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REVIEW ARTICLE

Exploring Protein Aggregation in Biological Products: From Mechanistic Understanding to Practical Solutions

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Abstract

Proteins are vital for the regulation of several cellular functions, including the synthesis of structural components. The change in environmental conditions will impact conformational stability and result in aggregation. Protein aggregation involves different states of proteins, like nonnative, unfolded, and native states, which make them complex processes. The proper understanding of protein aggregation pathways involving the role of thermodynamically unfavoured lag phase, soluble protofibrils triggered polymerization through an exponential phase, and depleted free monomers owing to the saturation phase resulted in the leveling off of the polymerization process. The aggregated therapeutic proteins can induce deleterious immune responses in patients, and control of the aggregation is essential for better therapeutic protein stability and targeting with the help of stable protein structures and function. Protein–protein interactions (PPIs) are important for protein stability, aggregation rate, and solubility, while advanced computational and biophysical methods have been developed to characterize therapeutic protein aggregation better. Hence, an effective strategy for controlling, monitoring, and reproducing protein aggregation propensities of the polypeptide chains is required. An in-depth understanding of protein aggregation mechanisms, characterization, and combat strategies will counter the issues of protein aggregation. It will also reduce the cost of the product, time constraints, stable & effective product availability, and potential immunogenicity.

Keywords Aggregation · Anti-drug antibodies · Immunogenicity · Neutralizing antibody · Protein denaturation

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Introduction

Therapeutic protein aggregation

Biotherapeutics has witnessed remarkable progress in recent years, revolutionizing healthcare by offering targeted and precise treatments for various diseases. These pharmaceutical, biological products derived from living organisms have opened new medical avenues, providing innovative solutions for complex medical challenges. Among the diverse range of biotherapeutics, protein-based pharmaceutical products have emerged as the cornerstone of modern medicine, presenting a promising arsenal that includes vaccines, monoclonal antibodies (mAbs), gene therapy, cytokines, and enzyme therapies [1].

mAbs have garnered immense attention for their precision in targeting specific antigens. These engineered proteins mimic the body's natural antibodies, exhibiting high specificity and affinity toward their targets [2, 3]. mAbs have revolutionized the treatment landscape, particularly in oncology, where drugs such as trastuzumab (Herceptin®) and rituximab (Rituxan®) have significantly improved outcomes in breast cancer along with non-Hodgkin lymphoma, respectively [4]. Other types of protein-based biological products include cytokines and enzyme therapies. Cytokines are involved in the regulation of the immune system for the modulation of inflammatory responses. They treat conditions such as cancer and autoimmune diseases [5–7].

Enzyme replacement therapy involves delivering functional enzymes to individuals with enzyme deficiencies, as seen in lysosomal storage disorders such as Gaucher disease and Fabry disease [8]. While advances in biotherapeutics have revolutionized healthcare, the development of stable and effective protein-based formulations remains a significant challenge. Stability is a critical attribute of biotherapeutics, ensuring that the product retains its desired properties over its shelf life and during storage and administration [9].

Stable protein formulations have multiple benefits, including extended shelf life, enhanced efficacy, and improved patient safety [10]. One of the major challenges in achieving stable protein-based formulations is protein aggregation. Protein aggregation refers to the spontaneous association of proteins into larger, nonnative structures, leading to the formation of aggregates with distinct physicochemical properties compared to their native counterparts [11]. Such aggregation can profoundly impact biological product safety, efficacy, and stability, making it a critical concern in the biopharmaceutical industry [12].

The three-dimensional structure and inherent thermostability of therapeutic proteins play a pivotal role in maintaining their biological activity and desired therapeutic effects. However, temperature variations can induce protein misfolding or unfolding, forming physical aggregates. When proteins aggregate, they may expose new epitopes or conformational changes not present in their native form. The immune system recognizes these altered structures as foreign, producing antibodies against the aggregates [13].

The antibodies or anti-drug antibodies (ADA) can have variations in the effects on the therapeutic protein. In some cases, ADAs may bind to the therapeutic protein and neutralize its activity, reducing its efficacy in treating the targeted condition [14]. This can lead to suboptimal treatment outcomes and diminish the therapeutic benefits for the patient. The immune system may recognize the therapeutic protein-ADA complex. The immune system can recognize the therapeutic protein-ADA complex as that of the foreign entity, which results in the clearance of the therapeutic protein from the body [15].

The interplay between protein aggregation and immunogenicity highlights the importance of understanding and controlling protein aggregation in therapeutic formulations. It is also worth mentioning that proteins exhibit diverse self-assembly mechanisms, creating intricate and specific complexes that play pivotal roles in various biological processes [16].

These protein complexes often involve intermolecular solid interactions, requiring delicate conditions to maintain their native state and functionality. Understanding the rates of the various stages of the aggregation process significantly influences the population and size distribution of diverse aggregates, which is crucial for elucidating their behavior and impact on biological products (Fig. 1) [17]. The nature of protein aggregates can vary based on concentration, pH, and ionic strength. Reversible protein aggregates can readily dissociate upon dilution or slight adjustments in environmental conditions, enabling their separation and characterization outside of their original environment [18]. However, a significant challenge arises when reversible aggregates transform into unstable, irreversible derivatives within practical concentration ranges. These irreversible aggregates resist dissociation upon dilution and may grow further in response to fluctuations in pH or ionic strength [7]. The transformations leading to irreversible aggregates often involve changes in the secondary or tertiary structures of the constituent monomers, presenting complex challenges during the formulation development process [19].

To address this challenge, it is vital to understand the factors contributing to protein aggregation. Intrinsic factors, such as protein structure and sequence, dictate aggregation propensity [20]. External factors, including temperature and various processing steps during manufacturing (e.g., fermentation, unfolding/refolding, purification, freeze–thaw, shaking, and shearing), significantly impact protein aggregation [20, 21].

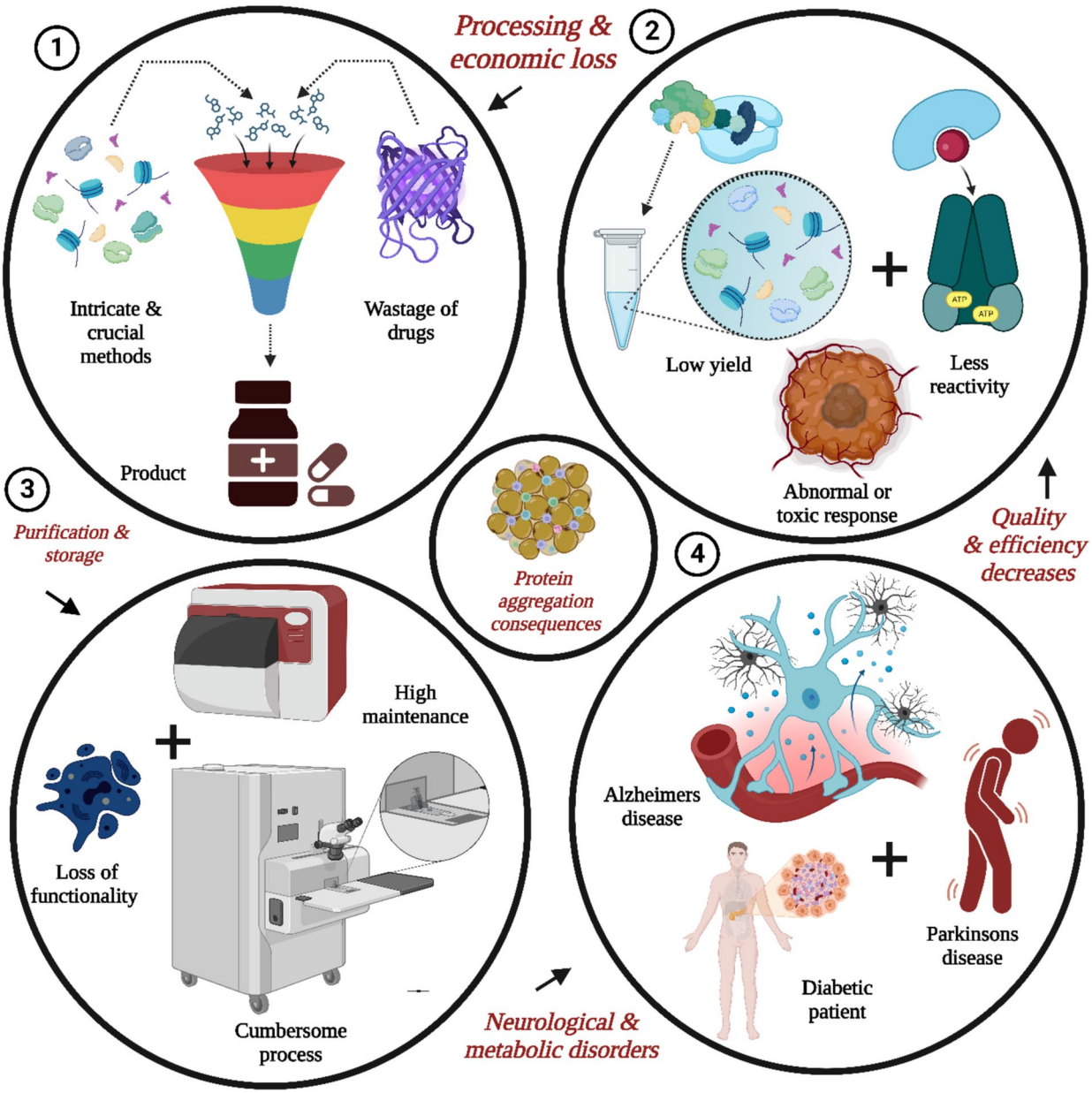


Fig. 1 Summary of the consequences of protein aggregation. 1. Processing and economic loss are induced due to intricate and crucial methods for product preparation, while protein aggregates can cause drug waste. 2. The protein aggregates also impacted on the quality and efficiency of protein with less yield, less reactivity, and abnormal toxic response. 3. It also involves some cumbersome processes in high maintenance. The loss of functionality has been found in protein aggregates. 4. Protein aggregates play a major role in neurological and metabolic disorders such as Alzheimer's disease, Parkinson's disease, and diabetes patients

Several factors like pH, ionic strength, type of excipients, protein concentration, and contact surfaces influence the aggregation of the therapeutic proteins. Other factors include container systems, irradiation, and light [19, 22, 23]. Recently, a trend has been observed in developing and utilizing advanced characterization techniques like analytical ultracentrifugation and size exclusion chromatography. It also includes dynamic light scattering and electron microscopy to assess protein aggregation and guide formulation optimization [24]. Moreover, various mitigation strategies are being explored to minimize immunogenic risks associated with protein aggregation. Rational drug design, engineering specific amino acid sequences to reduce aggregation-prone regions (APRs), and utilizing formulation additives that stabilize the protein in its native state are among the approaches to mitigate immunogenic side effects. Additionally, manufacturing processes are optimized to minimize the exposure of therapeutic proteins to conditions that promote aggregation [25, 26].

This review paper aims to comprehensively understand protein aggregation mechanisms, characterization techniques, and effective mitigation strategies in biological products. By exploring the complex interplay between protein aggregation and biotherapeutics' safety, efficacy, and stability, this review intends to equip researchers, formulation scientists, and developers with valuable insights to enhance the success of therapeutic protein formulations and ultimately benefit patients worldwide.

Mechanisms of Protein Aggregation

The aggregation of proteins can result in the formation of both soluble and insoluble aggregates, with the latter often precipitating out of solution. The morphology of these insoluble aggregates is influenced by the intrinsic properties of the protein and its surrounding environment and generally manifests as either amorphous or fibrillar structures. Fibrillar aggregates are highly ordered, characterized by extensive β-sheet–rich architecture, and are commonly associated with amyloid formation. In contrast, amorphous aggregates are structurally disordered and lack defined secondary or tertiary organization. Both forms pose significant challenges to protein stability and can compromise therapeutic efficacy. Therefore, a clear understanding of their formation mechanisms and morphological characteristics is essential for rationalizing stable formulations and optimizing biopharmaceutical development processes [10, 27].

Illustrative Mechanisms of Native and Non-Native Aggregation

Reversible Association of the Native Monomer

Native protein monomers can self-assemble into oligomers via attractive electrostatic interactions or covalent bonds generated between hydrophilic and hydrophobic residues on the protein surface [28, 29]. Individual monomers begin to self-associate, resulting in small reversible oligomers. Multiple "sticky" or complementary patches may exist on the monomer surface. These may produce a variety of interfaces, resulting in the formation of oligomers with varying patterns and conformations. Larger oligomers form as protein concentrations rise, owing to the law of mass action, while the larger aggregates generally convert irreversibly over time. The larger aggregates involve the formation of covalent bonds, e.g., disulfide linkages. For instance, insulin is a therapeutic protein that forms reversible oligomers under physiological and formulation conditions. Insulin also shows how such an association could have substantial bioactivity implications and how association via mutation has resulted in significant new products [29]. Notably, Insulin lispro and aspart contain substitutions at ProB28, disrupting dimer formation and promoting monomeric forms, though they can form reversible hexamers in the presence of phenolic excipients, which dissociate rapidly after injection. In contrast, insulin glulisine features substitutions at B3 (Asn → Lys) and B29 (Lys → Glu), preventing hexamer formation through steric and electrostatic effects, while a salt bridge between the N-terminus and B29E enhances monomer stability [30]. At high concentrations, interleukin-1 receptor antagonists (rhIL-1RA) undergo reversible dimerization, followed by the formation of an irreversible type of dimer along with the trimers. The mechanisms of protein aggregation are illustrated in Fig. 2 [31].

Aggregation of Conformationally Altered Monomer

In contrast to the previous mechanism, the native monomer has a very low propensity to form reversible aggregates. The transient conformational changes into the native protein resulted in a nonnative monomer with altered conformation and strong association. Since the initial step in Mechanism 2 is a conformational change to a nonnative state and the fraction of protein in the aggregation-prone nonnative state is relatively low, this represents a crucial difference between Mechanisms 1 and 2. Heat or shear is responsible for the initial conformational change [32]. Two therapeutics have reported this mechanism: recombinant human interferon-γ (rhIFN-γ) and G-CSF [33, 34].

Aggregation of Chemically Modified Products

Chemical instability, such as methionine oxidation, deamidation, or proteolysis, can alter the covalent structure of a protein, while the disruption of non-covalent interactions (e.g., hydrogen bonds, ionic interactions) can also induce conformational changes in the native protein, ultimately leading to aggregation. The chemical modifications induce the new sticky patch on the surface or show differences in the electric charge. The latter induces the decreased electrostatic attraction between the monomers [9]. The chemically different species are occasionally not degradable but a typical variety within the bulk medicinal product, such as an under- or unglycosylated fraction prone to glycoprotein aggregation. The chemically modified aggregates could be enriched (Fig. 2). Native monomers are sometimes recruited into aggregates by chemically modified monomers. As a result, the aggregate fraction sometimes consists of more than modified monomers (although not shown in Fig. 2). When this mechanism is active, improving chemical stability will also reduce aggregation, whereas improving conformational stability may not. Additionally, chemically altered protein aggregates may be immunogenic [35]. Mechanism 3 is explicitly described as a variant of Mechanism 2. In

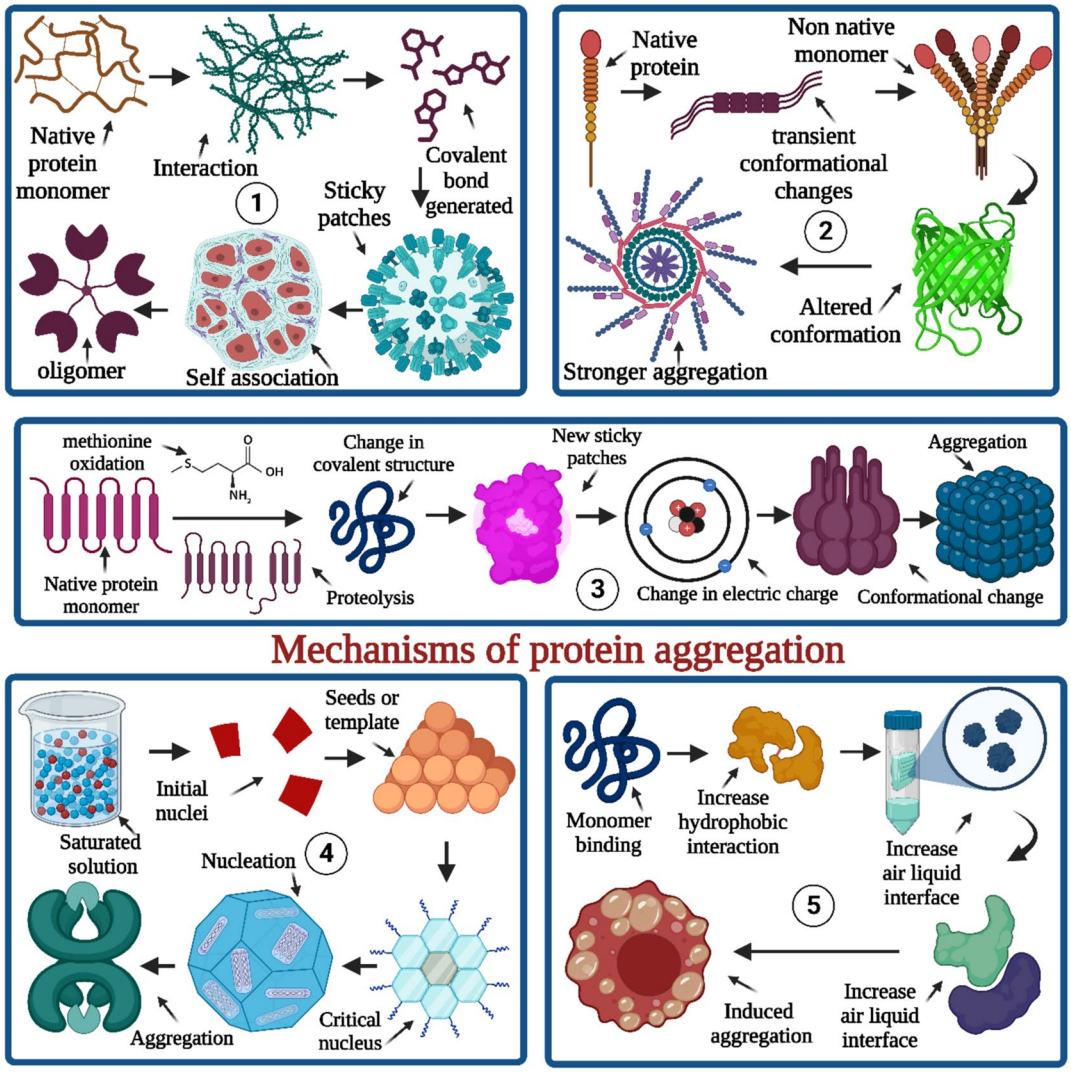


Fig. 2 Mechanisms of protein aggregation. Five mechanisms are involved in protein aggregation. The first involves a change in monomer interaction and its impact on protein aggregation. The second deals with the aggregation of conformationally altered monomers, while the third relates to chemically modified product aggregation. The fourth mechanism is coordinated with a nucleation-controlled aggregation system. The last mechanism is surface-induced aggregation, which involves hydrophobic and air–liquid interactions in protein aggregation

Mechanism 3, chemical modifications alter protein conformation, creating new aggregation-prone surfaces, thus making chemically altered monomers aggregate similarly to conformationally altered monomers (Fig. 2).

