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Physicochemical stability of monoclonal antibodies: a review

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ABSTRACT

Monoclonal antibodies (mAbs) are subject to instability issues linked to their protein nature. In this work, we review the different mechanisms that can be linked to monoclonal antibodies instability, the parameters and conditions affecting their stability (protein structure and concentration, temperature, interfaces, light exposure, excipients and contaminants, and agitation) and the different analytical methods used for appropriate physicochemical stability studies: physical stability assays (aggregation, fragmentation and primary, secondary and tertiary structure analysis), chemical stability assays and quantitative assays . Lastly, data from different published stability studies of mAbs formulations, either in their reconstituted form, or in diluted ready to administer solutions, was compiled. Overall, the physicochemical stability of mAbs is linked to numerous factors such as formulation, environment and manipulations, and must be thoroughly investigated using several complementary analytical techniques, each of which allowing specific characterization information to be harvested. Several stability studies have been published, some of them showing possibilities of extended stability. However, those data should be questioned due to potential lacks in study methodology.

KEYWORDS

Physicochemical stability; monoclonal antibody; drug; biopharmaceutical; protein.

ABBREVIATIONS

Asp: Aspartate
AUC: Analytical ultracentrifugation
AFM: Atomic Force Microscopy
CD: Circular Dichroism
CDR: Complementary Determining Region
CEX: Cation Exchange Chromatography
CE: Capillary Electromigration
CIEF: Capillary Isoelectric Focusing
CZE: Capillary Zone Electrophoresis
CGE: Capillary Gel Electrophoresis
DNA: DesoxyriboNucleic Acid
Fab: Antigen binding fragment
Fc: Crystallizable fragment
FT-IR: Fourier-transformed Infrared
HOS: Higher Order Structure
ICH: International Council for Harmonisation
INN: International Nonproprietary Name
IV: Intravenous
mAb: Monoclonal Antibody
MALDI: Matrix-Assisted Laser Desorption Ionization
MS: Mass Spectrometry
PAGE: PolyAcrylamide Gel Electrophoresis
Ph. Eur.: European Pharmacopeia
pI: Isoelectric point
PMF: Peptide Mass Fingerprint
PVC: Polyvinylchloride
RPLC: Reversed-Phase Liquid Chromatography
SDS: Sodium Dodecyl Sulfate
SEC: Size-Exclusion Chromatography
TOF: Time Of Flight
Tm: Thermal unfolding temperature
UV: Ultraviolet

1 Introduction

The use of biopharmaceuticals has drastically expanded since the 80s with the development of recombinant DNA technology[1]. Monoclonal antibodies (mAbs) are a major class of biopharmaceuticals with indications now covering a large panel of diseases, from cancer to asthma, including central nervous system disorders, infectious and cardiovascular diseases. Monoclonal antibodies are immunoglobulins (or fragments of immunoglobulins) with a precise target, produced from a single cellular clone[2]. They are proteins composed of 4 chains – two light chains and two heavy chains –linked together with disulfide bridges. In those chains can be found two different types of domains: constant or variable; the three complementary determining regions found in each variable domain are responsible for specificity of antibody binding to its target. Finally, the whole quaternary structure can be divided into three fragments: two antigen-binding fragments (Fab, corresponding to the "arms" of the antibody) containing one light chain, one variable heavy domain and one constant heavy domain, and one crystallizable fragment (Fc, corresponding to the "base" of the antibody) containing the rest of both heavy chains[3]. Figure 1 summarize those different structures.

Antibodies are divided into 5 isotypes (IgG, IgA, IgM, IgE and IgD), different in structure and function. The IgG isotype can be further divided into subtypes, differing in the number of disulfide bonds, especially in hinge region. IgG_1 is the most commonly used subtype for drug manufacturing, although IgG_2 and IgG_4 may be found (IgG_4 subtype, for example, being used in the design of immunotherapies for its different anti-inflammatory mechanisms)[4,5].

