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Nucleation in Protein Aggregation in Biotherapeutic Development: A look into the Heart of the Event

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ABSTRACT

In spite of extensive research, protein aggregation still remains one of the most difficult phenomena to be understood in the field of biologics research and development. Protein aggregation is a complex process which results in the formation of a variety of supramolecular protein structures. Nucleation is the core step that initiates the cascade of molecular events leading to the formation of protein aggregates. Understanding and characterizing nucleation is therefore crucial to avoid undesired protein aggregation. Here we review the state of the art on protein aggregation in biotherapeutics, primarily focusing on the nucleation events, stimulating discussions about key open questions, and clarifying the peculiarities of aggregation process relative to other protein phase separation processes, such as crystallization. We summarize recent progress in the identification of the sources of protein aggregation and in the development of analytical tools to characterize this process. Moreover, we discuss significant gaps in the analysis and understanding of nucleation in non-native aggregation of biologics.

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Protein aggregation in biologics development

Protein-based biologics represent an important class of drugs owing to their efficacy, specificity and safety. However, their complex structure makes them susceptible to a variety of chemical and physical degradation processes, including aggregation.[1-6] Protein aggregation is largely dependent on the surface hydrophobicity and charge as well as its environment including the solution conditions such as pH and ionic strength. Additionally, protein aggregation can also be triggered by chemical degradation.[7,8] In spite of extensive research on this topic, and more than 10,000 published research articles, protein aggregation still remains one of the most difficult phenomena to be understood in the field of biologics research and development. This topic has been recently covered in excellent reviews.[1,9,10] This work aims at critically assessing the state of affairs in this area of research especially focusing on nucleation events, stimulating discussions about key open questions, and clarifying the peculiarities of aggregation relative to other protein phase separation processes, such as crystallization.

Protein aggregation is a complex process which results in the formation of a variety of supramolecular protein structures that span a broad range of sizes from a few tens of nanometers to several hundreds of microns[11,12] and can differ widely in terms of protein secondary and tertiary structures, reversibility, and morphology.[13,14] Moreover, the different properties of aggregates can have different effects and consequences on the biological activity, safety and immunogenicity of the protein drug.[5] The term “aggregate” can therefore refer to many different species and terminology can differ in different research fields. For example, protein aggregates in micron or larger size ranges often are termed protein particles or protein particulates in biotherapeutic development. Some of these terminologies relevant to the discussions in this article are presented in Table 1. For additional details on many different aggregate types and how they are made, see tables in these articles.[13,14] The apparent discrepancy in terminologies also points to the much broader knowledge gaps in the field, namely (a) analytical quantitation challenges across the size continuum (nanometer to millimeter) of aggregates, (b) limitations in the characterization of morphology, physical structure and chemical composition of aggregates, and (c) scarcity of knowledge about biological consequences due to aggregates. As a result, the biologics developers are forced to use a very conservative approach for control and limits

In Memoriam of Wim Jiskoot, a colleague, friend, and mentor who passed away recently.

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(specifications) of aggregates and particles in biotherapeutic products for human use.

We also note that biologics, such as monoclonal antibodies, growth factors and interleukins, can undergo self-association (although to a different extent) and exhibit a population of monomers that may remain in equilibrium with a fraction of reversible dimers and/or oligomers.[15] These equilibrium oligomers have been shown to influence important quality attributes, such as viscosity at high protein concentration.[16]

The broad spectrum of aggregate structures is a consequence of the cascade of microscopic events that underlie protein aggregation and that involve possible conformational changes as well as different nucleation and growth reactions.[17-20] All of these microscopic events are specific to the combination of the protein molecule, its formulation, contact surfaces and external (stress) factors including temperature. This complexity results in several challenges: i) it is difficult to establish generic behaviours and trends, and every new molecule has to be characterized under the specific conditions (formulation and external factors); ii) it is challenging to predict aggregation over long-term storage from accelerated studies[21,22]; iii) it is currently challenging to correlate aggregation propensity with any individual biophysical properties of the molecule. A global aggregation "risk score" could be estimated by these parameters that can provide a directional guide.[23-25] Recently there has been great progress in the development of *in silico* tools to predict aggregation propensity and solubility,[26-28] to predict aggregation rate and to generate aggregation-related databases,[29,30] as recently reviewed in ref. [31] Moreover, at least a certain degree of qualitative correlation is observed between native protein-protein interactions (commonly quantified via the second virial coefficient or the diffusion self-interaction parameter) and solubility,[27,32] liquid-liquid phase separation[33] and viscosity at high protein concentration.[34] However, a reliable prediction of aggregation propensity especially over longer time scale remains a major challenge, and combined with the uncertainties of adverse impact on product quality and safety, protein aggregates continue to be designated by default as critical quality attributes.

