UNPAD

Indonesian Journal of Pharmaceutical Science and Technology
Journal Homepage : http://jurnal.unpad.ac.id/ijpst/
Review Article

The Stability of Protein Therapeutics: A Significant Challenge in the Formulation of Biopharmaceuticals

Rina F. Nuwarda[1*], Zelika M. Ramadhania[2], Mia T. Novianti[3]

[1]Department of Pharmaceutical Analysis and Medicinal Chemistry, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang, Indonesia

[2]Department of Biological Pharmacy, Faculty of Pharmacy, Universitas Padjadjaran, Sumedang, Indonesia

[3]Research Center for Molecular Biotechnology and Bioinformatics, Universitas Padjadjaran, Bandung, Indonesia

Submitted 04 July 2024; Revised 10 August 2024; Accepted 12 August 2024; Published 14 August 2024

*Corresponding author: rina.nuwarda@unpad.ac.id

Abstract

Protein-based therapies have already brought about a significant transformation in the field of medicine, and their use continues to grow. Protein therapeutics has revolutionized the treatment of various diseases, such as cancer, autoimmune disorders, viral infections, cardiovascular disease, myocardial infarction, etc. Consequently, it is crucial to ensure the stability of formulations, given the rapid growth of biotherapeutic products. Preserving the structural integrity of protein-based medicines is a significant challenge in developing a stable and high-quality formulation. This challenge arises during various stages, including manufacturing procedures, storage, handling, distribution, and delivery due to proteins' intricate and delicate nature. Hence, it is crucial to thoroughly understand the degradation mechanisms that impact protein stability to enhance different variables and minimize the formation of degradation products which could potentially have clinical implications. This review provides an important step in understanding the process of protein degradation and offers a beneficial approach to investigate the degradation of proteins, specifically aggregation, through several analytical and biophysical methods. Understanding factors affecting protein stability and how to observe the change are important for facilitating the further advancement of protein-based therapies.

Keywords: Biologics, biotherapeutics, protein aggregation, protein formulation stability.

Stabilitas Protein Terapeutik: Tantangan Signifikan dalam Formulasi Biofarmasi

Abstrak

Terapi berbasis protein telah membawa perubahan signifikan dalam bidang kedokteran, dan penggunaannya terus berkembang. Terapi protein telah merevolusi pengobatan berbagai penyakit, seperti kanker, gangguan autoimun, infeksi virus, penyakit kardiovaskular, infark miokard (serangan jantung akut), dan lain-lain. Oleh karena itu, sangat penting untuk memastikan stabilitas formulasi, mengingat pertumbuhan produk terapeutik protein yang pesat. Menjaga kestabilan struktural obat-obatan berbasis protein merupakan tantangan besar dalam mengembangkan formulasi yang stabil dan berkualitas tinggi. Tantangan ini muncul dalam berbagai tahapan, termasuk pada saat pembuatan, penyimpanan, penanganan, distribusi, dan pengiriman karena sifat protein yang kompleks dan rapuh. Oleh karena itu, sangat penting untuk memahami secara menyeluruh mekanisme degradasi yang berdampak pada stabilitas protein untuk meningkatkan berbagai variabel dan meminimalkan pembentukan produk degradasi yang berpotensi mempunyai implikasi klinis. Tulisan ini memberikan langkah penting dalam memahami proses degradasi protein dan merumuskan pendekatan yang bermanfaat untuk memahami degradasi protein, khususnya agregasi, melalui beberapa metode analitis dan biofisik. Memahami faktor-faktor yang berpengaruh pada kestabilan protein, serta bagaimana mengidentifikasinya penting untuk memfasilitasi kemajuan lebih lanjut dari terapi berbasis protein.

Kata Kunci: Agregasi protein, bioterapeutik, produk biologi, stabilitas formulasi protein.

1. Introduction

Protein therapeutics are a form of targeted therapies that utilize specific medications to target pathogenic substances such as proteins or genes selectively. Therapeutic proteins have usually been modified using hybridoma cells or recombinant DNA technologies to be used as pharmaceuticals.[1] Some notable examples of therapeutic agents include monoclonal antibodies, interferons, and cytokines, which have been approved by the FDA for treating many diseases, such as cancer, diabetes, anemia, and infections. Over 100 of these agents have received FDA approval.[2] These proteins, classified as antibodies, enzymes, hormones, and growth factors, provide targeted therapies that address specific protein deficits and offer customized approaches. Compared to conventional medications, they provide better selectivity by binding specifically to pathways associated with the disease.[1,2]

Furthermore, therapeutic proteins offer sophisticated functionalities with little risk of disrupting biological processes and a decreased likelihood of triggering immune reactions. These therapeutic agents break down undesirable molecules, restore protein deficiencies, or modulate inaccurate signaling or immunological responses. For example, Rituximab is a monoclonal antibody that specifically targets CD20, a protein present on the surface of B-cells. By attaching to CD20, Rituximab signals these cells for elimination by the immune system, employing mechanisms such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). This action is especially effective in the treatment of B-cell lymphomas and autoimmune disorders.[3] Due to the large size of protein, they mostly focus on cell surface receptors or chemicals found outside of cells. Engineering these proteins involves utilizing techniques that improve their efficacy, such as glycosylation to amplify the size and stability or PEGylation to prolong their half-life. Therapeutic proteins are a notable breakthrough in medical science, providing targeted therapies that result in fewer adverse effects and better patient outcomes.[4]

2. Classification and examples of protein-based therapeutics

Therapeutic proteins can be categorized based on their pharmacological features, clinical usage, and function.[1] They are often classified into four categories: those that the FDA has approved (Group I and II), and those that are currently being investigated in vivo or in vitro (Group III and IV).[4,5]

