Your guide to efficiently develop antibody-based therapeutics

How to measure stability, why it's important, and where it belongs in the workflow

About this eBook

The use of a monoclonal antibody (mAb) as a therapeutic has been around for over 30 years with the first approval by the FDA in 1986. The efforts to further develop and optimize better mAb-based biotherapeutics continue to rise. MAb-based therapeutics are becoming increasingly popular for the treatment and prevention of many diseases, including cancer and autoimmune disorders. In the “Quality by Design” approach for mAb-based therapeutics development, stability characterization—the probability of a protein unfolding or denaturing—is performed to ensure that structure and function are preserved throughout development and manufacturing. Antibodies can be adversely affected by extreme heat, cold, light, reagent concentrations, and many other factors during their journey to become a therapeutic. Stability characterization assays are pivotal, therefore, to help researchers to develop and determine the most appropriate formulations and storage conditions for each individual therapeutic.

In this eBook, we will present various techniques used to measure the stability of biotherapeutics, address the ins-and-outs of monoclonal antibody formulation, present a typical timeline for Investigational New Drug (IND) and New Drug Application (NDA) filing, and delve into how storage conditions affect mAb stability. Continue reading this eBook if you are interested in learning more about biotherapeutic characterization or tasked with performing storage and formulation studies.

1

Methods that measure the stability of biotherapeutics

Ensuring that a mAb or antibody fragment therapeutic will become an effective drug requires continuous monitoring of its stability throughout the development and manufacturing processes. MAb stability is affected by many factors, including storage temperature, protein structure, concentration in solution, exposure to light or heat, and storage buffer. Some of these factors, when encountered during process development and manufacturing, can cause protein aggregation and chemical or thermal unfolding of the biotherapeutic — which can lead to impaired function and an ineffective or even harmful drug when administered to patients. Here are some of the more commonly used methods to monitor conformational and colloidal stability of a protein.

Aggregation

Protein aggregation affects the safety and efficacy of a biotherapeutic. Higher-order aggregates, for example, potentially cause serious immune reactions through T-cell independent pathways. Aggregation can occur throughout the mAb manufacturing process, from detection of expression in early cell culture to when the therapeutic is on the shelf, ready for administration. Scientists must use analytical methods that are highly sensitive to detecting aggregation when performing stability studies such as size-exclusion chromatography.

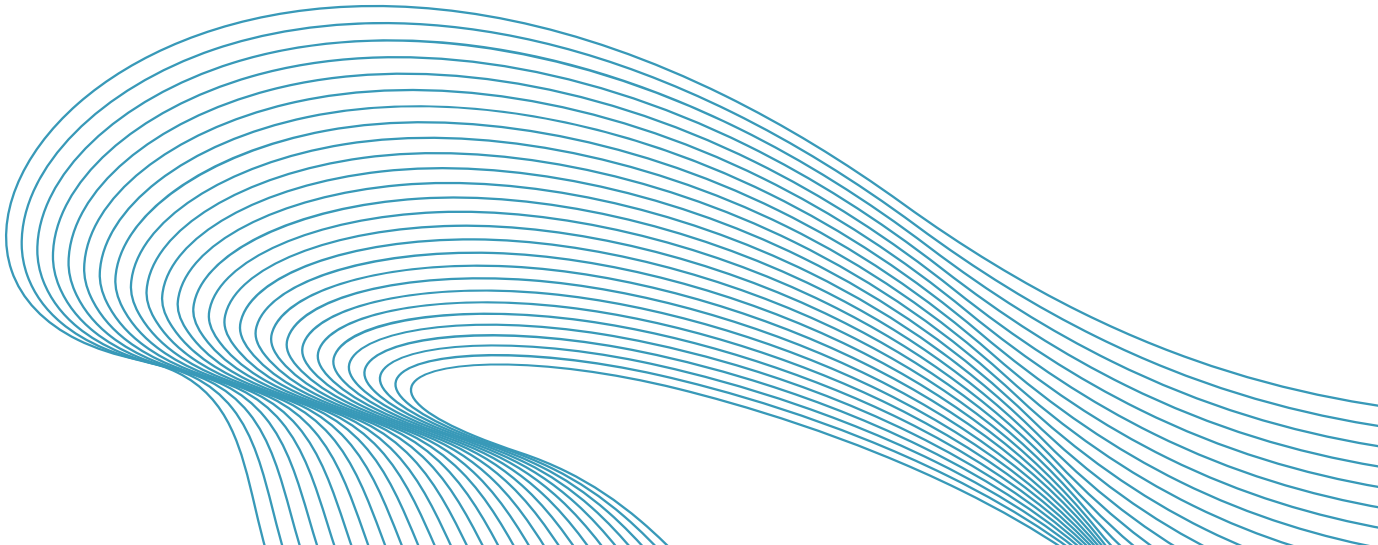
Size-exclusion chromatography (SEC) is used for the analysis and quantitation of soluble aggregates. It measures the presence of protein particles, dimers, and higher-order structures. SEC is usually performed using high-performance liquid chromatography (HPLC) or ultra-performance liquid chromatography (UPLC) with a size-exclusion column, using an absorption wavelength of 280 or 214 nm for the detection of the eluted fractions. Researchers commonly detect several peaks, with each peak representing protein particles, monomers, dimers, or higher order structures. Preferably, most mAb will be monomers as this represents a soluble and more stable protein state.

