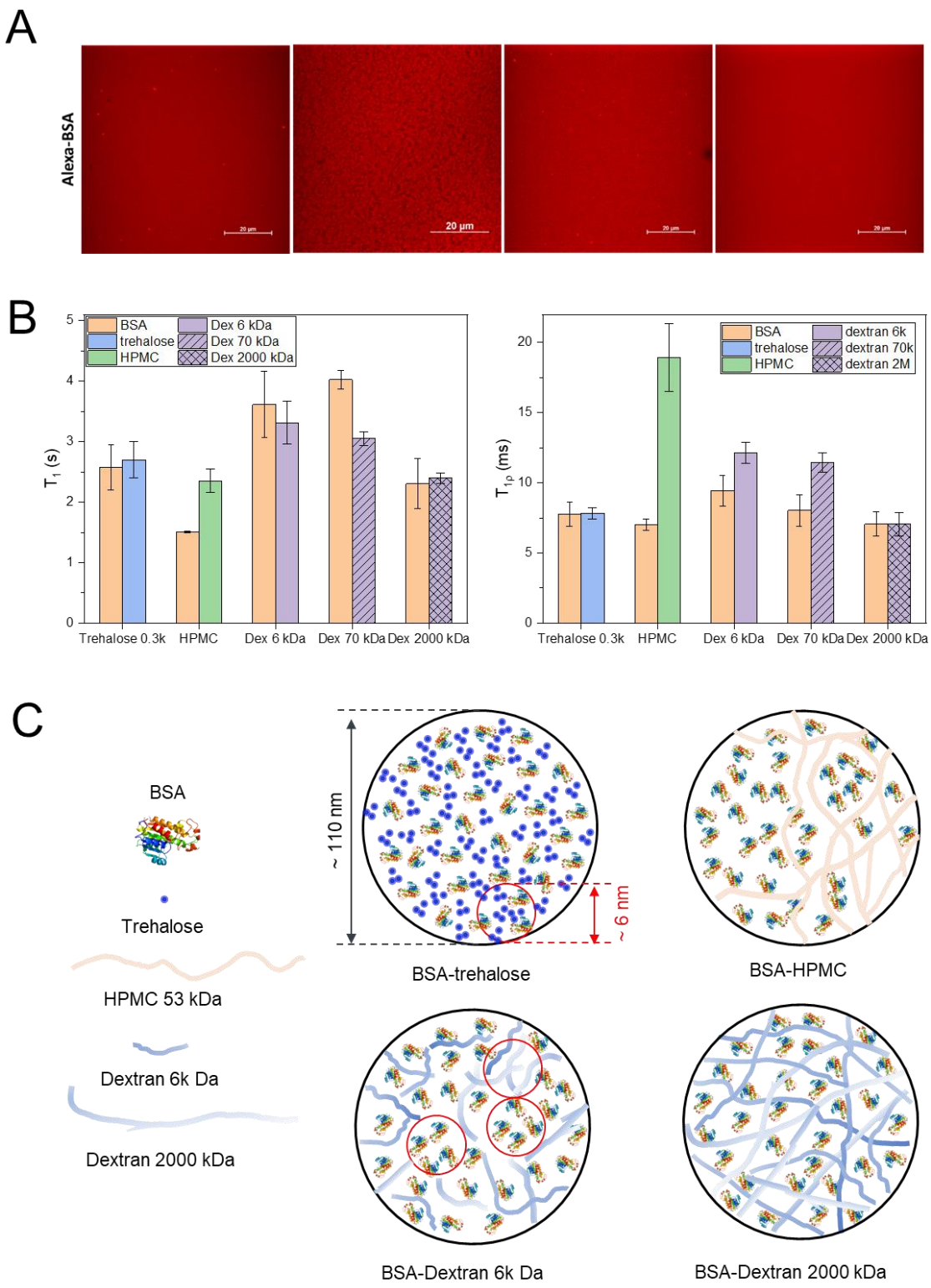


Figure 14. Impact of sugar molecular weight on protein-excipient miscibility. (A) Fluorescence images of BSA films containing trehalose, dextran 6 kDa, dextran 70 kDa, and dextran 2000 kDa; (B) T_1 (left) and $T_{1\rho}$ (right) measurements of lyophilized BSA samples (30% w/w BSA) with different additives; and (C) Proposed models of protein-sugar miscibility.[182]

Aside from the stabilization consideration in formulation design, the drying process efficiency also plays a critical role in the formulation optimization. Though it is well known that sucrose is traditionally utilized as a sugar stabilizer in solid protein formulation, from a process perspective, sucrose has some limitations in the drying steps including low glass transition of 60 ºC and collapse temperatures of -34 to -25 ºC. Consequently, these formulations require low primary drying temperatures making the lyophilization cycle time-consuming and costly. Additionally, vitrification can be problematic when the moisture content cannot be kept low. A combination of larger oligosaccharides or polysaccharides which has higher T_g with disaccharides can be an alternative strategy to mitigate this issue to increase the T_g of dried products. Dextran can function as an amorphous bulking agent and facilitate a rapid lyophilization process with the formation of a strong and elegant cake structure. In a study on a model protein BSA, Haeuser *et al.* found the influence of dextran with different molecule weights on the thermal properties and cake appearance in the solid state. Such a binary mixture of sucrose and dextran in an adequate ratio might potentially be superior to pure sucrose formulation allowing faster freeze-drying cycles resulting in elegant lyophilizates and good protein stability. Moreover, the 1:1 mixture of sucrose and dextran between 10-500 kDa showed about 5-fold reduced aggregation than pure dextran formulations and stability is inversely proportional to the increase of dextran molecular weight. A similar observation was found in the study using dextran 6 kDa and 70 kDa in insulin stabilization.[49] Remarkably, the insulin formulation with dextran 6 kDa was even less stable than the formulation of insulin only (no sugar). In contrast, the dextran 70 kDa formulation showed a more stabilizing effect than when the smaller dextran was used in freeze-dried protein formulations. The stabilizing capacity of dextran 70 kDa was substantially improved through a 1:1 weight ratio mixture.