The main reason of the complexity of prediction is that aggregation of protein biologics commonly involves a certain level of conformational change at the level of the monomer or of the oligomers, which typically confers irreversibility to the process.[19] This is a major difference with respect to other phase separation processes of biologics, such as protein crystallization and liquid-liquid phase separation (LLPS) in which proteins typically retain their native structure and activity. Indeed, crystallization can be used as a way to formulate biologics that ultimately produces native monomers once the crystals are solubilized, while protein aggregation is unwanted (unless the intentional molecular design involves a quaternary state). With "native" we indicate the most prominent structures in the folding landscape of the protein under given conditions. From a theoretical point of view, as proteins are known to be flexible molecules and conformationally heterogeneous even in the native state, some degree of conformational differences can in principle exist in structures such as crystals, reversible aggregates and in LLPS states. Therefore, it is very challenging to define the specific type and extent of conformational changes that will confer irreversibility (and hence transition to non-native structure) to the aggregation process. The issue is further compounded because currently no available biophysical techniques can systematically probe all small and larger conformational differences and in the timescale of their occurrences.

The presence of non-native structures mediating protein aggregation largely contributes to the challenges mentioned above due to the complexity of the protein unfolding landscape and the deep interconnection between conformational stability and colloidal stability,[35,36] which are further convoluted with a variety of interfacial instability events.[37] Chemical or conformational changes in the protein structure modify intermolecular interactions, which, in turn, can trigger aggregation, or in some cases, lower aggregation propensity. For instance, exposure of hydrophobic patches or other "hot-spots" upon unfolding is a common risk of protein aggregation.[35] However, some proteins could be only marginally stable and require no or only minor perturbations or stresses to promote aggregation, which in this case occurs mainly under native conditions, especially when they carry major hydrophobic patches in native state itself. This may also lead to native precipitation related to low solubility, without requiring the presence of conformational changes. Depending on the extent of protein unfolding required for aggregation, native protein-protein interactions may[38-43] or may not[33,41,42,44-46] be a good predictor of aggregation, as discussed in ref. [33] However, although native protein-protein interactions may not directly lead to aggregation, they could facilitate aggregation triggered by thermal, pH or interfacial stress.

Similarly to native interactions, conformational stability (typically indicated by the melting temperature) does not correlate with the aggregation propensity of different molecules,[23,24,46-48] although it may be useful to rank formulations of the same molecule.[47-49] Moreover, thermally-accelerated stability studies (e.g., at 25°C, 40°C or higher temperatures) may provide worse-case aggregation events but such data are not reliable to determine aggregation rate in refrigerated conditions (e.g., at 5°C).[20,50]

This balance of conformational and colloidal stability is highly affected by many intrinsic and extrinsic factors. Several sources of protein aggregation have been identified at different stages of the biologic product life cycle, including candidate selection, processing, formulation and long-term storage.[36,51] Bulk stresses[51] include chemical reagents such as an oxidizing agent, changes in temperature, pH and ionic strength and presence of cell culture impurities. Additional common risks are exposure to interfaces and heterogeneous nuclei,[52-56] which can occur both in the presence and absence of flow,[57,58] as well as cavitation,[59,60] which can induce aggregation via changes in temperature and formation of radicals.

Nucleation in protein aggregation

From a mechanistic point of view, irreversible aggregates are formed via a cascade of microscopic reactions that include possible conformational changes, primary nucleation and growth either by monomer addition or by cluster-cluster aggregation.[17-19,61-64] In this cascade, nucleation is a first key step towards the formation of aggregates. This step leads to the formation of nuclei from monomers, which can further grow over time (Fig. 1).

A universal definition of nucleus is very difficult, due to the broad range of properties (size, reactivity, reversibility, type of intermolecular interactions involved in their formation, level of conformational change of the monomer), which can largely vary from one system to another and within one system depending on the specific stress and formulation.