Once their patent expires, therapeutic proteins become open to development and manufacture by other companies. However, the term "generic" is inappropriate, as the new product wouldn't be produced by the exact same cell line, and therefore cannot be duplicated identically. For example, there may be post-translational differences (glycosylation) or altered higher order structures (HOS). The term "biosimilar" is preferred, short for "similar biological medicinal product", and is defined by the European medicine agency as "a biological medicine highly similar to another biological medicine already approved in the EU (called 'reference medicine') in terms of structure, biological activity and efficacy, safety and immunogenicity profile (the intrinsic ability of proteins and other biological medicines to cause an immune response)"[6], the U.S. Food and Drug Administration having a similar definition[7]. The approval requirements for marketing include comparability studies with the reference biological medicine, both analytical (physicochemical and biological) and clinical (pharmacokinetics, pharmacodynamics, safety, and potency)[8]. After approval and marketing, other indications may be extrapolated from the reference biological medicine only based on extensive justification of comparability and without further clinical studies[8]. However, extrapolation of clinical data can be challenging, especially in cancer indications[9]. Likewise, complex proteins like mAbs unavoidably have

microheterogeneities and contain co- and post-translationally modified variants, which in combination with possible excipient changes and different manufacturing conditions make extrapolation of stability data a complex process[10].

During therapeutic antibody production via mammalians cells such as Chinese hamster ovary, a multitude of parameters can be the cause of unwanted modifications, such as variability of the cell line, the number of cell subcultures performed over a time, cell passages over a time or environmental cell culture conditions[11]. Post-translational modifications, like glycosylations (eg. N-linked glycosylation) can alter biological activity[12], as for example altered fucosylation levels impacting antibody-dependent cell-mediated cytotoxicity activities[13], as well as mAb stability[14]. For example, Mimura *et al.* found that it may play an important role in thermal stability of mAbs[15], while Gaza-Bulseco and Liu showed that oligosaccharides reduced the fragmentation rate at a pH of 4, but not between 5 and 9[16].

Later in its life, a mAb may encounter various situations at risk of causing instability, like during repackaging, accidental freezing or just normal dilution in intravenous (IV) bags and administration through IV-lines[17]. Also, in certain situations, based on published stability studies, users may wander from the summary of product characteristics recommendations concerning diluted bags and reconstituted vial conservation time. However, this should be a careful practice, as the reliability of those stability studies is uncertain if they do not explore every aspect of protein stability, and their conclusions may not be transposable to every situation, being then at risk of mAb instability. Clinical consequences of such events are still under investigation, especially with regards to the immunogenicity potential[18], and available data is not always reassuring, as several publications have shown an enhanced immune response due to aggregates, even though the relationship is not fully understood and seems dependent on the degradation pathway[19–22].

Despite there being many excellent reviews on the overall subject of protein stability[23–28], the objective of this work is to propose an up-to-date review about mAb stability and a compilation of data from different published stability studies of mAbs, either in their reconstituted form, or in diluted ready to administer solutions, in order to give readers a comparative overview of post-marketing stability studies and to point out their potential lack of compliance to recommendations for protein stability studies through a gradation proposal. For a complete comprehension, we will first briefly describe the different instability mechanisms mAbs are subjected to and the parameters and conditions affecting their stability, before going into a description of the different analytical methods used for appropriate physicochemical stability studies, and eventually present the compilation itself. It is to be noted that, despite being an important subject, microbiological considerations will not be addressed in this paper.

2 Mab instabilities mechanisms

Protein degradation can result from many different instability mechanisms that can be divided into chemical and physical instabilities. Those instabilities are closely intertwined, as chemical reactions can lead to physical instability[29] and physical instability may give access to chemically susceptible residues or close the gap between residues that may interact[28], even if it hard to know what was the original cause of instability. For example, Luo *et al.* showed the presence of several chemical modifications in aggregates, however they didn't conclude about wither those modifications existed or not before aggregation[30].

2.1 Chemical instabilities

Oxidation, including disulfide bond formation, is one of the most frequent chemical degradation. It may happen in presence of oxidants (like peroxides, light or metals) or without, then referred to as auto-oxidation[28]. Some residues are particularly susceptible to oxidation, including methionine, histidine and cysteine residues[28,31]. Disulfide bond formation is one of the consequences of cysteine oxidation that occurs between two oxidized free residues, with a thiolate anion intermediate[24]. The formation of those bridges may be intra or intermolecular, and is enhanced in basic environment[28].

Another major chemical degradation process of proteins is deamidation, affecting mostly asparagine and, in a lesser extent, glutamine residues. It is an acid-base reaction, facilitated by the presence of specific nearby residues that may act as proton donors (threonine or serine for example), that results in the formation of a cyclic imide intermediate that can cause a distortion in the polypeptidic structure[25,32,33]. In the case of Asparagine, the succinimide intermediate spontaneously hydrolysed into Aspartic acid or Isoaspartic acid[34].