In practice, as long as protein formulation maintains vitrified, sugars that are smaller and molecularly more flexible experience less steric hindrance, enabling them to establish a greater number of hydrogen bonds with the protein and subsequently exhibit enhanced stabilization of the protein. A combination of disaccharides and polysaccharides can be a potential strategy to provide a reduction in mobility with adequate interaction between sugar and protein and increase the T_g of solid formulation simultaneously. However, some precaution is still needed for dextran application as the glycation by free terminal glucose of dextran can lead to aggregations.[245] As such, it is important to exercise caution regarding the amount and ratio of these components.

5.1.2 Sugar crystallinity

As illustrated in **Section 4.2**, the miscibility of protein and excipient is crucial in maintaining protein stability. Excipient crystallization during the drying process can compromise the interaction between protein and sugar limiting the protein motion. As such, it is crucial for excipients as amorphous during the freezing and drying process to ensure sufficient hydrogen bonding interaction between protein and stabilizing agents. The capacity of sugar to form intermolecular hydrogen bonding can be diminished and loss of its stabilization function if the sugar crystallizes.[246] For example, though trehalose is considered as best sugar for protein stabilization as amorphous, crystallization can occur during the sublimation process when trehalose is formulated at high concentrations and subsequently, hydrogen bonding with protein is no longer possible. Carpenter *et al.* showed that the FTIR spectrum of lysozyme with crystalline trehalose was similar to the sugar-free enzyme lyophilized sample with limited frequency of amide II band which indicated decreased interaction between trehalose and protein when trehalose crystallized at high concentration.[82]

With this design principle to retain the amorphous form of sugar stabilizer, studies have explored formulation optimization to overcome excipient crystallization during the protein drying process. Suryanarayanan and coworkers investigated the influence of sucrose in trehalose dihydrate crystallization[247] and the effective inhibition of mannitol crystallization using pharmaceutical-acceptable additives, including salts and polysorbate 80.[248] Additionally, buffers also play a role in affecting the crystallization of bulking sugar agents in freeze-dried formulations. For example, potassium phosphate buffer was observed to hinder the mannitol crystallization and inhibit the protein structural changes during freeze-drying.[249] Similarly, the inhibition of mannitol crystallization was found with the presence of sodium phosphate and sodium citrate.[250] Furthermore, the formulation composition including the ratio of trehalose to proteins and the process parameter such as the slow cooling rate was found also play a critical role in inhibiting trehalose crystallization.[251] Similarly, for sugar which has a crystallization tendency, the concentration and percentage of sugar used in the formulation are the critical factors that drive the risk of crystallization and require optimization during the formulation development. For example, leucine used at 50% weight percentage in the BSA formulation was observed to lead to significant crystallization post spray-drying and subsequently worse BSA storage stability.[21] In contrast, no crystallization was found when leucine was only used at 20% and good protein stability was observed. Among the sugars studied by Chen *et al*,[21] leucine exhibited the highest propensity of crystallization following spray drying observed from ^{13}C MAS NMR. Overall, the stabilizing capability of sugars varies which largely depends on the sugar types, crystallinity, and drying process. Elucidation of the interplay between sugar type and drying regime would be valuable for formulation optimization.

5.1.3 Sugar to protein ratio

The water replacement theory discussed in **Section 3.1** stated the importance of stoichiometry of sugar to protein in providing adequate hydrogen bonding interaction for protein stabilization. The effectiveness of sugars as stabilizers for proteins can be subject to the ratio of sugar to protein in the mixture. Sugars might not provide an adequate stabilization effect during the process if the ratio of excipient to protein is not sufficient. The final mass ratio of sugar to protein determines the stabilization power for protein protection during drying.[11, 40, 221, 252] Cleland *et al.* recent study has demonstrated that a 360:1 molar ratio which is about 1:1 w/w of sugar to protein was essential to ensure good storage stability of protein.[94] In general, the optimal weight ratio of 3:1 to 5:1 of sugar to protein would be ideal for inhibiting lyophilization-induced unfolding.[34] However, the optimal ratio between sugar and protein for protein stabilization can be sugar-type dependent. Lu *et al.* found that the denature temperature gradually increased with an increase sucrose to protein ratio while decreasing with an increase in trehalose, xylitol, and lactitol to protein ratio.[253] Later on, a more comprehensive study have been done to investigate the impact of sucrose levels on protein stability from both thermodynamic and kinetic aspects. The study found a sucrose to protein ratio saturation point that the extent of five protein's structure protection from FTIR by sucrose reached a plateau after reaching a mass ratio of 1:1, though the stabilization effect limiting the aggregation behavior is sucrose concentration dependent. Interestingly, the fast local dynamic measured NS as $\langle u^2 \rangle$ demonstrated linear correlation with the log scale of aggregation rate constant over the entire sucrose range studied. As such, it is critical to evaluate the sugar to protein ratio on the protein's physical stability from both structure preservation and fast dynamic changes during the formulation composition optimization.

