Mechanisms of Protein Aggregation

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Abstract: Aggregation or reversible self-association of protein therapeutics can arise through a number of different mechanisms. Five common aggregation mechanisms are described and their relations to manufacturing processes to suppress and remove aggregates are discussed.

Keywords: Aggregation, self-association, mechanism, nucleation.

INTRODUCTION

Protein aggregation can occur through a number of distinct mechanisms or pathways. These mechanisms are not mutually exclusive, however, and more than one can occur for the same product. While it is certainly not essential that one understand the aggregation mechanism for a particular protein in order to develop an appropriate manufacturing process, a good formulation, or a method to suppress and remove aggregates, some mechanistic understanding can help point the way to solving aggregation issues (or at least to avoiding excipients and processes that are likely to make things worse). Fig. (**1**) schematically illustrates five important mechanisms for protein aggregation. How these mechanisms and the types of aggregates relate to process and formulation development will be discussed in the last section.

MECHANISM 1: REVERSIBLE ASSOCIATION OF THE NATIVE MONOMER

In Mechanism 1 the tendency to reversibly associate (aggregate) is intrinsic to the native form of the protein. The surface of the native protein monomer is self-complementary so it will readily self-associate to form reversible small oligomers. As illustrated here there may be multiple "sticky" or complementary patches on the monomer surface. Those can then produce different types of interfaces, potentially producing multiple conformations for oligomers of the same stoichiometry and different patterns of oligomer growth. As the protein concentration rises and larger and larger oligomers form (driven by the law of mass action), over time these larger aggregates often become irreversible (sometimes through formation of covalent bonds such as disulfide linkages). Insulin is just one example of a therapeutic protein which readily (and normally) associates to form reversible oligomers [1]. Insulin also illustrates that such association can have important consequences for bioactivity, and how manipulation of that association via mutation has produced important new products [2]. Interleukin-1 receptor antagonist (rhIL-1RA) is an example of a product that undergoes reversible dimerization at high concentrations, followed by formation of irreversible dimers and trimers [3].

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MECHANISM 2: AGGREGATION OF CONFORMATIONALLY-ALTERED MONOMER

In contrast to Mechanism 1, for Mechanism 2 the native monomer has a very low propensity to reversibly associate. However after it transiently undergoes a conformational change or partial unfolding the resultant altered conformation of monomer associates strongly (in a manner similar to Mechanism 1). Thus a key difference between Mechanisms 1 and 2 is that in Mechanism 2 the first step is a conformational change to a non-native state, and at any given time the fraction of protein in that aggregation-prone non-native state will usually be quite small. For Mechanism 2 aggregation will be promoted by stresses such as heat or shear that may trigger the initial conformational change. A further (and important) consequence is that aggregation will be inhibited by excipients or conditions that stabilize the native conformation.

This aggregation mechanism does appear to be the dominant one for many proteins and has been discussed in several reviews [4-6]. Two therapeutics where this mechanism has been reported are interferon-γ [7] and G-CSF [8, 9].

MECHANISM 3: AGGREGATION OF CHEMICALLY-MODIFIED PRODUCT

Mechanism 3 is really a variant of Mechanism 2 where the change in protein conformation that precedes aggregation is caused by a difference in covalent structure. Usually this difference is caused by chemical degradation such as oxidation of methionine, deamidation, or proteolysis. Chemical changes may for example create a new sticky patch on the surface, or change the electric charge in a way that reduces electrostatic repulsion between monomers. In some cases however the chemically different species is not a degradant but rather it is a normal variant within the bulk drug product--for example in glycoproteins there might be a unglycosylated or under-glycosylated fraction that is prone to aggregation.