Fragmentation in mAbs may occur on disulfide bonds or peptides[28]. Disulfide bond disruption results in full chain fragments (eg. "one-arm" mAb, free light chain)[28]. Peptide bond cleavage results in low molecular weight species of different nature and size, and may be caused by enzymatic or non-enzymatic mechanisms. Due to its flexibility and accessibility, the hinge region is especially susceptible to cleavage, even if the instability mechanisms are incompletely characterized[28,33]. For example, Cordoba *et al.* studied mAb cleavage on the papain site in the hinge region, but found it to be unchanged by the addition of protease inhibitors, thus concluding to a non-enzymatic mechanism[35]. Asparagine and aspartic acid residues seem to be particularly

susceptible to spontaneous hydrolysis, possibly through the succinimide intermediate. However, this degradation pathway should only be observed in conditions not normally encountered during a therapeutic mAb product lifetime (highly acidic conditions and high temperature)[28,31] and prevented by appropriate formulation[31].

Sugars are used in mAb formulation as stabilizing excipients, as well as dilution solvent in IV bags (5% dextrose). Glycation, also known as Maillard reaction, occurs between reducing sugars and proteins, through the formation of a Schiff base that may undergo an Amadori rearrangement forming a stable ketoamine, affecting protein's structure and function and responsible of a browning[24,36]. It may happen at several times during mAb's lifetime, from cell culture production to administration[37]. As for excipients, non-reducing sugars are now mostly the only ones used. However, reducing sugars may still be found as degradation products from non-reducing sugars[38].

The impact of chemical modifications in mAbs highly depends on their location[28,39]. For example, deamidation occurring in the Fc fragment may have only few effects, while if located in the CDR region of Fab fragments, it may cause a reduction in binding affinity and mAb potency[39]. Oxidation may have the same consequences, and may also reduce binding affinity to FcRn if located in the Fc fragment, reducing affinity for macrophages or increasing mAb clearance[31,40]. Also, several studies have shown that chemical instabilities can lead to conformational modifications and aggregation[41]. For example, Burkitt *et al.* showed that methionin oxidation was suscptible to destabilise secondary structure[42].

Chemical modifications of mAbs can result in charge heterogeneity by changing their isoelectric pH (pI) values. An increase of overall negative charges (decreasing pI values), as seen with deamidation[31], results in acidic variants, while an increase of overall positive charges (increasing pI value), as seen in oxidation or succinimide formation, results in basic variants. Major modifications in pI (one unit or more) may be responsible of an alteration of pharmacokinetics[43]. Interestingly, several studies have showed that an increase in pI may provoke a decrease in a mAb's serum half-life, partly from an increase in tissue uptake, and seems to alter subcutaneous bioavailability[44,45]. On the other hand, a decrease in pI seemed to be responsible for an overall increase in mAb's whole-body clearance[45].

2.2 Physical instabilities

Protein denaturation refers to the loss of higher order structure through unfolding. It may result from previously described chemical instabilities, or from environmental conditions such as extreme temperatures or pH. The consequences of unfolding can be direct perturbation of the mAb's function, for example a decrease in hinge flexibility, or promotion of aggregation[28].

Aggregation is the main physical instability[46]. It is the assembly from initially native and folded proteins into high molecular weight species (multimers), regardless of their size or the nature of bonds linking them together[29]. Aggregates can be formed solely from weak non-specific bonds (Van der Waals interactions, hydrogen bonding, hydrophobic and electrostatic interactions) with no change in primary structure and the phenomenon is called physical aggregation or self-association, or contain covalent bonds, including disulfide bonds, and is then called covalent aggregation[24,29]. Both mechanisms can lead to the formation of soluble aggregates or insoluble precipitating aggregates.

Aggregation is often irreversible, especially at later stages, and aggregates often contain high levels of proteins with a non-native conformation[24,27,47]. Irreversible aggregation is a multistep process that can be described by the Lumry-Eyring model (equation 1), however other aggregation pathways exist such as described by Uchiyama[27].