Nucleation-Controlled Aggregation

In this mechanism, the monomer produces small and medium-sized oligomers, while adding monomers to these smaller aggregates is not thermodynamically preferred. Nevertheless, if a large enough aggregate is formed, monomer addition to this so-called "critical nucleus," followed by expansion, and the formation of a giant aggregate becomes rapid. The essential nuclei are also referred to as "seeds" or "templates" for aggregate formation because the procedure is comparable to growing enormous crystals by adding microcrystal "seeds" to a saturated solution [16, 36].

A lag phase distinguishes a nucleation-controlled process. No particles or precipitates are visible for a long time (perhaps months), but then aggregation appears and accumulates. Particles may appear in different vials at different times because the length of the lag phase varies from vial to vial within a single production lot. Thus far, the process has been dubbed "homogeneous nucleation," with product aggregation as the critical nucleus. The critical core (seed) in the second variant of this mechanism is not a product particle but rather originates from external particulates, a process referred to as heterogeneous nucleation [36]. The nucleation

phase generally initiates with monomeric species and is categorized as either primary or secondary nucleation, contingent upon the absence or presence of preformed aggregates, respectively. Primary nucleation can proceed through one of two distinct mechanisms: nucleated polymerization (NP) or nucleated conformational conversion (NCC). The NP mechanism predominates at relatively low protein concentrations, where monomers progressively assemble into oligomeric nuclei characterized by ordered β-sheet structures. Alternatively, the NCC mechanism, described for proteins such as the yeast prion Sup35, involves a two-step process. Initially, disordered oligomers with low β-sheet content are formed, which then slowly undergo conformational rearrangement into β-sheet–rich prefibrillar oligomers [37, 38].

Surface-Induced Aggregation

According to this mechanism, the aggregation process begins with binding the native monomer to a surface. Hydrophobic interactions are likely the driving force for binding at an air–liquid interface, but favorable electrostatic interactions could also be involved in the case of a container. Earlier studies have demonstrated that proteins, including BSA and HSA, exhibit greater diffusion rates and mobility when adsorbed onto hydrophobic surfaces in contrast to hydrophilic surfaces. The conformation of the monomer changes after this initial reversible binding event (for instance, to enhance the contact area with the surface).

In Mechanism 5, the conformationally altered monomer aggregates similarly to Mechanism 2; however, this time, it may occur on the surface or after the altered monomer is released back into the solution. Although Mechanism 5 can also result from aggregate formation at the surfaces of ice crystals or excipient crystals, other processes, such as pH changes, can also result in freeze/thaw loss. Notably, Mechanism 4 is a subset of Mechanism 5, with the surface of the crucial nucleus as the surface that induces aggregation (Fig. 2) [39].

Factors Responsible for Protein Aggregation

Intrinsic Factors-Protein Structure and Sequence

Several hydrophobic residues in the protein sequence, when exposed due to partial unfolding or conformational changes, play a pivotal role in driving aggregation by promoting intermolecular hydrophobic interactions [40, 41]. The occurrence of glycosylation and structures of glycans in the CH_2 domains could impact protein stability. While an increased degree of glycosylation helps stabilize the native conformation of a protein by enhancing internal noncovalent interactions and reinforcing structural rigidity, the expansion of glycan molecular size appears to reduce the stability of the unfolded state, potentially altering the protein's overall folding dynamics [42]. It has been determined that glycan-protein interactions are essential for the stability of CH_2 domains within the Fc region of IgG antibodies, as deglycosylation severely weakens the CH_2 domain and eliminates its capacity to attach to low-affinity FcRs [43]. CH_2 domain sequences that promote N-glycan conformational flexibility and expose the hydrophobic inner surface of the CH_2 domain can aggregate IgG antibodies and therapeutic protein-Fc fusions. Figure 3 summarizes intrinsic and extrinsic factors.

Extrinsic Factors

Temperature

Thermodynamic instability, hydrophobic interactions, protein transport, and chemical reactions that promote aggregation are just a few of the effects of high temperatures on proteins. Higher temperatures may also change the shape of polypeptide chains within protein structures and cause them to unfold, promoting aggregation [44, 45]. The size and stage of the aggregation affect reversibility [46]. The protein melting point (Tm) and thermodynamic stability have a positive relationship [47]. When the temperature approaches the Tm, proteins partially unfold and form aggregates. A rise in temperature above the Tm frequently results in faster aggregation by contact with hydrophobic residues [48]. A protein with a greater Tm appears to have a lower tendency to aggregate [18].

Effect of Processing Steps

Protein aggregation can occur during several processing stages, including unfolding/refolding, freeze-thawing, purification, fermentation/expression, pressurization, shaking/shearing, and drying. The preparation and administration of protein drug delivery systems also influences aggregation [20, 49, 50].

Fermentation/expression The initial step in large-scale protein manufacturing is fermentation or cell culture [51]. Proteins can be expressed in various cell systems and under several process circumstances. Several proteins cluster into inclusion bodies in bacterial expression [52]. In these systems, aggregate formation competes with correct folding. The host systems profoundly influence the glycosylated state/pattern of the produced proteins, resulting in various protein aggregation behaviors [53].

Unfolding/refolding The unfolding and subsequent refolding of proteins are pivotal steps in large-scale protein

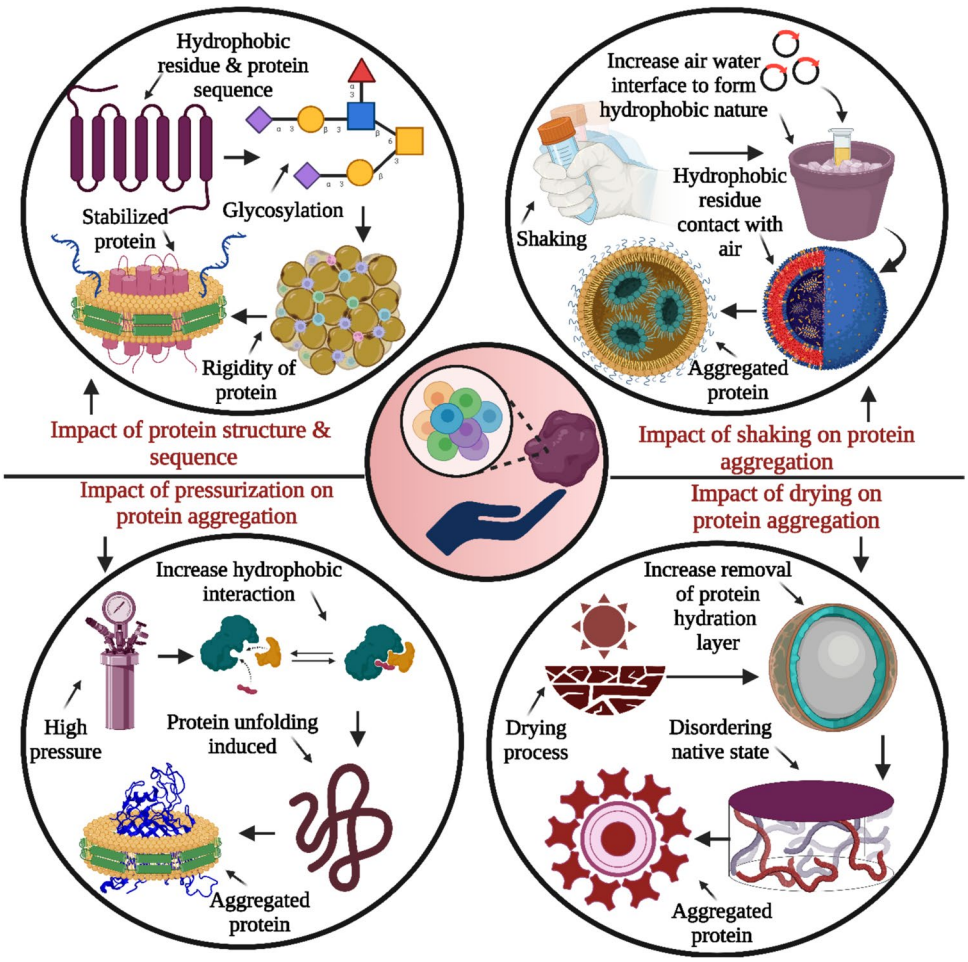


Fig. 3 Intrinsic factors such as protein structure and sequence greatly impact protein aggregation by using hydrophobic residues. Extrinsic factors such as shaking, shearing, pressurization, and drying significantly affect protein aggregation. Shaking influences the air–water interface due to the hydrophobic nature of air. The increase in hydrophobic residue contacts with air further initiated the aggregation of proteins. While drying accelerates the removal of the protein hydration layer and induces a disordered nature state, the generated disordered native state induces protein aggregation

production using microbial expression systems. During the refolding process, proteins traverse multiple intermediate conformational states, making them highly susceptible to misfolding and aggregation. As a result, protein aggregation has significantly reduced the yield of correctly folded biologically active proteins during refolding [54].

Purification Residual host cell protein and impurities can be removed through the purification stage used in protein manufacturing. The purification conditions may cause issues of protein aggregation with the involvement of pH, temperature as well as conductivity [55, 56]. One of the protein purification methods is affinity chromatography, which requires a low pH for the protein elution, and it can induce protein aggregation issues during the elution [57]. The mild purification conditions also triggered the protein aggregation, which can be countered by proper control through the addition of additives to the protein purification conditions. These include cosolvents (e.g., sugars, polyhydric alcohols) that promote native protein stability; chaotropic agents that disrupt intermolecular interactions; kosmotropes that favor the native folded state; reducing agents (such as DTT and β-mercaptoethanol) to prevent improper disulfide bond formation; and specialized agents like ethanol and detergents that assist in stabilizing folding intermediates or membrane proteins [58]. The impact of the purification on the protein aggregation is presented in Fig. 4. Many antibody purification methods are involved in the affinity purification technique like ammonium sulfate precipitation, immobilized protein A, G, A/G or L utilization, immobilized antigen, antibodies, ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography, multimodal chromatography, immunoprecipitation along with the co-immunoprecipitation [59–61].

Freeze–thaw method The type of protein and the concentration of the protein, along with other excipients during the freezing process, can induce protein aggregation. The solute concentration, low temperature, phase separation, pH changes, and ice water interfaces cause the protein aggregation in the freezing process. The freezing temperature can induce cold denaturation, where proteins may spontaneously unfold at low temperatures [62]. The freezing rate impacts the ice crystals' size and the ice-water interface's surface

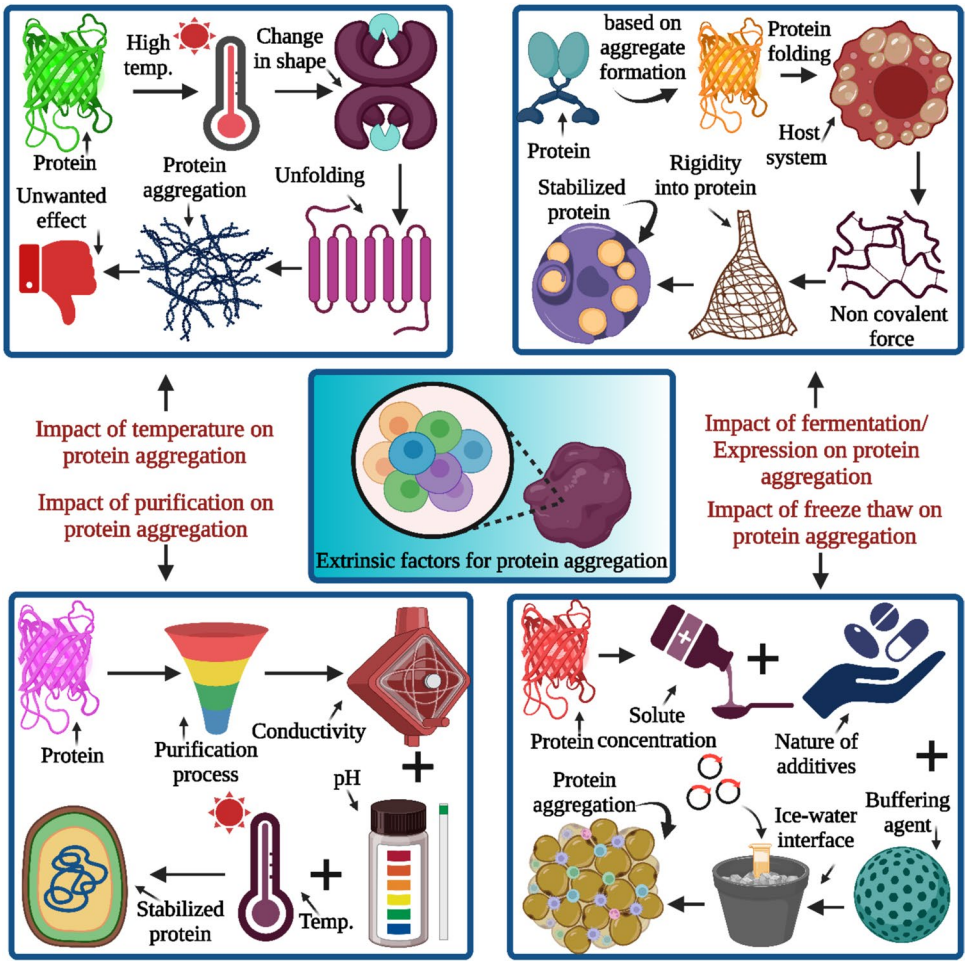


Fig. 4 The impact of extrinsic factors on protein aggregation. The influence of temperature, purification, fermentation, expression, and freeze–thaw cycles has been studied by different researchers across the globe. The increase in temperature induces faster protein aggregation with the help of contact with hydrophobic residues. In the case of fermentation, the rise in glycosylation degree enhances the noncovalent force, which further results in protein rigidification correlated with protein stability. The purification and freeze–thaw impact are studied along with several other factors, such as pH, conductivity, temperature, excipients, protein concentration, and protein type

area. The selective crystallization of the specific buffering agents triggered through the freezing-induced pH change resulted in protein aggregation. The container configuration and solution pH before the freezing/thawing are also important for protein aggregation [63]. The inhibition of freeze–thaw-induced protein aggregation can be done by properly selecting formulation excipients by reducing protein-ice interactions and suppressing preferential interactions and pH change. The inhibition of excessive protein–protein interactions and uncontrolled increase in solution viscosity can decrease the protein aggregations [64].

Shaking and Shearing The shaking and shearing encountered during shipment and protein synthesis can favor protein aggregation. The intensity of the stress given by these factors, along with the extent of exposure, is crucial for the extent of protein aggregation. The protein aggregation at the interface begins with the shaking process by generating the air-to-water interfaces to develop a hydrophobic nature of the air compared to that of the water [7]. The exposure of the hydrophobic regions or the protein domains by the shaking can lead to aggregation. High shear stress can induce protein aggregation to a larger extent than moderate shear. The protein aggregation induced by the shearing and shaking can be reduced through the surfactant through competition with the protein molecules for the hydrophobic surfaces. A controlled reduction in the protein mobility, achieved by enhancing solution viscosity through stabilizing excipients, and their direct attachment with the protein surface can reduce the protein aggregations, for example, hydroxypropyl-beta-cyclodextrin [65, 66]. The bioprocessing of biological materials like proteins and cells involves shear and stress induced by different forces like mechanical forces, hydrodynamic forces, and interfacial phenomena. The shear effects are necessary to produce the antibodies along with the enzymes, while the fermentation, formulation, and purification procedures are required for the protein pharmaceutical products [67–69]. Nikolai F. Bunkin et al. demonstrated two effects of shaking using immunoglobulin G (IgG) dispersions in water and ethanol/water combinations (36.7 vol%). First, it increases the rate of IgG macromolecule aggregation. Second, it generates bubbles with a different size spectrum in each solvent. Aggregation is facilitated in ethanol and water combinations due to IgG denaturation.

IgG aggregates in ethanol and water are 300 nm in size and 900 nm in water [70].

Pressurization Lower pressures to moderate pressures in the filtration process can cause protein aggregation. The high pressure increases the hydrophobic interactions, resulting in protein unfolding and aggregation [71, 72]. The pressure treatment of moderate concentrations of the chaotropic agents induces the chemically linked type of aggregates. At the same time, protein disaggregation is promoted by solubilizing aggregates with the high-pressure treatment. Hawley et al. predicted that proteins had a closed stability contour in P−T space, the key characteristic of the thermodynamics of pressure-induced protein transitions [73]. With some exceptions, many monomeric proteins undergo pressure-induced denaturation at pressures of approximately 400 MPa at temperatures ranging from 25 to 37 °C. It has been demonstrated that moderate pressures of roughly 200 MPa can dissociate native oligomers from nonnative aggregates and separate protein molecules from them, allowing the molecules to fold into their native conformations. When the pressure is released, the proteins can refold adequately, resulting in less aggregation [74, 75]. Richard J et al. demonstrated that low, nondenaturing guanidine hydrochloride (GdmHCl) concentrations and high hydrostatic pressures (1–2 Kbar) promote the disaggregation and refolding of aggregated and denatured human growth hormone, lysozyme, and lactose inclusion bodies. Application of hydrostatic pressure up to 2 kbar to suspensions containing aggregates of recombinant human growth hormone (up to 8.7 mg/ml) in the presence of 0.75 M GdmHCl enables complete disaggregation and facilitates the recovery of the protein in its fully folded, native conformation with 100% efficiency [76].

