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Protein Aggregation and Its Impact on Product Quality

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Abstract

Protein pharmaceutical products are typically active as folded monomers that are composed of one or more protein chains, such as the heavy and light chains in monoclonal antibodies that are a mainstay of current drug pipelines. There are numerous possible aggregated states for a given protein, some of which are potentially useful, while most of which are considered deleterious from the perspective of pharmaceutical product quality and performance. This review provides an overview of how and why different aggregated states of proteins occur, how this potentially impacts product quality and performance, fundamental approaches to control aggregate formation, and the practical approaches that are currently used in the pharmaceutical industry.

Introduction

Protein based pharmaceuticals are arguably the fastest growing sector of the innovator pharmaceutical industry, including many of the prospective treatments for autoimmune diseases and various forms of cancer that are currently in clinical trials [1]. All pharmaceutical products that are approved through regulatory agencies such as the U.S. Food and Drug Administration are held to strict standards of chemical and physical purity, both at the time of manufacturing and throughout the often multi-year shelf life of the product. In addition to standards set by regulatory agencies for products to be safe and effective in clinical trials, there are also practical constraints in terms of options for effectively and reliably delivering proteins to patients, and for enabling self-dosing by patients.

Unlike their small-molecule counterparts, it is not currently viable to deliver the necessary doses of protein pharmaceuticals via oral routes such as capsules and tablets [2,3]. As a result, proteins are almost invariably delivered via liquid injections (intravenous IV, intramuscular IM, or sub-cutaneous SC), although delivery via other routes is an active area of research [4]. Many of the most recently approved protein pharmaceuticals, as well as those in clinical trials, are manufactured and stored as liquids, although historically more

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products were developed as solid formulations that were reconstituted to a liquid state just prior to injection [5].

Many of these proteins require a relatively large dose, in terms of the mass or protein per unit mass of the patient body weight. IM and SC injections are preferred for patient convenience and compliance, as well as for use in autoinjection devices. The maximum volume that can be delivered per dose in such cases is approximately 1 mL, while the target dose of protein to the patient can be over 200 mg, therefore requiring product concentrations that are on the order of 10^2 mg/mL. While proteins are inherently prone to form certain types of aggregates over time, even at much lower concentrations, these much higher protein concentrations pose further issues via a range of different aggregated states. Depending on the protein in question, aggregates may or may not pose problems from the perspective of product quality – specifically, product safety, efficacy, delivery or dosing, and marketability.

This review focuses on an overview of how and why proteins aggregate; how this can impact product quality; and approaches to control or mitigate aggregation for proteins in general. It closes with an overview of what practical approaches are currently used for that purpose in the pharmaceutical industry, and the lucrative current and future avenues of research.

Why and how do proteins aggregate?

Proteins are typically required to be folded in order to function effectively as drug molecules. The fundamental forces and interactions that drive folding include: van der Waals and hydrophobic attractions between side-chain and backbone atoms; maximizing hydrogen bonding; minimizing steric clashes and energetically unfavorable bond torsional angles; maximizing chain entropy; minimizing (maximizing) electrostatic repulsions (attractions); and minimizing unfavorable interactions between amino acids and the solvent (water) and its co-solutes. These same types of interactions that occur between amino acids within the protein also exist between amino acids in neighboring proteins [6,7]. Therefore, it is perhaps not surprising that proteins at finite concentrations have a tendency to form aggregated states in addition the monomeric state that they would necessarily adopt in the limit of infinite dilution. What is often much less well appreciated is that there is a diverse array of different types of aggregated states for proteins – some of which co-exist with the protein in its monomeric state to a greater or lesser degree. Figure 1 provides a schematic overview of the different states and how they relate (roughly) to one another, with images reproduced from elsewhere [8,9]. These are enumerated in more detail in subsections below, which are separated into reversible and irreversible aggregates based on how that behavior then relates to product properties and quality attributes in later sections. Figure 1 uses a monoclonal antibody as its basis, but the general behaviors that are shown are not specific to antibodies. The figure simply uses them as a prime example that is of current interest in the pharmaceutical industry. There are aspects of protein stability that are particularly relevant to antibodies, such as fragmentation due to chemical degradation of the hinge region, but that is not depicted in the figure and is only mentioned briefly in the caption.