Equation 1, Lumry-Eyring model (N : Native ; U : Unfolded ; D : Deactivated):

$$N \leftrightarrow U \rightarrow D$$

The native protein first undergoes a reversible conformational change to a transition state of higher free energy that can change back to the native protein or form an intermediate more susceptible to aggregation[29,47], due to a decrease of free energy after every new aggregate formed[24]. Also, unfolding reveals the hydrophobic residues that are mostly hidden in the native conformation, reducing solubility in hydrophilic buffer and increasing subsequent self-association then aggregation[48]. However, the unfolding of mAbs occurs one domain after the other, as a multistep process. The Lumry-Eyring model still applies, but there may be several intermediate states between native and unfolded state[27].

Aggregation is suspected to cause a stronger immunogenicity of protein therapeutics in patients[24,49]. Two pathways have been proposed: presentation to T-helper cells (T-cell dependent response) and activation of cytokines (T-cell independent response), both by cross-linking with B-cell receptor, and both inducing B-cells proliferation and antibody production[50,51]. The capability to create an immune response seems to increase with the aggregate size, but is also impacted by the degree of glycosylation, origin of the product and presence of contaminants[52]. Immunogenicity can lead to the neutralization of the mAb, implying a loss of efficacy, but also to cross-reactions with an endogenous counterpart, leading to IgE-mediated immediate hypersensitivity and anaphylaxis[52]. However, actual models are only *in vitro* or preclinical (mice) and only predictive of the risk of immunogenicity of protein aggregates in human, and the exact mechanism needs to be elucidated[53].

As we discussed above, in certain conditions aggregates may precipitate and form insoluble particles. These particles may consist of proteins alone, or be heterogeneous (containing excipients, leachables, contaminants). Parenteral drug solutions, mAbs or not, should be practically free from visible particles[2,54], and limits for subvisible particles have been set for particles above 10 µm and 25 µm[55].

As it will be discussed in the following sections, many factors can impact both physical and chemical stability, and formulation and usage of the right excipients (some protecting the mAb) is a tremendous challenge for manufacturers. Also, different mAbs may have different sensitivity to different stresses, some having globally low vulnerability compared to others[56].

3 Stability influencing factors

3.1 Protein structure

All structure levels of proteins have their impact on its stability. The amino acid sequence (primary structure) gives an important clue on whether a protein will be prone or not to aggregation. For example, a low isoelectric point (pI) of CDR seems to promote soluble aggregates formation by enhancing electrostatic interactions between mAbs, whilst a high pI of CDR leads preferentially to insoluble aggregates, especially if in contact with negatively charged surfaces[57].

Subtle variations in mAb sequence and structure can also greatly influence stability when exposed to stress conditions. Pisupati *et al.* compared the behavior of infliximab reference product (Remicade®) and biosimilar (Remsima®) in a forced degradation study and found it to be similar, despite small differences in products' profiles and manufacturing processes, concluding that the primary sequence was the main factor impacting stability[58]. Moreover, Skamris *et al.* showed that three model mAbs with identical variable regions, representative of IgG1, IgG2 and IgG4 subclasses, behaved differently after low pH treatment (pH = 3.3) and subsequent neutralization. Whilst IgG1 remained monomeric, IgG2 and IgG4 exhibited a two-phase oligomerization process, which lead to aggregation for IgG4 after return to normal pH[59]. Such a behavior could be linked to an increased flexibility of the hinge region, shielding access to the hydrophobic patches. IgG2 and IgG4 oligomerization was attributed to the initial unfolding in the Fc region induced by the acidic treatment, which causes the exposure of residues or patches that are otherwise covered. The results are in accordance with earlier findings which classified different subclasses of IgG according to their aggregation potential (IgG1 < IgG2 < IgG4) for a pH range of 4 to 7[60]. In addition, attachments (such as glycans) to residues, especially the aggregation prone ones, may reduce the aggregation potential of the whole mAb[61].

Tertiary structure can have a tremendous influence on aggregation, depending on the degree of unfolding, since several studies showed that partially unfolded proteins are more prone to aggregation than both native and completely unfolded proteins[24,47].

3.2 Protein concentration and self-association potential

High protein concentrations have been shown to impact aggregation. Higher protein concentrations also seem to increase the viscosity of solutions, which itself may increase the aggregation potential of proteins by enhancing protein-protein interactions and self-association[27,62]. This concentration-dependent tendency to aggregation is an increasing concern considering the extended use of subcutaneous administration of mAbs which require highly concentrated solutions. However, the real impact of high protein concentrations is complex, as can be seen, for example, in the work published by Hauptmann *et al.* where they showed that high concentrations increased smaller particles concentrations while decreasing bigger ones[63], while Nicoud *et al.* showed an increase in aggregation rate with concentration[64]. However, other studies have reported more mitigated results, for example where increasing protein concentration decreased the rate of aggregation[24].