Drying Many drying methods involve preparing solid protein products, including freeze-drying, vacuum drying, and spray drying. The drying process removes the protein hydration layers, resulting in the disordered native state of the protein, inducing varying degrees of protein aggregation. Vacuum drying induces rapid dehydration, which triggers protein aggregation [77, 78]. Both the drying and freezing stresses influence protein aggregation due to the involvement of covalent and noncovalent bonds. The inhibition of protein aggregation can be done by adding protein stabilizers like sugar into the drying process [79]. Another well-studied method for protein dehydration is spray drying. Proteins are briefly subjected to higher temperatures and a significant air–water interface rise during atomization. Surfactants are frequently effective at inhibiting protein aggregation [80–83].

The requirement of high temperatures gets eliminated into spray freeze during the method, but the air–water interfaces are still formed, which could contribute to protein aggregation to a greater extent than simple spray drying [84].The protein aggregation can also be induced by the supercritical fluid drying [85–87]. The presence of other excipients, reconstitution methodologies, and mediums can induce varying degrees of protein aggregation into the reconstituted dried protein products [88].

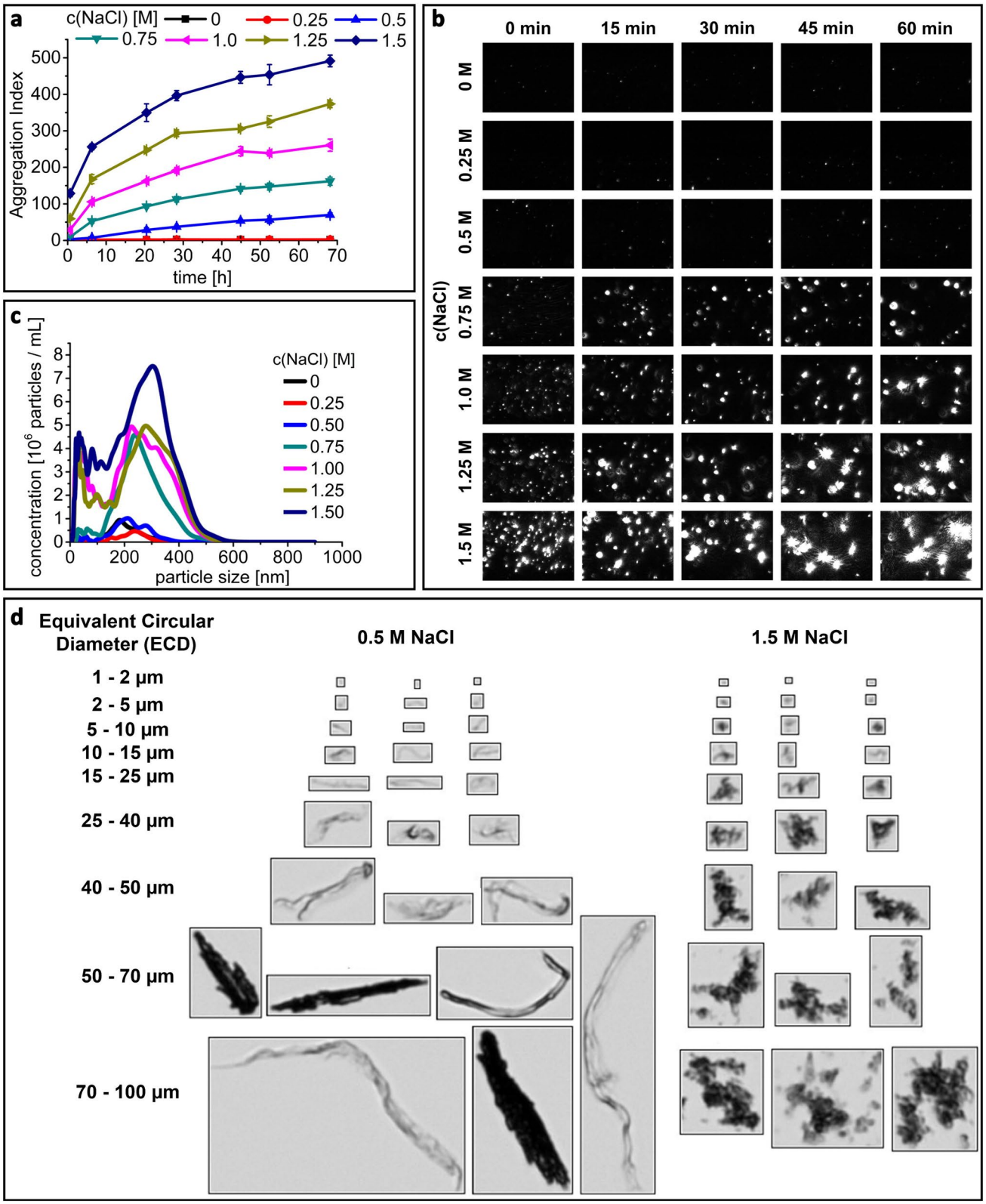
Miscellaneous processes Other processes that destabilize proteins and cause protein aggregation have also been reported. Filtration: Protein supersaturation and high solvent velocity (shear) in the concentrated layer close to the membrane surface play a role in protein aggregation. The protein molecules on the membrane surface are likely unfolded by solvent flow shear, which causes particle collisions to cause flocculation [89]. Filling: Shear forces may exist when pharmaceutical solutions are loaded into dosing equipment and containers. Such shear forces may hurt shear-sensitive substances, lowering the quality and yield of the finished pharmaceutical product [90]. Nebulization: A solid substance or liquid droplet must be dispersed or suspended in a gaseous medium to generate an aerosol. This process involves physical stressors that could cause changes in protein conformation and may induce aggregation [91, 92]. Protein labeling: While grafting small radio labels has minor impacts on protein properties, oxidizing conditions used in many labeling reactions can result in protein aggregation and formulation development. During formulation development stages, protein aggregates include excipient screening, concentrating, purification, freeze-thawing, and storage [93].

Impact of the Environmental Factors on Protein Aggregation

Many environmental factors can influence protein denaturation along with protein aggregation. Some crucial factors involved are the concentration of proteins, solution conditions, container/closure system and surfaces, and light irradiation.

Effect of general solution conditions on protein The impact of solution conditions on protein aggregation is presented as follows,

pH. The surface charges on proteins are determined by solution pH and affect intramolecular and intermolecular folding interactions, resulting in protein unfolding. The lesser protein charge-charge repulsion is observed at minimal protein solubility at the isoelectric pH [94, 95]. The greater reduction in protein aggregation by selecting the solution pH away from the protein isoelectric pH is not fruitful in every attempt, while the change in the pH of the solution can induce the indirect effect of the rate-limiting


a
c(NaCl) [M]
0
0.25
0.5
0.75
1.0
1.25
1.5
Aggregation Index
time [h]
b
0 min
15 min
30 min
45 min
60 min
c(NaCl)
0 M
0.25 M
0.5 M
0.75 M
1.0 M
1.25 M
1.5 M
c
concentration [10^6 particles / mL]
particle size [nm]
c(NaCl) [M]
0
0.25
0.50
0.75
1.00
1.25
1.50
d
Equivalent Circular Diameter (ECD)
1 - 2 µm
2 - 5 µm
5 - 10 µm
10 - 15 µm
15 - 25 µm
25 - 40 µm
40 - 50 µm
50 - 70 µm
70 - 100 µm
0.5 M NaCl
1.5 M NaCl

◂**Fig. 5** An investigation was conducted into mAb1's aggregation behavior at pH 3.5 and varying salt concentrations. **a** Aggregation Kinetics: We examined mAb1 aggregation kinetics at a 1 g/L concentration, reporting mean values and standard deviations from triplicate measurements across three independent samples. **b** Nanoparticle Tracking Analysis (NTA): Representative frames from NTA measurements were shown, conducted at 0.1 g/L mAb1 concentration, with variations in NaCl concentration and incubation times. **c** Initial NTA Analysis: An initial NTA analysis was performed immediately after adding NaCl to a 0.1 g/L mAb1 sample at t = 0 min. **d** Field Imaging Microscopy (FIM): Representative FIM images depicted particles categorized by size after a 1-h incubation of 1 g/L mAb1 under two NaCl concentrations: 0.5 M and 1.5 M. Copyright permission was obtained from [105]

steps/mechanisms involved in the protein aggregation [96, 97]. Despite the availability of various buffering agents for pH adjustment, protein stability, and aggregation behavior could vary considerably among buffer systems and concentrations. Buffer-binding effects have been blamed for the various effects [98].

Ionic strength. The ionic strength showed a dependent impact on protein aggregation. The destabilization into the protein can be done through enhanced ionic strength owing to the hydrophobic patches. The increase in protein aggregation can be done by charge induction on the neutralizing protein surface for the promotion of protein stability or the folding [94]. The solution's pH and the glycosylation state of the protein can induce protein aggregation with the help of ionic strength. The *in vivo* and *in vitro* stability of the protein therapeutics can be achieved through the utilization of glycans by manipulation of the glycosylation parameters with the help of glycoengineering [42]. Glycosylation is one of the most promising methods to reduce or prevent protein instability. It is generally believed that altering important glycosylation factors like glycosylation degree, glycan size, and glycal structural composition allows the protein's molecular stability to be tuned to the right level.

Glycosylation has been demonstrated to concurrently stabilize a wide range of proteins against nearly all the key physicochemical instabilities encountered throughout their pharmaceutical development [99, 100]. Protein pharmaceuticals are frequently administered intravenously rather than orally because proteases in the digestive system chemically degrade them. However, proteins delivered via other routes are extremely vulnerable to proteolytic destruction due to the systemic production of proteases. As a result, protein therapeutics' in vivo molecular stability and therapeutic potency are intimately connected to their resistance to proteolytic degradation. Glycosylation protects proteins from protease degradation. For example, G-CSF (GRANOCYTE®, Chugai Pharma), lipase (MERISPASE®; Meristem Therapeutics), protein C (XIGRIS®; Eli Lilly), ribonuclease (ONCONASE®; Alfacell), and thyroid-stimulating hormone (THYROGEN®; Genzyme) are a few examples [101–103]. Ionic strength could influence protein aggregation indirectly by impacting the rate of chemical reactions or the effect of other aggregation-inducing agents [96, 104].

Fabian Bickel et al. [105] studied the aggregates and factors affecting the reversible aggregation induced by sodium chloride (NaCl) into that of the monoclonal antibody at lower pH conditions. The assessment of protein conformation before salt addition and after salt addition in pH values from 2 to 7 into 1.5 molar NaCl through size exclusion chromatography and turbidity measurement. Other assessment studies include nanoparticle tracking analysis, flow imaging microscopy, circular dichroism, FTIR, and differential scanning fluorimetry. The high level of ionic strength and lower pH value supported the aggregation propensity, while the thermal stability decreased with pH. A pH value less than 3.5 showed no significant changes in the secondary structure, while protein precipitation reversed into monomers in a salt-free buffer, as presented in Fig. 5. Trehalose provided fewer protein aggregates, while the experimental results showed that with fewer pH conditions, there were limited changes in the tertiary structure of the protein, resulting in reversible aggregations. The salt addition induces colloidal instability due to electrostatic repulsion.

Excipients/additives. Few additives destabilize proteins and encourage aggregation, whereas others stabilize them. The preferential exclusion of excipients/additives from proteins can accomplish protein stabilization. The enhanced protein folding rate, increment into the solvent viscosity, reduced solvent accessibility, and conformational mobility are other mechanisms for the proposed protein stabilization [106]. The binding into the additives like amino acids, amines, polymers, surfactants, polyols, and sugars to that of the intermediate aggregation can accomplish protein stabilization [64]. Numerous minor neutral compounds influence protein stability or aggregation. A primary class of these neutral excipients is sugars/polyols. These sugars/polyols naturally stabilize and prohibit protein aggregation in various practical conditions [107]. These excipients are known as osmolytes or chemical chaperones because they can keep proteins in the correct conformation. Depending on the formulation or solution requirements, these excipients are typically used in 5–10% concentrations to stabilize the protein in solution and reduce Fc-Fc or Fc-Fab and air–water phase interactions. Christoffer Olsson et al. has studied the role of the polyols along with the sugar on that of the protein–protein interactions along with aggregation-induced from the small-angle neutron scattering (SANS) by using myoglobin in the aqueous solutions of the sucrose along with the trehalose. The distinctive protein–protein distance has been caused by sucrose and trehalose, which further supported their role as inhibitors of protein–protein interactions and protein aggregation [108]. In another work, Gregory

A. Bowden et al. showed the formation of *the Escherichia coli* inclusion bodies into the periplasm by using the overproduction of the secreted protein called β-lactamase. The lactamase aggregation was inhibited with the help of non-metabolizable carbohydrates like raffinose and sucrose in the growth medium. The amount of the soluble protein was enhanced fourfold under the ideal conditions [109–111].

The inhibition of protein aggregation can be done by hydroxyproline, glycine, lysine, arginine, histidine, proline, betaine, putrescine, spermidine, sulfobetaine, and imidazole. Some compounds have negatively impacted protein aggregation, including histidine, glycine, dopamine, and trimethylamine-N-oxide. The excipients have shown stabilization and destabilization effects on the protein molecules based on their concentration use [112, 113]. The protein denaturation or aggregation can be caused by the solid hydrophobic/electrostatic interaction between the polymer and protein, which is later addressed by applying surfactants to the protein solutions. The nonionic surfactants have a negative effect on protein aggregation, as observed through accelerated stability studies [114, 115]. The surface protein binding of the nonionic surfactant induces protein aggregation. Antioxidants, reducing agents, organic solvents, and preservatives are examples of formulation additives/excipients that may affect protein aggregation.

Protein concentration. The protein concentration has an impact on the protein aggregation. The enhanced concentration of the protein could induce three different outcomes, including reduction in the aggregation due to crowding, elevation of the aggregation due to possible association, and precipitation due to the solubility limit. The crowded intracellular environment is described by the term "macromolecular crowding" [116]. The increase in the probability of protein–protein association leading to protein aggregation was observed through enhanced protein concentration. Solution conditions influence the protein solubility limit. The proteins may get aggregated, precipitated, and crystallized due to protein concentration exceeding the limit [117].

Contact surfaces and container/closure systems. The protein tends to bind to a wide range of interfaces/surfaces. Protein denaturation changes into structural integrity, and protein adsorption can trigger aggregation. Protein adsorption reduces the energy barrier to aggregate the seed development. Protein aggregation increases with protein adsorption into multiple layers. The surfactant may or may not impact the protein adsorption depending on the surface and the proteins' nature [118]. The effect of the protein adsorption can be reduced due to surfactants through accelerated surfactant mobilization to the interface and high surfactant affinity at the interface compared to the protein or through the development of the surfactant–protein complex [119]. Surface-related protein aggregation can be induced through container/closure systems, especially in the case of metal containers/surfaces. The effect of the surface material on the protein aggregation can be increased under stressful conditions like mechanical stress occurring during agitation, tumbling, dropping, and freeze–thaw cycling at different temperatures. Thus, the protein aggregation can be monitored by container and closure chemistry [118, 120].

Mechanical movement and fluid interactions at the interface of rubber stoppers or the syringes can aid in protein aggregation, while the presence of the residual tungsten extract in the syringe manufacturing process has presented an impact on the protein aggregation [121]. The effect of the agitation stress on the rapid aggregation of the therapeutic monoclonal antibodies (mAb1) was studied by Sharvan Sreenivasan et al. using interfacial agitation with the help of the air-bubbling method. Rapid bubbling was induced into the phosphate-buffered saline solution using a peristaltic pump, and the flow rate was 11.5 ml per minute. The sample assessment was done through cell-based activity assays, dynamic light scattering, infrared spectroscopy, and circular dichroism. A protein loss of 53% at 240 min was found in the mAb1 sample under stress conditions with increased turbidity. The visible aggregates were observed with bubbling time, while the secondary structure alteration and greater hydrophobicity were shown during the aggregates' assessment. The later effects resulted in a reduction of 40% reduction in activity. At the same time, the impact of the sample volume, protein concentration, air flow rate, temperature, and surfactant role can be explored to identify the degree of protein aggregation, as presented in Fig. 6. Air bubbling is a convenient method for interfacial stress degradation in mAb1 and can be implemented in further studies [122].

Light and irradiation. The ultraviolet light (UV) can affect the protein after exposure and fuelled the protein unfolding, which further results in the protein aggregation or inhibition of the photocrosslinking reactions, leading to covalent aggregation [121]. The UV radiation showed crosslinking induction into the cysteine and the tryptophan residues. It produces the photolysis of the native disulfide bonds to form or exchange new disulfide bonds. The UV light can oxidize the tryptophan residues, forming the thiol radicals and the new disulfide bonds. In addition, amino acid residues like tyrosine and phenylalanine are also prone to undergoing oxidative reaction [123–125]. Gamma radiation generates ceruloplasmin and hemoglobin aggregation; thus, light protection is necessary for light-sensitive biotherapies [126].