Thermal and chemical unfolding

Thermal unfolding experiments rely on forced degradation assays, where biotherapeutics are subjected to increasingly high temperature causing them to unfold. This process results in a prediction of the melting temperature (T_m) at which 50% of the mAb population is unfolded[1]. Chemical unfolding experiments work in a similar way, but use chemical denaturants such as guanidine hydrochloride or urea to fully denature proteins. From the unfolding curves, researchers can calculate the change in the Gibbs free energy of unfolding, ΔG, and the inflection point, $C_{1/2}$. Theoretically, the greater the ΔG, $C_{1/2}$, or T_m, the more structurally or conformationally stable the molecule[2]. Next are the most utilized techniques to measure thermal and chemical unfolding of biotherapeutics.

Differential scanning calorimetry (DSC) a microcalorimetry thermal unfolding technique that measures heat capacity as a function of temperature; it predicts thermal stability. Usually, researchers place a mAb solution into a fixed sample cell and a corresponding buffer into a reference cell. They then compare the heat capacity (Cp) signal from the sample cell to the reference cell. As temperature increases, the temperature difference between reference and sample cells is continuously measured and calibrated to power units. The technique is considered the standard for testing unfolding and stability, but requires large sample volume.

Differential scanning fluorimetry (DSF) measures mAb thermostability through



thermal unfolding. Researchers add an extrinsic fluorescence dye (usually SYPRO orange) to the sample and measure the fluorescence intensity of a polarity sensitive probe at gradually increasing temperatures to obtain transition temperatures of exposure of the hydrophobic regions of proteins (Th). The change in fluorescence intensity correlates directly with protein unfolding levels and temperatures[3].

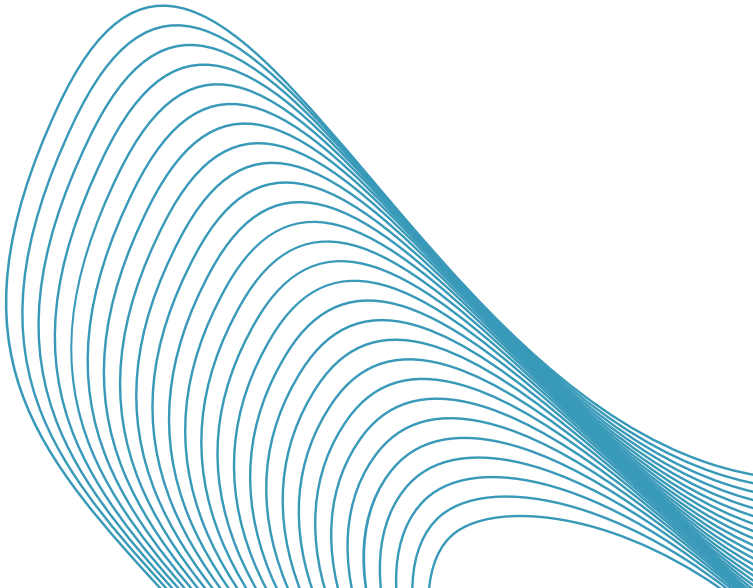
Circular dichroism (CD) measures thermal or chemical unfolding. It is a spectroscopic technique where the difference between the absorption of left-handed be εL and right be εR, circularly polarized light is measured during temperature or chemical increase. It can determine the van't Hoff enthalpy (ΔH) and entropy (ΔS) of unfolding, the midpoint of the unfolding transition (T_m or $C_{1/2}$), and the free energy (ΔG) of unfolding. Sample volume is high, and resolving the spectra of complex proteins can take several hours [4].

nanoDSF is a modified form of DSF that does away with the need to add an extrinsic dye. It measures the intrinsic fluorescence from tryptophan or tyrosine residues to get a readout to monitor either thermal or chemical protein unfolding. The fluorescence intensity ratios at 350 nm and 330 nm detect any changes in protein structure from thermal or chemical denaturation. With nanoDSF, scientists can get precise unfolding temperatures (T_m and T_{onset}), critical denaturant concentrations (Cm), and free folding energy (ΔG and ΔΔG), while using very little sample, which can accelerate the biotherapeutics development process.