A decrease in protein concentration can lead to dissociation of weakly bonded aggregates[29,65]. However, increasing the dilution without modifying the excipient to mAb ratio decreases the excipients concentration, including the protecting ones (such as for example surfactants, sugars and arginine (see infra)), and modifies the electrolytic composition (influencing pH and ionic strength), which may decrease the chemical stability[17].

Independently from any other factor, proteins have a certain propensity to self-associate, which has been shown to be particularly implicated in aggregates growth[66]. This phenomenon seems to be governed by electrostatic interactions end by dipole moments between protein surfaces[67], and might engage preferentially Fab-Fab interactions[68]. Self-association highly impacts solution viscosity and is dependent on the ionic strength of the solution[68]. Yadav *et al.* also showed that viscosity may be maximized at pH near the isoelectric point of the protein, as charges are then reduced and repulsive electrostatic interactions decreased[69]. Self-association potential may be predicted by the osmotic second virial coefficient (measure of the solution non-ideality) and by the diffusion interaction parameter (quantification of intermolecular interactions, component of the osmotic second virial coefficient), which can be determined by several techniques (including dynamic light scattering (DLS) and self-interaction chromatography)[70]. If strongly negative, the osmotic second virial coefficient may reflect a higher probability

for the protein to form multimers as negative values of this coefficient show the existence of net attractive forces between solute molecules present[71,72]. Likewise, zeta potential can be a good indicator of the surface charges that may lead to electrostatic and van der Waals interactions[62].

3.3 Temperature

MAbs, like other therapeutic protein, can be exposed to temperature variations during their processing, storage and transportation[24,49]. High temperatures can perturb the native protein conformation to a sufficient degree to promote aggregation, but it starts at temperatures well below the equilibrium melting temperature (Tm) of the protein. The aggregation rate is increased at high temperature, as other protein reactions[47]. Heat induced unfolding usually leads to irreversible conformational changes[29,49]. High temperatures also accelerate chemical reactions, such as deamidation and oxidation[24,29].

Each and every protein has a specific Tm, which is the temperature where 50% of proteins are unfolded. This temperature is in most cases between 40 and 80°C and manufacturers generally recommend storing biopharmaceuticals between 2 and 8°C, well below this Tm[29].

Low temperatures can also induce protein denaturation, especially during freezing and freeze-thawing cycles, linked to the combination of multiple stress factors (drop of buffer pH due to crystallization, solute molecules cryoconcentration, water-ice interface formation…), affecting both colloidal and conformational stability of proteins[73]. Freezing mAbs solutions may happen willingly, for example for freeze-drying[74] or bulk storage[75], or accidently during refrigerated storage. Cold caused unfolding and aggregation is usually reversible, as the mAb mostly stays in a native conformation[24,49]. However, the number of freeze-thaw cycles seems to have an impact on the aggregation potential of mAbs, as well as the mAb concentration[76–80]. For example, when 0.5 mg/mL bevacizumab solutions were subjected to 1 to 30 freeze-thaw cycles, the bevacizumab monomer peak (analysed by size exclusion chromatography (SEC)) decreased with an increasing number of cycles. In the same study, for a fixed number of freeze-thaw cycles (10 cycles), the monomer peak decreased with bevacizumab concentration, indicating an improved stability to freeze-thawing cycles at higher concentrations[76]. On the other hand, Rayfield *et al.* showed an opposite result with aggregation increasing with concentration. They also demonstrate the importance of formulation in preventing aggregation from freeze-thawing[80] (among other factors, as will be presented in section 3.6).

Heating or cooling rate are also important factors, as extreme rates seeming to lead to instability[74,79,81]. However, mAbs have a good stability and resistance to moderate thermal stress, when compared to other proteins[23,32]. This fact is of crucial importance in case of accidental short-term temperature excursion, justifying a safe use of the exposed products. As another

example, lyophilisation processes with a high freezing temperatures (ie. near the equilibrium freezing point) seem to have a protective effect against further stresses when compared to lower freezing temperature, as the cooling rate is decreased[82].