In the context of non-native aggregation of biologics, nuclei can be defined as the irreversible species with the highest energy level on-pathway towards larger aggregates, after which the aggregation process is "downhill" and energetically favoured (Fig. 1).

From an operative point of view, it could be more practical to characterize and refer to the nucleation process itself, e.g., the series of microscopic events that starting from a homogeneous solution of protein monomers lead to the formation of a new condensed protein phase (which can be represented by a small oligomer or a large particle or precipitate).

Based on the definition noted above, in a limiting case scenario a nucleus can be represented also by a single unfolded or chemically modified monomer. However, the formation of nuclei typically starts with the assembly of monomers into oligomers, which can be defined

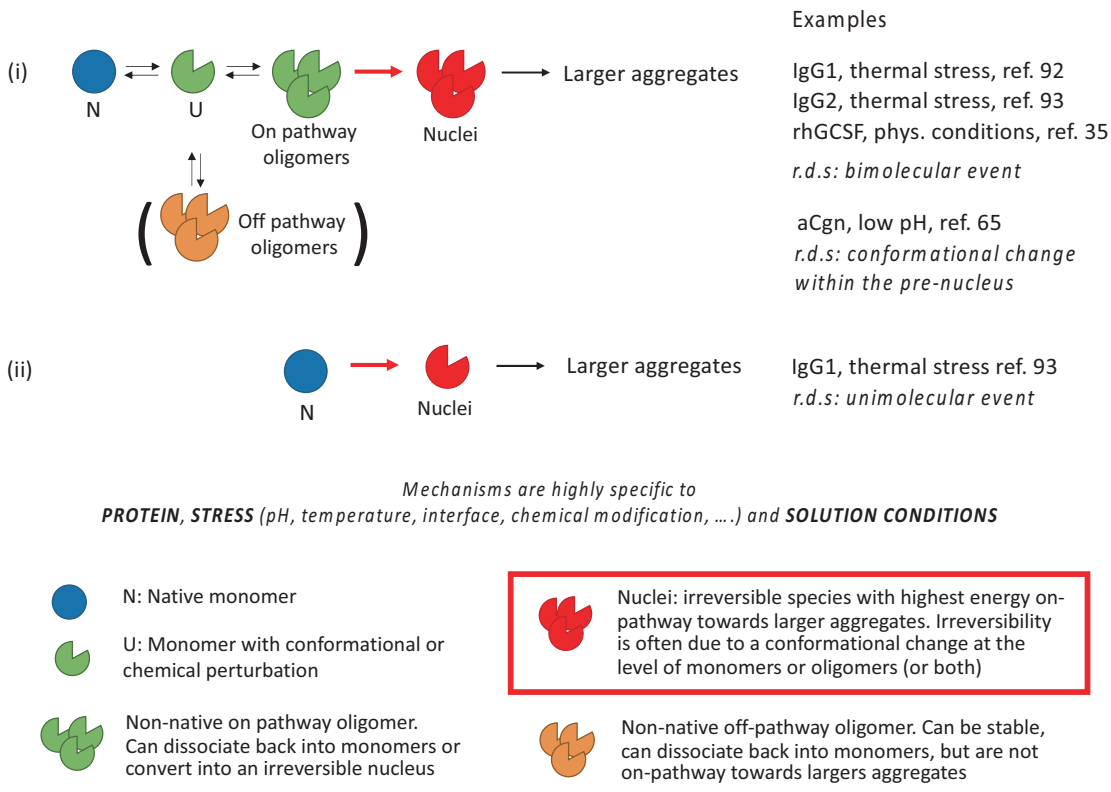

Fig. 1. Schematic of possible nucleation mechanisms in irreversible protein aggregation in biotherapeutic development. Depending on the characteristic time scales of the individual steps, as well as on the extent of misfolding required to promote aggregation, the identification of all species and microscopic events involved in the nucleation cascade can be challenging. The measurement of the apparent reaction order, however, provides important information on the rate determining step (r.d.s) (conformational changes vs colloidal stability), which is highly relevant to identify the source of aggregation and to suggest mitigation strategies.

as clusters of evolving structures containing a limited number of monomeric units (approximately 2-10).[65] The level of unfolding of the monomers in the resulting initial oligomers can be broad-ranging, therefore the intermediate or ultimate species can have a diversity in size, reversibility and morphology. Nuclei are the smallest irreversible species of these initial oligomers, after which the aggregation process is energetically favoured. The irreversibility is almost always connected to a certain level of conformational change, which may occur at the level of the monomer or oligomers (or both) (Fig. 1).