2.1. Group I: Therapeutic proteins with enzymatic activity

These proteins exhibit enzymatic properties that facilitate specific biochemical reactions in the body, resulting in therapeutic effects.[6] Group I has three main categories:

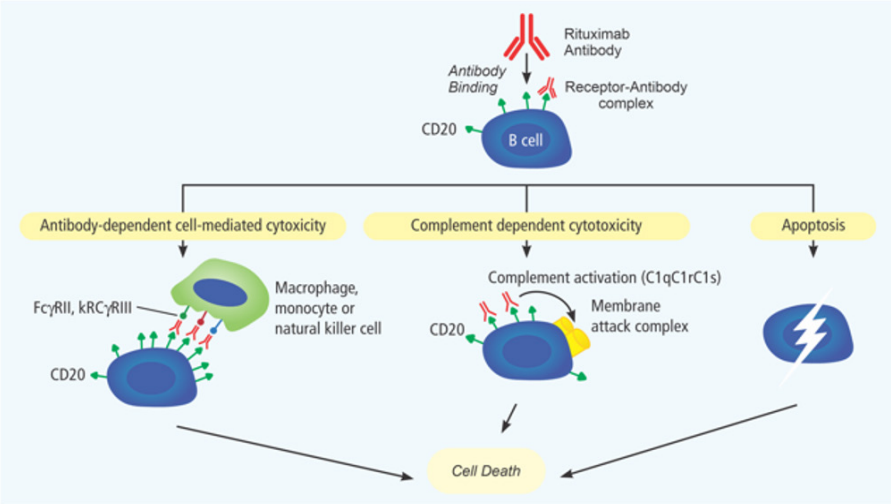

Figure 1. Rituximab binding to a CD20 protein on the surface of a B-cell. It also show immune cells, like natural killer cells, attacking the marked B-cell (Figure adapted from reference 3)

those that replace missing proteins or enzymes that lead to illnesses or diseases, boost the impact or adjust the timing of a typical protein's expression, and provide a new function or activity.[1]

Several well-known examples include lactase, which aids individuals who have difficulty digesting lactose, and β-glucocerebrosidase, used to treat Gaucher's disease. Pancreatic enzymes are beneficial in the treatment of cystic fibrosis and pancreatic insufficiency. Alteplase helps to dissolve blood clots in cases of myocardial infarction. Botulinum toxin type A is used for treating dystonia and for cosmetic purposes. Collagenase is utilized to manage dermal ulcers and burns, while human deoxyribonuclease I aids in managing cystic fibrosis by enhancing lung mucus clearance.[4]

2.2. Group II: Therapeutic proteins with special targeting activity

Therapeutic proteins Group II fall into two categories: first, proteins that disrupt other bodily molecules, and second, molecules that are transported to a particular location within the body. Monoclonal antibodies (mAbs) are commonly used in both categories.[7] They can imitate the immune system's reaction to pathogens and cancer cells as well as identify and attach to specific molecules, particularly proteins, that are either connected to cells or released into the body. This is possible because mAbs can adapt their structure to match the variable region of the target molecule, allowing them to bind to a particular antigenic domain consistently.[8]

The development of mAbs as therapeutic agents has resulted in several approaches to manage autoimmune disorders in preclinical and experimental phases and to induce anti-tumor effects. In 1986, the first monoclonal antibody (mAb) called muromonab-CD3 was used to treat allogenic transplant rejection. With its anti-CD20 action, Rituximab became the first monoclonal antibody (mAb) authorized for therapeutic use in 1997.[8]

2.3. Group III: Therapeutic proteins as vaccines

Three approaches comprise the group III category of therapeutic proteins: prevention of harmful agents, treatment of autoimmune disorders, and cancer treatments. Various technological strategies are employed for this objective, including vector- and cell-based vaccines and molecular-based vaccines such as peptide/protein, DNA, and mRNA vaccines.[9] To reshape the host's immune response for disease eradication and long-lasting memory, these vaccines are designed to induce or enhance cell-mediated and humoral immunity.[10] The current focus of research is the development of therapeutic protein-based vaccines that capitalize on cellular responses to improve their efficacy against complex infections.[11] Furthermore, recent developments have encompassed a broad spectrum of diseases, such as solid tumors like lung, breast, colorectal, liver, and gastric cancers, infectious diseases like HIV, HBV, and HPV-induced diseases, and chronic conditions like hypertension, diabetes, and dyslipidemia, illustrating the diverse applications of therapeutic vaccines in contemporary medicine.[5]

2.4. Group IV: Therapeutic proteins as diagnostics

Some therapeutic proteins have been used in medical diagnostics in addition to their therapeutic applications. For instance, secretin is employed to diagnose gastrinoma and identify the ampulla of Vater. Satumomab pendetide is utilized to detect ovarian and colon cancer by employing a monoclonal antibody specific for tumor-associated glycoprotein (TAG-72) and labeled with indium-111. Nofetumomab is a technetium-labeled antibody that is specifically designed for the detection and staging of small-cell lung carcinoma. Hepatitis C antigens are employed to diagnose hepatitis C exposure by detecting antibodies to hepatitis C. Apcitide is a synthetic technetium-labeled synthetic peptide that binds to GPIIb/IIIa receptors on activated platelets, providing imaging for acute venous thrombosis4. Classification and examples of therapeutic proteins are listed in Table 1.