3.4 Interfaces

Proteins are surface active molecules, and have a tendency to adsorb to hydrophobic surfaces and interfaces. Throughout their life, they encounter those in many different forms[83,84].

The adsorption phenomenon is especially relevant when the preparation is highly diluted and/or when the contact surface is important. Contact of the drug with various surfaces can affect stability and drug recovery and can lead to unacceptable product loss, decreased potency and potential under-dosing[81,85]. Adsorption has been shown to be concentration-dependent with saturation occurring at some point. It is function of various other parameters like the number of protein layers, pH and ionic strength, but when it occurs, desorption of adsorbed mAbs can lead to denaturation and aggregation[86,87]. Focusing on the hydrophobicity of surfaces, Couston *et al.* demonstrated that mAbs predominantly adsorbed onto hydrophilic surfaces, but that their secondary structure was less altered than with hydrophobic surfaces[88].

These interactions can be observed whatever the container material is. Type I glass, the most used primary packaging material in vials, and a relatively inert one, can adsorb relatively large amounts of proteins during the shelf life of a drug, mostly due to electrostatic interactions[84]. Glass and silica microparticles have also been shown to cause adsorption of humanized mAbs to their surface, in about 4 nm thick layers, as visualized by atomic force microscopy (AFM), but without mediating any changes in secondary structure[89], which is consistent with earlier studies and studies with other proteins[90,91]. Interactions with glass can also cause analytical problems, as adsorption to the glass walls of certain chromatography vials has been shown to influence analytical reproducibility during size exclusion chromatography[92]. Interestingly, such a phenomenon seems to increase in importance with the concentration of the analyzed mAb solutions (minimum impact at concentrations lower than 5 mg/mL) which is contrary to usual content-container interactions (higher impact for lower concentrations, as for a same amount of adsorption the higher the overall adsorption percentage will be), and also to the shape of glass vial used (and maybe the nature of the glass). The material used for the IV bag delivery can also impact concentration of the mAb monomer and particle formation. Kumru *et al.* showed that incubating 1 mg/mL IgG4 solutions in either polyvinylchloride (PVC) or polyolefin IV-bags for 1 to 6 hours caused an up to 25% decrease of mAb concentration for the solutions stored in the PVC bags and increased sub-visible particle formation and overall turbidity, when compared to polyolefin bags. The addition of polysorbate 20 greatly reduced particles in both IV-bags, with a particle count near the negative control[93]. The same observation was made in

another study by Sreedhara *et al.*[17]. However, the effect of polysorbate 20 alone was not evaluated. Indeed, polysorbates are known to increase plasticizers leaching from PVC, which may interact with proteins to form heterogeneous particles[94]. In another study, repackaged bevacizumab solutions in plastic syringes (no precise data on the exact nature of the plastic is available) from three different American compounding pharmacies were compared to solutions obtained directly from the original vial. Total immunoglobulin content varied significantly between samples from the different compounding pharmacies and from the original solution, with the concentration from one sample have a 50% loss in detectable IgG content[95]. That sample also contained the highest amount of particles, the biggest reaching a diameter of nearly 19 µm. These phenomena are particularly important in drug delivery systems, but can also be observed during the manufacturing process or during the analysis time, for example onto chromatographic columns of multiple natures[87]. Lastly, Zhang *et al.* quite recently showed the interaction of a mAb's Fab fragment with a rarely studied material, polystyrene, forming a single protein layer at the surface[96].

Proteins may adsorb at the air-water interface, forming layers, because of the relative hydrophobicity of air compared to water[24]. It has been shown than a rupture of these layers leads to the formation of protein aggregates in the solution, linked to surface tension forces at the interfaces perturbing the protein structure[83]. Also, Sreedhara *et al.* showed that removing headspace from IV-bags decreased agitation-induced aggregates formation independently of polysorbate concentration[17].