We note that irreversibility should also be contextualized since a broad range of reversibility tests exist, starting from dilution-induced reversibility to solubilizing agent-induced reversibility to mild denaturant-induced reversibility to redox-agent mediated reversibility to essentially total irreversibility (i.e., covalently-modified aggregates).

Although the formation of nuclei always occurs via initial oligomers, some of these can be “off-pathway” and represent a kinetically metastable or thermodynamically stable state that does not convert into nuclei, therefore increasing their concentration in solution over time. These off-pathway oligomers can be seen as small aggregates, with morphology and structure which may differ from the larger aggregates formed via the nuclei. Other oligomers could dissociate back into the monomers.

Schematics of possible mechanisms of nucleation in (non-native) irreversible aggregation are shown in Fig. 1. As discussed in the first paragraph, some proteins can self-assemble or aggregate under largely native conditions. Depending on the specific proteins and the conditions, proteins can self-assemble into oligomers containing few monomeric units or precipitates into larger aggregates. The latter case resembles the thermodynamic instability of hydrophobic colloids in water (e.g., polymeric latexes), which leads to the formation of distributions of aggregates via collision events as described by population balance equation models.[62] In this context, the concept of nucleus becomes less critical, since monomers, oligomers and aggregates of all size have similar reactivity.

This type of protein precipitation differs from protein crystallization, although both processes occur under native conditions. In protein crystallization the concept of nucleus is more relevant and clearly defined according to the Classical Nucleation Theory (CNT)[66,67]: the nucleus is the oligomer with the critical size at which the loss of energy required to create the solid-liquid interface is compensated by the gain of internal energy generated by intermolecular protein-protein interactions. Any cluster with a size larger than this critical size is energetically favourable and further growth is promoted by the decrease in free energy. An important consequence is that nuclei are very reactive, i.e., the growth rate is much faster compared to the nucleation rate. As soon as they form, they grow. Therefore, nuclei are transient and it is very difficult to isolate and characterize them.

Another important protein phase separation process under native conditions is liquid-liquid phase separation (LLPS), which can occur either via nucleation and growth mechanism or via spinodal decomposition.[68] In some cases, LLPS can promote crystallization via a two-step nucleation process.[69]

The fate of the protein under specific conditions is determined by the boundaries of the different states in the phase diagram (homogeneous solution, LLPS, precipitation or crystallization), which are highly specific to both the protein and the solution conditions.[70]

In crystallization and LLPS nuclei largely involve native structures of proteins that represent the dominant conformer in the folding landscape. In contrast, in non-native aggregation of therapeutic

Table 1
Description of a few selected terminologies* for protein aggregation and related phenomena in biotherapeutic development

