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Protein aggregation, particle formation, characterization & rheology

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ABSTRACT

In this review, we attempt to give a concise overview of recent progress made in mechanistic understanding of protein aggregation, particulate formation and protein solution rheology. Recent advances in analytical techniques and methods for characterizing protein aggregation and the formed protein particles as well as advancements, technique limitations and controversies in the field of protein solution rheology are discussed. The focus of the review is primarily on biotherapeutics and proteins/antibodies that are relevant to that area. As per the remit of *Current Opinion in Colloid and Interface Science*, here we attempt to stimulate interest in areas of debate. While the field is certainly not mature enough that all problems may be considered resolved and accepted by consensus, we wish to highlight some areas of controversy and debate that need further attention from the scientific community.

1. Introduction

The development of stable protein-based formulations with controlled rheological response is an area of high interest for the high-growth biotherapeutic industry, as well as for more traditional industrial sectors such as foods. Although the final applications in these two industrial sectors are very different, the complex self-assembly and particle formation processes under various formulation conditions (pH, ionic strength, buffer salts, temperature) must be well-understood, characterized, and controlled. This then allows the development of formulations which remains stable with long shelf life and that exhibits rheological properties that enhance/optimize the application performance — e.g. processing, delivery through injection in the case of therapeutic proteins, and texture/sensory features in the case of foods. The early detection and characterization of protein particles or aggregates — their size, structure, morphology, interactions and rheology in therapeutic protein formulations are critical to reduce safety issues (e.g. immunogenic response in biologics) and to ensure stability and optimized delivery etc. [1–4]. In food based systems, the food protein self-assembly, microstructure and resulting rheological properties must be characterized and controlled in order to ensure optimized textural/sensory experiences for the consumer and ensure issue-free processing [5,6]. Due to the multiple length scales and time scales of interest in protein aggregate formation, the need arises for different techniques that span these wide ranging length and time scales. This article will review the progress made in the understanding of protein particle formation and advances made in analytical techniques and analysis methods that allow the development of new insights into the formation of protein particles and their corresponding properties-size, structure, microstructure, and rheology.

2. Native and non-native aggregation: reversible and (effectively) irreversible aggregates

Proteins can self-assemble in a number of ways. They can form highly specific, structured complexes such as receptors with ligands [7], multimeric native states with or without metal complexation [8,9], and multi-protein "machines" such as the ribosome [10]. Those types of protein complexes typically have sufficiently strong inter-protein interactions that one must work at extremely dilute conditions in order for the complex to not be the natural or "native" state. We do not review such systems explicitly here, as a majority of pharmaceutical proteins currently or recently in development do not associate so strongly unless it is via non-native conformers [11–13].

When self-association of native or folded proteins occurs in pharmaceutical products or model proteins that mimic pharmaceuticals, it primarily occurs via transient and relatively weak interactions that require one to work at high concentrations (on the order of 10^{-3} M or larger) [14–18]. In this case, one might consider an array of possible aggregate species (dimers, trimers, tetramers, etc.) that interchange with one another dynamically. These species are typically easily reversed simply by moving to lower protein concentrations and/or slightly shifting the solution pH or ionic strength to alter the charge–charge interactions between monomers [14,15,17,19]. As a result, one should anticipate that

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aggregates of this type that are isolated (e.g., via purification) or characterized with ex situ methods that require dilution and/or a change in solvent conditions (cf. discussion below) will likely not be quantitatively, or possibly qualitatively, representative of the aggregate population(s) that exists in situ.

For purely reversible aggregates, one often can ignore the precise mechanism – i.e., the detailed steps and the order in which they occur in at a molecular level – if the time scales for equilibration of the aggregate population are short compared to that for production and storage of protein products such as pharmaceuticals. That is, one may only need the equilibrium aggregate size distribution, or equivalently the concentration of each species (monomer, dimer, trimer, tetramer, etc.) if the system equilibrates quickly [20]. For a simple diffusion-limited biomolecular reaction $M + M_j \leftrightarrow M_{j+1}$ (M = monomer, M_j = oligomer composed of j monomers), the characteristic time scale for equilibration of such a "reaction" may be expected to be too small ($\ll 1$ s) to resolve with many experimental techniques that are in current practice (cf. discussion below). However, this is an important consideration when selecting techniques to monitor/detect/quantify aggregation, and when interpreting the results. Depending on the choice of experimental technique and analysis methods, one can reach quite different conclusions regarding the size and concentration of different oligomers or "clusters" [14,16,21]. In general, one requires systematic and detailed experimental characterization over a wide range of protein concentrations in order to refine even simple mass-action or multimer-equilibrium models with any quantitative certainty [17,20].