2

What's in a formula? Developing antibody formulations with stability in mind

Protein stabilization during mAb manufacturing and long-term storage presents a challenge for pharmaceutical scientists. The antibody drug must be formulated to ensure that the proper dose of active antibody reaches the correct site of action in the body upon administration, and that the drug is stable during storage. Formulation development should be initiated as soon as product is available—even product from process development activities that may not fully represent the final process[1]. Generally, product is available 4-5 months after drug discovery; at this point pre-formulation development takes place, followed closely by clinical formulation development. Mixtures of several excipients (pharmacologically inert substances that may provide benefits when used with active ingredients) are generally required to stabilize mAb. Therefore, scientists must test several formulations when producing potential therapeutics using various pH conditions, ionic strengths, buffering agents, and stabilizing agents.



Stages of formulation development

During preclinical drug development (before Investigational New Drug [IND] filing), characterization and product testing must be defined to ensure that the final product is safe, effective, and consistent between batches. These activities are known as CMC: chemistry, manufacturing, and control. In general, CMC for mAb takes place over approximately 18 months. Activities include the following[5].

1. Analytical methods development
2. Cell line selection and optimization
3. Purification process development
4. Formulation development
    a. Pre-formulation development occurs approximately 4-5 months after drug discovery. Pre-formulation is the phase of research and development when researchers characterize a drug's physical and chemical properties.
    b. Clinical formulation development occurs after pre-formulation development, approximately 8-9 months after drug discovery. This is the phase when scientists finalize the formula that will be used in clinical trials.
5. Analytical assay qualification and transfer to Quality Control
6. Product characterization
7. Drug substance (DS) production
8. Preliminary DS stability studies
9. Preclinical drug product (DP) production
10. Preliminary DP stability studies
11. IND filing

Formulation factors

As mAb are usually administered by injection, they are commonly formulated as lyophilized powders for reconstitution, or as solutions for direct injection. Various excipients are required to stabilize mAb during manufacturing, processing, fill and finish, and storage. Appropriate excipients enable development of novel therapies and robust pharmaceutical products. When deciding which excipients to add to biologic formulations, researchers must take the following factors in these general ranges into account[6].

- Buffer (type and concentration): 5-100 mM
- pH range: from 4.0-8.0
- Stabilizer: e.g., cryoprotectants 1-10%
- Salt concentration: 0-300 mM
- Surfactant concentration: 0.01-0.1% (w/v)

Types of excipients

Excipients have several roles in mAb therapeutic formulations, from enhancing solubility, process, and shelf-life stability, to controlling pH and toxicity[7]. New excipients are always being considered; The International Pharmaceutical Excipients Council (IPEC)—an industry association that contributes to the development and harmonization of international excipient standards—provides guidance concerning excipients. Excipient categories to consider when developing mAb and other biologic formulations include[6]

- Buffers: Acetate, Succinate, Citrate, Histidine, Phosphate, Tris
- Amino acids: Arginine, Aspartic Acid, Glutamic Acid, Lysine, Proline, Glycine, Histidine, Methionine
- Stabilizers and bulking agents: Lactose, Trehalose, Dextrose, Sucrose, Sorbitol, Glycerol, Albumin, Gelatin, Mannitol, Dextran
- Surfactants: Tween 20, Tween 80, Pluronic F68
- Preservatives: Benzyl alcohol, m-cresol, Phenol, 2-phenoxyethanol
- Chelators: Ca^{2+}, Zn^{2+}, EDTA
- Others: Salts, Polyanions, Cyclodextrin-based excipients

During the formulation development process, researchers test several conditions at various time points and temperatures to achieve the final formulation.

3

Journey of a mAb through IND and NDA filing

Stability studies at the Investigational New Drug (IND) stage provide impurity profiles for investigational products used during non-clinical toxicological studies. These tests ensure that the quality and safety of the investigational drug is acceptable throughout the clinical trial period. At the New Drug Application (NDA) stage, stability studies establish appropriate retest or expiration guidelines, long-term storage conditions, and provide evidence of the effect of various environmental conditions on the quality of the drug substance (active ingredient) and drug product (finished dosage form).