A diagnostic feature of this mechanism is that the aggregates will be enriched in the modified form. (Although this is not illustrated in the figure, the modified monomers are sometimes able to recruit normal monomers into the aggregates, so the aggregate fraction will not necessarily contain

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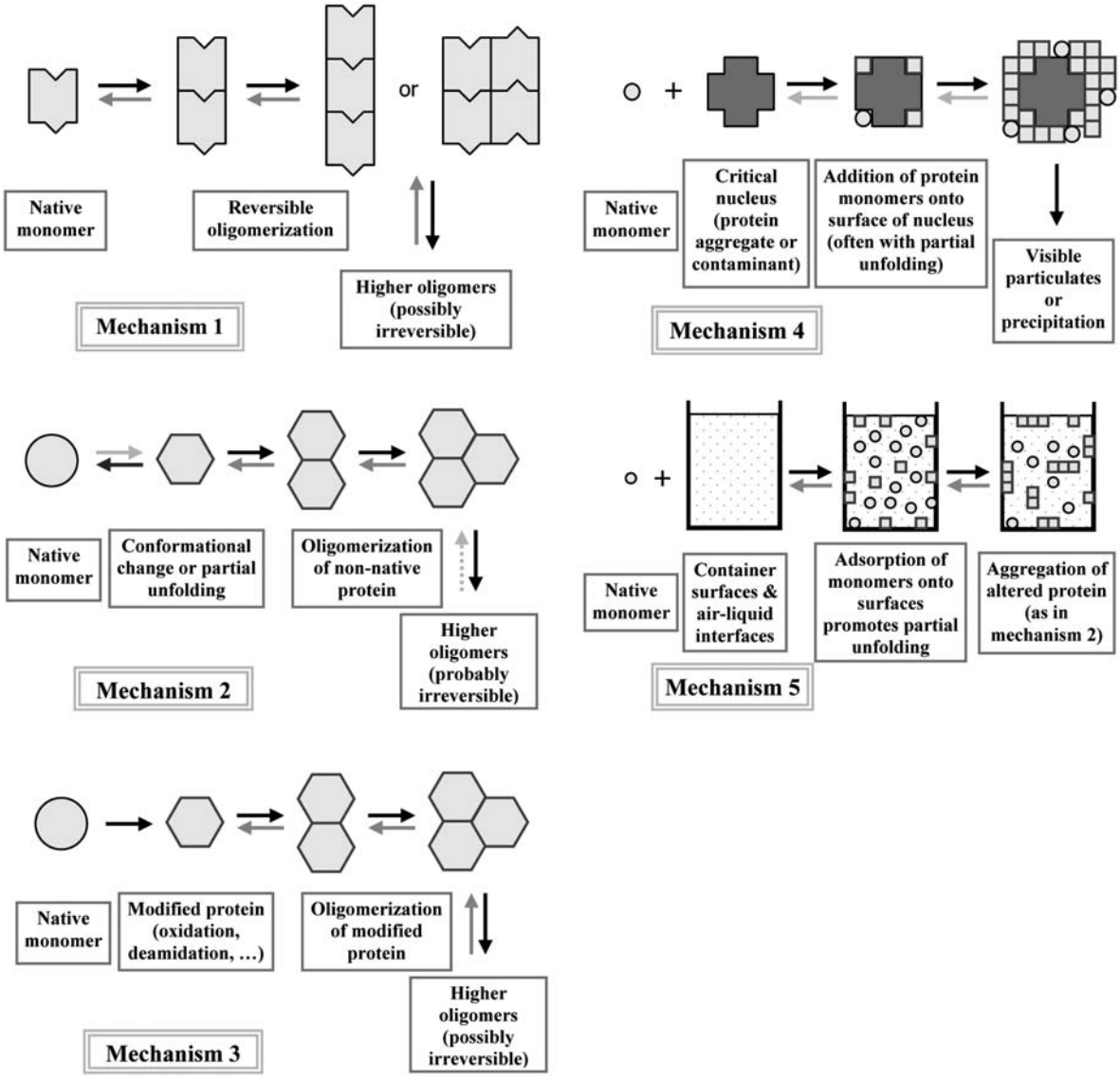

Fig. (1). Shematic illustrations of five common aggregation mechanisms.

only modified monomers.) Clearly when Mechanism 3 is operative improving the chemical stability will also reduce aggregation, and conversely attempts to improve the conformational stability of the monomer may not reduce aggregation. It is also worth noting that aggregates of chemically altered protein can be particularly immunogenic [10].