5.2 Surfactants

We have extensively reviewed the critical role of sugars as effective stabilizers protecting protein against stress during the freeze-drying process and storage. However, they cannot overcome all liabilities inducing protein instability, such as interfacial stress. As discussed in **Section 4.5**, the protein adsorption at the ice-water interface formed during the freezing step can disrupt such interactions between protein and water, which can subsequently induce interfacial stressed-related protein aggregation. The primary goal of the formulation design is to achieve sufficient hydrogen bonding between protein and water during the freezing step to preserve the natural structure of proteins prior to the drying process which sugar can subsequently replace the water to interact with protein to retain protein stability in the dried state. To overcome such interfacial stress and ensure the adequate interaction between water and protein, blending of surfactants such as polysorbates 80 and 20 in the formulation is a common strategy if biologic drug is sensitive to interfacial interactions.

Surfactant excipients preferentially coat surfaces and prevent proteins from unfolding at the interfaces. It can prevent protein aggregation by competing with proteins for adsorption at the air-water interfaces. Other distinct mechanisms of surfactant improve the protein stability by directly interacting with the target protein. Several mechanisms for surfactant stabilization have been proposed in the literature. Nonionic surfactants like polysorbates 80 and their hydrophobic segments can target the hydrophobic region of unfolded protein, thereby decreasing intermolecular interactions and helping protect them from self-association. Studies have also shown that an optimal composition of trehalose and polysorbates 80 can protect the lysozyme and retain more than 92% of the activity compared to the native enzyme.[254] The exposed hydrophobic regions of the unfolded proteins can be protected via polysorbate 80 adsorption. With the presence of surfactant, local interaction and surface tension can be altered to modulate the adsorption loss and enable protein disaggregation and refolding by coating the interface and involved in protein-surfactant associations as stated by Lee *et al.*[255] Furthermore, Surfactant can also increase the free energy of protein and potential serve as a chemical chaperone which provides sterically hindering in intermolecular interaction leading to aggregation and supporting folded protein.[250,254, 256 257]

Warne *et al.* reviewed recent high-concentration biologics solutions and lyophilized formulations and found 16 of the 21 antibody formulations contain either polysorbate-20 or polysorbate-80.[258] Learning from the commercial examples, the inclusion of surfactant in the protein formulation is highly recommended and the amount of surfactant to include in the products to protect from interfacial stress is the critical parameter to optimize. Concentration selection of polysorbate 80 in protein formulations can mitigate the stability issue on protein thermal and photostability demonstrated by Agarkhed *et al*.[10] Besides the benefits of using surfactants to provide additional stabilization for protein stabilization, there have been concerns regarding the residual amounts of peroxide which might induce oxidation risk which can lead to the loss of protein potency and stability during storage.[259]

5.3 Physicochemical attributes in frozen solution: Buffer, Sugar, and Protein Phase and Dynamics

During the freezing process and in the frozen state, the drug substance encounters a variety of stresses. Freeze concentration, resulting from ice crystallization, can cause a significant increase in both viscosity and local ionic strength. Investigating the physicochemical properties of pharmaceutical formulations upon freezing is technically challenging due to their multiphase and multicomponent nature. In recent years, ssNMR and Synchrotron X-ray diffraction (SXRD) have been utilized as advanced techniques for this purpose, as summarized in **Figure 15**. [27, 260-263] This includes the development of ssNMR

as an in-situ method to analyze microenvironmental properties across the entire freezing temperature range, from room temperature to ultracold temperatures (**Figure 15A**). For example, the freezing point and residual water content of Dupilumab formulations have been readily quantified (**Figure 15B**). This capability enables a comprehensive investigation of physicochemical attributes in freeze concentrates as elaborated below.

Maintaining pH control with an appropriate buffer system is crucial in lyophilized formulations to minimize protein degradation and aggregation.[227, 264, 265] One factor to consider is freezing can concentrate the buffer salt and drive the buffer salt precipitation and pH shift which subsequently induces protein aggregation. Therefore, the pH in the final dry protein may not retain the pH value as it is prepared in the solution. It has been reported that a large pH shift was seen for several buffers when the temperature was lowered.[226, 227] The activity and stability of a protein can be affected by the ionization state of the protein itself. The stability of lyophilized proteins can be effectively regulated through the thorough selection of buffers and controlling the initial pH.[266] Evidence has indicated that buffers play a significant role in influencing the characteristics of freeze-dried protein formulations, which can be molecule-specific. It was found that histidine outperformed phosphate and citrate as a buffer for stabilizing lyophilized lactate dehydrogenase.[267] In another study, it was discovered that sodium citrate buffer offered superior stability compared to sodium phosphate buffer for freeze-dried IL-1 receptor antagonists (IL-1ra) at a pH of 6.5.[268] The stability of IL-1ra was found to be higher when sodium citrate buffer was used as a stabilizer as opposed to sodium phosphate buffer.