Table 1. Classification and examples of therapeutics proteins

Group	Category and Examples	Commercial Brand Names	Status
I	**Therapeutic Proteins with Enzymatic Activity[12]**		
	Lactase (digestive aid for lactose intolerance)	Lactaid®, Dairy Ease™	Approved, On the Market
	β-Glucocerebrosidase (Gaucher's disease treatment)	Cerezyme®, VPRIV®	Approved, On the Market
	Pancreatic Enzymes (cystic fibrosis, pancreatic insufficiency)	Creon®, Pancreaze®, Zenpep®	Approved, On the Market
	Alteplase (blood clot dissolver for myocardial infarction)	Activase®, Cathflo®	Approved, On
	Botulinum Toxin Type A (dystonia treatment, cosmetic use)	Botox®, Dysport®, Xeomin®	Approved, On the Market
	Collagenase (management of dermal ulcers and burns)	Santyl®	Approved, On the Market
	Human Deoxyribonuclease I (cystic fibrosis management)	Pulmozyme®	Approved, On the Market
II	**Therapeutic Proteins with Special Targeting Activity[8]**		
	Muromonab-CD3 (treatment for allogenic transplant rejection)	Orthoclone OKT3®	Approved, On the Market
	Rituximab (anti-CD20 mAb for autoimmune disorders, cancer)	Rituxan®, MabThera®	Approved, On the Market
	Other monoclonal antibodies (various targets and diseases)	Various (e.g., Herceptin®, Avastin®, Keytruda®)	Clinical Studies, Approved
III	**Therapeutic Proteins as Vaccines[5,9]**		
	mRNA Vaccines (COVID-19 vaccines)	Comirnaty® (Pfizer-BioNTech), Spikevax® (Moderna)	Approved, On the Market
	Peptide/Protein Vaccines (various diseases)	various	Clinical Studies
	DNA Vaccines (cancer, infectious diseases)	various	Clinical Studies
IV	**Therapeutic Proteins as Diagnostics[13]**		
	Secretin (diagnosis of gastrinoma, ampulla of Vater identification)	ChiRhoStim®	Approved, On the Market
	Nofetumomab (detection and staging of small-cell lung carcinoma)	Verluma®	Approved, On the Market
	Apcitide (imaging for acute venous thrombosis)	AcuTect®	Approved, On the Market
	Capromab pendetide (imaging of prostate cancer)	ProstaScint®	Approved, On the Market

3. Challenges for Therapeutic Protein Development

Rapid progress has been made in the last decades in developing engineered proteins to treat several life-threatening conditions. The success of therapeutic proteins depends on their efficacy, quality, stability, and immunogenicity. Those four factors often pose significant challenges in developing protein therapeutics.[4]

Protein therapeutics development is a multi-step process with substantial quality control challenges. Good quality

protein therapeutics products with minimal heterogeneousness and impurities are essential for safety, efficacy, as well as approval by the FDA14. Because proteins are susceptible to various physical and chemical degradation processes, developing formulations to ensure their stability is becoming increasingly important, given their rapid growth. Due to proteins' complex nature and fragile structural stability, developing stable protein formulations frequently requires more time and resources than small molecule drugs.[15] Proteins are large macromolecules composed of a specific arrangement of amino acids, exhibiting a distinct three-dimensional conformation corresponding to their biologically functional state. The conformation of a protein molecule is formed by an intricate arrangement made up of different interactions, including covalent bonds, hydrophobic interactions, electrostatic attractions, hydrogen bonding, and van der Waals forces. Both intra-protein and protein-solvent interactions are crucial in preserving proteins' structural integrity and stability. Protein degradation, aggregation, and inactivation can be induced by any change in the protein's environment, as even slight differences in stability between the folded and unfolded states of proteins make them susceptible to changes in the protein's surroundings. These changes can substantially impact, reducing efficacy, increasing immunogenicity, and even causing undesirable immune responses.[16,17] Thus, preserving protein-based therapeutics' functionality and structural integrity is paramount during drug development.

Maintaining the integrity of protein-based medicines presents significant obstacles during routine manufacturing steps, storage, handling, distribution, and their ultimate delivery to the patient. To achieve this goal, developing a formulation that demonstrates exceptional stability with no detectable changes in the protein's chemical and physical properties is necessary. Recognizing the inherent challenges in achieving absolute formulation stability, the primary focus is ensuring the product's safety and effectiveness are always upheld. To effectively accomplish this objective, it is necessary to understand the various degradation pathways that impact proteins. Additionally, it is essential to have access to a wide range of analytical methods and possess the necessary expertise in utilizing the equipment and techniques involved. Formulation development focuses on comprehensively analyzing and identifying potential degradation pathways. By thoroughly evaluating the importance of each pathway, formulation work aims to optimize various variables to minimize the generation of degradation products that may have clinical consequences.[15] Figure 2 shows the schematic diagram illustrating the aggregation pathways of protein.[18]

4. Protein Degradation Mechanism

Understanding protein degradation mechanisms is critical for developing stable formulations, ensuring therapeutic efficacy, and maintaining product safety throughout its shelf life. Temperature and pH have the most influence on both chemical and physical protein stability. High temperatures can cause thermal denaturation and subsequent aggregation, as well as accelerate chemical

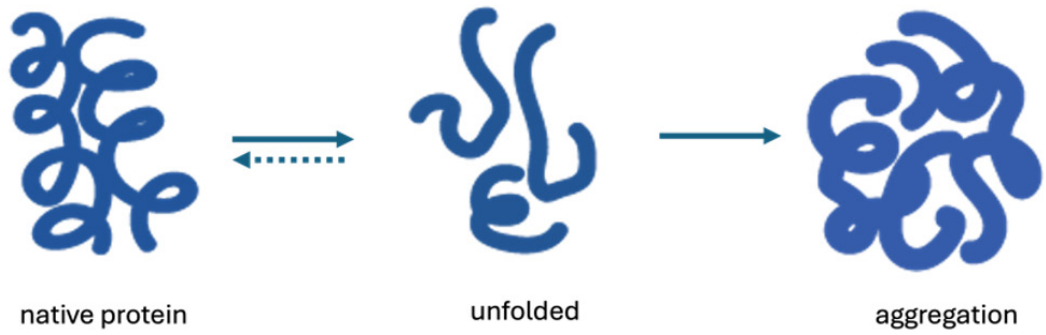

Figure 2. The schematic diagram illustrating the aggregation pathways of protein (adapted from reference 18).