Terminologies	Meaning in protein therapeutic development	Commonly used analytical techniques** and approaches for detection/characterization
High Molecular Weight (HMW) species, or, small aggregates, or, 'soluble aggregates', or, oligomers, or, agglomerates	Typically, these terms refer to nanometer sized protein aggregates, from approximately 5-10 nm size to many tens of nanometer (range depends on the size of starting protein monomer).	SEC (DLS, AUC and others)
Nucleation (of aggregation)	Nucleation is a first key step in the cascade towards the formation of aggregates. More details are included in the following section.	A variety of approaches that monitor time-resolved changes in physicochemical properties and amounts of protein monomers/aggregates
Precipitation	Phase transition resulting in protein (in native or denatured forms) separated from solution phase, typically as amorphous content.	Precipitates can be often detected visually.
Crystallization	Phase transition resulting in crystalline protein (generally in native form) separated from solution phase.	Crystals can be often detected visually or by light microscope.
Native protein	Refers to the most prominent structures in the folding landscape of the protein under given conditions.	A wide range of protein analytical techniques are available (details are out of scope for this paper).
Physical degradation	Refers to degradation processes that cause major changes in physical size, such as aggregation and fragmentation.	Several techniques included in this table (see also Table 2 and Figure 2 in reference [51], as an example)
Self-association	Proteins that exhibit net attractive forces can associate with each other resulting in a loosely-associated state or a tightly-bound state, depending on strength of the attractive forces.	Interaction parameter (kD) by DLS
Reversible aggregation/association	Generally, it refers to weaker associations of the constituent protein monomers. 'Reversible' can be a subjective term unless applied conditions to effect form changes are defined clearly (e.g., dilution in water/buffer/ saline, or, application of mild agents that can disrupt association).	Comparative data of various techniques (e.g., SEC, DLS, AUC, AF4) can provide clue for reversible aggregation process (also see Appendix A in reference[127]).
Irreversible aggregation/association	Generally, it refers to stronger associations (i.e., tightly bound) of the constituent protein monomers. 'Irreversible' also can be a subjective term unless applied conditions for making form changes are defined clearly (e.g., dilution, or, application of mild agents, or, applying strong agent such as SDS, urea that can disrupt association).	Comparative characterization data of various techniques (e.g., SEC, CGE etc) can aid in understanding extent of irreversibility.
Morphology of aggregates	Often this refers to coarse-level structural features, i.e., shape, of aggregates (or particles), such as spherical, rod-like, fibrous etc.	Microscopy-based techniques (static- and flow-imaging)
Quantity of protein aggregates	Typically aggregates are quantified as total mass (relative to total initial protein mass or absolute quantity).	SEC (for both relative and absolute quantities)
Quantity of protein particles	Typically measured as particle count (total mass can be approximated from counts).	Quantifying absolute mass of particles is very challenging as different techniques give different counts.
Submicron particles/particulates	Typically, these refer to sizes from approximately 50-100 nm to 1000 nm.	Lack of robust techniques for this size range. DLS, NTA, RMM, RPS, laser diffraction analyzer, microfluidics and others are used.

*For additional terminologies including various size ranges, different morphologies, types of aggregates made in laboratory, covalent vs noncovalent etc, see references in this paper including.[11-14]
**SEC, size-exclusion chromatography; CGE, capillary gel electrophoresis; AUC, analytical ultracentrifugation; AF4, asymmetric flow-field-flow-fractionation; DLS, dynamic light scattering; NTA, nanoparticle tracking analysis; RMM, resonant mass measurement; RPS, resistive pulse sensing.

proteins the recruitment of monomers may become important only when a non-native nucleus is present.

Non-native aggregation of biologics can take several concepts from the related field of amyloid formation, which often involves conformational changes at the level of the monomer or the oligomers (or both).[67,71] In these systems often monomers form oligomers that are metastable and redissolve into monomers. Only a minor fraction of these oligomers converts into nuclei via a conformational change promoting the beta-sheet formation.[72] After this conversion occurs, nuclei are very reactive and rapidly elongate. In analogy to crystallization, the elongation rate is orders of magnitude higher than the nucleation rate and therefore nuclei are very difficult to identify and isolate (in monomer equivalent they typically do not exceed 2% of the proteins in the system[72,73]).

In addition to primary nucleation events, secondary nucleation processes involving existing aggregates are frequent in amyloids[74] and crystallization,[75] while they have been rarely reported in biologics, i.e., in most conditions the addition of aggregates in solutions of native conformers does not promote formation of aggregates from monomers.

Experimental approaches to directly or indirectly characterize nucleation events in biologics development

Due to the low concentration and the typical high reactivity of the nuclei, their experimental characterization remains a formidable challenge, and detection and isolation of short-lived nuclei are not practically feasible on a routine basis. Yet, capturing the formation of nuclei for a protein of interest would be key to determine the mechanism of aggregation of that protein under a specific condition or stress, as well as to develop an effective control strategy. From an operational point of view, it is more practical and more relevant to characterize the entire nucleation process rather than a specific species, e.g., by analysing samples at different time points.

Several experimental techniques allow to monitor early aggregation events, potentially in combination with reversibility tests

Table 2
Description of a few selected analytical tools for monitoring nucleation and early aggregation events in the context of biologics development. We focus on techniques which do not require labelling of the protein with fluorophores or other tags.