Not all aggregates are reversible. In some cases, what might be thought of as otherwise reversible aggregates can convert to stable species that are "bound" together so strongly that they are effectively irreversible on practical time scales and concentration ranges. In practice, this typically manifests as aggregates that do not dissociate appreciably upon multi-fold dilution or upon shifts in solution pH or ionic strength — although, the latter can cause aggregates to grow dramatically [22,23]. Furthermore, creation of such aggregates typically involves changes in the secondary and/or tertiary structures of the constituent monomers in a given aggregate species. These structural changes do not need to involve more than a (small) portion of the overall monomer chain(s) [24–26]. In the case of small proteins, there is often a marked increase beta-sheet content [27–30], but in general it remains unclear precisely what structural changes are required to create net-irreversible aggregates. High concentrations of chemical denaturants (urea, guanidinium, ionic surfactants, etc.) or high pressures ($>10^3$ bar) are sometimes able to dissociate such aggregates [31–33]. In such cases, small aggregates (dimers, etc.) may initially form as reversible species, but ultimately one often recovers or detects only the net irreversible species in most experimental techniques that resolve the different species from one another. In such cases, the mechanism(s) of aggregation become important because changes in the relative rates of different steps in the overall aggregation process can dramatically shift the population (concentration) of different sized aggregates, as well as potentially affecting the structure/morphology of the aggregates that are detected [34]. The next section provides additional details regarding illustrative aggregation mechanisms as a context for the discussion below regarding the importance of mechanism(s) and what controls them when one is considering how best to monitor and quantify protein aggregates.

3. Illustrative mechanisms of non-native aggregation

This section provides a brief overview of some the mechanisms by which non-native aggregates form. It is not realistic to exhaustively enumerate all conceivable aggregation mechanisms within the available space, nor is it necessary, as the examples below illustrate key conceptual approaches that aid when interpreting experimental results for aggregating systems. In what follows, the term non-native aggregate will be synonymous with net-irreversible aggregate, although reversible intermediates can also be involved (see below). Net-irreversible protein aggregation is described without explicit formation of new covalent bonds. While changes in covalent bonds can promote aggregation [35–37], the rate limiting step(s) in many cases involve formation of non-covalently linked aggregates prior to covalent linkages forming that further stabilize the initial aggregates [38]. That notwithstanding, aggregation mechanisms are conceivable in which non-covalent bond formation is rate-limiting, and therefore can be important from both the perspectives of kinetics and the resulting aggregate morphology [39,40].

Many of the recent studies with pharmaceutical proteins that form larger aggregates do not require covalent bonds to form between proteins, although some examples for mimics of food systems show a mix of behaviors [39,40]. The discussion below does translate, in qualitative terms, to aggregates that form by covalent linkages, although the detailed kinetics and time scales involved can be quite different [34]. To try to maintain as much generality is possible, most of the discussion below is cast in terms of relative rates of different steps, as it is only the relative rates that ultimately determine which competing pathway(s) are ultimately observed for a given protein and a given solution condition or storage environment.

Fig. 1 shows a schematic representation of a number of the key steps involved in competing pathways of protein aggregation that have been shown or speculated in the recent literature (see also, Figure caption), and adapted from [41,42]. Alternative representations are also possible, and many of the published mechanisms that have been validated in detail are similar to or essentially the same as in Fig. 1 [34]. Double arrows for any steps in the diagram indicate net reversible steps. Single line arrows represent net irreversible steps, with ellipsis indicating a series of similar or analogous steps. Block arrows indicate steps that may be poorly or only qualitatively defined to date, and may involve multiple steps that are lumped into one block arrow.

Starting with folded monomer protein (blue), the monomers could conceivably form weak, easily reversible folded dimers or small oligomers (Fig. 1). Alternatively, a folded monomer is able unfold or partially unfold (red) and refold dynamically while in solution. The partly unfolded monomers expose more hydrophobic amino acid sequences that can help to drive initially reversible dimer or oligomerization (Fig. 1), and ultimately if the different protein chains can find ways to form both strong hydrophobic contacts and satisfy their hydrogen bonding needs (e.g., with inter-protein beta sheets) then they can "lock" into net irreversible, non-native oligomers that can stay as just dimers/oligomers or can grow through different mechanisms. If one considers sufficiently high concentrations then it may be feasible that otherwise weakly bound native oligomers will become sufficiently populated to be the faster pathway for transitioning from reversible oligomers to irreversible ones (Fig. 1) [43], although that would require a rather complex process of a native oligomer sufficiently unfolding and then misfolding as a cluster to form the non-native oligomer(s) that remain stable or grow to much larger sizes.

In qualitative terms, growth can first be categorized as dominated by monomer addition or by aggregate–aggregate coalescence (cf. labels in Fig. 1). In the former case, electrostatic repulsions between aggregates are sufficiently large that aggregates do not aggregate with one another except if one exhausts the available monomer pool [30,41,44,45]. In the latter case, aggregates are sufficiently attracted to one another that monomers are only consumed by the creation of new dimers/small oligomers, and those small aggregates rapidly coalesce with one another to form larger aggregates that propagate the aggregate coalescence process [44–48]. In the extreme, interactions between aggregates can become so favorable that the aggregates undergo bulk phase separation to form macroscopic and microscopic/subvisible particles [22,23]. Of course, these mechanisms can also occur simultaneously and so the behavior can change over the course of time as a sample is stored [44,45].

If one also considers aggregate formation via bulk interfaces, then the following qualitative features summarize key findings from a