degradation pathways that can result in aggregation. Furthermore, most proteins are generally stable only in a narrow pH range.[19] The isoelectric point of a protein is an essential property because it is the least soluble and thus unstable. The critical point is that the protein is soluble below and above the isoelectric point (isoelectric pH).[20,21,22] In addition, protein in near-neutral pH is crucial to maintain their structure, support enzyme activity, and thrive in the cellular environment.[17] Protein degradation in therapeutics can be divided into chemical and physical degradation mechanisms, contributing to product activity and potency loss.[23,24]

4.1. Chemical Instability

Chemical instability in protein-based therapeutics refers to the propensity of these proteins to undergo chemical modifications that can compromise their structural integrity, functional activity, and safety. These modifications can occur during manufacturing, storage, or administration, leading to degradation and loss of therapeutic efficacy.[25] The primary types of chemical instability include oxidation, deamidation, hydrolysis, glycation, and isomerization.[26]

a. Oxidation

Oxidation occurs when reactive oxygen species (ROS) interact with amino acid side chains, especially those containing sulfur (cysteine and methionine) and aromatic residues (tryptophan, tyrosine, histidine).[27] Oxidative modifications can cause the formation of disulfide bonds, sulfoxides, or other oxidative products, which alter the protein's conformation and function. Oxidation can cause aggregation, reduced solubility, and loss of biological activity.[28] Possible prevention measures include using antioxidants (e.g., ascorbic acid, methionine, EDTA), careful control of oxygen levels during manufacturing and storage, and limiting light exposure, which can all help reduce oxidation.[29]

b. Deamidation

Deamidation is the hydrolytic removal of amide groups from asparagine (Asn) and glutamine (Gln) residues, resulting in aspartic acid (Asp) and glutamic acid (Glu). This process frequently involves a succinimide intermediate.[30] Deamidation alters the protein's charge and conformation, potentially causing altered activity, increased immunogenicity, and decreased stability. The rate of deamidation is influenced by the residues' local environment, which includes pH, temperature, and ionic strength.[31] During protein engineering, deamidation can be mitigated by optimizing buffers to maintain a neutral pH and lowering storage temperatures that can avoid forming Asn and Gln residues. In addition, replacing susceptible residues with more stable amino acids (site-directed mutagenesis) is used to produce an analogous protein, resulting in a more resistant or active species than the wild type of the protein.[17,32]

c. Hydrolysis

Hydrolysis involves the cleavage of peptide bonds within the protein backbone, typically accelerated under extreme pH conditions or in the presence of catalytic residues. Hydrolysis causes protein fragmentation, resulting in structural integrity and biological activity loss. The fragments may also aggregate, compromising the therapeutic product.[33,34] Preventive methods for minimizing hydrolytic degradation include maintaining a neutral pH, stabilizing excipients (buffers, salts, amino acids, polyols/disaccharides polysaccharides and surfactants), and avoiding harsh processing conditions.[35]

d. Glycation

Glycation is the non-enzymatic attachment of reducing sugars to lysine amino groups or protein N-terminal residues, forming a Schiff base that can rearrange into more stable advanced glycation end products (AGEs). Glycation can cause structural and functional changes to proteins, promote aggregation, and increase immunogenicity. AGEs are particularly problematic because they resist proteolytic degradation and accumulate over time.[36,37,38,39] Avoiding

reducing sugars in formulations, using alternative stabilizers (e.g., surfactants, carbohydrates, amino acid-based stabilizers, polymers, or ionic liquids), and keeping temperatures low can help reduce glycation.[40]

e. Isomerization

Isomerization involves converting amino acid residues into their isomeric forms, such as transforming aspartic acid (Asp) into isoaspartic acid. Isomerization can alter the protein's conformation and function, potentially resulting in activity loss and increased degradation.[41,42] To reduce isomerization, optimizing pH and temperature conditions, as well as using stabilizing agents such as surfactants, carbohydrates, amino acids-based stabilizer, and ionic liquids, can be utilized.[43]

4.2. Physical Instability

Physical instability in protein-based therapeutics refers to structural changes that disrupt their native conformation, resulting in loss of function, aggregation, and degradation. Chemical instability involves covalent modifications of amino acid residues, whereas physical instability primarily concerns non-covalent alterations such as denaturation, aggregation, and adsorption. These structural changes can significantly impact the protein's biological activity and its recognition by degradation pathways.[23,44] The sugars generate a hydrophilic environment, which helps prevent aggregation and denaturation.[47]

a. Denaturation

Denaturation is the process when a protein loses its native three-dimensional structure as a result of non-covalent interactions such as hydrogen bonds, hydrophobic interactions, van der Waals forces, and ionic bonds. Temperature changes, pH shifts, and mechanical forces are all potential stressors that can trigger this process.[45] Physical factors that can cause denaturation include extreme pH, high temperatures, mechanical stress, and exposure to organic solvents or detergents. Sugars (e.g., trehalose, sucrose) and polyols (e.g., glycerol) can be used to protect formulations from denaturation.[46]

b. Aggregation

Protein aggregation occurs when misfolded or partially unfolded proteins bind together to form soluble oligomers or insoluble fibrils. This can occur through either non-specific hydrophobic interactions or specific intermolecular interactions. Aggregates can reduce product efficacy, elicit immunogenic responses, and complicate administration. Aggregation is a feature of many neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's disease.[48,49] Conditions that promote aggregation such as high protein concentrations, temperature fluctuations, and agitation during processing and storage can all contribute to aggregation.[15] Mitigation strategies include using surfactants such as polysorbates, controlling protein concentration, employing agitation-free manufacturing processes, and optimizing formulation components to help prevent aggregation.[50,51]

c. Adsorption

Protein adsorption involves binding proteins to surfaces such as glass, plastic, or other biomaterials. This interaction can lead to conformational changes in the protein structure. Adsorption can cause protein denaturation and subsequent aggregation. In pharmaceutical formulations, protein adsorption to container surfaces can reduce the effective concentration of the active protein.[52] Factors that affect adsorption are surface properties (hydrophobicity, charge), protein concentration, and environmental conditions (pH, ionic strength) influence adsorption. Coating surfaces with inert materials, using non-adsorptive containers and adding stabilizing agents can minimize adsorption.[53]