Prediction and Mitigation Approaches for Protein Aggregation

Proteins, the most prevalent biomolecules essential to various biological functions, rely heavily on natural intrachain and interchain connections that govern their folding,

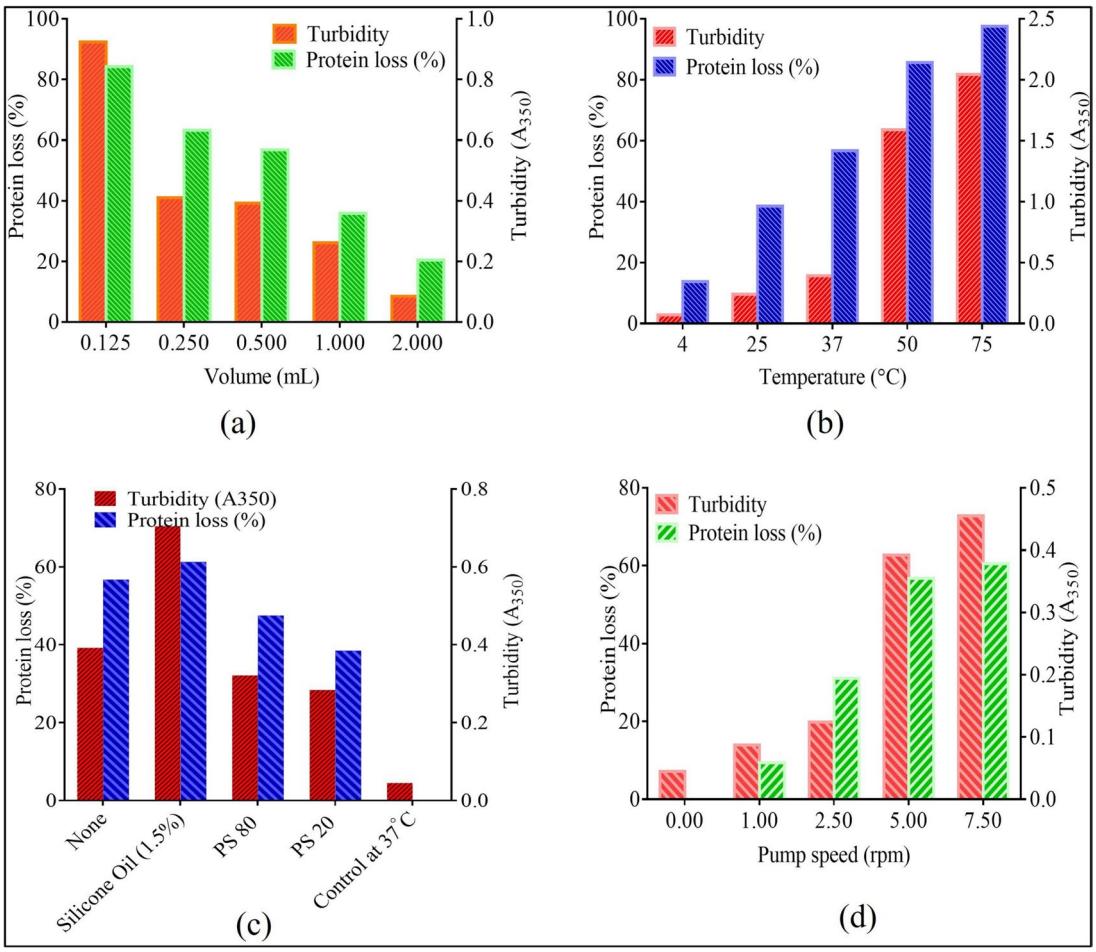


Fig. 6 Illustrates the effect of several factors on the stability of the 1 mg/ml of mAb1 in the PBS when subjected to bubbling for 240 min. The stability assessment factors involve **a** sample volume, **b** temperature, **c** the presence of silicon oil or polysorbate, and **d** the bubbling rate. Turbidity (A350) and protein loss (%) were used as measurements. Copyright permission was obtained from [122]

binding, and functional activity [127]. The conformational changes of proteins within living cells are driven by achieving the state with the lowest free energy. During this process, native contacts are crucial in agglomerating the protein surface. Unfortunately, nonnative intermolecular interactions can also occur, forming amorphous, nonstructured aggregates, negatively impacting protein function and increasing toxicity [128, 129]. The aggregation of proteins into insoluble moieties is a significant obstacle in developing protein-based therapeutics and biotechnological products. Production processes often subject proteins to nonnative stresses and concentrations, promoting aggregation and reducing final product yield and activity [130]. Furthermore, this phenomenon can trigger unpredictable immune responses in patients. Understanding the molecular basis of abnormal protein oligomerization in the absence of native control has been the focus of extensive research [131]. Factors such as protein conformational stability, colloidal stability in solution, and environmental stressors during transit and storage significantly influence the quality of protein products. Protein aggregation can be encouraged at solid–liquid or air–liquid interfaces through adsorption, which is attributed to electrostatic interactions or hydrophobic effects on material surfaces [131–133].

Developing effective techniques to prevent protein aggregation in these applications is critical for advancing protein- and biologics-based therapeutics. One of the most widely adopted strategies to hinder protein aggregation in solution involves incorporating compounds that impede the process [134]. Such compounds include amino acids, sugars, urea, polyols, and specific polymers. For instance, polyethylene glycol has successfully prevented the binding of interferon and tissue plasminogen activators during refolding by forming hydrophobic bonds with unfolded proteins and folding intermediates. Consequently, reducing the unfolded protein's free energy increases the final yield of active protein [135]. Sugars such as sucrose have also been found to prevent the nonnative unfolding of protein

molecules by increasing the unfolding free energy barrier, thereby reducing the population of intermediates and slowing aggregation [136]. Another promising anti-aggregation additive is the amino acid arginine, which appears to exert its effect by preferentially increasing the protein association barrier [137]. Despite their resource-intensive nature, time-consuming aspect, and dependence on detailed mechanistic knowledge, empirical approaches, although successful, have certain limitations. The following section focuses on recent strides in computational methodologies for forecasting therapeutic protein aggregation and innovative strategies to counter these challenges.

Computational Methods to Predict Protein Aggregation

The primary structure of proteins harbors intrinsic factors that significantly influence their aggregation tendencies. Among these factors, hydrophobic interactions play a major driving force in protein aggregation. Regions with high hydrophobicity in the protein sequence promote intra-chain and interchain contacts, thereby leading to aggregation [138]. Conversely, hydrophilic regions may act as gatekeepers, counteracting aggregation by preventing hydrophobic residues from interacting with each other [41].

The local and net charges of protein residues can also influence aggregation. Electrostatic repulsion between charged residues can prevent aggregation, whereas opposite charges or the absence of repulsive forces may facilitate aggregation [94]. Numerous aggregates exhibit a cross-β fold, wherein β-sheet structures play a critical role. Amino acids with high β-sheet propensity are favored in aggregation, while β-sheet-breaking residues such as proline (Pro) and α-aminoisobutyric acid (AIB) can counteract aggregation [139]. Notably, the aggregation propensity of a protein is not uniformly distributed along the sequence but tends to concentrate in specific short linear stretches referred to as APRs. These regions are sufficient and necessary for nucleating protein aggregation and are often enriched in hydrophobic residues [140]. In globular proteins, APRs are commonly found in the hydrophobic core, but they can also be exposed in regions involved in catalysis and binding [141]. Furthermore, external factors such as pH, ionic strength, temperature, and environmental conditions can modulate aggregation propensities, leading to polymorphism, where different aggregates may form under varying conditions. The linear code of protein sequences not only governs folding into native globular states but also determines the propensity for partial or complete unstructured conformations, as observed in intrinsically disordered proteins [142, 143].

This convergence between folding and aggregation codes has facilitated the development of computational methods to predict aggregation propensities from protein sequences. Over 20 prediction algorithms have been designed to recognize linear APRs in polypeptides. These algorithms employ diverse metrics to derive aggregation propensity, solubility, and thermodynamic stability [144].

All prediction methods use protein sequences as input, but their APR identification and quantification strategies differ, relying on either phenomenologically or theoretically derived parameters [140]. Table I summarizes different computational methods (with examples) used to predict protein aggregation. Figure 7 illustrates computational approaches employed in predicting protein aggregation, focusing on intrinsic determinants, sequential features, and the evaluation of aggregation propensity using AI and machine learning. The utilization of structure-based algorithms is currently constrained to a limited subset of structures available in the protein data bank. This constraint arises from a notable practical limitation in the predictive methodologies employed. Specifically, these algorithms demonstrate satisfactory accuracy solely when a high-resolution experimental structure is accessible [145]. Consequently, when dealing with intricate molecular models, the predictive accuracy of these algorithms may be significantly compromised. Nevertheless, these methods represent a significant step toward understanding and predicting protein aggregation, but further research is required to overcome challenges and enhance the reliability of in silico predictions [146]. Additionally, it is imperative to advance in silico models to improve their capability to predict potential adverse effects and immunogenicity associated with protein aggregation in biopharmaceuticals. These efforts will significantly contribute to developing safer and more effective biopharmaceutical products [147]. Furthermore, computational tools aimed at identifying APRs continue to face notable constraints, including elevated false-positive rates, dependence on sequence length, and restricted applicability across diverse protein classes. In parallel, the trajectory of predictive methodologies has strikingly advanced, progressing from initial machine-learning approaches to intricate deep-learning architectures that incorporate sequence embeddings and pretrained language models. Pivotal breakthroughs, exemplified by AlphaFold's revolutionary contributions to structural prediction and the emergence of sophisticated protein language models (PLMs), have facilitated previously unattainable precision in elucidating the relationships between protein sequences and their corresponding structures [148].

Development of Aggregation Resistance in Proteins

In addition to optimizing formulation factors (e.g., protein concentration, pH, buffer components and other cosolutes, temperature, etc.), achieving aggregation resistance in

Table I Computational methods to predict protein aggregation

Type	Overview	Example	Reference
Sequence-based methods	These methods predict protein aggregation by analyzing the primary amino acid sequence. They utilize experimentally determined aggregation propensity scales for different residues or employ specific physicochemical properties, such as hydrophobicity and β-sheet propensity. By identifying APRs in the sequence, they calculate aggregation susceptibility, solubility, and thermodynamic stability to predict the likelihood of protein aggregation	AGGRESCAN	[149]
		Waltz	[150]
		TANGO	[151]
		PASTA 2.0	[152]
		SecStr	[153]
		BETASCAN	[154]
		STITCHER	[155]
		GAP	[156]
Machine-learning methods	These methods employ algorithms to predict protein aggregation based on data-driven patterns and correlations derived from training datasets. These approaches use features extracted from protein sequences or structures and learn to classify sequences as either aggregating or non-aggregating based on these features. Machine-learning models, such as neural networks, random forests, and support vector machines, offer more sophisticated and accurate predictions by considering multiple factors and interactions. They are valuable for analyzing complex molecular models and improving prediction accuracy	ANuPP	[157]
		NetCSSP	[158]
		Budapest	[159]
		CORDAX	[160]
		AgMata	[161]
Consensus methods	Consensus methods enhance predictive accuracy by combining the outputs of multiple prediction algorithms. These approaches integrate results from various sequence-based, machine-learning, or other aggregation predictors to generate a consensus prediction for protein aggregation. By leveraging the strengths of different methods and mitigating individual method limitations, consensus methods offer more robust and reliable predictions. They are precious when dealing with diverse protein sequences and structures	Amylpred 2 (combines Aggrescan, AmyloidMutants, Amyloidogenic, Pattern, Average Packing Density, Beta-strand contiguity, Hexapeptide Conformational Energy, NetCSSP, Pafig, SecStr, Tango, and Waltz)	[162]
		MetAmyl (combines SALSA, PAFIG, Waltz, and FoldAmyloid)	[163]
3D structure-based methods	These methods utilize three-dimensional structural information of proteins to predict aggregation propensity. By considering the influence of protein tertiary structures on aggregation, they offer a more comprehensive assessment of aggregation tendencies. However, their accuracy is currently constrained to a subset of structures available in the protein data bank, as they heavily rely on high-resolution experimental structures. 3D structure-based methods are valuable for understanding the spatial arrangement of residues and their impact on aggregation propensity	SolubiS 1.0	[164]
		Aggscore	[165]
		AGGRESCAN3D 2.0	[166]
		Camsol	[167]

therapeutic proteins by employing mutational design methods represents a novel promising avenue. By introducing specific amino acid substitutions or deletions, researchers can strategically modify the protein's sequence to disrupt APRs and enhance its stability and solubility [117].

These targeted mutations aim to reduce the likelihood of unfolding and the formation of reactive monomers, critical precursors to irreversible aggregation [168]. Mutational design methods for achieving aggregation resistance include two key strategies: directed evolution and high-throughput screening. It involves creating diverse mutant libraries through random mutagenesis or DNA shuffling. These libraries are then subjected to iterative rounds of selection and amplification to identify variants with improved aggregation resistance [169]. High-throughput screening methods enable the rapid evaluation of a large number of mutants, allowing the identification of aggregation-resistant proteins with enhanced stability and solubility [170].

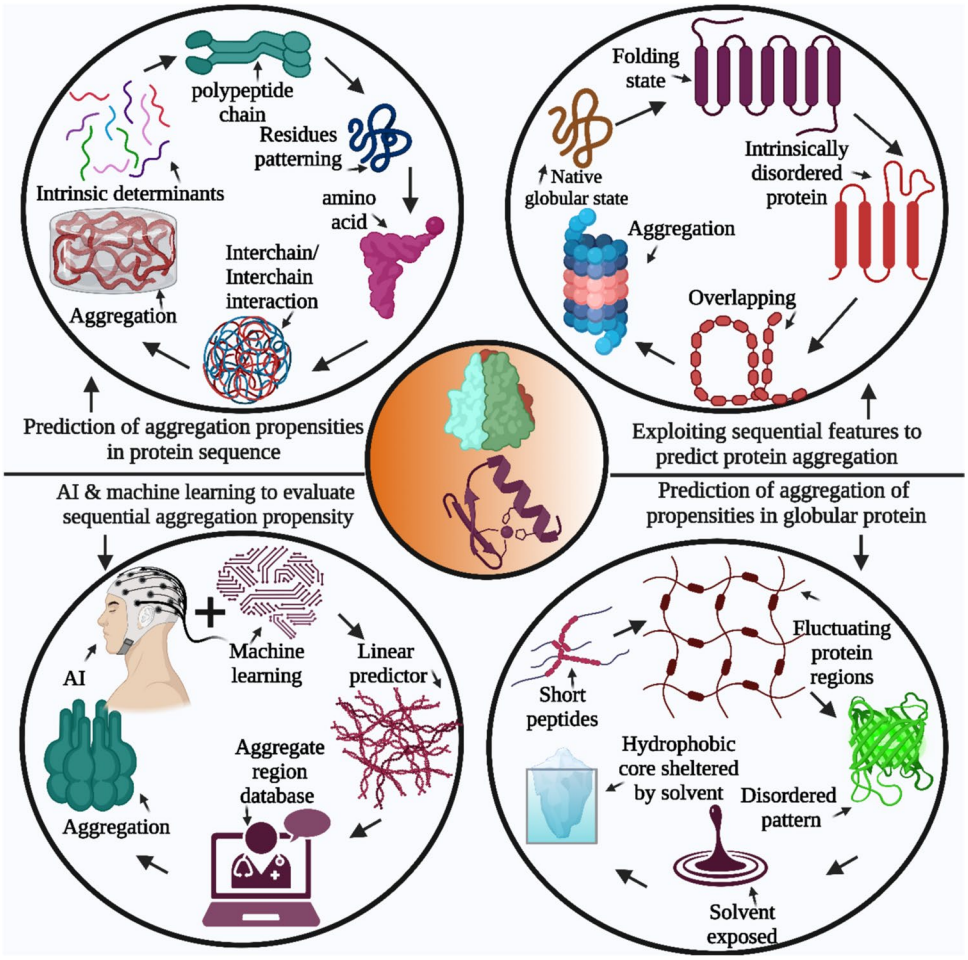


Fig. 7 Computational approaches employed in predicting protein aggregation, focusing on intrinsic determinants, sequential features, and the evaluation of aggregation propensity using artificial intelligence (AI) and machine learning. First, the prediction of protein aggregation propensities relies on intrinsic factors inherent to the protein sequences. Second, sequential features are harnessed to enhance the prediction of protein aggregation. This involves leveraging AI and machine learning techniques to assess sequential aggregation propensity. Last, the aggregation propensities are predicted based on their manifestation in globular protein structures. These methodologies collectively contribute to the comprehensive understanding and prediction of protein aggregation phenomena

The successful application of this approach relies on the careful design of selection criteria and screening assays, coupled with the availability of robust analytical tools to assess aggregation propensity [171].

- Knowledge-based or informatics approaches: These approaches harness existing information on homologous proteins to discern sequence or structural characteristics associated with aggregation rates. Leveraging bioinformatics tools and databases, researchers can analyze protein sequences and structures to identify key determinants influencing aggregation propensity [172].
- By understanding how specific amino acid substitutions or sequence patterns influence protein stability and conformational dynamics, rational choices for mutational design can be made to enhance the protein's resistance to aggregation. This method demands a comprehensive understanding of protein structure–function relationships and accurate bioinformatics tools for data analysis [173, 174].
- Chemistry- and physics-based design approaches: Often referred to as rational design strategies, these approaches employ molecular simulations and computational modeling to study protein–protein interactions, folding kinetics, and thermodynamic stability. By elucidating the fundamental principles governing protein aggregation, researchers can make rational modifications to disrupt aggregation-prone regions and stabilize the protein's native conformation [175].
- These approaches provide valuable insights into the impact of specific mutations on the intrinsic aggregation propensity (IAP) of proteins, aiding in the development of aggregation-resistant proteins with improved structural integrity and reduced aggregation potential [176].
- This approach requires expertise in computational biophysics, accurate force fields, and sophisticated simulation algorithms to model protein dynamics and interactions accurately [177].

The rational design approach can be used to control protein aggregation and achieve enhanced effectiveness. The conformational stability of the protein can be achieved through proper efforts at the unfolding stage [178]. The minimization of the generation of the "reactive" monomers

into the solution, which usually increases the propensity of the interaction with each other, can lead to the formation of stable irreversible aggregates. The rational design can focus primarily on introducing the specific mutations that enhance the electrostatic repulsion among the monomers [179, 180]. The reduction into the reversible oligomer formation can mitigate the progression of protein aggregation [181]. Furthermore, the rational design strategy can be implemented in the nucleus formation stage, identification along with the disruption of the intrinsic aggregation-prone regions which are involved in the interprotein solid contacts like amorphous or fibril-like interprotein beta sheets leads to nucleation as well as overall growth of the aggregates [179].

The protein's IAP can be diminished through targeted disruption of these APRs. The IAP refers to the protein's inherent inclination to aggregate when these APRs are fully solvent-exposed, i.e., in an unfolded or intrinsically disordered state [182]. By employing this strategy, nucleation rates from reactive monomers can be effectively reduced compared to wild-type (WT) proteins, even if the monomers become "reactive" due to unfolding [29]. Particularly noteworthy is the application of the APR identification strategy for native human proteins associated with various human diseases. In this context, the discovery of peptide-based inhibitors targeting these APRs holds immense promise. Such inhibitors could impede the formation of toxic aggregated species, thereby mitigating the onset or progression of related human diseases (e.g., amyloid formation/fibrillation) [183–185].

Advanced Formulation Techniques to Maintain Protein Stability

Numerous nontraditional technologies have been developed or are under development to tackle the challenging issue of protein aggregation in biological products.

