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Stabilization of proteins in solid form☆,☆☆

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ABSTRACT

Immunogenicity of aggregated or otherwise degraded protein delivered from depots or other biopharmaceutical products is an increasing concern, and the ability to deliver stable, active protein is of central importance. We review characterization approaches for solid protein dosage forms with respect to metrics that are intended to be predictive of protein stability against aggregation and other degradation processes. Each of these approaches is ultimately motivated by hypothetical connections between protein stability and the material property being measured. We critically evaluate correlations between these properties and stability outcomes, and use these evaluations to revise the currently standing hypotheses. Based on this we provide simple physical principles that are necessary (and possibly sufficient) for generating solid delivery vehicles with stable protein loads. Essentially, proteins should be strongly coupled (typically through H-bonds) to the bulk regions of a phase-homogeneous matrix with suppressed β relaxation. We also provide a framework for reliable characterization of solid protein forms with respect to stability.

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1. Introduction

While there are many potential applications for targeted drug delivery of therapeutic proteins, protein instability [1,2] associated with process, storage, and delivery-related stresses remains the primary roadblock for most of these applications. However, nature has found ways to ameliorate stresses similar to many of those encountered in preparation of drug delivery depots. In nature, some organisms protect proteins under desiccation [3] and freezing stress [4] through elevated local concentrations of sugars or sugar-alcohols. Crowe and Carpenter found they could apply this strategy by lyophilizing proteins or lipids [5] in the presence of disaccharides with nearly 100% recovery of protein function or membrane integrity. This approach is now commonly used in the biopharmaceutical industry, and has facilitated formulation development of many dried therapeutic protein products for human use. The use of disaccharides as stabilizers in drug delivery applications has been relatively limited, but has shown promise [6–10].

Since the work of Crowe and Carpenter, scores of biopharmaceutical products have been administered from a lyophilized state to millions of patients with very few obvious adverse health effects. However, it now appears that it is not uncommon for patients to develop antibodies to the therapeutic proteins over time [11], reducing their efficacy [11,12] or occasionally inducing dangerous immunogenic responses [11]. The appearance of anti-drug antibodies has been associated with the presence of protein aggregates [13], which occur at low levels even for these minimally processed biopharmaceutical products lyophilized in the presence of disaccharides [14].

Recent reviews have pointed out potential immunogenicity of various components of delivery vehicles themselves [15] and the enhanced ikelihood of finding immunogenic protein aggregates associated with injectable protein delivery systems [16]. Stresses that could lead to protein aggregation or other protein degradation products can occur at many stages of producing a drug delivery product, including encapsulation-related processing, storage, and delivery or release. As with conventional biopharmaceutics, efforts have been made in drug delivery applications to reduce process-related degradation and improve stability of the protein to be delivered. Among these are the use of systems that are amenable to all-aqueous processing such as chitosan [17] and gels [18]. Proteins and nucleic acids have also been pre-encapsulated in sugars, preventing their direct exposure to solvents [10,19,20], and this has also been effective in preventing burst release [10]. Damage at time of release associated with low pH from degrading polymer has been addressed by Schwendeman and colleagues [21]. These improved approaches will no doubt be helpful in efforts to deliver active, stable protein. However, in addition to employment of a protein-friendly delivery platform, an appropriate level of stabilization generally requires significant formulation effort for each payload protein, involving characterization of intended biopharmaceutical payload as well as degradation products that may accumulate [12,16]. Here we focus on characterizing key properties of dry protein formulations and explore the potential of these properties as reliable metrics for predicting protein stability.

Regulatory and other pressures have driven development of methods for detailed characterization of biopharmaceutical products in order to minimize adverse effects on patients, reduce product loss, ensure reproducibility, and predict stability. Thus, even though the connection between degraded protein and immunogenicity is just emerging, there is an established analytical infrastructure in place for characterizing products with respect to stability. Within this infrastructure, a range of analytical methods is in common use, but the field is still developing as a quantitative science. The utility of most analytical methods used in this context is judged on correlation between metrics and functional outcomes. As we will see below, many of these correlations are not completely reliable, demonstrating at least that additional variables are important. The various characterization methods are inspired by decades-old hypotheses regarding causal factors in protein degradation. These hypotheses are reasonable, and probably each holds some validity, but further careful exploration and critical evaluation will likely lead to analytical approaches that are more reliable and possibly quantitatively predictive of protein stability in dried form.

1.1. Potential mechanisms of stabilization

Two hypotheses for how a solid matrix (sugars in particular) can stabilize dry-state proteins were articulated soon after the discovery that sugar-glass can stabilize proteins. One idea is that sugars substitute for water at the protein surface, conferring thermodynamic stability [22]. Another idea is that, upon vitrification, sugars simply impede degradation processes [23]. It has also been suggested that water trapped by sugars at the surface of the proteins mediates either the proposed dynamic [24,25] or thermodynamic [26,27] stabilization. It has been difficult to cleanly discriminate among these hypotheses, for practical and fundamental reasons. Among these reasons is the fact that the hypotheses are not mutually exclusive. It is possible that both could play important roles. Also, it is practically impossible to try to disprove the thermodynamic stabilization hypothesis by performing thermodynamic studies on these vitrified systems since the required system ergodicity may not be obtained on a practical timescale. Adding further difficulty is that these questions have been addressed in several different fields, from biophysics to pharmaceutics, and the notion of what "protein stability" means can differ significantly from one report to another. In spite of these difficulties, sufficient data now exist to allow (a somewhat overdue) refinement of the hypotheses. Here we will focus on work that addresses stability against aggregation and chemical degradation.

2. Thermodynamic considerations

It has been hypothesized that a good matrix material can provide stability against degradation by substituting the thermodynamic role typically played by water and stabilizing the native protein conformation [22]. It is difficult to directly test this hypothesis because it is not feasible to perform thermodynamic conformational studies for proteins in anhydrous sugars, due to the very slow dynamics of these glassy systems. However, we can glean useful information from studies performed for proteins in anhydrous solvents. It has been known for decades that small proteins can perform enzymatic functions in anhydrous media when introduced as a suspension of lyophilized powder [28]. Whether this activity is a result of residual water in the lyophilized powder is not entirely clear. However, when dissolved in anhydrous hydrophilic solvents, protein conformation [29] and often substrate specificity [29,30] change dramatically, indicating that these solvents really cannot be said to replace water. There has been one example of a protein retaining native conformation in anhydrous solvent, and that is lysozyme in glycerol [31]. However, lysozyme is denatured in other hydrophilic solvents [29], and glycerol is known to induce conformational changes in other small proteins such as alcohol dehydrogenase [32]. Thus, it appears that in almost all cases, anhydrous hydrophilic solvents do not replace water by stabilizing the native conformation of even small proteins, although glycerol may do better than most solvents. We can tentatively extend the results of these solvent studies to sugars since enthalpic interactions between sugars and proteins are comparable to those between glycerol and protein, both being weaker than enthalpic interactions between water and proteins.[33,34].

While it is unclear whether stabilization of the native state *per se* is required for achieving good functional stabilization, or is even generally possible, it *is* clear that the thermodynamic interactions between the solid matrix and the protein play a critical role in functional stabilization. Crowe and Carpenter [22] first showed that dehydration-induced shifts in the positions of amide I and amide II infrared absorption bands for lysozyme could be partially or fully reversed if the protein was dried in the presence of sugars. They suggested that sugars formed hydrogen bonds with the native surface groups of the protein, helping to stabilizing the protein in a near-native state. Later, Allison et al. [35]