Reversible aggregation

Starting at the folded monomer state (center of Figure 1), proteins can reversibly form small dimers and oligomers that are stable complexes that can be separated from the monomer or are in dynamic equilibrium with the monomer state. For example, the monomer-dimer equilibrium dissociation constant ($K_d = [\text{monomer}]^2/[\text{dimer}]$) for insulin is $\sim 10 \mu\text{M}$ in the absence of zinc [10], while typical dosing requires insulin to be formulated at much higher concentrations; as a result insulin exists as a stable dimer unless one considers extremely low pH (below approx. pH 2) [11]. If one works at concentrations close to the value of K_d for a given protein then monomer and dimer will coexist at similar amounts in solution. If one works at concentrations well below K_d then dimers will be metastable relative to monomers. In this case, one will not be able to directly observe dimers as they will be too transient to isolate without reversing monomers, and/or will be too poorly populated to detect in vitro with the monomer present [12]. The same reasoning extends to protein oligomers, except that there is a different K_d value for each step – e.g., monomer-to-dimer, dimer-to-trimer or dimer-to-tetramer, etc.

Pharmaceutical proteins such as monoclonal antibodies (MAb) typically have large K_d values, if they can even be measured experimentally [13]. As such, folded or native dimers and oligomers are not observed until very high concentrations ($\sim 10^2 \text{ mg/mL}$), or are not observed at all within the range of concentration up the protein solubility limit [14,15]. In some cases, stable dimers and oligomers are a predominant and long-lived species [13]. In other case they may be small transient clusters [15,16], although there remains debate as to the limitations and interpretation of the available experimental techniques [17–20]. In the opposite extreme, large clusters can be stable and isolated for imaging, providing one can avoid difficulties with metastable or trapped aggregate states [21].

In most of the above examples, the aggregates do not grow to macroscopic dimensions, and as such may be termed “molecular” aggregates, in that it is useful to distinguish between a dimer, trimer, tetramer, ..., up to an “n-mer” (even with $n \sim 10^2$). But as “n” becomes very large the aggregates that form are essentially macroscopic particles (for crystals or amorphous solid) or droplets (for liquid-liquid phase separation, LLPS). Macroscopic phase separation does occur for proteins. The most obvious illustration is the fact that all proteins have a solubility limit, although it not always clear whether the “condensed” phase that forms is crystalline or amorphous[22]. Pharmaceutical proteins can form crystals – although with more difficulty than model proteins [23,24] – or undergo LLPS [25], and may show opalescence if the solution conditions are close to the limit of phase separation but it remains as a single phase [26,27].

Irreversible aggregation

All of the examples summarized in the preceding section involve only folded proteins, and are relatively easy to reverse simply by dilution (e.g., by a factor of 10 – 100) or shifting the solution conditions such as pH or salt concentration. In addition, proteins can aggregate to form net-irreversible species that are not readily dissociable unless one exposes them to extremely high concentrations of chemical denaturants [7,28,29], pressure [30–32], and/or temperature [28]. In these cases, the proteins within the aggregates have lost some or all of

their native secondary and/or tertiary structure; doing so allows them to create multiple very strong contacts (e.g., hydrophobic interactions and hydrogen bonds) between proteins by inter-digitating chains from multiple proteins.

Figure 1 illustrates that the initiation process for such “non-native” aggregates involves two or more proteins “misfolding” together to “nucleate” a stable, net irreversible species that can then grow via different mechanisms,[33–35] and the molecular aggregates can ultimately phase separate as macroscopic particles [9,35,36]. Nucleation is usually a kinetically limited process that can require extremely long time scales (from minutes to years) when compared to typical timescales for protein folding ($\sim \mu\text{s}$ to s) under native-favoring conditions where most proteins are stored and delivered. In many cases, partial unfolding of protein monomers appears to be the first step, followed by slow association of two or more chains to create the nuclei [37], although at high protein concentration it has been inferred that association occurs before unfolding [38,39], and in some cases unfolding is clearly rate-limiting. If growth occurs by monomer addition, then the (partially) unfolded monomers can add more easily to the existing aggregates [34,35,40]. In either case, changing the interactions between the different species in solution (monomers, different sized aggregates) changes the rates of each step in the process. The overall process is net under kinetic control, and unlike the case of reversible aggregation, the relative rates of the different processes determines the concentrations of different aggregate species, and these change with time (e.g., during product storage) [37]. In addition, irreversible or stable aggregates can cause unexpected changes in the solution viscosity as a function of protein concentration [41].