Chemical Conjugation

Chemical conjugation or protein fusion has primarily been devised to extend the in vivo half-life and promote a longer duration of action for therapeutic proteins. This approach involves attaching specific moieties, such as polyethylene glycol (PEG), glycans, or other hydrophilic substances, to the protein [186]. PEG reduces protein aggregation by inducing repulsive interactions between the PEG-modified surface, and it also enhances the thermal stability of proteins. PEGs, in particular, are widely utilized as conjugation agents due to their biocompatibility and demonstrate effectiveness in stabilizing proteins against various stresses, including thermal stability, pH-induced or protease-induced degradation, and oligomerization [187].

Glycosylation of proteins with maltodextrins, lactose, dextran, and modified trehalose polymers has also shown promise in inhibiting protein aggregation [188, 189]. For instance, glycosylation of ovalbumin with more hydrophilic substituents enhanced its thermal stability by altering intermolecular interactions [190].

Similarly, protein fusion with more stable partner proteins has been shown to enhance the stability of the fused protein, reducing aggregation and improving overall drug characteristics. For example, fusion of the MpAFP698 protein with maltose-binding protein (MBP) significantly increased the thermal stability of the resulting MBP-MpAFP698 fusion protein compared to MBP alone [191].

However, both chemical conjugation and protein fusion introduce additional complexities into the drug substance manufacturing process, necessitating extra processing steps. Furthermore, the larger size of the final drug candidate poses challenges in characterization and stability monitoring. Additionally, careful consideration must be given to selecting appropriate conjugation moieties and partner proteins to avoid potential immunogenicity or other adverse effects.

Employing Polar Organic Solvents

The use of polar organic solvents presents another avenue for addressing protein aggregation. Although traditionally, adding a miscible organic solvent to an aqueous protein solution could lead to protein unfolding and instability, modifying the solvent properties can reduce protein solution viscosity, such as dimethyl sulfoxide or dimethylacetamide [192].

While they effectively reduce the viscosity of protein solutions, the melting temperatures of the protein drop with increasing concentrations of these solvents, potentially compromising the storage stability of the protein. However, using a neat organic solvent could significantly change the interaction of proteins with the solvent, enabling the preparation of nonaqueous suspensions to reduce the viscosity of protein preparations [193].

Protein therapeutics suspended in various organic solvents showed significantly reduced viscosities compared to their aqueous solutions, with viscosity reductions depending on the properties of the solvent, particularly its hydrogen-bonding characteristics. Nevertheless, the toxicity and tolerability of organic solvents for human use pose concerns [194]. Careful selection of suitable solvents and thorough toxicological evaluation are crucial before considering this approach for clinical use.

Developing Protein Suspensions

Suspensions have yet to be widely explored as commercial dosage forms for proteins. However, proteins can hypothetically be prepared as crystalline or amorphous particulate

suspensions to stabilize proteins and facilitate high-concentration formulations [195].

Crystalline suspensions of mAbs have been successfully prepared, retaining their structural integrity and biological activity. For example, crystalline suspensions of the mAbs rituximab, trastuzumab, and infliximab were designed with no detectable structural alteration and exhibited a reduced viscosity compared to their soluble counterparts. The shorter $t_{1/2}$ of soluble infliximab was doubled after SC injection when administered as a crystalline suspension, showcasing the potential of this approach to improve drug delivery [196]. However, several challenges must be addressed when considering crystalline suspensions. The solution conditions optimal for crystallization may not be suitable for long-term protein storage [197]. Additionally, any additives that promote protein crystallization must be safe for injections. The crystalline suspension drug products filling during the manufacturing also produces challenges owing to the constant settling of the crystals [198].

Developing Non-Freeze-Dried Formulations

Non-freeze-dried solid formulations have traditionally been prepared to accommodate unstable proteins in solutions. While freeze-drying has been a popular method, alternative drying techniques such as spray-drying, spray freeze-drying, supercritical fluid drying, and vacuum foam drying have been explored [83, 199]. The spray drying method has shown great promise as an effective tool for producing solid formulations with the advantages of cost and scalability. The protein can induce stress during the spray drying due to drying temperature and air–water interfaces. The spray drying also showed a reduction in the structural integrity of some proteins [80].

These issues can be countered by using spray freeze drying use, which further eliminates the drying process, but it also creates the air–water interfaces, which can cause protein aggregation [200]. The spray freeze-drying technique was used for darbepoetin α with the help of ultrasonic atomization at the specific frequencies, generating minimal insoluble aggregates as compared to the conventional spray-drying [201]. The protein aggregation issues are also found with supercritical fluid (SCF) drying, which can get critical due to the involvement of the stringent requirements of instrumentation, process-induced excipient crystallization with the protein reconstitution into the same [85]. Vacuum foam drying has shown several advantages for process efficiency reduction without the propensity of excipient crystallization. The high drying efficiency through vacuum foam drying requires stringent control of the process conditions [202].

Developing Protein Nanoclusters

The crowder-like trehalose involves nanoclusters, densely packed protein molecules showing equilibrium hydrodynamic diameters without the gelation at high concentrations [203]. The nanocluster dispersion is suitable for subcutaneous injection due to its reduced viscosity and the improvised syringeability [204, 205]. It is plausible that interprotein distances within nanoclusters are significantly reduced compared to those in bulk solution. The shorter distance could enhance protein–protein interactions and potentially lead to stability issues. Nonetheless, the nanocluster concept is relatively new and requires further evaluation to understand its implications and potential applications in biopharmaceuticals fully [88, 206, 207].

Protein Aggregation and Clinical Immunogenicity

Protein aggregates comprise several monomers, repetitive patterns, or sequences of amino acids that may mimic a ligand of scavenger and other receptors, resulting in increased uptake by antigen-presenting cells (APCs) [28]. They must interact with immune cells to become immunogenic biotherapeutics. In general, biotherapeutics can induce immune responses in patients in one of two ways. In the first case, biotherapeutics may be suitably foreign, must be recognized by the patient's immune system, and must elicit an adaptive immune response [208, 209]. As a result, these biotherapeutics could be internalized, processed, and presented by antigen-presenting cells (APCs), resulting in CD4 and T-cell responses and antibody elaboration, as shown in Fig. 8 [210–212].

However, suppose the biotherapeutics exhibit a more significant complete homology with endogenous proteins to which the patient is immunologically tolerant. In that case, B-cell tolerance must be broken for the antibody to be effective [213–216]. The primary effector mechanism in the immune response to biotherapeutics is antibody generation. While anti-drug antibodies (ADAs) may be clinically ineffective, side effects such as loss of drug efficacy and anaphylaxis reactions may occur [14, 217].

After processing by APCs, ADA generation against biotherapeutics is proposed via T-cell-dependent and T-cell-independent pathways [218]. Furthermore, APCs use major histocompatibility complex (MHC) molecules to process and present protein fragments to T-cell receptors (TCRs) on naive T-cells [211, 219, 220]. Most importantly, once the binding between TCRs and MHC is recognized, APCs can form secondary connections with T cells via costimulatory molecules such as CD86. CD80, and B7 [221]. This

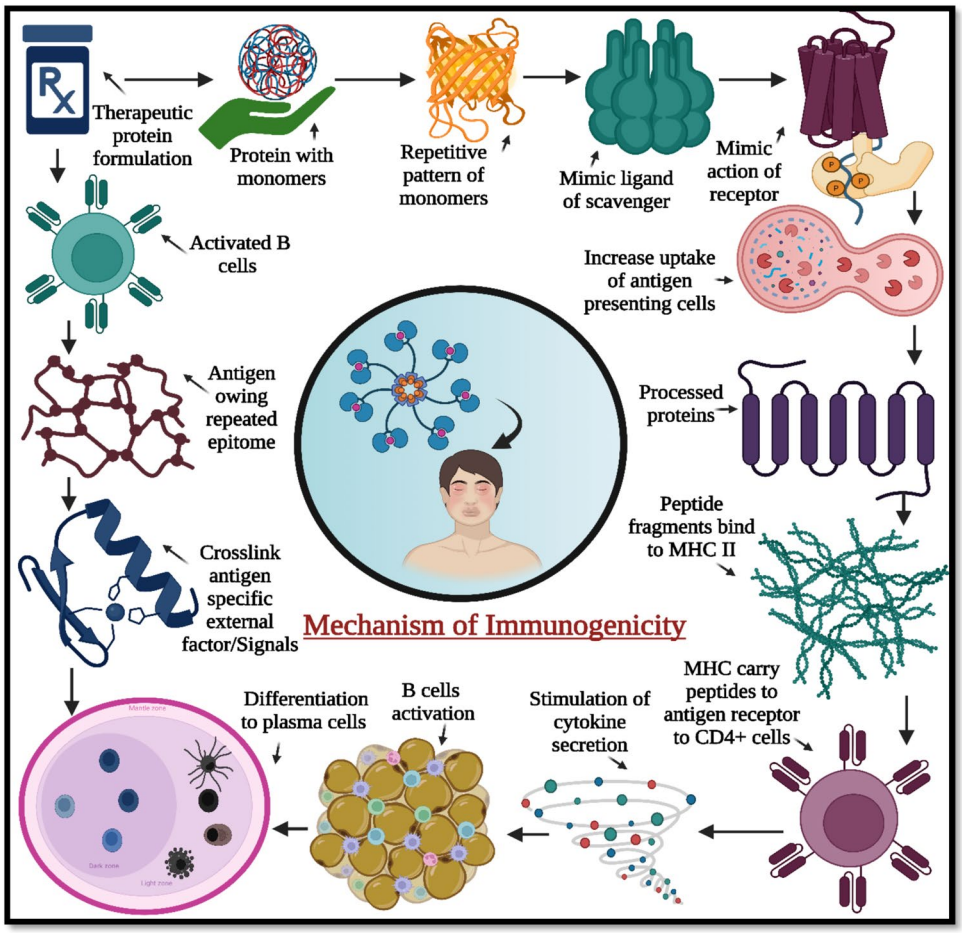


Fig. 8 B-cell activation mechanisms, classical response: Biotherapeutics are internalized by APCs and processed to peptide fragments that bind to MHC II, followed by recognition by CD4 + cells, which stimulate cytokine secretion and B-cell activation, followed by differentiation to plasma cells. Breakdown of B-cell tolerance: B cells can be activated to plasma cells via antigen-owning repeated epitopes that cross-link antigen-specific Bc. External factors/signals may play a role in this process. They were adopted from the reference

activated T-cell then proliferates and releases cytokines, which may be involved in communication between activated T and B cells [222].

Furthermore, mature B cells secrete memory B cells and long-span plasma cells, which can generate ADAs, primarily IgG1 [214]. However, in the T-cell-independent pathway, protein aggregates could have directly activated B cells, resulting in the generation of short life span cells (mostly IgG2b, IgG3, or IgM) and subsequent ADAs [223]. In general, these ADAs are categorized as neutralizing and nonneutralizing subtypes. Neutralizing ADAs interferes with a drug at the active site; therefore, it could reduce the therapeutic efficacy. Conversely, neutralizing ADAs bind to the epitopes, which do not interfere with drug-target interactions [224]. The development and validation of ADA detection assays is a broad topic beyond the scope of this discussion, which has focused on regulatory aspects of aggregation and immunogenicity. However, it can be found in the FDA's final guidance for the industry issued in January 2019 [225]. A comprehensive summary of FDA-approved biologic therapeutics is provided in Tables II and III, detailing antibody-based agents and other biologics, respectively, along with their reported immunogenicity profiles as documented in regulatory prescribing information [226].

(Note: Products are designated recombinant human (rHu), plasma-derived (PD), or recombinant (r). Companies that obtained a majority stake in any companies that performed the initial drug development of the biologics listed are indicated in brackets).

Methods for The Characterization of Protein Aggregates and Their Regulatory Requirements

Proteins can aggregate during development and production at any moment, such as through bioprocessing, purification, formulation, packaging, and storage [227]. As a result, characterizing protein aggregates is as essential as detecting them, as it aids in the successful development and manufacturing of biopharmaceuticals. Molecular

Table II FDA-approved antibody therapeutics (adapted from http://www.fda.gov/BiologicsBloodvaccines/ucm133705.htm, [226] Displaying the level of immunogenicity observed in patients (prescribing information available at http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm.)

Antibody name	Company	Type	Target	Indication(s)	Reported Immunogenicity*
Muromanab (OKT3)	Ortho Biotech	Murine	CD3	Allograft rejection	25% (24)
Abciximab (Reopro)	Centocor (Johnson & Johnson)	Chimeric Fab	GPiib/iiia	PTCA adjunct	6%–44% (36)
Rituximab (Rituxan)	Genentech (Roche)/Biogen Idec	Chimeric	CD20	Non-Hodgkin lymphoma	11% (2578)
Daclizumab (Zenapax)	Hoffman Laroche	Humanized	IL2r	Transplant rejection	14–34%
Trastuzumab (Herceptin)	Genentech (Roche)	Humanized	Her2/neu	Breast cancer	<1%
Palivizumab (Synagis)	Medimmune (Astra Zeneca)	Humanized	rSv F	rSv prophylaxis	0.7%–2% (1002–639)
Basiliximab (Simulect)	Novartis	Chimeric	IL2r	Transplant rejection	1–2% (138–339)
Infliximab (Remicade)	Centocor (Johnson & Johnson)	Chimeric	TNFα	RA/Crohn	10–15%
Arcitumomab (Cea-Scan)	Immunomedics	Murine	CeA	Colorectal cancer	<1% (3/400)
Canakinumab (Ilaris)	Novartis	Human	IL-1β	Cryopirin-associated periodic syndrome	0% (64)
Fanolesomab (Neutrospec)	Palatin Tech	Murine	CD15	Imaging for appendicitis	0–16.6% (30–54)
Imciromab (Myoscint)	Centocor (Johnson & Johnson)	Murine	Myosin	Cardiac imaging for Mi	<1% (914)
Capromab (Prostascint)	Cytogen	Murine	PSMA	Prostate cancer diagnostic	8%–19% (27–239)
Nofetumomab (Verluma)	Boehringer Ingelheim	Murine	40 kDa Glycoprotein	Detection of SCLC	6% (53)
Gemtuzumab (Mylotarg)	Wyeth Pharma (Pfizer)	Humanized	CD33	Acute myeloid leukemia	0% (277)
Alemtuzumab (Campath)	Ilex Pharma (Genzyme)	Humanized	CD52	B-cell chronic lymphocytic leukemia	1.9–8.3% (133–211)
Ibritumomab (Zevalin)	Idec Pharma (Biogen Idec)	Murine	CD20	Non-Hodgkin lymphoma	1.3% (446)
Adalimumab (Humira)	Abbott	Human	TNFα	rA/Crohn/PsA/JiA Ankylosing spondylitis/ plaque psoriasis	2.6%–26%
Omalizumab (Xolair)	Genentech (Roche)	Humanized	IgE	Asthma	<0.1% (1723)
Efalizumab (Raptiva)	Genentech (Roche)	Humanized	CD11a	Psoriasis	6.3% (1063)
Tositumomab (Bexxar)	Gsk	Murine	CD20	Non-Hodgkins lymphoma	11% (230)
Cetuximab (Erbitux)	Imclone (Eli Lilly)	Chimeric	EGFR	Colorectal cancer	5% (1001)
Bevacizumab (Avastin)	Genentech (Roche)	Humanized	VEGF	Colorectal, breast, renal, and NSCL cancer	0% (~500)
Panitumumab (Vectibix)	Amgen	Human	EGFR	Colorectal cancer	4.6% (613)
Ranibizumab (Lucentis)	Genentech (Roche)	Humanized	VEGF	Macular degeneration	1–6%
Eculizumab (Soliris)	Alexion Pharma	Humanized	C5	Paroxysmal nocturnal hemoglobinuria	2% (196)
Natalizumab (Tysabri)	Biogen Idec	Humanized	α–4 integrin	MS & Crohn	9% (627)
Golimumab (Simponi)	Centocor (Johnson & Johnson)	Human	TNFα	rA/PsA/Ankylosing spondylitis	4% (1425)
Cetolizumab Pegol (Cimzia)	Ucb	Humanized	TNFα	rA/Crohn	8% (1509)
Ofatumumab (Arzerra)	Gsk	Human	CD20	CLL	0% (79)
Ustekinumab (Stelara)	Centocor (Johnson & Johnson)	Human	IL-12/IL-23	Plaque psoriasis	3–5% (743–1198)
Tocilizumab (Actemra)	Genentech (Roche)	Humanized	IL-6r	rheumatoid arthritis	2% (2876)
Denosumab (Prolia)	Amgen	Human	RANKL	Osteoporosis	<1% (8113)

*The frequency of anti-drug antibody responses (neutralizing and nonneutralizing antibodies) observed in patients is expressed as a percentage, with the size of the patient group evaluated in immunogenicity studies indicated in brackets

weight, conformation, and size are a few physicochemical characteristics that must be identified. Recent technical breakthroughs have greatly accelerated the speed of protein characterization, and as a result, many analytical techniques for characterizing protein aggregates are now available. Several methods exist for quantifying and estimating the size of protein aggregates and characterizing them [29, 228]. However, there could be discrepancies in

Table III FDA approved other biologics (adapted from http://www.fda.gov/BiologicsBloodvaccines/ucm133705.htm, [226] Displaying the level of immunogenicity observed in patients (prescribing information available at http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm)

Biologic name	Company	Type	Target	Indication(s)	Reported Immunogenicity
Prolastin	Talecris biotherapeutics	PD	α1-proteinase inhibitor	α1-antitrypsin deficiency	None reported
Aralast	Baxter Healthcare	PD	α1-proteinase inhibitor	α1-antitrypsin deficiency	None reported
Zemaira	Aventis Behring (CSL Behring)	PD	α1-proteinase inhibitor	α1-antitrypsin deficiency	None reported
Kogenate FS	Bayer (Bayer Schering Pharma)	rHu	Factor viii	Hemophilia A	15%
Refactor	Genetics Institute (Wyeth)	rHu	Factor viii	Hemophilia A	30%
Zyntha	Wyeth (Pfizer)	rHu	Factor viii	Hemophilia A	2.2% (89)
Novoseven	Novo Nordisk	rHu	Factor Fvii	Hemophilia	< 1%
Benefit	Wyeth (Pfizer)	rHu	Factor iX	Hemophilia B	3%
Atryn	GTC Biotherapeutics	rHu	Anti-thrombin	Thromboembolism	None reported
Babybig	California Department of Health Services	PD	Botulism immune Globulin intravenous Human	infant botulism	
Berinert	CSL Behring	rHu	C1 esterase inhibitor	Angioedema	
Cinryze	Lev Pharmaceuticals	rHu	C1 esterase inhibitor	Angioedema	
Rhophylac	CSL Behring	PD	Rho(D) immune Globulin	ITP	0% (447)
Evithrom	OMriX Biopharmaceuticals	rHu	Thrombin, Topical	Coagulation	3.3%
Recothrom	ZymoGenetics	rHu	Thrombin, Topical	Coagulation	1.2–1.5%
Wilate	Octapharma	PD	von willebrand Factor	Coagulation	1.5–3%
Cerezyme	Genzyme	rHu	ß-glucocerebrosidase	Gacher Disease	15%
Exenatide Or Byetta	Amylin Pharmaceuticals/ eli Lilly	r	Glucagon Like Peptide-1	Type ii diabetes	6%
Introna	Schering Corp (Bayer Schering Pharma)	rHu	IFNα	Leukemia, Kaposi sarcoma, hepatitis B/C	< 3–13%
Betaseron	Bayer Schering Pharma	rHu	IFNβ	Multiple sclerosis	16.5–25.2%
Novolog	Novo Nordisk	rHu	Insulin analog	Type ii diabetes	Transient antibodies
Leukine	Genzyme	rHu	GM-CSF	Preventing infection in cancer	2.3%
Neupogen (Filgrastim)	Amgen	rHu	G-CSF	Preventing infection in cancer	3%
Retavase	PDL Biopharma	rHu	TPA	Myocardial infarction, pulmonary embolism	0% (2400)
Humatrope	Eli Lilly	rHu	Growth hormone	Dwarfism	1.6%
Adagen	enzon Pharmaceuticals	Bovine	ADA Adenosine deaminase	inherited immunodeficiency	Not reported (SCiD)
Pulmozyme	Genentech (Roche)	rHu	DNase i	Cystic fibrosis	2–4%
Procrit	Amgen	rHu	EPO	Anemia in chronic renal disease	
Proleukin	Novartis	rHu	IL-2	Oncology	< 1%

the reported mean size, size distributions, and amount of an aggregate species for a given sample due to the inherent variances in what is being measured and the specifications of the majority of these methods for data evaluation.