For every 5,000-10,000 compounds that enter the research and development (R&D) pipeline, ultimately only one receives approval[8]

Year 1

Target discovery & validation - Hit screening, lead validation, and optimization

Year 2

Animal Pharmacology and Toxicology Studies - Preclinical data to permit an assessment as to whether the product is reasonably safe for initial testing in humans

High throughput, low volume requirement (e.g., nanoDSF, DSF, CD, SEC)

IND Investigational New Drug Application

Year 4

FDA: Center of Drug Evaluation and Research (CDER) evaluates the IND and allows clinical trials to proceed, or issues a "clinical hold" if more information is needed

Full complement of stability assays: (e.g., nanoDSF, DSF, CD, SEC

Clinical Trials
- Phase 1
- Phase 2
- Phase 3

Nearly 14% of all drugs in clinical trials eventually win approval from the FDA[9]

General stability requirements for IND

- Information sufficient to support stability of the drug substance during the toxicological studies and the planned clinical studies

NDA New Drug Application

Year 8-9

FDA: CDER reviews NDA to provide doctors and patients with the information they need to use medicines wisely

Full complement of stability assays

Year 10-11

The cost per single therapeutic developed and approved can range from $19 million to $5 billion[10]

Only 30% or less of initial drug candidates make it to approved NDA status

Approved or Not Approved

Post-marketing safety monitoring

Year ~30

Full complement of stability assays

Patent expires 20 years after approval

General stability requirements for NDA

- Minimum of 12 month (long-term) and 6-month (accelerated) for three primary batches
- Any available supporting stability data
- Appropriate statistical analysis, if needed
- Stability data on reconstitution/ dilution/admixing at initial and final time points
- Data from forced degradation and photostability studies
- Appropriate post approval stability commitments

4

In storage conditions we trust

Inappropriate storage conditions and exposure to extreme temperatures during manufacturing have a detrimental effect on an antibody's structure. Scientists in the pharmaceutical industry routinely test the stability of mAb after they have been stored at various temperatures for different lengths of time when developing new mAb therapeutics. Forced degradation studies test the effects of severe storage conditions on mAb; the assays test temperatures and chemical concentrations that are more extreme than a mAb might come across during storage or manufacture. Degradation testing is pivotal during biotherapeutics manufacturing; the assays generate degradation products caused by antibody unfolding and aggregation that can be studied to determine the stability of the molecule. Early stage stability testing often makes use of high-throughput assays, where a small number of focused variables are measured, such as aggregation, purity, and unfolding.

Going to extremes: mAb unfolding

Antibodies are composed of 6 to 70 immunoglobulin (Ig) fold domains, often connected by inter-domain disulfide bonds. Separated single immunoglobulin fold domains can be refolded through complex methods consisting of solubilization with detergents, removal of solubilizing reagents and, for disulfide-containing proteins such as antibody fragments, oxidation of sulfhydryl groups. Low concentrations of aggregation suppressors or folding-assisting agents are included in the refolding solvent to enhance refolding yield[11]. However, when pairs of domains become denatured, this is irreversible[12]. Having an understanding of the unfolding, solubilization, and aggregation properties of target candidates leads to better development of mAb biotherapeutics.

Each Ig domain starts unfolding at different temperatures. For example, the constant heavy chain 3 (CH3) domain of IgGs—such as monoclonal antibodies used as therapeutics—unfolds at a higher temperature than its CH2 domain. At certain temperatures, antibody solutions have a mixture of folded and unfolded structures. Co-existence of folded and unfolded domains in a single polypeptide chain may increase the tendency to aggregate, which inactivates the antibody[13]. IgG denaturation becomes irreversible at temperatures higher than 65 °C, and IgG almost completely loses its antigen-binding activity after heat treatment at 90 °C for several minutes[13].

Forced degradation studies

Forced degradation demonstrates the specificity of stability-indicating methods and also provides insight into degradation pathways and degradation products of the drug substance. The assays also help elucidate the structures of the degradation products and show the chemical behavior of the molecule. This, in turn, helps in developing formulation and packaging[12].