Organic substances are often used in pharmaceutical compounding and material manufacturing. There are a lot of publications reporting interactions between those substances and proteins. An example of an organic product that may interact with the solution is silicone oil, used as a lubricant for syringe plungers and vial stoppers. The adsorption of proteins onto the silicone oil phase can lead to the formation of viscoelastic gel layers stabilized by many noncovalent interactions. This phenomenon has been shown to be faster with the protein concentration increasing. If the gel is ruptured by mechanical stresses, aggregates and sub-visible particles are to be released in the solution. Those particles are of irregular morphologies and sizes and generally insoluble[83,97]. Thirumangalathu *et al.* observed that silicone oil was responsible of monolayer adsorption of mAbs at the interface, but principally as an enhancing factor, needing another destabilization parameter (eg. agitation or unoptimal pH) for a significant loss of monomer to occur. Also the addition of surfactant suppressed agitation-induced monomer loss with silicone oil[98]. In another study, Lubiniecki *et al.* only detected a small raise in particles over 10 µm in silicone oil lubricated prefilled syringes when compared to in vial product[99]. However, depending on the container material, silicone oil coverage can be a protection towards adsorption to highly reactive surfaces[100].

MAbs solutions may interact with in-line filters in several ways. If aggregates of sufficient size are present in solution, they will be trapped in the filter, decreasing the dose administered to the patient, and possibly clogging the filter[101], but overall adsorption to filters should not be a problem, except in some cases specified by manufacturers[101]. However, Besheer showed that

several mAbs adsorbed to positively charged polyethersulfone and positively charged polyamide when diluted to 0.01 mg/mL in 0.9% sodium chloride. Interestingly, the use of 5% glucose reduced protein adsorption to the previously cited filters, and with only the neutral PES filters tending to adsorb the proteins under the investigated conditions[102]. It must be however noted that the high dilution factor applied (of 2500 to 15000) makes extrapolation to clinical conditions very difficult.

In a drug formulation, mAbs are not the only substance that may undergo ad- or absorption. This phenomenon may affect excipients (function of their physicochemical characteristics and therefore their affinity for the surfaces), and depending on their role in the formulation (buffer, surfactant, preservative, other stabilizers,…), a loss in their concentration may lead to a condition where requirements for mAbs' stability are not met anymore.

Beside the adsorption phenomenon, every surface the solution gets in contact with is highly susceptible to release foreign substances or even particles into the solution, especially when subjected to a mechanical coercion. Other stress conditions (eg. temperature variations, photodegradation) may favour leaching from materials. In addition to the potential toxicity of such leachable for patients, this causes an increase of adsorption-competent surface[90].

A recent development in preventing protein interactions with surfaces has been coating those surfaces, also called surface passivation. Several polymers (eg. Oligo(ethylene glycol) and poly(ethylene oxide)), techniques and architectures have been used. Coatings can be divided into two groups: monolayer coatings (the most studied) and multilayer coatings (less controllable)[84,103]. Also, it seems that using polymers that have a reduced number of hydrogen bond donors and an increased number of hydrogen bond acceptors, and that are polar and neutrally charged leads to a decrease in protein adsorption[104,105].

3.5 Light

Protein medications are exposed to light at many points during their life. For example, during production they are generally purified by column chromatography and then are exposed (briefly) to the ultraviolet (UV) light of the detector, but the main exposure to light occurs during storage and during administration to patients with IV bags. The exposition may be even more important if the primary container isn't stored within an opaque secondary container. Proteins, especially their aromatic residues, are very sensible to light, inducing photodegradation, mainly through photooxidation and formation of oxygenated radicals, but also fragmentation and crosslinking[106]. Antibodies, containing a large number of aromatic

residues (especially tryptophan residues), are particularly sensible to this phenomenon, confirming the greater impact of UV light when compared to white light. On the other hand, light exposure doesn't seem to directly alter mAbs secondary and tertiary structures[107]. Liquid forms also seem to be more sensible than lyophilized forms[108].

When exposing high concentration (100 mg/mL) IgG solutions to intense light (30 to 78 hours under 8000 lux white fluorescent light, or under International Council for Harmonisation (ICH) Q1B conditions), a yellow coloration appeared, increasing with exposure time, and which wasn't present when only the formulation buffer was exposed. Overall, photoexposition decreased purity, reduced monomer proportions, oxidized multiples amino acids and in the worst cases reduced biological activity to 30% of reference standard activity[109]. However, these light exposure conditions are very harsh and do not reflect the reality of mAb exposure to light during manufacturing and use[110]. Recently, Sreedhara *et al.* confirmed the photoinstability of 5 different mAb formulations after exposure to ICH lighting conditions, reporting solution coloration, formation of high molecular weight species in proportions superior to 10% and photo-oxidation of amino acids (generally Trp and Met) in the Fc or Fab regions. When exposed to milder conditions, similar to the ambient lighting encountered during the mAb formulation process, 2 of the tested mAb formulations showed only limited modifications, whilst the other 3 formulations remained unchanged and stable. As such, the authors conclude that ambient light stability studies would be more relevant stress models than ICH conditions for mAb photostability testing[111]. Luis *et al.* assessed the photostability of two mAbs through both the ICH Q1B recommendations (1.32 million lux hours) and custom ambient light exposure assays (0.24 million lux hours), and exhibited drastic differences concerning mAbs degradation. They also showed that mAbs photostability depends on the overall amount of light exposure rather than light intensity[112].