The lack of a common approach to quantify submicron particles makes one of the biggest problems with protein aggregation analysis the fact that no one analytical method presently exists to cover the whole size range in which aggregates may arise. However, only a tiny portion of the overall protein mass, protein aggregates, are particularly noteworthy because of their potential to play a role in immunogenicity [217]. Due to their small size, ranging from a few nanometers to hundreds of micrometers to massive visible particles, several analytical techniques must be utilized to detect these minute aggregate fractions. The summary of methods for characterization is tabulated in Table IV.

Table IV The methods of characterization for protein aggregate inclusion bodies [228]

Sr. No	Method	Applications in Protein Characterization	Specifications
1	Analytical Ultracentrifugation (AUC)	Determination of protein size, shape, and sedimentation behavior	Particle size range: Nanometers to micrometers; Concentration range: Low µM to mg/mL
2	Size Exclusion Chromatography (SEC)	Separation and quantification of protein aggregates by size	Particle size range: Nanometers to micrometers; Resolution: < 1 nm
3	Dynamic Light Scattering (DLS)	Measurement of particle size and monitoring of protein aggregation	Particle size range: Nanometers to micrometers; Concentration range: 0.1 mg/mL—100 mg/mL
4	Microscopic Flow Imaging	Visualization and characterization of protein aggregates	Imaging resolution: Submicron; Particle size range: Micrometers to millimeters
5	Zeta Potential	Study of stability and interaction of protein aggregates in different dispersing media	Measures surface charge and potential of aggregates; Concentration range: Low µM to mg/mL
6	Gel Electrophoresis (Native, SDS−PAGE)	Separation and analysis of proteins and aggregates based on charge and size	Separation based on charge and size; Concentration range: ng/mL to mg/mL
7	Cryo-Transmission Electron Microscopy	Characterization of the morphology and internal structure of inclusion bodies	Resolution: Subnanometer; Sample preparation: Frozen-hydrated samples
8	Atomic Force Microscopy	Investigation of morphology and conformation and detailed visualization of fibril morphology, stiffness, chirality, and conformational changes during incubation	Resolution: Subnanometer; Imaging mode: Contact or tapping mode
9	Differential Scanning Calorimetry (DSC)	Determination of thermal stability and transition temperatures of protein aggregates	Temperature range: Room temperature to 200 °C; Concentration range: mg/mL
10	Nanoparticle Tracking Analysis	Identification of heterogeneous mixtures through simultaneous measurement of particle size and scattering intensity	Particle size range: Nanometers to micrometers; Concentration range: Low µg/mL to mg/mL
11	Fluorescence Spectroscopy	Monitoring changes in fluorescence properties during protein aggregation	Wavelength range: Ultraviolet and visible; Sensitivity: Fluorescence intensity changes
12	Circular Dichroism (CD)	Assessment of changes in protein secondary structure due to aggregation	Wavelength range: Ultraviolet and visible; Sensitivity: Down to 0.1 mdeg
13	ATR-FTIR Spectroscopy	Study of the kinetics of protein aggregation during inclusion body formation. It helps determine the secondary structure of protein for information related to intramolecular vibrations of compounds	Infrared spectral range; Resolution: Down to single bonds
14	Raman Spectroscopy	Characterization of molecular vibrations and structural changes in protein aggregates	Raman shift range: Infrared to visible; Sensitivity: Structural vibrational changes
15	X-ray Diffraction	Analysis of crystalline structures and periodicity in protein aggregates	Resolution: Atomic-level; Requires crystalline samples
16	Protein Thermal Shift (PTS)	Evaluation of protein stability and aggregation propensity under various conditions	Temperature range: Room temperature to 95 °C; Sensitivity: Fluorescence intensity changes
17	Nuclear Magnetic Resonance (NMR)	Detailed investigation of protein aggregate structure and dynamics	Atomic-level resolution: Requires isotopic labeling
18	Wettability	Investigate surface properties, protein adsorption, and interaction of different inclusion bodies	Contact angle measurements; Surface tension and hydrophilicity analysis

FDA Regulatory Guidelines on Protein Aggregation and Immunogenicity Assessment for Therapeutic Proteins

The development and approval of biologics, which include monoclonal antibodies, cytokines, and other therapeutic proteins, undergo thorough evaluation by regulatory agencies like the FDA in the United States. Protein aggregation and immunogenicity are crucial factors that are assessed during this process, as they can significantly affect the safety and efficacy of these drugs.

Protein aggregation refers to the clumping together of protein molecules that occurs unintentionally. This can compromise the stability, bioactivity, and safety of therapeutic proteins. Aggregates may happen at different stages of biologics manufacturing, storage, and administration, leading to adverse effects like decreased efficacy and increased immunogenicity. Therefore, regulatory guidelines mandated by the FDA stress the importance of controlling and assessing protein aggregation throughout the lifecycle of biologics. The FDA recommends a comprehensive approach to determining protein aggregation that begins with understanding the underlying causes and mechanisms of aggregation. Manufacturers must use analytical techniques like size-exclusion chromatography, dynamic light scattering, and imaging methods to detect and quantify product aggregates. These methods help identify aggregates' size, morphology, and concentration, enabling the assessment of their potential impact on safety and efficacy. Additionally, manufacturers are encouraged to implement strategies to minimize protein aggregation during formulation, storage, and handling. This may involve optimizing pH, buffer conditions, and excipient selection to maintain protein stability and prevent aggregation. Appropriate storage conditions, such as temperature control and stabilizing agents, are essential to minimize aggregation risk during product storage and distribution.

Immunogenicity is another critical consideration for regulatory evaluation, referring to the ability of a protein to induce an immune response in patients. Immune responses can lead to adverse reactions or loss of efficacy over time. The FDA requires manufacturers to assess and mitigate the immunogenic potential of therapeutic proteins through preclinical and clinical studies. Preclinical assessments usually involve evaluating the protein sequence for potential immunogenic epitopes and conducting animal studies to assess immune responses. Clinical studies play a crucial role in characterizing immunogenicity in human populations, focusing on sensitive detection methods for anti-drug antibodies (ADAs) and neutralizing antibodies (NAbs). These studies help identify patient populations at risk of developing immune responses and inform risk mitigation strategies.

To address immunogenicity concerns, manufacturers may employ various approaches, including protein engineering to reduce immunogenic epitopes, co-administration of immunomodulatory agents, or implementation of patient monitoring programs. Post-marketing surveillance also allows for ongoing immunogenicity assessment in real-world patient populations, facilitating early detection of safety concerns and informing risk management strategies [216].

The investigational new drug (IND) application should include a risk assessment and justification for testing. The risk assessment includes in-depth analyses and discussions of therapeutic protein factors that may influence immunogenicity. The FDA recommends that biological drugs with a positive ADA assay result be tested for neutralizing antibodies. Titer, isotype, and domain specificity of the ADA may be assessed in addition to PK, PD, and clinical outcomes [216]. Overall, the FDA regulatory guidelines on protein aggregation and immunogenicity assessment for therapeutic proteins emphasize the importance of a systematic and proactive approach to ensure the safety and efficacy of biological drugs. Manufacturers can minimize patient risks and maximize the therapeutic benefit of these important treatment modalities by understanding and controlling protein aggregation and immunogenicity throughout the product lifecycle.

Conclusion and Summary

Protein aggregation is a complex, multifactorial phenomenon influenced by a range of solution conditions and processing variables, including pH, ionic strength, excipients, shear stress, and thermal or mechanical stress during manufacturing and storage. Aggregation can occur via chemical degradation, physical unfolding, or self-association, and it proceeds through heterogeneous pathways that yield diverse aggregate species, soluble, insoluble, reversible, or irreversible. Controlling protein aggregation requires an understanding of the causes of aggregate formation as well as the use of appropriate analytical techniques. Critically no single analytical method exists to cover the entire size range or type in which aggregates may appear due to the wide size range and structural diversity of protein aggregates. While methods such as SEC, SDS-PAGE, and light scattering (SLS, DLS) are routinely employed, each has inherent limitations in resolution, detection range, and data comparability. Therefore, an orthogonal, multi-technique approach remains essential for accurate aggregate profiling, particularly in the development of biopharmaceutical formulations. Moving forward, the development of standardized, cross-platform analytical workflows and data interpretation guidelines is paramount. Integration of advanced tools such as microfluidics, AI-driven modeling, and high-resolution imaging may offer deeper insights into aggregation kinetics and structural transitions. Additionally, rational design of stabilizers, including amino

acids, sugars, polyols, and surfactants, should be guided by mechanistic understanding of protein, additive interactions specific to structural motifs. In summary, a holistic strategy combining stress-specific stabilization, predictive analytics, and complementary analytical techniques will be crucial in mitigating protein aggregation and ensuring the safety and efficacy of therapeutic proteins. Continued research into aggregation pathways and innovative analytical platforms holds the key to overcoming current limitations and advancing biopharmaceutical stability science.

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References

1. Dimitrov DS. Therapeutic proteins. In: Voynov V, Caravella J, editors. Therapeutic proteins. Methods in Molecular Biology, vol 899. Totowa (NJ): Humana Press; 2012. pp. 1–26.
2. Quinteros DA, Bermúdez JM, Ravetti S, Cid A, Allemandi DA, Palma SD. Therapeutic use of monoclonal antibodies: general aspects and challenges for drug delivery. In: Andronescu E, Grumezescu AM, editors. Nanostructures for drug delivery. Amsterdam: Elsevier; 2017. pp. 807–833.
3. Lu R-M, Hwang Y-C, Liu I-J, Lee C-C, Tsai H-Z, Li H-J, et al. Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci. 2020;27:1.
4. Yasunaga M. Antibody therapeutics and immunoregulation in cancer and autoimmune disease. Semin Cancer Biol. 2020;64:1–12.
5. Propper DJ, Balkwill FR. Harnessing cytokines and chemokines for cancer therapy. Nat Rev Clin Oncol. 2022;19:237–53.
6. Chavda VP, Sugandhi VV, Pardeshi CV, Patil RJ, Joshi M, Patel B, et al. Engineered exosomes for cancer theranostics: Next-generation tumor targeting. J Drug Deliv Sci Technol. 2023;85:104579.
7. Li J, Krause ME, Chen X, Cheng Y, Dai W, Hill JJ, et al. Interfacial stress in the development of biologics: fundamental understanding, current practice, and future perspective. AAPS J. 2019;21:44.
8. Laptoš T, Omersel J. The importance of handling high-value biologicals: physico-chemical instability and immunogenicity of monoclonal antibodies. Exp Ther Med. 2018;15:3161–8.
9. Krause ME, Sahin E. Chemical and physical instabilities in manufacturing and storage of therapeutic proteins. Curr Opin Biotechnol. 2019;60:159–67.
10. Wang W, Roberts CJ. Protein aggregation – Mechanisms, detection, and control. Int J Pharm. 2018;550:251–68.
11. Hamrang Z, Rattray NJW, Pluen A. Proteins behaving badly: emerging technologies in profiling biopharmaceutical aggregation. Trends Biotechnol. 2013;31:448–58.
12. Tao Y, Chen Y, Howard W, Ibrahim M, Patel SM, McMahon WP, et al. Mechanism of insoluble aggregate formation in a reconstituted solution of spray-dried protein powder. Pharm Res. 2023;40:2355–70.
13. Pang KT, Yang YS, Zhang W, Ho YS, Sormanni P, Michaels TCT, et al. Understanding and controlling the molecular mechanisms of protein aggregation in mAb therapeutics. Biotechnol Adv. 2023;67:108192.
14. Ratanji KD, Derrick JP, Dearman RJ, Kimber I. Immunogenicity of therapeutic proteins: Influence of aggregation. J Immunotoxicol. 2014;11:99–109.
15. Hindryckx P, Novak G, Vande Casteele N, Khanna R, Laukens D, Vipul J, et al. Incidence, prevention and management of anti-drug antibodies against therapeutic antibodies in inflammatory bowel disease: a practical overview. Drugs. 2017;77:363–77.
16. Amin S, Barnett GV, Pathak JA, Roberts CJ, Sarangapani PS. Protein aggregation, particle formation, characterization & rheology. Curr Opin Colloid Interface Sci. 2014;19:438–49.
17. Kohler V, Andréasson C. Reversible protein assemblies in the proteostasis network in health and disease. Front Mol Biosci. 2023;10:1155521.
18. Zapadka KL, Becher FJ, Gomes dos Santos AL, Jackson SE. Factors affecting the physical stability (aggregation) of peptide therapeutics. Interface Focus. 2017;7:20170030.
19. Roberts CJ. Therapeutic protein aggregation: mechanisms, design, and control. Trends Biotechnol. 2014;32:372–80.
20. Wang W, Nema S, Teagarden D. Protein aggregation—Pathways and influencing factors. Int J Pharm. 2010;390:89–99.
21. Akbarian M, Chen S-H. Instability challenges and stabilization strategies of pharmaceutical proteins. Pharmaceutics. 2022;14:2533.
22. Ko SK, Berner C, Kulakova A, Schneider M, Antes I, Winter G, et al. Investigation of the pH-dependent aggregation mechanisms of GCSF using low resolution protein characterization techniques and advanced molecular dynamics simulations. Comput Struct Biotechnol J. 2022;20:1439–55.
23. Hipper E, Lehmann F, Kaiser W, Hübner G, Buske J, Blech M, et al. Protein photodegradation in the visible range? Insights into protein photooxidation with respect to protein concentration. Int J Pharm X. 2023;5:100155.
24. Courtois F, Schneider CP, Agrawal NJ, Trout BL. Rational design of biobetters with enhanced stability. J Pharm Sci. 2015;104:2433–40.
25. Castañeda Ruiz AJ, Shetab Boushehri MA, Phan T, Carle S, Garidel P, Buske J, et al. Alternative excipients for protein