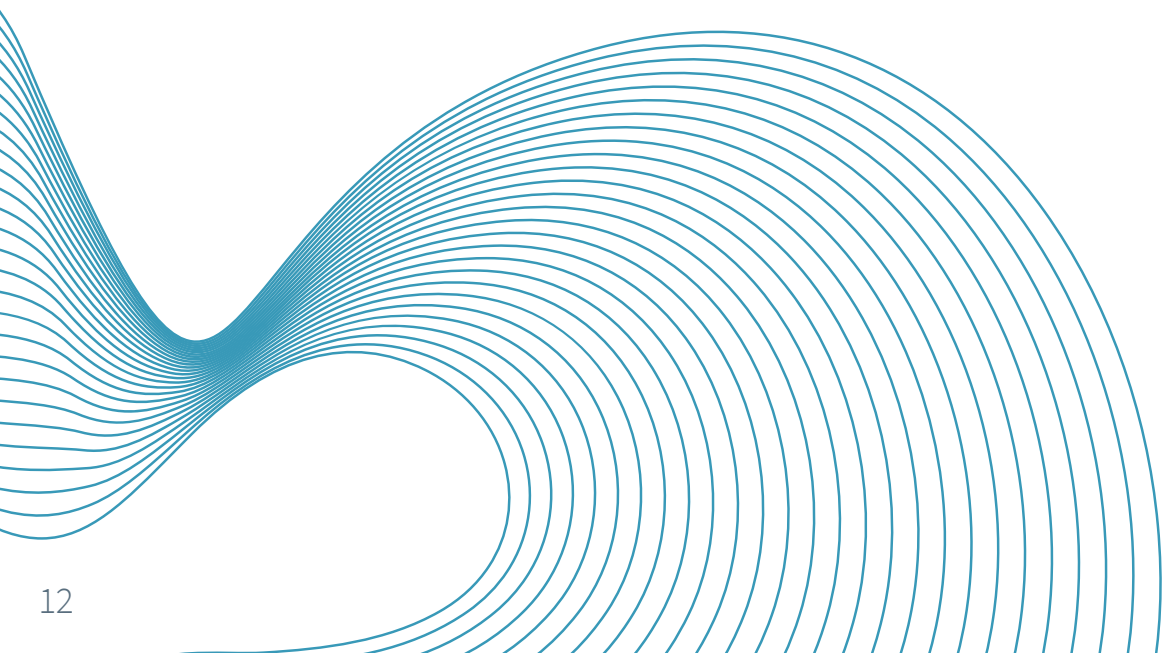
Forced degradation studies are performed on the drug substance (DS)—the active ingredient that is intended to furnish pharmacological activity or other direct effects in the diagnosis, cure, mitigation, treatment, or prevention of disease, or to affect the structure or any function of the human body, but does not include intermediates used in the synthesis of such ingredient [21 CFR 314.3]. They are also performed on the drug product (DP)—a finished dosage form, for example, tablet,

capsule, or solution, that contains a drug substance, generally, but not necessarily, in association with one or more other ingredients [21 CFR 314.3][14]. Forced degradation means degrading the new drug substance or product at conditions more severe than those typically encountered.

Commonly used forced degradation conditions include high temperature, freeze-thaw, agitation, high pH, low pH, light exposure, oxidation, and glycation. These are all conditions that may be present during processing, packaging, shipping, and handling. Although these conditions are relatively severe compared to real-life storage and manufacturing conditions, they bring about degradation products and elucidate degradation trends within a fairly short time period[14].

High-throughput assays for testing degradation

For clarifying the effects that various conditions have on antibody stability, researchers can use high-throughput testing. This is particularly useful in the early stages of mAb development when degradation studies may be constrained by limited material and time. High-throughput testing is also preferable for screening multiple drug candidates before lead candidate selection. Scientists should use methods that are sensitive to degradation products, including aggregation, deamidation, and oxidation products. Examples of high-throughput methods include capillary electrophoresis-SDS (CE-SDS) to measure purity, CE-isoelectric focusing (CE-IEF) to measure changes in charge profile, and nanoDSF to measure aggregation and unfolding[14].



Conclusion

To date, over 79 therapeutic mAb have been approved by the US FDA as marketable drugs[15]. The research devoted to developing more effective mAb-based biotherapeutics continues to grow. In addition to supporting the identification and discovery of potential new targets, it is equally important to monitor the formulation for stability and aggregation of the mAb target molecules early on in the drug development process. While there are many methodologies available to monitor stability, it is important to select the one(s) that best address your needs. Some key features to consider are sample size, the addition of an extrinsic reagent, the time to results, throughput needs, and assay costs. Additionally, proper formulation needs to be optimized to keep the drug product stable during storage.

Collectively, selecting the best methodology, along with a clear understanding of the various factors that impact stability, helps to accelerate the mAb development process and bring more effective biotherapeutic drugs to patients sooner.

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