Protein degradation in therapeutic products presents a multifaceted challenge that affects their stability, efficacy, and safety. Both chemical and physical degradation mechanisms contribute to the overall stability of protein-based products. The stability of

these biologics can be improved through careful formulation development, optimized manufacturing processes, and stringent storage and handling protocols, ensuring their full therapeutic potential is realized. Understanding and mitigating protein degradation mechanisms is critical for successfully developing and commercializing stable and effective protein-based therapeutics.

5. Biophysical and analytical methods to assess the stability of protein therapeutics

Aggregation is the most common factor in the physical degradation of the protein. Protein aggregation can potentially induce serious consequences of immunogenic reactions, posing considerable challenges to biologics research and commercialization. Aggregation occurs at various protein product development and manufacturing stages, including formulation. Fluorimetry-based methods (differential scanning fluorimetry), spectroscopy-based techniques (UV/Vis spectroscopy, fluorescence spectroscopy), separation-based methods (size exclusion chromatography) and scattering-based methods (static and dynamic light scattering) have been extensively utilized in the qualitative and quantitative evaluation of protein aggregation.[54]

a. Differential scanning fluorometry

Biophysics plays a crucial role in modern drug discovery research by enabling fast and high-throughput data collection to screen extensive chemical libraries and uncover novel bioactive compounds.[55] Differential Scanning Fluorimetry (DSF) is a biophysical technique widely used to assess protein stability.[56] It is a cost-effective, parallelizable, feasible, and easily accessible method.[55] DSF is commonly used in the early-stage screening of proteins, including different mutations, as well as in the later-stage evaluation of final product formulations to determine shelf life and product quality.[57] This method is utilized to monitor protein folding state and thermal stability, serving as a reliable tool for studying protein unfolding by gradually raising the temperature under set conditions. The denaturation of a stable protein necessitates a high temperature, but an unstable protein can be denatured at a lower temperature. DSF is employed to analyze the unfolding of proteins when the temperature rises, disrupting noncovalent interactions responsible for protein folding. Thus, it is also referred to as a thermal shift assay. The process of protein denaturation can be observed by monitoring alterations in fluorescence emission that occur as the temperature increases.[58]

The conventional DSF assay utilizes the fluorescence of the dye Sypro Orange to indicate the extent to which hydrophobic regions are exposed as the protein unfolds at higher temperatures. Sypro Orange fluorescence significantly intensifies in a nonpolar environment. Elevated temperature can induce protein aggregation, resulting in a decrease in fluorescence.[58,59] The most recent technology, Nanoscale Differential Scanning Fluorometry (nanoDSF®), distinguishes itself from conventional DSF by utilizing dye-free techniques to detect changes in the fluorescence signal released by the Trp residues of a protein in response to changing temperatures. Exposure to this generates a change in the Trp λmax from 330 nm to 350 nm, known as a redshift (emission maxima in the direction of longer wavelength), due to a change in the polarity of the Trp residues' surrounding environment. The NanoDSF approach uses back-reflection technology to assess protein aggregation. This technique identifies the temperature at which protein aggregation starts, crucial for evaluating the protein's colloidal stability, particularly under elevated temperature conditions.[60]

b. Fluorescence spectroscopy

Fluorescence spectroscopy utilizes the occurrence of electron excitation caused by collisions with high-energy particles such as photons and other excited electrons, resulting in the emission of photons as their energy falls to the ground state. Compounds that exhibit fluorescence activities, such as fluorophores, can serve as physical indicators in the

biophysical evaluation of macromolecules, such as proteins and nucleic acids, for their structural analysis. The fluorophores may be extrinsic, like dyes, or intrinsic, such as specific amino acids within protein sequences. Proteins contain intrinsic fluorophores from the amino acids tryptophan, tyrosine, and phenylalanine.[61] Tryptophan and tyrosine are commonly used in biophysical studies to monitor alterations in the tertiary structure of proteins to a greater extent than phenylalanine, as phenylalanine yields lower fluorescence than the other two amino acids. Fluorescence intensity changes can be observed depending on the surroundings of Trp and Tyr residues inside the protein. The presence of Trp and Tyr will enhance the fluorescence signal, leading to a redshift, indicating that the tryptophan residues were located within hydrophobic pockets that were concealed through proper protein folding.[62]

Proteins can be labeled with external fluorophores using fluorescence dyes such as 8-Anilinonaphthalene-1-sulfonic acid (ANS) and Thioflavin T (ThT). These dyes specifically bind to the hydrophobic regions of proteins that become exposed during the unfolding process. Extrinsic fluorescent dyes are used in various areas of protein research, playing a crucial role in studying folding intermediates, quantifying surface hydrophobicity, and detecting aggregates. Extrinsic dyes can either create covalent bonds with amino acids, such as by attaching to the α-amino group of the N-terminus, or the thiol group of cysteine, or they can interact through non-covalent means, such as hydrophobic or electrostatic interactions.[63]

c. Size exclusion chromatography (SEC) and multi-angle light scattering (MALS)