Finally, the creation of nuclei does not necessarily occur readily in bulk solution. It is also possible that adsorption of proteins to hydrophobic interfaces such as those between water and air or silicone oil may promote unfolding and subsequent aggregation [42–44]. Adsorption to charged interfaces such as water in contact with container materials such as glass and stainless steel can also promote non-native aggregation [45,46].

How can aggregates impact product quality?

In a small number of cases, native reversible aggregation is a major benefit to product quality, as the native oligomers have higher stability and potentially higher solubility than their monomeric counterparts – insulin in the presence of zinc ions is the canonical example of this situation for a protein pharmaceutical product [11,47]. However, in most cases aggregation is considered a potential detriment to product quality. If the protein remains folded upon aggregation (and hence is likely undergoing reversible aggregation), then major concerns with aggregation are typically one or more of the following: (i) limited solubility, which then may require low protein concentrations, and therefore may limit either the maximum dose that can be delivered via IM or SC, or will force the use of IV delivery; (ii) changes in solution viscosity that may impact IM or SC delivery. For case (ii), if the aggregates are compact (e.g., microcrystals or dense amorphous nanoparticles [21]), viscosity is expected to decrease as aggregation occurs, because the proteins have lower net excluded volume. But if one considers aggregates that behave more like extended structures that can entangle with one another, then viscosity is expected to rise as a result of aggregate

formation [15,41]. This highlights that the “type” of aggregates that are formed are important for even qualitatively predicting whether aggregation cause positive or negative effects for product quality. If the aggregates are irreversible, then at a minimum they are considered a degradation product that must be controlled at very low levels (typically a few percent or lower, based on total protein mass). Beyond that, there are growing concerns about the risks of patients developing immune responses to the drug if the partially folded, irreversible aggregates persist in the blood stream and invoke an immune response [48]. If this occurs, patients can become “immune” to the drug, or in worst-case (and extremely rare) scenarios the patient can develop an autoimmune disease [49]. Finally, visible aggregates are often considered a liability from a marketing perspective, as patients and clinicians often expect injectable products to be transparent and particle-free [12].

How can one control or mitigate aggregation?

While it is not currently possible to predict *a priori* when protein aggregation will occur for a given protein, there are a number of factors that influence whether and/or how quickly protein solutions will aggregate. The major factors include [5]: solution conditions (pH, salt concentration and which salt type, the amount and type of osmolytes present, and amphiphilic molecules such as surfactants; temperature; pressure; air-water interfaces and other bulk interfaces with water such as stainless steel. These are all factors for a given protein, with each protein responding differently to changes in these “environmental” variables. There is also the option to modify the molecular structure of the protein itself – i.e., protein engineering – if the protein is produced recombinantly. Box 1 provides an high level overview of why a change in a given environmental variable – e.g., temperature (T), pressure (p), pH, ionic strength, and concentration of hydrophilic or amphiphilic co-solutes – is expected to influence different types of aggregation.

Box 1

Why does aggregation depend on the protein environment?

Changes in solution T , p , pH, ionic strength, and concentration of different co-solutes inherently shift the chemical potential (μ) or partial molar Gibbs free energy of folded and unfolded monomeric protein states, as well as each distinct aggregated protein state summarized in Figure 1. For reversible aggregation, thermodynamics governs which states are preferred as one changes environmental conditions. Increasing T at fixed p and composition promotes states with higher enthalpy and entropy, and therefore typically disfavors the various types of reversible aggregation in Figure 1 [50]. For irreversible aggregates, kinetics tend to dominate what species form, and decreasing T slows the rates of aggregation [51–53]. Similarly, increasing p favors states with higher net density. This promotes dissociation of aggregates and protein unfolding because it forces water molecules to “squeeze” into molecular-scale cavities with folded structures and within the interfaces between proteins [54]. Changing pH alters the net charge on the proteins, and increasing ionic strength causing electrostatic repulsions and attractions to only be “felt” between proteins at short inter-protein distances. Typically, shifts in pH towards the isoelectric point and increases in ionic strength favor aggregation unless aggregation is driven by electrostatic attractions [14,21]. At very high salt concentrations, there are