MECHANISM 4: NUCLEATION-CONTROLLED AGGREGATION

Nucleation-controlled aggregation is a common mechanism for formation of visible particulates or precipitates [5]. In this mechanism the native monomer has a low propensity for formation of small and moderately-sized oligomers (the addition of monomers onto these smaller aggregates is not thermodynamically favored). However if an aggregate of sufficient size manages to form, then the growth of this so-called "critical nucleus" through addition of monomers is strongly favored and the formation of much larger species is rapid. This type of process is similar to growing large crystals by adding micro-crystal "seeds" to a saturated solution, and thus the critical nuclei are also sometimes called the "seeds" or "templates" for aggregate growth.

A characteristic feature of a nucleation-controlled process is that the rate of formation of the large particles or precipitates usually exhibits a lag phase. That is, no particles or precipitates can be detected for a long period of time (perhaps months) but then rather suddenly the large species appear and accumulate. The length of the lag phase can vary in a stochastic manner from one vial to another within a single manufacturing lot, so particles may first appear in individual vials over a wide range of times.

Thus far what has been described is called "homogeneous nucleation" where the critical nucleus is itself a product aggregate. In a second variant of this mechanism the critical nucleus (seed) is not a particle made of the product protein but rather a particle of an impurity or contaminant. This second variant is called "heterogeneous nucleation". Two examples of contaminants reported to have served as seeds for

aggregation are silica particles shed by product vials [11] and steel particles shed by a piston pump used for filling vials [12]. Anecdotal evidence also implicates silicone particles introduced by tubing used in manufacturing or as lubricants for syringes, and vacuum pump oil particles introduced during lyophilization.

MECHANISM 5

The last mechanism to be discussed here is surface-induced aggregation (Mechanism 5). This aggregation process starts with binding of the native monomer to a surface. In the case of an air-liquid interface that binding would probably be driven by hydrophobic interactions, but for a container favorable electrostatic interactions might also be involved. After this initial reversible binding event the monomer undergoes a change in conformation (for example to increase the contact area with the surface). Like in Mechanism 2, it is then that conformationally-altered monomer which aggregates, but in this case that aggregation might occur either on the surface or perhaps after the altered monomer is released back into the solution. Freeze/thaw damage can also arise from aggregation at the surfaces of ice crystals or crystals of excipients, and thus can occur through Mechanism 5, but freeze/thaw damage can also involve other mechanisms such as changes in pH.

It is interesting to note that Mechanism 4 could be considered a special case of Mechanism 5 where the surface that induces aggregation is the surface of the critical nucleus. A second point about this mechanism is that during accelerated stability testing the tests that involve agitation or that try to induce shear forces by moving balls through the liquid (will produce conformational stress and therefore may induce aggregation through Mechanism 2), but also may simultaneously produce significant exposure to surfaces, so it may be unclear which stress is actually inducing the aggregation.

WHY DOES MECHANISM MATTER?

The principal advantage of an understanding of the mechanism of aggregation is that this can help guide process development and/or the formulation effort. There are a number of ways such understanding might help with either upstream or downstream process development. When proteins are marginally stable against partial or complete unfolding, care must be exercised to avoid both mechanical stress and exposure to air or solid surfaces that may lead to adsorption-induced unfolding. In such cases, processes that minimize the surface exposure should reduce aggregation. Certain proteins are sensitive to mechanical stresses such as agitation and hence may aggregate during chromatography or filtration due to shear strain, requiring extra care during these operations. Aggregation during production steps can also sometimes be prevented by adding appropriate stabilizing co-solutes, provided that such additives do not interfere with the purification processes.