Given the complex nature of protein therapeutics, it is necessary to use several complementary approaches to evaluate important features of intermediary drug components accurately. One aspect being examined involves a quantitative assessment of the aggregation phenomenon of the native protein. Due to concerns about the potential implications for safety and effectiveness, examining and quantifying dimers, trimers, and larger aggregates of proteins in protein-based therapeutic products has become routine. Multiple approaches have been developed to observe protein aggregation. Still, SEC has been the favored option for regular and validated studies because of its substantial advantages in terms of speed and reliability.[64] Proteins are exposed to greater stresses beyond actual conditions to predict forthcoming stability issues. Consequently, the degradation products produced due to these stresses are examined. The results obtained from these "accelerated stability studies" can be useful for estimating the rate at which degradation processes occur in practical situations when real-time data may be unavailable due to time and resource constraints.[65]

SEC is utilized for protein characterization and measurement of molecular mass, as it separates proteins based on their size. However, the accuracy of mass estimate by SEC can be limited at times since it depends on the protein's retention time, which its hydrodynamic radius can influence. The possible interactions between the protein sample and the stationary phase can also affect retention time. SEC-MALS, which combines SEC with MALS, is a valuable technique for accurately analyzing the protein's molar mass, oligomeric states, and hydrodynamic radius, irrespective of the protein's retention time.[66] MALS is based on static light scattering to measure the amount of light scattered at multiple angles. The measured intensity is directly proportional to molecular weight (Mw) and the concentration of the protein. Combining these two techniques enables the separation of individual protein molecules from contaminants such as aggregates or fragments. This facilitates the detection of these impurities, providing essential data that can be utilized to evaluate the quality of the product.[31]

d. Other approaches

Various other methods can also be employed to enhance understanding of

protein unfolding and aggregation. These approaches include differential scanning calorimetry (DSC), dynamic light scattering (DLS), electron microscopy (EM), and time-resolved fluorescence spectroscopy.[57,58] Small-angle X-ray scattering (SAXS) is increasingly believed to be a reliable method for characterizing intermediates in protein solutions. It may be utilized to observe changes in the structure of macromolecules, interactions between proteins, forms of assembly like oligomerization and aggregation, and the attraction and repulsion between molecules. Thus, SAXS can potentially be a valuable supplementary technique for studying the dynamics of protein unfolding and aggregation.[69]

5. Conclusion and future direction

The discovery and development process of protein-based therapeutic drugs can be quite challenging and time-consuming. Therefore, exploring innovative ideas or approaches that could enhance this process is crucial. The rapid development of protein therapeutics necessitates optimizing the pharmacology-related properties and ensuring the product's stability. Protein therapeutic products face substantial challenges in terms of stability due to alterations in protein folding. These alterations can cause the accumulation of partially unfolded or misfolded proteins, ultimately resulting in protein aggregation. Hence, it is crucial to understand the process of protein degradation, particularly aggregation, using various analytical and biophysical methods. This would greatly aid in the advancement of biological therapeutic development.

The advancement in molecular biotechnology and structural biology has greatly contributed to the growth of structural information on biological macromolecules. Therefore, computational techniques have become essential in drug discovery and development initiatives. In-silico aggregation predictors have assisted and directed experimental aims to understand the molecular pathways responsible for protein aggregation-related disorders. In addition, they have advanced the design of engineered protein variants by improving their solubility and stability. This has resulted in time and cost savings in producing therapeutic proteins. Various computational toolboxes have been created and made available to predict protein aggregation tendencies, pinpoint areas susceptible to sequential or structural aggregation, evaluate the impact of mutations on aggregation, and identify prion-like domains. Navarro and Ventura categorize these tools into sequence-based methods, such as AGGRESCAN, Zyggregator, PASTA 2.0; machine-learning methods, such as ANuPP and Pafig; and 3D structure-based methods, such as Aggscore and AGGRESCAN3D 2.0. By integrating different approaches, methods, and knowledge, the development and manufacturing of protein-based therapeutics can be greatly enhanced to ensure better stability.

References

1. Leader B, Baca QJ, Golan DE. Protein therapeutics: a summary and pharmacological classification. Nature Reviews Drug Discovery. 2008;7(1):21-39.
2. Dimitrov DS. Therapeutic proteins. Methods Mol Biol. 2012;899:1-26.
3. Wood C, McKay G, Fisher M. Rituximab. Practical Diabetes. 2017;34(7):258-9a.
4. Singh DB, Tripathi T. Protein-based Therapeutics: Springer Nature Singapore; 2023.
5. Gandhi RP, Mehetre GD, Gandhi SJ, Wadichar SP. A systematic review of therapeutic proteins as a promising approach to treat various diseases. GSC Biological and Pharmaceutical Sciences. 2023;22(1):157-69.
6. Zbar NS. A Review Article: Protein Engineering of Therapeutic Enzymes. International Journal for Research in Applied Sciences and Biotechnology. 2022.
7. Lagassé HA, Alexaki A, Simhadri VL, Katagiri NH, Jankowski W, Sauna ZE, et al. Recent advances in (therapeutic protein) drug development. F1000Res.