Analytical method	Advantages	Challenges that require attention during method development
Size exclusion chromatography (SEC) coupled with static and/or dynamic light scattering	Resolve monomers from dimers, trimers, small oligomers and fragments; Good throughput; Routinely applied and standardized	Possible changes in the oligomer distribution during fractionation; Poor separation resolution (dynamic range) for species larger than approx. 30 nm
Dynamic light scattering (DLS)	Routinely applied and standardized; Highly sensitive in detecting onset of aggregation; Availability of instruments with high-throughput multi-well format	Only average size of the population is measured; Bias towards larger sizes; Sensitive to particle impurities
Analytical ultracentrifugation (AUC)	Resolution of oligomer distribution with excellent resolution	Low throughput
Asymmetrical flow-field-flow fractionation (AF4) coupled with static and/or dynamic light scattering	Good resolution in separating species in the hundreds of nm – submicron size range	Optimization of elution conditions needed; interactions with membrane (mass recovery); maintenance
Raman, infrared and nuclear magnetic resonance spectroscopy (NMR)	Information on conformational changes and metastable species if these are present in significant quantities; Can be applied in combination; with the techniques mentioned above	Direct detection of nuclei (very small fraction of protein) generally would not be possible by these techniques

(Table 2). Size exclusion chromatography (SEC) coupled with light scattering enables the detection of monomer conversion as well as the formation of dimers, trimers and larger oligomers, together with the information on average size and molecular weight of the aggregates.[61,76,77] However, the analysis should take into account possible changes in the oligomer distribution during fractionation upon dilution or interactions with the stationary phase,[78] which can be partially mitigated by the choice of mobile phase.

Asymmetrical flow-field-flow fractionation (AF4) is an alternative fractionation method to separate aggregates in the submicron range and has potential to monitor aggregation events during early stages.[61,79,80] Dynamic light scattering (DLS) is an *in situ* technique which is highly sensitive to the formation of aggregates and therefore is very suitable to detect aggregation during early stages, although it cannot resolve size distribution in heterogeneous samples since the signal is strongly biased towards large particles.[12] Analytical ultracentrifugation (AUC) can resolve oligomer distribution with high resolution,[81-83] although the technique could suffer from low throughput.

Raman,[84,85] infrared[86-88] and nuclear magnetic resonance spectroscopy (NMR)[89,90] are non-destructive techniques which can report also on conformational changes and metastable species if these are present in significant quantities, and can be applied in combination with the techniques mentioned above. For example, Amide I band (in Raman and IR spectra) can detect protein conformational changes. Direct detection of nuclei (very small fraction of protein) generally would not be possible by these techniques. For discussions about sample needs, resolution and limitations of these different techniques we refer to the book chapter[91] and references therein.

In addition to direct measurements, a powerful approach to obtain indirect information on a nucleation event is to exploit the experimental data by applying chemical kinetic analysis. The strategy consists of acquiring time-resolved data on aggregation and compare them with model simulations that consider different microscopic mechanisms.[92,93] If a sufficient number of experimental constraints is provided (in particular by performing experiments at different initial protein concentrations), this comparison allows to identify the aggregation scheme compatible with the experimental observation. Depending on the characteristic time scales of the individual steps of the nucleation cascade (Fig. 1), as well as on the extent of misfolding required to promote aggregation, a microscopic picture of the nucleation process can be obtained in addition to the overall kinetic scheme (Fig. 1).

This strategy has been applied to a large variety of biologics and has revealed a broad range of aggregation mechanisms,[44,61,62,65,77,92-98] highlighting the challenge to identify generic behaviours. However, even if generic behaviour cannot be obtained, for individual systems this approach allows to obtain a lot of valuable information, including the identification of global reaction orders and rate-limiting steps for aggregation. For instance, a reaction order of one indicates that the rate-limiting step of aggregation is a unimolecular reaction, such as a conformational change of the monomer.[65,93] In contrast, reaction order equal to or larger than two would indicate association between monomers or larger species as rate-limiting step.[36,92,93] Interface-induced aggregation often exhibits saturation effects which lead to an aggregation rate only weakly dependent on bulk monomer concentration.[58,99]

The characterization of the kinetic steps along with understanding of the factors controlling them are important to identify mitigation approaches based on the use of solution conditions, excipients, and stabilizers. For instance, for two immunoglobulins, sugars affected mainly the conformational stability of the proteins, while salt modified both unfolding and colloidal stability.[100]

A caveat of this kinetic approach is the need to acquire a sufficient amount of experimental data as a function of time and to observe monomer conversions larger than approximately 20% in order to discriminate between different models (multiple models provide similar results in the low conversion regime). This can be challenging for aggregation studies over long-term storage at low temperature, due to the very low conversion rates typically observed.