An understanding of aggregation mechanisms is particularly useful during formulation, with respect to the addition of "generic protein stabilizers" such as sucrose, polyols and certain amino acids and salts. These co-solutes increase the stability of native protein structure against various environmental stresses that cause unfolding. The co-solute interactions with the protein surface are thermodynamically unfavorable, which favors a minimal surface area and hence the native structure (see Chapter 4.1 for further discussion of this stabilization mechanism). Such co-solutes should therefore reduce aggregation that is caused by Mechanism 2.

Mechanism 2 is certainly an important one that has been reported for a number of proteins [7, 9, 13, 14], and one which has received particular emphasis by John Carpenter, Ted Randolph and co-workers at the University of Colorado [5]. Antimicrobial preservatives added to multi-dose formulations often increase protein aggregation, and it was shown that benzyl alcohol drives aggregation through Mechanism 2 by promoting transitions to partially-unfolded states [15]. However many scientists do not realize that addition of "generic structure stabilizers" is not a universal cure for aggregation problems, and in fact will usually significantly increase aggregation arising via Mechanisms 1, 4, and 5. As already noted these co-solutes tend to drive the protein to a state of minimum surface area exposed to the co-solute. One way to minimize the surface area per monomer is for the native monomer to self-associate (Mechanisms 1 and 4). An alternative way to minimize protein surface area exposed to co-solute is to leave the solution by adsorbing to a surface (Mechanism 5).

Conversely, for mechanisms 1, 4 or 5 adding co-solutes that weakly bind to the protein may reduce aggregation. Co-solutes that strongly bind to the protein would also reduce aggregation via this mechanism, but may also destabilize the folding and hence enhance aggregation via Mechanism 2. Such destabilizing co-solutes, e.g., urea, guanidine hydrochloride or strong detergents, are strong protein solubilizing compounds, as briefly described in chapter 4.1. Among weakly binding co-solutes, arginine is especially effective in reducing protein aggregation that occurs due to Mechanisms 1, 4 and 5. The mechanism of aggregation suppression by arginine is described in detail in chapter 4.2 and 4.3.

This discussion of mechanisms also helps in understanding whether addition of a surfactant will help to reduce aggregation. It is fairly obvious that surfactants should reduce aggregation through Mechanism 5 by reducing exposure of the protein to the surfaces. Surfactants may also help reduce nucleation-controlled aggregation (Mechanism 4) by covering the surface of the critical nuclei. Typically however surfactants are not helpful for Mechanisms 1, 2, or 3 (and indeed the impurities commonly found in polysorbates may increase chemical degradation and therefore drive Mechanism 3).

A third way in which mechanism may matter is that some forms of aggregates may be worse than others. We see that for Mechanisms 2-5 the aggregates are primarily made from non-native monomers. This makes it more likely they will have altered potency as well as altered immunogenicity (because the altered monomers present different epitopes). On the other hand, because Mechanism 1 aggregates are native-like, if their larger size does induce an immune response it is more likely those antibodies will cross-react with (and potentially neutralize) the native monomer. While the formulation or process development scientist may not be able to control which type of aggregates are dominant for a particular pro-

tein, such considerations might be important when different pathways dominate under different formulation conditions.

Understanding aggregation mechanisms may also help developing an optimal chromatography step to remove aggregation (Chapter 5.1, 5.2, 5.3 and 5.4) or for pressure-induced aggregate disruption (Chapter 5.5). When high hydrostatic pressure is used to dissociate aggregates, understanding the aggregation mechanism may help in designing optimal solvent and temperature conditions, as high pressure mainly disrupts hydrophobic interactions. A lightly chaotropic co-solvent may enhance dissociation conferred by pressure. It is obvious that chromatography chosen to remove aggregates should not generate new aggregates and hence understanding the mechanism should be helpful; e.g., aggregate removal chromatography that generates shear strain should not be used when the protein is susceptible to shear stress. In addition, knowing the type of aggregates may help design a better chromatography method for aggregate removal. Hydrophobic interaction chromatography may be most effective, for example, when conformational change and hence exposure of hydrophobic surface that cause Mechanism 2 aggregation are extensive.

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