2017;6:113.
8. Hafeez U, Gan HK, Scott AM. Monoclonal antibodies as immunomodulatory therapy against cancer and autoimmune diseases. Curr Opin Pharmacol. 2018;41:114-21.
9. Tian Y, Hu D, Li Y, Yang L. Development of therapeutic vaccines for the treatment of diseases. Mol Biomed. 2022;3(1):40.
10. Li T, Qian C, Gu Y, Zhang J, Li S, Xia N. Current progress in the development of prophylactic and therapeutic vaccines. Sci China Life Sci. 2023;66(4):679-710.
11. Jiang S, Gong M, Xu XN. Editorial: Research, Development and Clinical Trials for Peptide-Based Vaccines. Front Immunol. 2022;13:894989.
12. Jain M, Kumar A, Khan RJ, Jha RK, Singh E, Muthukumaran J, et al. Introduction to Protein Therapeutics. In: Singh DB, Tripathi T, editors. Protein-based Therapeutics. Singapore: Springer Nature Singapore; 2023:1-22.
13. S S, Xavier J, Saravankumar R. Emerging Trends, Challenges, and Opportunities in Protein Therapeutics. 2023:297-324.
14. Birch JR, Racher AJ. Antibody production. Adv Drug Deliv Rev. 2006;58(5-6):671-85.
15. Chang BS, Hershenson S. Practical approaches to protein formulation development. Pharm Biotechnol. 2002;13:1-25.
16. Rathore N, Rajan RS. Current perspectives on stability of protein drug products during formulation, fill and finish operations. Biotechnol Prog. 2008;24(3):504-14.
17. Akbarian M, Chen SH. Instability Challenges and Stabilization Strategies of Pharmaceutical Proteins. Pharmaceutics. 2022;14(11).
18. Upadhyay U, Barua C, Devi S, Kumar J, Singh R. Dynamics of unfolded protein aggregation2021.
19. Challener CA. Excipient Selection for Protein Stabilization. Pharmaceutical Technology. 2015;39(18):s35-s9.
20. Zhou H-X, Pang X. Electrostatic Interactions in Protein Structure, Folding, Binding, and Condensation. Chemical Reviews. 2018;118(4):1691-741.
21. Schröder C. Proteins in Ionic Liquids: Current Status of Experiments and Simulations. Top Curr Chem (Cham). 2017;375(2):25.
22. Vilg JV, Undeland I. pH-driven solubilization and isoelectric precipitation of proteins from the brown seaweed Saccharina latissima-effects of osmotic shock, water volume and temperature. J Appl Phycol. 2017;29(1):585-93.
23. 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 Advances. 2023;13(51):35947-63.
24. Clarkson BR, Schön A, Freire E. Conformational stability and self-association equilibrium in biologics. Drug Discov Today. 2016;21(2):342-7.
25. Krause ME, Sahin E. Chemical and physical instabilities in manufacturing and storage of therapeutic proteins. Curr Opin Biotechnol. 2019;60:159-67.
26. Gupta S, Jiskoot W, Schöneich C, Rathore AS. Oxidation and Deamidation of Monoclonal Antibody Products: Potential Impact on Stability, Biological Activity, and Efficacy. J Pharm Sci. 2022;111(4):903-18.
27. Andrés CMC, Pérez de la Lastra JM, Andrés Juan C, Plou FJ, Pérez-Lebeña E. Impact of Reactive Species on Amino Acids-Biological Relevance in Proteins and Induced Pathologies. Int J Mol Sci. 2022;23(22).
28. Lévy E, El Banna N, Baïlle D, Heneman-Masurel A, Truchet S, Rezaei H, et al. Causative Links between Protein Aggregation and Oxidative Stress: A Review. Int J Mol Sci. 2019;20(16).
29. Brovč EV, Pajk S, Šink R, Mravljak J. Protein Formulations Containing Polysorbates: Are Metal Chelators Needed at All? Antioxidants (Basel). 2020;9(5).
30. Jin Y, Yi Y, Yeung B. Mass spectrometric analysis of protein deamidation – A focus on top-down and middle-down mass spectrometry. Methods. 2022;200:58-66.

31. Gervais D. Protein deamidation in biopharmaceutical manufacture: understanding, control and impact. Journal of Chemical Technology & Biotechnology. 2016;91(3):569-75.
32. Lu X, Nobrega RP, Lynaugh H, Jain T, Barlow K, Boland T, et al. Deamidation and isomerization liability analysis of 131 clinical-stage antibodies. MAbs. 2019;11(1):45-57.
33. Ravuluri S, Bansal R, Chhabra N, Rathore AS. Kinetics and Characterization of Non-enzymatic Fragmentation of Monoclonal Antibody Therapeutics. Pharm Res. 2018;35(7):142.
34. Zhang S, De Leon Rodriguez LM, Li FF, Brimble MA. Recent developments in the cleavage, functionalization, and conjugation of proteins and peptides at tyrosine residues. Chem Sci. 2023;14(29):7782-817.
35. 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(12).
36. Twarda-Clapa A, Olczak A, Białkowska AM, Koziołkiewicz M. Advanced Glycation End-Products (AGEs): Formation, Chemistry, Classification, Receptors, and Diseases Related to AGEs. Cells. 2022;11(8).
37. Uceda AB, Mariño L, Casasnovas R, Adrover M. An overview on glycation: molecular mechanisms, impact on proteins, pathogenesis, and inhibition. Biophysical Reviews. 2024;16(2):189-218.
38. Kumar Pasupulati A, Chitra PS, Reddy GB. Advanced glycation end products mediated cellular and molecular events in the pathology of diabetic nephropathy. Biomol Concepts. 2016;7(5-6):293-309.
39. Sarmah S, Roy AS. A review on prevention of glycation of proteins: Potential therapeutic substances to mitigate the severity of diabetes complications. Int J Biol Macromol. 2022;195:565-88.
40. Wei B, Berning K, Quan C, Zhang YT. Glycation of antibodies: Modification, methods and potential effects on biological functions. MAbs. 2017;9(4):586-94.
41. Yi M, Sun J, Sun H, Wang Y, Hou S, Jiang B, et al. Identification and characterization of an unexpected isomerization motif in CDRH2 that affects antibody activity. MAbs. 2023;15(1):2215364.
42. Gibson K, Cooper-Shepherd DA, Pallister E, Inman SE, Jackson SE, Lindo V. Toward Rapid Aspartic Acid Isomer Localization in Therapeutic Peptides Using Cyclic Ion Mobility Mass Spectrometry. Journal of the American Society for Mass Spectrometry. 2022;33(7):1204-12.
43. Nugrahadi PP, Hinrichs WLJ, Frijlink HW, Schöneich C, Avanti C. Designing Formulation Strategies for Enhanced Stability of Therapeutic Peptides in Aqueous Solutions: A Review. Pharmaceutics. 2023;15(3).
44. Butreddy A, Janga KY, Ajjarapu S, Sarabu S, Dudhipala N. Instability of therapeutic proteins - An overview of stresses, stabilization mechanisms and analytical techniques involved in lyophilized proteins. Int J Biol Macromol. 2021;167:309-25.
45. Acharya V, Chaudhuri P. Modalities of Protein Denaturation and Nature of Denaturants. International Journal of Pharmaceutical Sciences Review and Research. 2021;69:19-24.
46. Solá RJ, Griebenow K. Effects of glycosylation on the stability of protein pharmaceuticals. J Pharm Sci. 2009;98(4):1223-45.
47. Muntu CM, Avanti C, Hayun H, Surini S. Impact of Excipients Blending on Sugar-Stabilized Therapeutic Proteins. Journal of Medicinal and Chemical Sciences. 2024;7:777-9.
48. Pukala TL. Mass spectrometric insights into protein aggregation. Essays Biochem. 2023;67(2):243-53.
49. Vidović M, Rikalovic MG. Alpha-Synuclein Aggregation Pathway in Parkinson's Disease: Current Status and Novel Therapeutic Approaches. Cells.