stabilization in protein therapeutics: overcoming the limitations of polysorbates. Pharmaceutics. 2022;14:2575.
26. Ebo JS, Guthertz N, Radford SE, Brockwell DJ. Using protein engineering to understand and modulate aggregation. Curr Opin Struct Biol. 2020;60:157–66.
27. Mahler H-C, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. J Pharm Sci. 2009;98:2909–34.
28. Pham NB, Meng WS. Protein aggregation and immunogenicity of biotherapeutics. Int J Pharm. 2020;585:119523.
29. Rajan R, Ahmed S, Sharma N, Kumar N, Debas A, Matsumura K. Review of the current state of protein aggregation inhibition from a materials chemistry perspective: special focus on polymeric materials. Mater Adv. 2021;2:1139–76.
30. Das A, Shah M, Saraogi I. Molecular aspects of insulin aggregation and various therapeutic interventions. ACS Bio Med Chem Au. 2022;2:205–21.
31. Alford JR, Kendrick BS, Carpenter JF, Randolph TW. High concentration formulations of recombinant human interleukin-1 receptor antagonist: II. aggregation kinetics. J Pharm Sci. 2008;97:3005–21.
32. Housmans JAJ, Wu G, Schymkowitz J, Rousseau F. A guide to studying protein aggregation. FEBS J. 2023;290:554–83.
33. Meric G, Robinson AS, Roberts CJ. driving forces for nonnative protein aggregation and approaches to predict aggregation-prone regions. Annu Rev Chem Biomol Eng. 2017;8:139–59.
34. Chi EY, Krishnan S, Randolph TW, Carpenter JF. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. Pharm Res. 2003;20:1325–36.
35. Gribar JJ, Ramachandra M, Hrycyna CA, Dey S, Ambudkar SV. Functional characterization of glycosylation-deficient human P-Glycoprotein using a vaccinia virus expression system. J Membr Biol. 2000;173:203–14.
36. Philo J, Arakawa T. Mechanisms of protein aggregation. Curr Pharm Biotechnol. 2009;10:348–51.
37. Almeida ZL, Brito RMM. Structure and aggregation mechanisms in amyloids. Molecules. 2020;25:1195.
38. Serio TR, Cashikar AG, Kowal AS, Sawicki GJ, Moslehi JJ, Serpell L, et al. Nucleated conformational conversion and the replication of conformational information by a prion determinant. Science. 2000;289:1317–21.
39. Rabe M, Verdes D, Seeger S. Surface-induced spreading phenomenon of protein clusters. Soft Matter. 2009;5:1039.
40. Kim W, Hecht MH. Generic hydrophobic residues are sufficient to promote aggregation of the Alzheimer's Aβ42 peptide. Proc Natl Acad Sci. 2006;103:15824–9.
41. Beerten J, Schymkowitz J, Rousseau F. Aggregation prone regions and gatekeeping residues in protein sequences. Curr Top Med Chem. 2013;999:43–9.
42. Solá RJ, Griebenow K. Effects of glycosylation on the stability of protein pharmaceuticals. J Pharm Sci. 2009;98:1223–45.
43. Zheng K, Bantog C, Bayer R. The impact of glycosylation on monoclonal antibody conformation and stability. MAbs. 2011;3:568–76.
44. Krishna TC, Najda A, Bains A, Tosif MM, Papliński R, Kapłan M, et al. Influence of ultra-heat treatment on properties of milk proteins. Polymers (Basel). 2021;13:3164.
45. Kerr D, Wizemann E, Senstius J, Zacho M, Ampudia-Blasco FJ. Stability and performance of rapid-acting insulin analogs used for continuous subcutaneous insulin infusion: a systematic review. J Diabetes Sci Technol. 2013;7:1595–606.
46. Gallardo P, Salas-Pino S, Daga RR. Reversible protein aggregation as cytoprotective mechanism against heat stress. Curr Genet. 2021;67:849–55.
47. Miotto M, Olimpieri PP, Di Rienzo L, Ambrosetti F, Corsi P, Lepore R, et al. Insights on protein thermal stability: a graph representation of molecular interactions. Bioinformatics. 2019;35:2569–77.
48. Robinson MJ, Matejtschuk P, Bristow AF, Dalby PA. T m -Values and unfolded fraction can predict aggregation rates for granulocyte colony stimulating factor variant formulations but not under predominantly native conditions. Mol Pharm. 2018;15:256–67.
49. Wang W, Li N, Speaker S. External factors affecting protein aggregation. In: Aggregation of therapeutic proteins. Wiley; 2010. pp. 119–204.
50. Psimadas D, Georgoulias P, Valotassiou V, Loudos G. Molecular nanomedicine towards cancer: 111In-labeled nanoparticles. J Pharm Sci. 2012;101:2271–80.
51. Espargaró A, Castillo V, de Groot NS, Ventura S. The in vivo and in vitro aggregation properties of globular proteins correlate with their conformational stability: the SH3 case. J Mol Biol. 2008;378:1116–31.
52. Hao Y, Chu J, Wang Y, Zhuang Y, Zhang S. The inhibition of aggregation of recombinant human consensus interferon-α mutant during Pichia pastoris fermentation. Appl Microbiol Biotechnol. 2007;74:578–84.
53. Zhang Y. Understanding the impact of Fc glycosylation on its conformational changes by molecular dynamics simulations and bioinformatics. Mol Biosyst. 2015;11:3415–24.
54. Englander SW, Mayne L. The nature of protein folding pathways. Proc Natl Acad Sci. 2014;111:15873–80.
55. Ito T, Lutz H, Tan L, Wang B, Tan J, Patel M, et al. Host cell proteins in monoclonal antibody processing: control, detection, and removal. Biotechnol Prog. 2024;40:e3448.
56. Liu HF, Ma J, Winter C, Bayer R. Recovery and purification process development for monoclonal antibody production. MAbs. 2010;2:480–99.
57. Shukla AA, Hinckley PJ, Gupta P, Yigzaw Y, Hubbard B. Strategies to address aggregation during protein a chromatography. Bioprocess Int. 2005;3:36–44.
58. Bondos SE, Bicknell A. Detection and prevention of protein aggregation before, during, and after purification. Anal Biochem. 2003;316:223–31.
59. Liu S, Li Z, Yu B, Wang S, Shen Y, Cong H. Recent advances on protein separation and purification methods. Adv Colloid Interface Sci. 2020;284:102254.
60. Structural Genomics Consortium, China Structural Genomics Consortium. Northeast structural genomics consortium, Gräslund S, Nordlund P, Weigelt J, et al. Protein production and purification. Nat Methods. 2008;5:135–46.
61. Doonan S. Protein Purification Protocols. New Jersey: Humana Press; 1996.
62. Bhatnagar BS, Bogner RH, Pikal MJ. Protein stability during freezing: separation of stresses and mechanisms of protein stabilization. Pharm Dev Technol. 2007;12:505–23.
63. Thorat AA, Munjal B, Geders TW, Suryanarayanan R. Freezing-induced protein aggregation - Role of pH shift and potential mitigation strategies. J Control Release. 2020;323:591–9.
64. Wöll AK, Hubbuch J. Investigation of the reversibility of freeze/thaw stress-induced protein instability using heat cycling as a function of different cryoprotectants. Bioprocess Biosyst Eng. 2020;43:1309–27.
65. Duerkop M, Berger E, Dürauer A, Jungbauer A. Impact of cavitation, high shear stress and air/liquid interfaces on protein aggregation. Biotechnol J. 2018;13:1800062.
66. Bekard IB, Asimakis P, Bertolini J, Dunstan DE. The effects of shear flow on protein structure and function. Biopolymers. 2011;95:733–45.

67. Thomas CR, Geer D. Effects of shear on proteins in solution. Biotechnol Lett. 2011;33:443–56.
68. Ghadge RS, Patwardhan AW, Sawant SB, Joshi JB. Effect of flow pattern on cellulase deactivation in stirred tank bioreactors. Chem Eng Sci. 2005;60:1067–83.
69. Rathore N, Rajan RS. Current perspectives on stability of protein drug products during formulation, fill and finish operations. Biotechnol Prog. 2008;24:504–14.
70. Bunkin NF, Shkirin AV, Ninham BW, Chirikov SN, Chaikov LL, Penkov NV, et al. Shaking-induced aggregation and flotation in immunoglobulin dispersions: differences between water and water-ethanol mixtures. ACS Omega. 2020;5:14689–701.
71. Patel HA, Singh H, Havea P, Considine T, Creamer LK. Pressure-induced unfolding and aggregation of the proteins in whey protein concentrate solutions. J Agric Food Chem. 2005;53:9590–601.
72. Fernández del Río B, Rey A. Behavior of proteins under pressure from experimental pressure-dependent structures. J Phys Chem B. 2021;125:6179–91.
73. Hawley SA. Reversible pressure-temperature denaturation of chymotrypsinogen. Biochemistry. 1971;10:2436–42.
74. Balny C. High pressure and protein oligomeric dissociation. High Press Res. 2002;22:737–41.
75. Lee S, Carpenter JF, Chang BS, Randolph TW, Kim Y. Effects of solutes on solubilization and refolding of proteins from inclusion bodies with high hydrostatic pressure. Protein Sci. 2006;15:304–13.
76. St. John RJ, Carpenter JF, Randolph TW. High pressure fosters protein refolding from aggregates at high concentrations. Proc Natl Acad Sci. 1999;96:13029–33.
77. Chen Y, Mutukuri TT, Wilson NE, Zhou Q. Pharmaceutical protein solids: drying technology, solid-state characterization and stability. Adv Drug Deliv Rev. 2021;172:211–33.
78. Bouman J. Drying and hydration of proteins at high concentration. Wageningen University; 2015. p. 161.
79. Mensink MA, Frijlink HW, van der Voort MK, Hinrichs WLJ. How sugars protect proteins in the solid state and during drying (review): mechanisms of stabilization in relation to stress conditions. Eur J Pharm Biopharm. 2017;114:288–95.
80. Pinto JT, Faulhammer E, Dieplinger J, Dekner M, Makert C, Nieder M, et al. Progress in spray-drying of protein pharmaceuticals: Literature analysis of trends in formulation and process attributes. Dry Technol. 2021;39:1415–46.
81. Lee G. Spray-drying of proteins. In: Carpenter JF, Manning MC, editors. Rational design of stable protein formulations. Vol 13, Boston, MA: Springer; 2002. pp. 135–58.
82. Jadhav K, Kole E, Abhang A, Rojekar S, Sugandhi V, Verma RK, et al. Revealing the potential of nano spray drying for effective delivery of pharmaceuticals and biologicals. Dry Technol. 2025;43:90–113.
83. Jadhav K, Kole E, Singh R, Rout SK, Verma RK, Chatterjee A, et al. A critical review on developments in drying technologies for enhanced stability and bioavailability of pharmaceuticals. Dry Technol. 2024;42:1415–41.
84. Adali MB, Barresi AA, Boccardo G, Pisano R. Spray freeze-drying as a solution to continuous manufacturing of pharmaceutical products in bulk. Processes. 2020;8:709.
85. O'Sullivan A, Ryan KM, Padrela L. Production of biopharmaceutical dried-powders using supercritical CO2 technology. J Supercrit Fluids. 2022;187:105645.
86. Jovanović N, Bouchard A, Sutter M, Van Speybroeck M, Hofland GW, Witkamp G-J, et al. Stable sugar-based protein formulations by supercritical fluid drying. Int J Pharm. 2008;346:102–8.
87. Cape SP, Villa JA, Huang ETS, Yang T-H, Carpenter JF, Sievers RE. Preparation of active proteins, vaccines and pharmaceuticals as fine powders using supercritical or near-critical fluids. Pharm Res. 2008;25:1967–90.
88. Wang W. Advanced protein formulations. Protein Sci. 2015;24:1031–9.
89. Birrenbach O, Faust F, Ebrahimi M, Fan R, Czermak P. Recovery and purification of protein aggregates from cell lysates using ceramic membranes: fouling analysis and modeling of ultrafiltration. Front Chem Eng. 2021;3:656345.
90. Randolph TW, Schiltz E, Sederstrom D, Steinmann D, Mozziconacci O, Schöneich C, et al. Do not drop: mechanical shock in vials causes cavitation, protein aggregation, and particle formation. J Pharm Sci. 2015;104:602–11.
91. Respaud R, Marchand D, Parent C, Pelat T, Thullier P, Tournamille J-F, et al. Effect of formulation on the stability and aerosol performance of a nebulized antibody. MAbs. 2014;6:1347–55.
92. Sugandhi VV, Mahajan HS. Development of vitamin B12 containing pullulan-bovine serum albumin microparticles designed dry powder inhaler: In-vitro and in-vivo study. J Drug Deliv Sci Technol. 2022;70:103212.
93. Cromwell MEM, Hilario E, Jacobson F. Protein aggregation and bioprocessing. AAPS J. 2006;8:E572–9.
94. Zhou H-X, Pang X. Electrostatic interactions in protein structure, folding, binding, and condensation. Chem Rev. 2018;118:1691–741.
95. Shaw KL, Grimsley GR, Yakovlev GI, Makarov AA, Pace CN. The effect of net charge on the solubility, activity, and stability of ribonuclease Sa. Protein Sci. 2001;10:1206–15.
96. Li R, Wu Z, Wangb Y, Ding L, Wang Y. Role of pH-induced structural change in protein aggregation in foam fractionation of bovine serum albumin. Biotechnol Rep. 2016;9:46–52.
97. Baler K, Martin OA, Carignano MA, Ameer GA, Vila JA, Szleifer I. Electrostatic unfolding and interactions of albumin driven by pH changes: a molecular dynamics study. J Phys Chem B. 2014;118:921–30.
98. Brudar S, Hribar-Lee B. Effect of buffer on protein stability in aqueous solutions: a simple protein aggregation model. J Phys Chem B. 2021;125:2504–12.
99. Vegarud G, Christensen TB. Glycosylation of proteins: a new method of enzyme stabilization. Biotechnol Bioeng. 1975;17:1391–7.
100. Vegarud G, Christensen TB, Sarvela J, Jänne J, Liaaen-Jensen S. The resistance of glycoproteins to proteolytic inactivation. Acta Chem Scand. 1975;29:887–8.
101. Weintraub BD, Stannard BS, Meyers L. Glycosylation of thyroid-stimulating hormone in pituitary tumor cells: influence of high mannose oligosaccharide units on subunit aggregation, combination, and intracellular degradation. Endocrinology. 1983;112:1331–45.
102. Carter CRD, Whitmore KM, Thorpe R. The significance of carbohydrates on G-CSF: differential sensitivity of G-CSFs to human neutrophil elastase degradation. J Leukoc Biol. 2004;75:515–22.
103. Wicker-Planquart C, Canaan S, Rivière M, Dupuis L. Site-directed removal of N-glycosylation sites in human gastric lipase. Eur J Biochem. 1999;262:644–51.
104. Sahin E, Grillo AO, Perkins MD, Roberts CJ. Comparative effects of pH and ionic strength on protein-protein interactions, unfolding, and aggregation for IgG1 antibodies. J Pharm Sci. 2010;99:4830–48.
105. Bickel F, Herold EM, Signes A, Romeijn S, Jiskoot W, Kiefer H. Reversible NaCl-induced aggregation of a monoclonal antibody at low pH: characterization of aggregates and factors affecting aggregation. Eur J Pharm Biopharm. 2016;107:310–20.

106. Bhojane PP, Joshi S, Sahoo SJ, Rathore AS. Unexplored excipients in biotherapeutic formulations: natural osmolytes as potential stabilizers against thermally induced aggregation of IgG1 biotherapeutics. AAPS PharmSciTech. 2022;23:26.
107. Vagenende V, Yap MGS, Trout BL. Mechanisms of protein stabilization and prevention of protein aggregation by glycerol. Biochemistry. 2009;48:11084–96.
108. Olsson C, Swenson J. The role of disaccharides for protein–protein interactions – a SANS study. Mol Phys. 2019;117:3408–16.
109. Bowden GA, Georgiou G. The effect of sugars on β-Lactamase aggregation in Escherichia coli. Biotechnol Prog. 1988;4:97–101.
110. Mojtabavi S, Samadi N, Faramarzi MA. Osmolyte-induced folding and stability of proteins: concepts and characterization. Iran J Pharm Res IJPR. 2019;18:13–30.
111. Kim S-H, Yan Y-B, Zhou H-M. Role of osmolytes as chemical chaperones during the refolding of aminoacylase. Biochem Cell Biol. 2006;84:30–8.
112. Despotović D, Longo LM, Aharon E, Kahana A, Scherf T, Gruic-Sovulj I, et al. Polyamines mediate folding of primordial hyperacidic helical proteins. Biochemistry. 2020;59:4456–62.
113. Schuster I, Bernhardt R. Interactions of natural polyamines with mammalian proteins. Biomol Concepts. 2011;2:79–94.
114. Cortés H, Hernández-Parra H, Bernal-Chávez SA, Del P-A, Caballero-Florán IH, Borbolla-Jiménez FV, et al. Non-Ionic surfactants for stabilization of polymeric nanoparticles for biomedical uses. Materials (Basel). 2021;14:3197.
115. La Mesa C. Polymer–surfactant and protein–surfactant interactions. J Colloid Interface Sci. 2005;286:148–57.
116. Treuheit MJ, Kosky AA, Brems DN. Inverse relationship of protein concentration and aggregation. Pharm Res. 2002;19:511–6.
117. Qing R, Hao S, Smorodina E, Jin D, Zalevsky A, Zhang S. Protein design: from the aspect of water solubility and stability. Chem Rev. 2022;122:14085–179.
118. Yoneda S, Maruno T, Mori A, Hioki A, Nishiumi H, Okada R, et al. Influence of protein adsorption on aggregation in prefilled syringes. J Pharm Sci. 2021;110:3568–79.
119. Otzen D. Protein–surfactant interactions: a tale of many states. Biochim Biophys Acta Proteins Proteomics. 2011;1814:562–91.
120. Movafaghi S, Daniels AL, Kelly MD, Witeof AE, Calderon CP, Randolph TW, et al. Hydrogel coatings on container surfaces reduce protein aggregation caused by mechanical stress and cavitation. ACS Appl Bio Mater. 2021;4:6946–53.
121. Richter C, Lipperheide C, Lipke U, Lamprecht A. Impact of extractables from rubber closures on protein stability under heat stress. Eur J Pharm Biopharm. 2018;130:22–9.
122. Sreenivasan S, Jiskoot W, Rathore AS. Rapid aggregation of therapeutic monoclonal antibodies by bubbling induced air/liquid interfacial and agitation stress at different conditions. Eur J Pharm Biopharm. 2021;168:97–109.
123. Teresa M, Petersen S, Prakash G. UV Light effects on proteins: from photochemistry to nanomedicine. Molecular photochemistry-various aspects. InTech; 2012. pp. 125–158.
124. Steinmann D, Mozziconacci O, Bommana R, Stobaugh JF, Wang YJ, Schöneich C. Photodegradation pathways of protein disulfides: human growth hormone. Pharm Res. 2017;34:2756–78.
125. Jiang S, Carroll L, Mariotti M, Hägglund P, Davies MJ. Formation of protein cross-links by singlet oxygen-mediated disulfide oxidation. Redox Biol. 2021;41:101874.
126. Lee Y-W, Song K-B. Effect of γ-irradiation on the molecular properties of myoglobin. BMB Rep. 2002;35:590–4.
127. Kuan SL, Bergamini FRG, Weil T. Functional protein nanostructures: a chemical toolbox. Chem Soc Rev. 2018;47:9069–105.
128. Faísca PFN, Nunes A, Travasso RDM, Shakhnovich EI. Nonnative interactions play an effective role in protein folding dynamics. Protein Sci. 2010;19:2196–209.
129. Ha J, Loh SN. Protein conformational switches: from nature to design. Chem A Eur J. 2012;18:7984–99.
130. Nicoud L, Owczarz M, Arosio P, Morbidelli M. A multiscale view of therapeutic protein aggregation: a colloid science perspective. Biotechnol J. 2015;10:367–78.
131. Chang BS, Hershenson S. Practical approaches to protein formulation development. In: Carpenter JF, Manning MC, editors. Rational design of stable protein formulations: theory and practice. Vol 13, Boston MA: Springer; 2002. pp. 1–25.
132. Shieh IC, Patel AR. Predicting the agitation-induced aggregation of monoclonal antibodies using surface tensiometry. Mol Pharm. 2015;12:3184–93.
133. Frachon T, Bruckert F, Le Masne Q, Monnin E, Weidenhaupt M. Insulin aggregation at a dynamic solid–liquid–air triple interface. Langmuir. 2016;32:13009–19.
134. Tosstorff A, Svilenov H, Peters GHJ, Harris P, Winter G. Structure-based discovery of a new protein-aggregation breaking excipient. Eur J Pharm Biopharm. 2019;144:207–16.
135. Mousavi SB, Fazeli A, Shojaosadati SA, Fazeli MR, Hashemi-Najafabadi S. Purification and efficient refolding process for recombinant tissue-type plasminogen activator derivative (reteplase) using glycerol and Tranexamic acid. Process Biochem. 2017;53:135–44.
136. Abe M, Abe Y, Ohkuri T, Mishima T, Monji A, Kanba S, et al. Mechanism for retardation of amyloid fibril formation by sugars in Vλ6 protein. Protein Sci. 2013;22:467–74.
137. Hadrovic I, Rebmann P, Klärner F-G, Bitan G, Schrader T. Molecular lysine tweezers counteract aberrant protein aggregation. Front Chem. 2019;7:657.
138. Xiao F, Chen Z, Wei Z, Tian L. Hydrophobic interaction: a promising driving force for the biomedical applications of nucleic acids. Adv Sci. 2020;7:16:2001048.
139. Bansal S, Maurya IK, Shenmar K, Yadav N, Thota CK, Kumar V, et al. Aβ 1–42 C-terminus fragment derived peptides prevent the self-assembly of the parent peptide. RSC Adv. 2017;7:4167–73.
140. Navarro S, Ventura S. Computational methods to predict protein aggregation. Curr Opin Struct Biol. 2022;73:102343.
141. Prabakaran R, Goel D, Kumar S, Gromiha MM. Aggregation prone regions in human proteome: insights from large-scale data analyses. Proteins Struct Funct Bioinforma. 2017;85:1099–118.
142. van der Lee R, Buljan M, Lang B, Weatheritt RJ, Daughdrill GW, Dunker AK, et al. Classification of intrinsically disordered regions and proteins. Chem Rev. 2014;114:6589–631.
143. Babu MM. The contribution of intrinsically disordered regions to protein function, cellular complexity, and human disease. Biochem Soc Trans. 2016;44:1185–200.
144. Santos J, Pujols J, Pallarès I, Iglesias V, Ventura S. Computational prediction of protein aggregation: advances in proteomics, conformation-specific algorithms and biotechnological applications. Comput Struct Biotechnol J. 2020;18:1403–13.
145. Pintado-Grima C, Bárcenas O, Bartolomé-Nafría A, Fornt-Suñé M, Iglesias V, Garcia-Pardo J, et al. A review of fifteen years developing computational tools to study protein aggregation. Biophysica. 2023;3:1–20.
146. Prabakaran R, Rawat P, Thangakani AM, Kumar S, Gromiha MM. Protein aggregation: in silico algorithms and applications. Biophys Rev. 2021;13:71–89.
147. Rajpoot K, Desai N, Koppisetti H, Tekade M, Sharma MC, Behera SK, et al. In silico methods for the prediction of drug toxicity. In: Tekade RK, editor. Pharmacokinetics and toxicokinetic considerations. Elsevier; 2022. pp. 357–83.