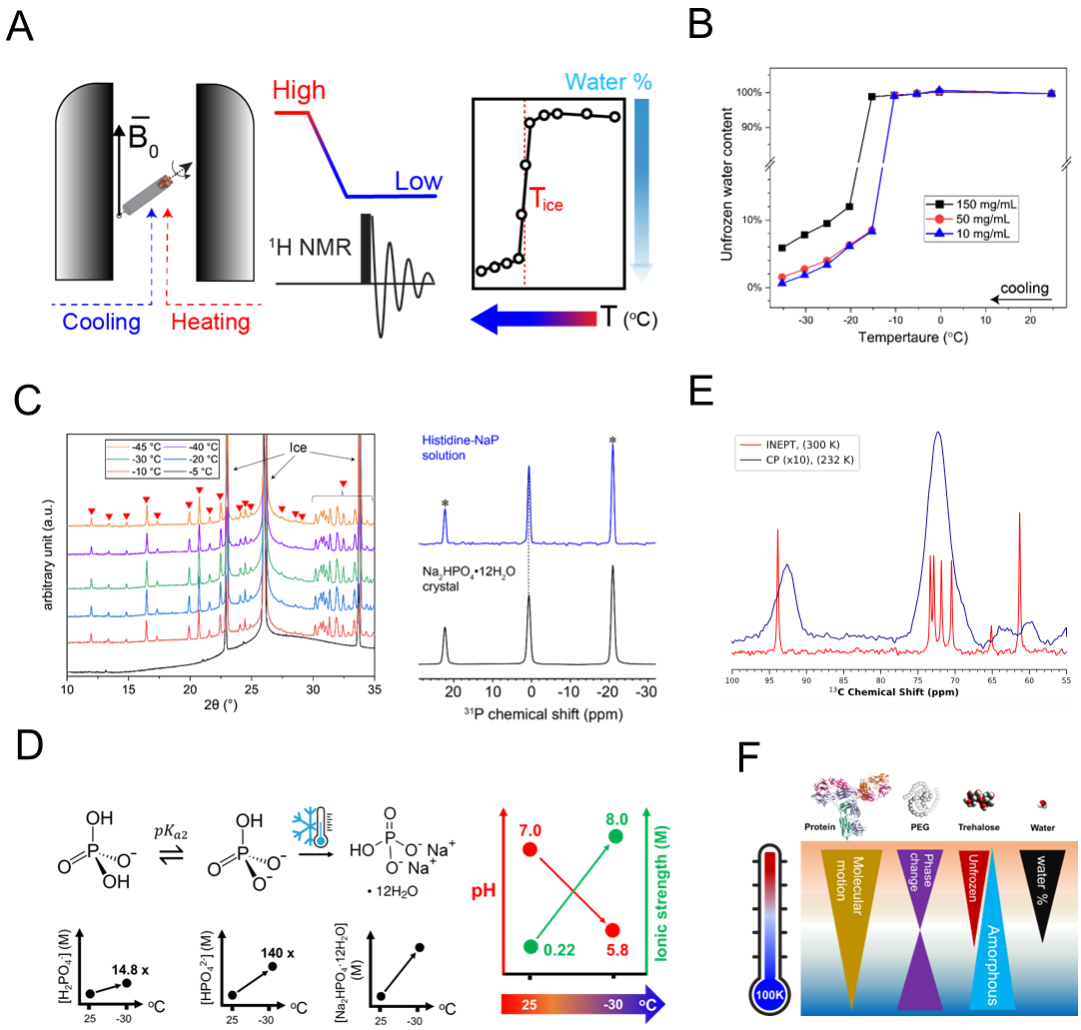


Figure 15. Physicochemical investigation of pharmaceutical frozen solution using ^{1}H, ^{13}C and ^{31}P ssNMR and Synchrotron X-ray diffraction (SXRD). (A) In situ ssNMR analysis of the frozen solution;[260] (B) ^{1}H ssNMR quantification of Dupilumab at different protein concentrations upon freezing;[260] (C) Identification of the crystallization of disodium phosphate hydrogen dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, DPDH) in freeze concentrates of phosphate buffer using SXRD (left) and ^{31}P ssNMR (right);[261] (D) Chemical equilibrium of sodium phosphate buffer in the freeze concentrate, with pH and ionic strength shifts;[27] (E) Amorphization of trehalose upon freezing observed by ^{13}C ssNMR; and (F) Transitions of molecular dynamics and phases of proteins and excipients upon freezing.[263]

As we discussed in **Section 4.4**, the microenvironmental pH of lyophilized formulation plays a critical role in protein stability. Buffer pH shift is typically caused by the certain buffer species precipitated from the solution. As such, there are formulation strategies to inhibit the buffer crystallization during the freezing process. For example, the buffer salt crystallization-induced pH shift can be adversely affected by adding non-crystallizing solutes e.g., sugar, polymer and proteins.[239] In a recent study, the role of pH shift from phosphate buffer on the aggregation behavior of two model proteins BSA and β-galactosidase (β-gal) was evaluated.[227] Phosphate buffer (100 mM) demonstrated a pH swing of 3.1 and 2.7 for BSA and β-gal respectively when cooling from 20 to -25°C. Moreover, it was also found that the addition of cellobiose not