additional non-idealities and the precise chemical identity of the salt species is important [55,56]. Adding hydrophilic co-solutes tends to promote states that are more compact because they have lower excluded volume, relative to water, and therefore cause less of an entropy penalty to the cosolutes [57]. This favors folded states [58] that then slows the rates of irreversible aggregation [12,37], but also can favor reversible aggregation in many cases. Adding amphiphilic co-solutes such as non-ionic surfactants can suppress aggregation of highly hydrophobic proteins such as membrane proteins [59,60] by “binding” to their solvent exposed hydrophobic domains, but also helps to slow the irreversible aggregation of hydrophilic proteins by adsorption to hydrophobic air-water interfaces [44].

Of the fundamental approaches to control aggregation, in industrial practice the most common strategies to control aggregation are to first adjust the solution pH and salt concentration to minimize both reversible and irreversible aggregation. This sometimes requires finding an optimum condition, since irreversible aggregation rates are extremely sensitive to changes in conformational stability of the folded state, and that often improves as one moves the pH towards the pI of a given protein [61]. Conversely, native aggregation tends to increase as one approaches the pI or adds significant amounts of salt [22]. For non-native irreversible aggregation, preferentially excluded solutes such as sugars and certain amino acids are often added to improve the protein conformational stability so as to counterbalance its sensitivity to pH, but the mechanism by which they promote folding can also lower solubility (cf., Box 1). Non-ionic surfactants are added if the conformational changes and aggregation are found to be sensitive to the presence of air-water interfaces or other hydrophobic interfaces. Lowering temperature typically has opposite effects for irreversible vs. reversible aggregation and phase separation (Box 1). Finally, one must also balance these physical degradation routes with their chemical counterparts such as deamidation [62] and oxidation [63,64], as well as fragmentation in the case of monoclonal antibodies [39,65]. In all cases, there is an argument for trying to engineer the protein (i.e., change its amino acid sequence) to have improved aggregation behavior, but historically protein engineering in industrial practice has focused to a larger extent in improving protein binding, specificity, or in vivo performance such as circulation half-life in the blood stream.

Summary and outlook

As the examples above illustrate, protein aggregation can manifest in a variety of ways for biopharmaceuticals and can have major impacts on product quality. There are an array of different “types” of aggregates, with limited current understanding of the link(s) between the physical and chemical properties of the different aggregate types and a given product attribute, and the field could benefit greatly from new technologies for experimental characterization of protein products. Current practices are effective in empirically controlling aggregation such that viable products continue to be developed and provided to patients. However, a major limitation is that there are very few, if any, truly predictive approaches to rapidly optimize or design the solution conditions and/or protein *a priori* to have the desired product quality attributes. As a result, there remains a large amount of phenomenological or empirical experimental screening that is needed to progress a protein

candidate through different stages of development in support of clinical trials. The net impact to patients is longer development timelines and cost, and development of new approaches to rapidly and accurately predict the array of aggregation behaviors remains an outstanding challenge that is the focus of much ongoing research.

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Highlights

1. Proteins aggregate in a variety of ways, most of which are unwanted in drug products.
2. Possible impacts on product quality include deliverability and immunogenicity.
3. Controlling aggregation is difficult and is currently primarily phenomenological.
4. Improved predictive approaches to control aggregation are needed.

Figure 1.

Schematic overview of a range of different aggregated states that proteins can adopt either as folded molecule or unfolded/partially-unfolded ones. The latter typically result in aggregates that are difficult to dissociate without extreme conditions (high pressure, temperature, and/or denaturant concentration). Images for liquid-liquid and liquid-crystal phase separation (bottom left) are reproduced with permission from Ref. [8], as are those for aggregate phase separation (bottom right) from Ref. [9].