2022;11(11).
50. Kim HL, McAuley A, McGuire J. Protein effects on surfactant adsorption suggest the dominant mode of surfactant-mediated stabilization of protein. J Pharm Sci. 2014;103(5):1337-45.
51. Katz JS, Chou DK, Christian TR, Das TK, Patel M, Singh SN, et al. Emerging Challenges and Innovations in Surfactant-mediated Stabilization of Biologic Formulations. J Pharm Sci. 2022;111(4):919-32.
52. Mitra SP. Protein Adsorption on Biomaterial Surfaces: Subsequent Conformational and Biological Consequences—A Review. Journal of Surface Science and Technology. 2020;36(1-2):07-38.
53. Schubert J, Chanana M. Coating Matters: Review on Colloidal Stability of Nanoparticles with Biocompatible Coatings in Biological Media, Living Cells and Organisms. Curr Med Chem. 2018;25(35):4553-86.
54. 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. Materials Advances. 2021;2(4):1139-76.
55. Gao K, Oerlemans R, Groves MR. Theory and applications of differential scanning fluorimetry in early-stage drug discovery. Biophysical Reviews. 2020;12(1):85-104.
56. Moreau MJJ, Morin I, Askin SP, Cooper A, Moreland NJ, Vasudevan SG, et al. Rapid determination of protein stability and ligand binding by differential scanning fluorimetry of GFP-tagged proteins. RSC Advances. 2012;2(31):11892-900.
57. Statistical Repeatability of Tm Values Highlights Utility of Protein Stability Screening Automation United Kingdom: Protein Stable; 2024 [Available from: https://photophysics.com/knowledge-hub/statistical-repeatability-of-tm-values-highlights-utility-of-protein-stability-screening-automation/.
58. Sun C, Li Y, Yates EA, Fernig DG. SimpleDSFviewer: A tool to analyze and view differential scanning fluorimetry data for characterizing protein thermal stability and interactions. Protein Sci. 2020;29(1):19-27.
59. Gooran N, Kopra K. Fluorescence-Based Protein Stability Monitoring-A Review. Int J Mol Sci. 2024;25(3).
60. Kim SH, Yoo HJ, Park EJ, Na DH. Nano Differential Scanning Fluorimetry-Based Thermal Stability Screening and Optimal Buffer Selection for Immunoglobulin G. Pharmaceuticals. 2022;15(1):29.
61. Garidel P, Hegyi M, Bassarab S, Weichel M. A rapid, sensitive and economical assessment of monoclonal antibody conformational stability by intrinsic tryptophan fluorescence spectroscopy. Biotechnol J. 2008;3(9-10):1201-11.
62. Misra G. Chapter 3 - Fluorescence spectroscopy. In: Misra G, editor. Data Processing Handbook for Complex Biological Data Sources: Academic Press; 2019:31-7.
63. Hawe A, Sutter M, Jiskoot W. Extrinsic Fluorescent Dyes as Tools for Protein Characterization. Pharmaceutical Research. 2008;25(7):1487-99.
64. Fekete S, Beck A, Veuthey J-L, Guillarme D. Theory and practice of size exclusion chromatography for the analysis of protein aggregates. Journal of Pharmaceutical and Biomedical Analysis. 2014;101:161-73.
65. Hong P, Koza S, Bouvier ES. Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and their Aggregates. J Liq Chromatogr Relat Technol. 2012;35(20):2923-50.
66. Amartely H, Avraham O, Friedler A, Livnah O, Lebendiker M. Coupling Multi Angle Light Scattering to Ion Exchange chromatography (IEX-MALS) for protein characterization. Scientific Reports. 2018;8(1):6907.
67. Housmans JAJ, Wu G, Schymkowitz J, Rousseau F. A guide to studying protein aggregation. Febs j. 2023;290(3):554-83.
68. Dudure R, Joshi R, Pritam P, Panda

AK, Jadhao M. Probing the interaction and aggregation of lysozyme in presence of organophosphate pesticides: a comprehensive spectroscopic, calorimetric, and in-silico investigation. J Biomol Struct Dyn. 2023:1-15.

69. Zhang F, Richter G, Bourgeois B, Spreitzer E, Moser A, Keilbach A, et al. A General Small-Angle X-ray Scattering-Based Screening Protocol for Studying Physical Stability of Protein Formulations. Pharmaceutics. 2021;14(1).