only mitigates the pH shifts but also inhibits buffer salt crystallization. Similar inhibition of buffer salt using trehalose and mannitol was observed.[269] Trehalose can completely inhibit the buffer salt crystallization and render the lyophilized solid completely amorphous, whereas mannitol can partially suppress the buffer salt crystallization and only can attenuate the magnitude of pH shift. The co-solutes of buffer salt with sugar can potentially mitigate the aggregation of proteins caused by pH shifts in phosphate buffered saline (PBS). Strategies and knowledge enabling pH stability during the process are critical to the drug development of proteins and vaccines.[270] Most recently, ^{31}P solid-state NMR was used to probe the physicochemical properties of phosphate salt species in equilibrium at subzero temperatures.[27] Phosphate species in frozen solution were directly quantified which provides insights into ionic strength and pH in the freeze concentration. Briefly, the crystallization of disodium phosphate hydrogen dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$, DPDH) can be probed by SXRD and readily identified by ^{31}P ssNMR (**Figure 15C**).[261] All phosphate species in solution and the frozen state in freeze concentrates can be quantified, enabling the calculation of pH and ionic strength (**Figure 15D**).[27] In addition, the type of buffer can alter the thermodynamic properties of the glass matrix of the protein formulation. Phosphate ion was found to increase the T_g values of both trehalose and sucrose, but the extent of this increase varied depending on the pH conditions.[271] A similar observation was seen with the increase in the T_g of trehalose in citrate buffer.[272] In contrast, a lowering effect of T_g of trehalose was observed with the presence of tris and other buffers.[273]

Moreover, there is spectroscopic evidence that phosphate salt crystallization can be mitigated by trehalose in the formulation.[263] The physical forms of excipients in the frozen matrix, by influencing their functionality, can significantly impact drug stability. For example, stabilizers (also called cryoprotectants) need to be in the amorphous state to exert their function. Therefore, cryoprotectant crystallization can be a major destabilizing factor in protein formulations. Thus, understanding the crystallization tendency of excipients is necessary to ensure stability and maintain the biological activity of proteins. As shown in **Figure 15E**, the amorphization of trehalose has been spectroscopically identified, as evidenced by the peak linewidth change from a narrow peak in solution at room temperature to a broad peak upon freezing. Very importantly, the in-situ ssNMR analysis enables the characterization of molecular dynamics and phase transitions of individual components in protein formulations upon freezing (**Figure 15F**). Specifically, the protein pembrolizumab has exhibited global and local motions at room temperature and, at temperatures below ~173 K, is nearing a globally rigid-lattice state.

5.4 Controlled ice nucleation

We have discussed the impact of formulation composition, e.g., excipients, buffer, and sugar, in protein stabilization in the solid state. The freeze-drying process itself can also play an important role in determining the stability and critical attributes of the final product. The rate of cooling during the freezing process determines the degree of supercooling, morphology, porosity, and surface area of solid cake, which subsequently affects the rate of primary drying and stability of protein. If high supercooling is generated, smaller nuclei would form, and the crystal growth is limited which in turn results in small pores and high surface area. This can lead to more resistance in heat transfer with a longer duration of primary drying to remove the water and higher interfacial stress. However, the process of ice nucleation using conventional lyophilization is stochastic and heterogeneous.[274] The ice can nucleate at different times/temperatures, or be triggered by impurities, which result in variability in crystal morphology, freezing time, and stability. Strategies to control ice nucleation can enable spontaneous and simultaneous nucleation to facilitate both the freezing and drying processes.[275, 276] Iyer *et al.* demonstrated that controlled nucleation could help

maintain the native structure of proteins.[277] Myoglobin structure with or without controlled nucleation was characterized by solid-state photolytic labeling mass spectrometry (ssPL-MS). Myoglobin lyophilized with controlled nucleation in the presence of sucrose showed greater pLeu incorporation and the fraction of intact protein labeled increased dramatically than for other conditions, suggesting greater protein-matrix interactions and enhanced stability. Similarly, Rui *et al.* found aggregation of human IgG and recombinant human serum albumin can be minimized with higher ice nucleation temperature (−5 vs. −12°C) which results in large ice crystals and limits the ice-water interface stress-induced protein adsorption and unfold.[262]

6. Understanding stabilizing and destabilizing interactions in new and evolving modalities and delivery of peptide and mAb Drugs

6.1 Peptide stability

In the past few decades, there has been an increasing number of therapeutic peptides entering the market. Unfortunately, peptide presents several critical challenges in stability where peptides are short amino acids that can easily degrade and aggregate. Aggregation is a significant challenge in the formulation and stability of parenteral peptide formulations.[278] Peptide aggregation is a common instability issue where individual peptide molecules join together to form larger structures such as dimers, oligomers, or insoluble particles. As such, peptides are often unstable in aqueous formulation and require lyophilization to make a stable solid formulation, such as GlucaGen R,[279] TERLIVAZ,[280] Anidulafungin,[281] Caspofungin acetate,[282] Human secretin,[283] Dactinomycin,[284] Oritavancin,[285] Rezafungin,[286] and Triptorelin.[287]

In addition to developing stable peptide drug products via the drying technique, it is important to understand the mechanism of peptide aggregation. This investigation could offer the opportunity to re-design the molecules and optimize the formulation with re-tuned propensity of aggregation. For example, glucagon and insulin regulate the blood glucose levels. While insulin remains stable for several days in its solution form, glucagon quickly forms fibrils when exposed to acidic pH necessary for solubility. Glucagon with an isoelectric point of 7.1, exhibits insolubility in water under physiological pH conditions (pH 4-8). When the pH is 3 or below, glucagon is initially able to dissolve, but after a few hours, it will aggregate together to create a gel. The gelled glucagon primarily consists of beta-sheets or fibril structures and has the potential to obstruct the thin tubing of an infusion set.[288] Early-stage aggregation of glucagon has been investigated by HDX MS and MD simulation (**Figure 16A**), suggesting the mechanistic role of a few critical intermolecular contacts.[289] Solid-state NMR was used to determine the atomic-resolution structure of fibrils of synthetic human glucagon grown at pharmaceutically relevant low pH, as shown in **Figure 16 (B-E)**. Unexpectedly, two sets of chemical shifts are observed, indicating the coexistence of two β-strand conformations. Those two conformations have distinct water accessibilities and intermolecular contacts, indicating that they alternate and hydrogen bond in an antiparallel fashion along the fibril axis. Two antiparallel β-sheets assemble with symmetric homodimer cross sections. This amyloid structure is stabilized by numerous aromatics, cation-π, polar and hydrophobic interactions, suggesting mutagenesis approaches to inhibit fibrillization to improve this important drug. Therefore, glucagon is so far produced as lyophilized powder and must be reconstituted in an acidic solution just before use.