148. Alanazi W, Meng D, Pollastri G. Advancements in one-dimensional protein structure prediction using machine learning and deep learning. Comput Struct Biotechnol J. 2025;27:1416–30.
149. Aggrescan: Prediction of "Hot Spots" of aggregation in polypeptides [Internet]. [cited 2024 Mar 17]. Available from: http://bioinf.uab.es/aap/
150. Waltz [Internet]. [cited 2024 Mar 17]. Available from: https://waltz.switchlab.org/
151. Tango [Internet]. [cited 2024 Mar 17]. Available from: http://tango.crg.es/
152. Aggregation Prediction with PASTA [Internet]. [cited 2024 Mar 17]. Available from: http://old.protein.bio.unipd.it/pasta2/
153. Biol.Uoa.Gr - AmylPred [Internet]. [cited 2024 Mar 17]. Available from: http://aias.biol.uoa.gr/AMYLPRED/
154. Betascan: Probable Beta-Structures Identified through Pairwise Probabilistic Analysis [Internet]. [cited 2024 Mar 17]. Available from: https://cb.csail.mit.edu/cb/betascan/
155. STITCHER 2.0: Primer design for overlapping PCR applications | Scientific Reports [Internet]. [cited 2024 Mar 18]. Available from: https://www.nature.com/articles/srep45349
156. GAP [Internet]. [cited 2024 Mar 17]. Available from: https://www.iitm.ac.in/bioinfo/GAP/
157. ANuPP [Internet]. [cited 2024 Mar 17]. Available from: https://web.iitm.ac.in/bioinfo2/ANuPP
158. Net-CSSP [Internet]. [cited 2024 Mar 17]. Available from: https://cssp2.sookmyung.ac.kr/
159. The Budapest Amyloid Predictor | PIT Bioinformatics Group [Internet]. [cited 2024 Mar 17]. Available from: https://pitgroup.org/bap/
160. Cordax [Internet]. [cited 2024 Mar 17]. Available from: https://cordax.switchlab.org/
161. Agmata [Internet]. [cited 2024 Mar 17]. Available from: https://bio2byte.be/b2btools/agmata/
162. AmylPred 2 [Internet]. [cited 2024 Mar 17]. Available from: http://thalis.biol.uoa.gr/AMYLPRED2/
163. Emily M, Talvas A, Delamarche C. MetAmyl: A METa-Predictor for AMYLoid Proteins. Tosatto SCE, editor. PLoS One. 2013;8:e79722.
164. SolubiS 1.0 [Internet]. [cited 2024 Mar 17]. Available from: https://solubis.switchlab.org/
165. Aggregation prediction with protein surface analyzer - Schrödinger [Internet]. [cited 2024 Mar 17]. Available from: https://newsite.schrodinger.com/life-science/learn/white-papers/aggregation-prediction-protein-surface-analyzer/
166. Aggrescan3D 2.0 [Internet]. [cited 2024 Mar 17]. Available from: https://biocomp.chem.uw.edu.pl/A3D2/
167. The CamSol method for protein solubility prediction [Internet]. [cited 2024 Mar 17]. Available from: http://www-vendruscolo.ch.cam.ac.uk/camsolmethod.html
168. Alam P, Siddiqi K, Chturvedi SK, Khan RH. Protein aggregation: from background to inhibition strategies. Int J Biol Macromol. 2017;103:208–19.
169. McLure RJ, Radford SE, Brockwell DJ. High-throughput directed evolution: a golden era for protein science. Trends Chem. 2022;4:378–91.
170. Ebo JS, Saunders JC, Devine PWA, Gordon AM, Warwick AS, Schiffrin B, et al. An in vivo platform to select and evolve aggregation-resistant proteins. Nat Commun. 2020;11:1816.
171. Kwan TOC, Kolek SA, Danson AE, Reis RI, Camacho IS, Shaw Stewart PD, et al. Measuring protein aggregation and stability using high-throughput biophysical approaches. Front Mol Biosci. 2022;9:890862.
172. Paxman JJ, Heras B. Bioinformatics tools and resources for analyzing protein structures. In: Keerthikumar S, Mathivanan S, editors. Proteome bioinformatics. New York, NY: Springer; 2017. pp. 209–20.
173. María Hernández-Domínguez E, Sofía Castillo-Ortega L, García-Esquivel Y, Mandujano-González V, Díaz-Godínez G, Álvarez-Cervantes J. Bioinformatics as a tool for the structural and evolutionary analysis of proteins. Comput Biol Chem. IntechOpen; 2020.
174. de Paiva VA, de Gomes IS, Monteiro CR, Mendonça MV, Martins PM, Santana CA, et al. Protein structural bioinformatics: an overview. Comput Biol Med. 2022;147:105695.
175. Courtois F, Agrawal NJ, Lauer TM, Trout BL. Rational design of therapeutic mAbs against aggregation through protein engineering and incorporation of glycosylation motifs applied to bevacizumab. MAbs. 2016;8:99–112.
176. Ebrahimi SB, Samanta D. Engineering protein-based therapeutics through structural and chemical design. Nat Commun. 2023;14:2411.
177. Tiwari MK, Singh R, Singh RK, Kim I-W, Lee J-K. Computational approaches for rational design of proteins with novel functionalities. Comput Struct Biotechnol J. 2012;2:e201204002.
178. Costanzo JA, O'Brien CJ, Tiller K, Tamargo E, Robinson AS, Roberts CJ, et al. Conformational stability as a design target to control protein aggregation. Protein Eng Des Sel. 2014;27:157–67.
179. Sahin E, Jordan JL, Spatara ML, Naranjo A, Costanzo JA, Weiss WF, et al. Computational design and biophysical characterization of aggregation-resistant point mutations for γD Crystallin illustrate a balance of conformational stability and intrinsic aggregation propensity. Biochemistry. 2011;50:628–39.
180. O'Brien CJ, Blanco MA, Costanzo JA, Enterline M, Fernandez EJ, Robinson AS, et al. Modulating non-native aggregation and electrostatic protein–protein interactions with computationally designed single-point mutations. Protein Eng Des Sel. 2016;29:231–43.
181. Austerberry JI, Dajani R, Panova S, Roberts D, Golovanov AP, Pluen A, et al. The effect of charge mutations on the stability and aggregation of a human single chain Fv fragment. Eur J Pharm Biopharm. 2017;115:18–30.
182. Villar-Piqué A, Ventura S. Protein aggregation propensity is a crucial determinant of intracellular inclusion formation and quality control degradation. Biochim Biophys Acta - Mol Cell Res. 2013;1833:2714–24.
183. Al Musaimi O, Lombardi L, Williams DR, Albericio F. Strategies for improving peptide stability and delivery. Pharmaceuticals. 2022;15:1283.
184. Wang X, Ni D, Liu Y, Lu S. Rational design of peptide-based inhibitors disrupting protein-protein interactions. Front Chem. 2021;9:682675.
185. Meric G, Naik S, Hunter AK, Robinson AS, Roberts CJ. Challenges for design of aggregation-resistant variants of granulocyte colony-stimulating factor. Biophys Chem. 2021;277:106630.
186. Kang MS, Kong TWS, Khoo JYX, Loh T-P. Recent developments in chemical conjugation strategies targeting native amino acids in proteins and their applications in antibody–drug conjugates. Chem Sci. 2021;12:13613–47.
187. Zuma LK, Gasa NL, Makhoba XH, Pooe OJ. Protein PEGylation: navigating recombinant protein stability, aggregation, and bioactivity. Biomed Res Int. 2022;2022:8929715.
188. Flores-Fernández GM, Pagán M, Almenas M, Solá RJ, Griebenow K. Moisture-induced solid state instabilities in α-chymotrypsin and their reduction through chemical glycosylation. BMC Biotechnol. 2010;10:57.
189. Mancini RJ, Lee J, Maynard HD. Trehalose glycopolymers for stabilization of protein conjugates to environmental stressors. J Am Chem Soc. 2012;134:8474–9.

190. Budhavaram NK, Miller JA, Shen Y, Barone JR. Protein substitution affects glass transition temperature and thermal stability. J Agric Food Chem. 2010;58:9549–55.
191. Qiu L-M, Ma J, Wang J, Zhang F-C, Wang Y. Thermal stability properties of an antifreeze protein from the desert beetle Microdera punctipennis. Cryobiology. 2010;60:192–7.
192. Kamerzell TJ, Pace AL, Li M, Danilenko DM, Mcdowell M, Gokarn YR, et al. Polar solvents decrease the viscosity of high concentration IgG1 solutions through hydrophobic solvation and interaction: formulation and biocompatibility considerations. J Pharm Sci. 2013;102:1182–93.
193. Soares CM, Teixeira VH, Baptista AM. Protein structure and dynamics in nonaqueous solvents: insights from molecular dynamics simulation studies. Biophys J. 2003;84:1628–41.
194. Srinivasan C, Weight AK, Bussemer T, Klibanov AM. Non-aqueous suspensions of antibodies are much less viscous than equally concentrated aqueous solutions. Pharm Res. 2013;30:1749–57.
195. Marschall C, Graf G, Witt M, Hauptmeier B, Friess W. Preparation of high concentration protein powder suspensions by milling of lyophilizates. Eur J Pharm Biopharm. 2021;166:75–86.
196. Yang MX, Shenoy B, Disttler M, Patel R, McGrath M, Pechenov S, et al. Crystalline monoclonal antibodies for subcutaneous delivery. Proc Natl Acad Sci. 2003;100:6934–9.
197. McPherson A. Introduction to protein crystallization. Methods. 2004;34:254–65.
198. Orehek J, Teslić D, Likozar B. Continuous crystallization processes in pharmaceutical manufacturing: a review. Org Process Res Dev. 2021;25:16–42.
199. Sharma A, Khamar D, Cullen S, Hayden A, Hughes H. Innovative drying technologies for biopharmaceuticals. Int J Pharm. 2021;609:121115.
200. Ishwarya SP, Anandharamakrishnan C, Stapley AGF. Spray-freeze-drying: a novel process for the drying of foods and bioproducts. Trends Food Sci Technol. 2015;41:161–81.
201. Burke PA, Klumb LA, Herberger JD, Nguyen XC, Harrell RA, Zordich M. Poly(Lactide-Co-Glycolide) microsphere formulations of darbepoetin alfa: spray drying is an alternative to encapsulation by spray-freeze drying. Pharm Res. 2004;21:500–6.
202. Jangle R, Pisal S. Vacuum foam drying: an alternative to lyophilization for biomolecule preservation. Indian J Pharm Sci. 2012;74:91.
203. Danielsen M, Hempel C, Andresen TL, Urquhart AJ. Biopharmaceutical nanoclusters: towards the self-delivery of protein and peptide therapeutics. J Control Release. 2022;347:282–307.
204. Yearley EJ, Godfrin PD, Perevozchikova T, Zhang H, Falus P, Porcar L, et al. Observation of small cluster formation in concentrated monoclonal antibody solutions and its implications to solution viscosity. Biophys J. 2014;106:1763–70.
205. Johnston KP, Maynard JA, Truskett TM, Borwankar AU, Miller MA, Wilson BK, et al. Concentrated dispersions of equilibrium protein nanoclusters that reversibly dissociate into active monomers. ACS Nano. 2012;6:1357–69.
206. Srinivasan AR, Lakshmikuttyamma A, Shoyele SA. Investigation of the stability and cellular uptake of self-associated Monoclonal Antibody (MAb) nanoparticles by non-small lung cancer cells. Mol Pharm. 2013;10:3275–84.
207. Laue T. Proximity energies: a framework for understanding concentrated solutions. J Mol Recognit. 2012;25:165–73.
208. Dunkelberger JR, Song W-C. Complement and its role in innate and adaptive immune responses. Cell Res. 2010;20:34–50.
209. Chaplin DD. Overview of the immune response. J Allergy Clin Immunol. 2010;125:S3-23.
210. Vishweshwaraiah YL, Dokholyan N V. mRNA vaccines for cancer immunotherapy. Front Immunol. 2022;13:1029069.
211. Hwang I, Huang J-F, Kishimoto H, Brunmark A, Peterson PA, Jackson MR, et al. T cells can use either T cell receptor or Cd28 receptors to absorb and internalize cell surface molecules derived from antigen-presenting cells. J Exp Med. 2000;191:1137–48.
212. Harryvan TJ, de Lange S, Hawinkels LJAC, Verdegaal EME. The ABCs of antigen presentation by stromal non-professional antigen-presenting cells. Int J Mol Sci. 2021;23:137.
213. Dingman R, Balu-Iyer SV. Immunogenicity of protein pharmaceuticals. J Pharm Sci. 2019;108:1637–54.
214. Tsai D-Y, Hung K-H, Chang C-W, Lin K-I. Regulatory mechanisms of B cell responses and the implication in B cell-related diseases. J Biomed Sci. 2019;26:64.
215. Noviski M, Mueller JL, Satterthwaite A, Garrett-Sinha LA, Brombacher F, Zikherman J. IgM and IgD B cell receptors differentially respond to endogenous antigens and control B cell fate. Elife. 2018;7:e35074.
216. FDA. Guidance for industry: Immunogenicity assessment for therapeutic protein products. 2014.
217. Lundahl MLE, Fogli S, Colavita PE, Scanlan EM. Aggregation of protein therapeutics enhances their immunogenicity: causes and mitigation strategies. RSC Chem Biol. 2021;2:1004–20.
218. Moussa EM, Panchal JP, Moorthy BS, Blum JS, Joubert MK, Narhi LO, et al. Immunogenicity of therapeutic protein aggregates. J Pharm Sci. 2016;105:417–30.
219. Tan H, Ramirez CM, Miljkovic N, Li H, Rubin JP, Marra KG. Thermosensitive injectable hyaluronic acid hydrogel for adipose tissue engineering. Biomaterials. 2009;30:6844–53.
220. Nakayama M. Antigen presentation by MHC-dressed cells. Front Immunol. 2015;5:672.
221. Buchbinder EI, Desai A. CTLA-4 and PD-1 pathways: similarities, differences, and implications of their inhibition. Am J Clin Oncol. 2016;39:98–106.
222. Bevington SL, Cauchy P, Withers DR, Lane PJL, Cockerill PN. T cell receptor and cytokine signaling can function at different stages to establish and maintain transcriptional memory and enable T helper cell differentiation. Front Immunol. 2017;8:204.
223. Goulet DR, Atkins WM. Considerations for the Design of Antibody-Based Therapeutics. J Pharm Sci. 2020;109:74–103.
224. Pedersen ME, Østergaard J, Glintborg B, Hetland ML, Jensen H. Assessment of immunogenicity and drug activity in patient sera by flow-induced dispersion analysis. Sci Rep. 2022;12:4670.
225. Bray-French K, Hartman K, Steiner G, Marban-Doran C, Bessa J, Campbell N, et al. Managing the impact of immunogenicity in an era of immunotherapy: from bench to bedside. J Pharm Sci. 2021;110:2575–84.
226. Baker M, Reynolds HM, Lumicisi B, Bryson CJ. Immunogenicity of protein therapeutics: the key causes, consequences and challenges. Self Nonself. 2010;1:314–22.
227. Rahban M, Ahmad F, Piatyszek MA, Haertlé T, Saso L, Saboury AA. Stabilization challenges and aggregation in protein-based therapeutics in the pharmaceutical industry. RSC Adv. 2023;13:35947–63.
228. Tatkiewicz W, Elizondo E, Moreno E, Díez-Gil C, Ventosa N, Veciana J, et al. Methods for characterization of protein aggregates. In: García-Fruitós E, editor., et al., Insoluble proteins methods mol biol. Humana Press: New York; 2015. p. 387–401.