conventionally referred to as soluble particles; (b) subvisible particles (1–100 μm in size); and (c) visible particles (>100 μm in size).

There are several models of aggregation. In the native-to-unfolded-to-aggregate model, denatured or unfolded molecules aggregate due to hydrophobic interactions. Aggregate formation driven by hydrophobic effect happen when the normally buried hydrophobic regions are exposed. The rate of reaction in this model increases with temperature since unfolding increases with temperature and reactions generally follow first-order kinetics.

In the model native-to-unfolded-to-aggregate wherein the intermediate yields the aggregate, the misfolded intermediates are often thermodynamically stable and can be part of the native-state ensemble. Therefore, aggregation is not an unnatural state of a protein and can occur even under conditions favoring the native state.

The mechanism of protein aggregation involves two stages: a nucleation process which is followed by growth of the nuclei in a critical mass. The level of aggregation can be monitored by the turbidity measurement. However, turbidity is not necessarily an indicator of aggregation. The assembly of initially native and folded proteins can result in irreversible nonnative structures that may contain high levels of nonnative intermolecular β-sheet structures. The onset, rate, and final morphology of the aggregate depend on solution conditions such as pH, salt species, salt concentration, cosolutes, excipients, and surfactants. The exact nature of an aggregate is a function of the relative intrinsic thermodynamic stability of the native state.

Because of the many physical and chemical manipulations required in upstream production and downstream processing, followed by formulation and filling operations, the aggregation of protein biopharmaceuticals can be induced during nearly every step of the process including at hold points, shipping, and long-term storage. Agitation (e.g., shaking, stirring, and shearing) of protein solutions, can promote aggregation at the air–liquid interfaces, where protein molecules may align and unfold, exposing their hydrophobic regions for the charge-based association. Agitation-induced aggregation has been observed in numerous protein products, including recombinant factor XIII, human growth hormone, hemoglobin, and insulin. Minimizing foaming caused by agitation during manufacture (as well as during product use) may be critical to preventing significant loss of protein activity or generation of visible particulate matter. The antimicrobial preservatives used in multidose formulations can also induce protein aggregation. For example, benzyl alcohol accelerates the aggregation of rhGCSF because it favors partially unfolded conformations of the protein. Increasing antimicrobial preservative levels may enhance the hydrophobicity of a formulation and could affect a protein's aqueous solubility. Phenol and *m*-cresol can considerably destabilize a protein: Phenol promotes the formation of both soluble and insoluble aggregates, whereas *m*-cresol can precipitate protein.