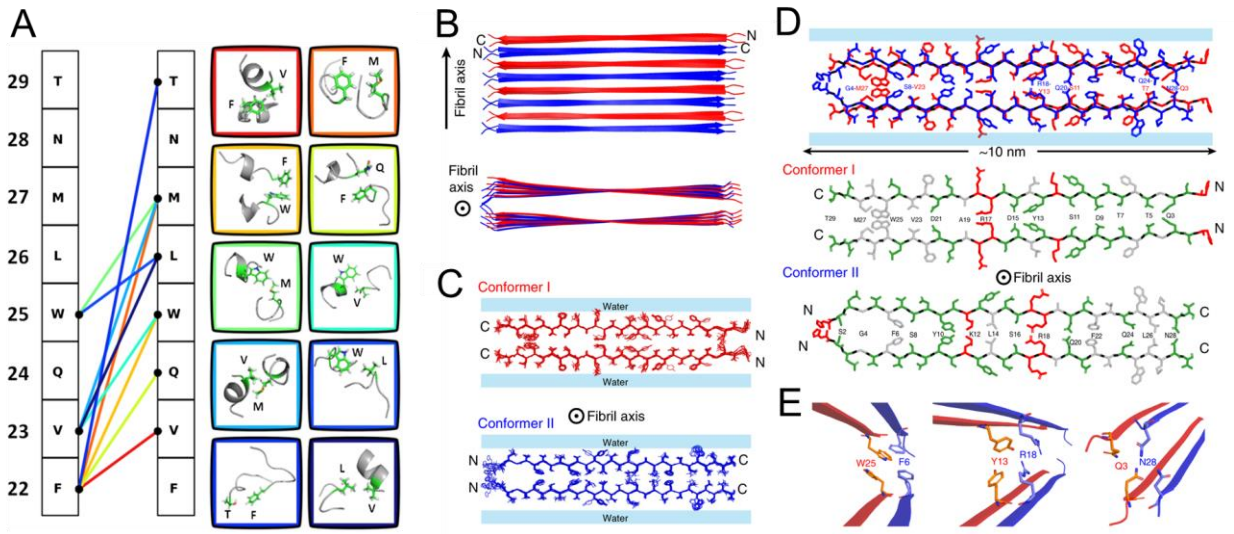


Figure 16. Molecular mechanism of glucagon aggregation investigated by HDX MS [289] and ssNMR.[22] (A) Critical intermolecular contacts for the C-terminal interactions in early stage glucagon fibrillation predicted from MD simulation that agrees with the HDX MS findings;[289] (B) Atomic structure of the glucagon fibril elucidated by multidimensional ssNMR;[22] (C) Conformer I (red) and conformer II (blue) alternate and hydrogen-bond in antiparallel in the atomic fibril structure; (D) Sidechain packing in the dimer-of-dimer subunit of glucagon fibrils; (E) Critical interaction including F6–W25, Y13-R18 and Q3–N28 sidechain packing, which stabilize the fibril structure.

6.2 Antibody drug conjugates (ADC) solid state stability

In recent years, ADC has been at the forefront of cancer therapy due to their target specificity. An ADC consists of 3 main components: an antibody attached to a linker system which is used to attach one or more individual molecules of a small cytotoxic agent to the antibody.[290, 291] To date, there are 13 ADCs approved by the United States food and drug administration (FDA) for the treatment of hematological and solid organ cancers.[292, 293] Though all FDA-approved ADCs are developed in lyophilized form, there is a lack of mechanistic understanding of the solid-state stability of ADCs from the literature.[291, 294-298]

The formulation development of ADCs is similar to that of proteins or antibodies but with more complicities with the addition of payload and linkers. The hydrophobic payloads affect overall ADC stability to a great extent, especially depending on the drug to antibody ratio (DAR) values.[299] The hydrophobic components of ADC molecules create entropically unfavorable states while in contact with water. Thus, ADC are at higher risk of aggregation in an aqueous environment.[300] Furthermore, the linker stability also plays an essential role in ADCs stability in the context of a process, in vivo systemic circulation and product storage.[301-304] The stability of ADCs is questionable if formulated in solution due to the risk factors associated with the linker system, particularly hydrazone linkers may undergo in vitro cleavage and release the cytotoxic drug during storage. [295-298, 305] Lyophilization may lower the degradation rates by lowering the mobility of molecules. Most of the stability evaluation of lyophilized formulation focuses on the in vivo activity of ADCs. Rowland *et al.* found lyophilized idarubicin antibody conjugate retained bioactivity for more than 100 days during storage at -20°C.[298] Jaime *et al.* found that lyophilized paclitaxel-antibody conjugate with trehalose as sugar stabilizer demonstrated the best pharmacological activity, whereas PEG alone or a combination of PEG and trehalose resulted in compromised

immunological and cytotoxic activities.[297] Barbour *et al.* studied the stability of lyophilized BR96-doxorubicin conjugate and found the acid-catalyzed hydrazone hydrolysis induced major degradation in solution formulation.[294] This study demonstrated improved long-term solid stability in the presence of lactose or sucrose after lyophilization using size exclusion chromatography (SEC). A recent report has also demonstrated the correlation between ADC storage stability and protein structure and matrix interaction characterized by ssHDX-MS under accelerated conditions.[291]