Mechanistic drivers of nucleation in biologics development

Nucleation can occur in bulk or at the interface between two phases (e.g., silicon oil-water, air-water, glass/metal-water or particle-water). Indeed, interaction between proteins and interfaces is a well-known risk factor that can promote potential unfolding of the protein and nucleation of aggregation.[52-55,101-108] When occurring in bulk solution, nucleation is homogeneous, while in the second case (interface) nucleation is heterogeneous. Heterogeneous nucleation also occurs when the nucleating agent does not consist purely of the target protein (e.g., metal leachate, rubber particles, glass shards, cell culture impurities including host cell proteins and DNA). Heterogeneous nucleation therefore describes situations in which the external agent is either the direct nucleating species (which remains incorporated in the aggregates) or a catalyst promoting the nucleation event

(in this case the agent facilitates the formation of protein aggregates but it is not included in the aggregates, which are fully protein-based). In non-native irreversible protein aggregation, both homogeneous and heterogeneous nucleations (when an external agent is the trigger of nucleation) require a dedicated step converting monomers into nuclei.

In bulk homogeneous nucleation, nucleus generation is typically triggered under conditions which favour the formation of conformationally altered species, i.e., high temperature and low pH.

High temperature has a double effect on protein aggregation: first, it can induce conformational changes promoting the formation of nuclei. Secondly, it accelerates the rate of all association and growth events that lead to the formation of larger aggregates from the initially formed irreversible nuclei. Nuclei formed at lower temperature can originate from perturbations that differ from conformational changes induced at higher temperature (e.g., 40°C). Therefore, although thermally accelerated studies can provide worst case scenarios, the relevance of the species formed at high temperature may be low even from the nucleation angle.[20]

Similarly, low and high values of pH can induce conformational changes. Such changes in pH are commonly encountered during downstream processes. Some of these protein conformers can reversibly interconvert during these pH switches, while in other cases, changes in pH (for instance during neutralization after viral inactivation at acidic pH[109]) lead to the formation of precipitates or particles. This process is dictated by a competition of time scales of (re)folding and association. For instance, at low pH electrostatic intermolecular repulsion can induce activation barriers for association, therefore increasing time scale of nucleation and preventing even largely unfolded monomers from nucleating. In contrast, if electrostatic intermolecular repulsive forces are screened by ionic strength (e.g., during neutralization) larger structures can be formed. [61,63,109-111]

Current status in biological product development and mitigation strategies for nucleation events

Protein aggregation is an undisputable challenge in every phase of biologic development. As a result, detection and control of nucleation events are considered among the most important tasks of the biopharmaceutical manufacturer during both development and manufacturing. In recent years, more emphasis has been placed on assessing aggregation propensity of new biologic candidates during discovery, drug product development, and process development for both drug substance and drug product. For example, in addition to other forms of stress that are commonly mentioned (i.e., thermal stress, extreme pH, etc.), interfacial stress is also highly prevalent in biologics manufacturing, shipping, and administration. Keys to overcoming the challenge of protein aggregation during product development are clear understanding of the mechanisms and good simulation by scaled-down models. In order to fulfil this need and effectively mitigate the challenge of protein aggregation, the industry's focus should be on early detection and mechanistic understanding that will enable the creation of an effective control strategy in both development and manufacturing. In practical terms, this begins with identifying the root cause, which requires timely detection. In order to determine the effect of each solution environment and/or process parameter on protein storage stability (shelf life), it is ideal to be able to monitor the transition from monomer to dimers and oligomers in real time. Conventional approaches monitor aggregation by taking a periodic "snapshot" of the sample at a specific point over a period of time. However, the lag time between sample generation and testing makes mechanistic insights more difficult to obtain. Therefore, there is a need for tools that can simultaneously induce stress while monitoring aggregation kinetics in real-time. Ideally, such tools would be coupled to analytical methods that do not disrupt the aggregates and be capable of detecting aggregates that are loosely associated. Finally, successful development and implementation of such technology will enable proper identification of the dominant mechanism(s) of aggregation/nucleation during both bioprocessing and storage.