Although there is growing interest in developing liquid formulations based on the increased knowledge and experience with ADCs and the improved physicochemical properties of the new generation of ADCs, few such feasibility studies have been reported in the literature to date.[306] It is desired to maintain payload attachment to antibodies over prolonged durations in solution. This emerging trend places significant emphasis on the design of linkers and payloads, conjugation methods, properties of the parent antibodies, as well as formulation and device development, all of which are crucial for preventing payload detachment.[306]

6.3 Crystalline and amorphous suspension formulations

Most biologics are formulated as parenteral drugs for intravenous and subcutaneous administration.[307] Subcutaneous injection, especially when combined with pre-filled syringes or auto-injectors, is gaining attention as it improves patient compliance and reduces medical costs, particularly for patients with chronic diseases who require frequent and chronic administration.[169, 308] However, to avoid pain at the injection site, the injection volume for subcutaneous administration is typically limited to 1-1.5 ml. This necessitates high-concentration protein formulations, which create an enhanced molecular crowding environment, presenting challenges such as high viscosity, protein aggregation, particle formation, and degradation.[309] **Figure 17** proposes formulation strategies to achieve high and ultrahigh protein concentrations while mitigating the risk of high viscosity.[310,311-313] Briefly, high protein concentration is often achieved via ultrafiltration in an aqueous setup. However, solution formulations can exhibit an exponential increase in viscosity as concentration rises.[314] To push protein concentrations further, dried protein particles, such as those obtained via spray drying, can be suspended in non-aqueous media, potentially presenting lower viscosity with minimized protein-protein contacts.[312] Additionally, using crystalline or amorphous suspension formulations may offer the advantage of lower viscosity compared to solution formulations of similar concentrations.[310] For instance, crystalline suspensions of monoclonal antibodies (mAbs) like Infliximab at a concentration of 150 mg/ml exhibit an acceptable viscosity of 26 cP, whereas a solution formulation at a comparable concentration increases viscosity approximately tenfold.[311] Moreover, the use of protein crystals in pharmaceutical applications offers advantages in terms of enhancing protein stability and improving the pharmacokinetics of drug delivery.[315-317]

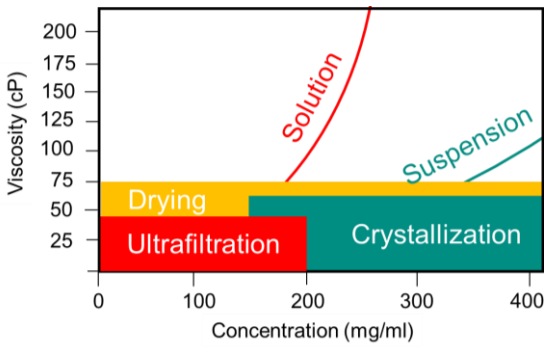


Figure 17. A schematic illustration of formulation strategies to achieve high (⩾100 mg/ml) and ultrahigh (⩾200 mg/ml) protein concentrations, specifically by utilizing dry protein powder in non-aqueous suspensions and crystalline or amorphous protein particles in aqueous suspensions.[310,169, 308, 311-313]

Using a crystalline or amorphous suspension formulation may offer the advantage of lower viscosity compared to solution formulations of similar concentration.[310] In addition, the utilization of protein crystals in pharmaceutical applications offers advantages in terms of enhancing protein stability and improving the pharmacokinetics of drug delivery.[315-317] Reichert *et al.* studied the crystallization processes for the purification, drug delivery, and storage of Pembrolizumab (Keytruda®).[318] The production of uniform crystalline suspensions, ranging from 1 to 5 μm in size, has been achieved using rotational mixers to minimize sedimentation and temperature gradients to induce and regulate crystallization. Through these techniques, it has been possible to obtain crystalline suspensions with desirable properties such as acceptable viscosity (less than 10 cP), suitable rheological characteristics, and syringeability, enabling the preparation of injectable formulations. An important hurdle in drug development is the limited availability of high-resolution characterization methods to assess the crystallinity and stability of microcrystals of monoclonal antibodies (mAbs) in their native formulations. Traditional analytical techniques face difficulties in evaluating the structural properties of mAb microcrystals within suspension due to factors such as the presence of visible particles, small crystal size, high protein concentration, and the complex composition of liquid formulations. Li *et al.* recently developed the first high-resolution characterization of mAb microcrystalline suspension using MAS NMR spectroscopy using pembrolizumab crystalline suspension as a model system, as shown in **Figure 18A-D**.[19] The remarkably narrow linewidth of approximately 29 Hz observed in the ^{13}C spectral analysis indicates a high level of crystallinity and conformational uniformity in pembrolizumab crystals. The evaluation focuses on assessing the effects of thermal stress and dehydration on the structure, dynamics, and stability of these monoclonal antibody (mAb) crystals within the formulation environment. Though a decrease in structural homogeneity was found when stress at 55 °C, pembrolizumab did not experience severe structural perturbations or thermal unfolding as the position of major peaks remained the same. In addition, the technique of isotopic labeling combined with heteronuclear ^{13}C and ^{15}N spectroscopies has been employed to detect and study the interaction between caffeine and pembrolizumab in the crystal lattice. The intermolecular interactions are suggested by the chemical shift changes of caffeine and good agreement with the structure elucidated by single-crystal X-ray diffraction. This approach offers valuable molecular insights into the co-crystallization process of the protein and ligand. Moreover, the polymorphs of the pembrolizumab-caffeine co-crystals (Form I and II) have been detected and differentiated by both SAXS and ssNMR, as shown in **Figure 18E** and **F**.