In recent years, there has been a drive to a transition from classical batch to continuous integrated manufacturing of therapeutic proteins across laboratory, clinical and commercial scales. The development of effective strategy to both detect and control nucleation is highly relevant to the industry because continuous integrated manufacturing strategy requires strong fundamental understanding of upstream and downstream processes as well as the impact of these processes on product quality. This trend is expected to drive the continual development and improvement of technologies for online and real-time monitoring of protein aggregation, which bodes well for those who seize the opportunity to improve product quality by leveraging emerging process analytical technologies (PATs).

Like all other biophysical properties, aggregation propensity is not an intrinsic property of the protein sequence but is strongly affected by the formulation composition.[23] For instance, arginine, sorbitol and sucrose are well known stabilizers, and surfactants can protect against interfacial stresses.[112,113] The pH value of the formulation is another important variable that influences aggregation propensity by modulating both conformational change and protein-protein interactions.[35,114] Design of optimal formulations can therefore represent a first important step towards mitigating risks of aggregation.[115,116]

Current trends

Notwithstanding the challenges noted earlier for detection and characterization of aggregates in a wide size range, we are experiencing tremendous progress in the identification of the sources of protein aggregation and in the development of analytical tools to characterize this process.

- In particular, techniques capable of analysing aggregates in the submicron range are emerging, based for instance on diffusion measurements, light scattering, mass measurement, microfluidics and other techniques.[117-127] This is important since these submicron species appear to be characterized by high immunogenic risks,[128,129] in addition to risks from micron size subvisible particles.[130-132]
- Similarly, progresses have been made in characterizing the micron size particles (often termed as subvisible and visible particles depending on approximate size threshold) using flow imaging techniques as well as other orthogonal methods. Significant advances have been made in getting compositional identification of particles using FTIR and Raman microscopes along with energy dispersive spectroscopy and scanning electron microscopy techniques.
- There has been important progress also in the development of theoretical models of protein aggregation in biologics, which together with experimental data allow one to obtain a detailed description of nucleation at the molecular level (Fig. 1).[2,17,73] This kinetic approach has been applied in many case studies,[20,61-63,65,92,94,98] which have highlighted the variety of possible aggregation mechanisms of biologics.
- Although it may be challenging to identify all individual species and events involved in the nucleation cascade (Fig. 1), measurement of the apparent reaction order provides crucial information on the rate limiting step responsible for the formation of aggregates. This information is important to understand the source of aggregation and to suggest mitigation strategies to decrease risks of aggregation.

Open challenges

Despite this progress, there are still some significant gaps in the analysis and understanding of nucleation in non-native aggregation of biologics. A few selected areas which are worth further investigation are highlighted below:

- From an analytical point of view, there is the need of better methods to improve the sensitivity and resolution of detection in the submicron size range, as well as to decrease analysis time and amount of sample required. Methods capable of characterizing heterogeneous aggregate size populations directly in bulk solution would be highly desirable, overcoming the limitations of size exclusion chromatography.[78]
- The majority of particle characterization techniques as well as traditional quantitative analytical techniques (e.g., SEC) cover only a limited size range of aggregates/particles. Therefore, even when a combination of techniques is used to analyse aggregates/particles over a wide size range, an analyst is forced to weave non-equivalent sizing data sets derived from various techniques - a task which often is not very meaningful. For example, the submicron particle characterization techniques produce particle counts that can vary many orders of magnitude between techniques (such as NTA, RMM, RPS, see Table 1).[124,127] A standardization approach for absolute count and total mass of particles is needed to rationalize data from various techniques.
- The nucleation process is highly specific to the protein under consideration and, for the given protein, to the specific stress and solution conditions. The identification of generic patterns and common mitigation strategies in the broad landscape of biologics remains a great challenge. This highlights the importance of developing novel experimental platforms, ideally high throughput, which can be rapidly applied to specific proteins and stresses. We envision that emerging approaches based on machine learning[133] will also be highly helpful.
- It remains difficult to reproduce nuclei in a reasonable time frame under conditions that are relevant for bioprocessing and storage (e.g., nuclei formed in accelerated studies at high temperature may not be relevant for long term storage conditions). This arises because often of nucleation in the process remain unclear (e.g., due to irreproducibility or sporadic events or the large number of possible factors), or the nucleation events occur over long-time scales of months. This limitation challenges the establishment of suitable standard models that would facilitate the development of theoretical and analytical methods.

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