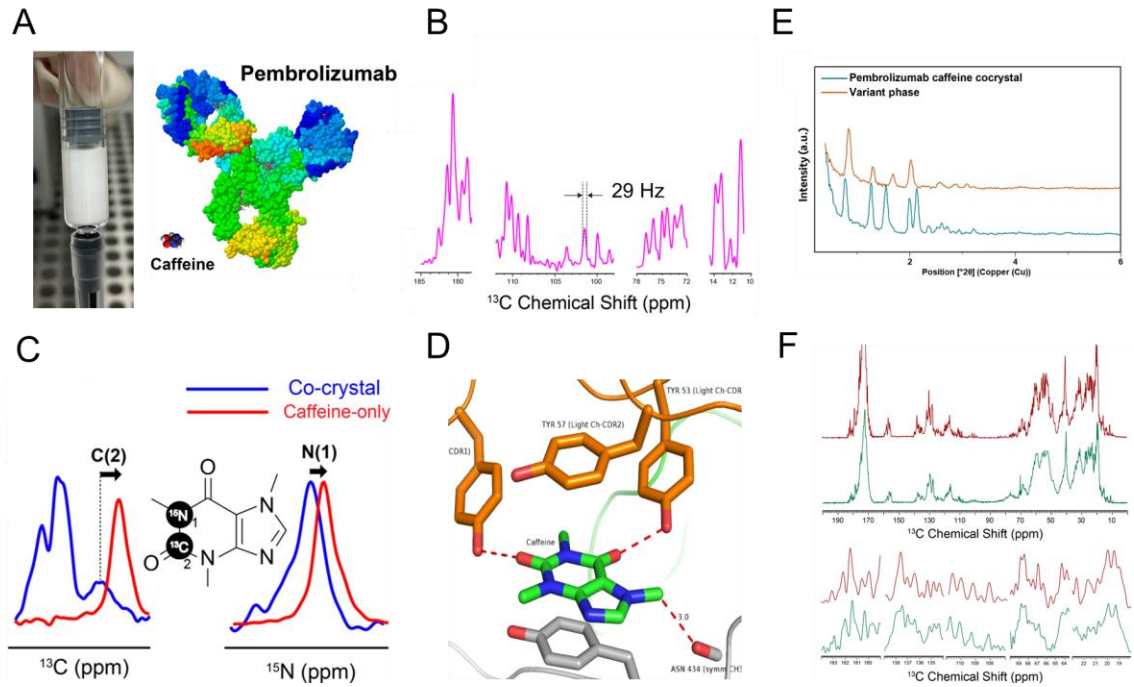


Figure 18. High crystallinity and structural order of crystalline pembrolizumab suspension formulation from ssNMR and Small Angle X-ray Scattering (SAXS).[19, 81] (A) Illustration of the pembrolizumab-caffeine co-crystalline formulation; (B) ^{13}C MAS NMR spectrum of the as-is crystalline formulation showing sharp linewidth; (C) Overlay of 1D ^{13}C and ^{15}N MAS NMR spectra of isotopically labeled caffeine bound to pembrolizumab in microcrystals (blue) with free crystalline caffeine as a reference (red); (D) Pembrolizumab-caffeine interactions from PDB 8SJK; and pembrolizumab-caffeine co-crystalline form I (red) and II (green) identified by SAXS (E) and ssNMR (F).

7. Conclusions

Solid formulation enhances the stability of biologics during storage by overcoming the challenges of biophysical and biochemical stability in the aqueous state. Fundamental investigations of protein structure and dynamics provide comprehensive insights into stabilization mechanisms at the molecular level. Protein stabilization in the glass state can be achieved through thermodynamic stabilization via water replacement and kinetic stabilization by limiting protein dynamics both locally and globally. The glass dynamics hypothesis, encompassing molecular motions relevant to solid-state proteins and dynamics pertinent to solid biologics, is thoroughly reviewed. Although characterization techniques for solid biologics formulations are limited due to their multi-component and amorphous nature, advanced high-resolution biophysical techniques such as DES, NS, and ssNMR are employed to characterize protein motion in the solid state and its correlation with protein stability. Additionally, molecular attributes and formulation factors impacting protein stability should be considered when selecting excipients like sugar stabilizers, buffers, and surfactants. Furthermore, we have reviewed protein structure, protein-excipient miscibility, protein-excipient interactions, and physicochemical attributes such as microenvironmental pH, packing density, and interfacial stress and their impact on protein stability. Looking forward, it is critical to further investigate these mechanisms at different resolutions and time scales and directly measure protein-sugar interactions at the molecular level. These insights can guide the design of novel excipients,

the rational selection of stabilizing agents, and facilitate the evaluation of the impact of the selection and optimization of drying processes.

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