Eventually, it is not to be forgotten that not only the mAb, but also the excipients in formulation may undergo light induced degradation. Photodegradation of polysorbates occurs through autoxidation and can lead to the formation of peroxides that intensify oxidation processes[112–115]. Photo-oxidized histidine may crosslink with a non-oxidized histidine residue, forming a protein-buffer adducts[106].

Concerns about photodegradation might however decrease due to the spread of light-emitting diode lighting. Indeed, the intensity of the UV radiation emitted by this kind of lighting is far weaker that what is observed with fluorescent lights. Associated with UV-blocking on windows, this may enhance security regarding drugs light exposure, especially for mAbs[116].

3.6 Excipients

Proteins are often stable against aggregation over narrow pH ranges and may aggregate rapidly in solutions with pH outside these ranges. Solution pH (controlled by the use of pH buffers) determines the type (positive or negative) and total charge on the protein, thereby affecting electrostatic interactions[24,29,47]. Lower pH may lead to protein cleavage and isomerization when higher pH may favour deamidation and oxidation reactions, both increasing the aggregation potential[29,117,118]. Brummitt *et al.* studied the aggregation mechanism of an IgG1 (pI = 9) with different buffer pH. They found aggregation to be mostly caused by monomer addition at low pH, but to evolve as the ph increases to become more of a condensation of the previously formed aggregates, leading to phase separation[119]. Concerning mAb fragmentation, it has been noticed that certain pH ranges (pH = 5-6) are generally more protective against fragmentation than higher pH ranges (pH = 7-8), but can also depend on the type of residue involved in the fragmentation process (for example Asp residue fragmentation will occur principally for a pH lower than 5)[120]. Also, some domains of mAbs seem to be more sensitive to certain pH ranges (eg. C_H2 domain more sensitive to low pH)[121].

Electrolytes have a complex effect on protein physical stability by modifying conformational stability, equilibrium solubility, intermolecular repulsion, and rate of formation of non-native aggregates. Salts bind to proteins, as the ions can interact with unpaired charged side chains on the protein surface. Binding of multivalent ions to these side chains can cross-link charged residues on the protein surface, leading to the stabilization of the protein native state. The net effect of salts on protein stability is protein-dependent and is a complex balance of the multiple mechanisms by which the ionic salt interacts with protein molecules, shielding charged solvent exposed residues and then potentially decreasing protein-protein long range electrostatic interactions[47,81,122]. This charge shielding may be responsible for a decrease in formulation viscosity, improving global colloidal stability. However, it may also favor short-range hydrophobic interactions at high protein concentration, reducing solubility[122]. The effect of salts depends on their nature and concentration, and may be divided between salting-in (stabilization) and salting-out (precipitation), high salt concentration (high ionic strength) being more in favor of aggregation salting-out[123,124]. The unchanged secondary structure analyses were however in favor of the retention of a native-like secondary structure even in the precipitated state, and mAb aggregation may be at least partially reversible by salt dilution. The type of salt also greatly influences the aggregation kinetics, with Na^+ being the ion causing the smallest increase in aggregation formation (when compared to Ca^{2+} or K^+, at pH 4), but in certain cases the addition of the correct anion (sulfate) restored an IgG2 to native configuration, at pH 3[125].

While the reconstitution solvent for powdered in vial mAb formulations is often water for injections or specific solvent provided along, further dilution use several different solvents, nature of which is of great importance for protein stability, as it can impact several parameters like solution pH. The dilution of excipients that stabilize the protein (e.g., surfactants) and the use of

destabilizing vehicle solutions (e.g., dextrose solution that may lead to protein glycosylation) can result in protein instability, especially when the drug formulation is added to an infusion bag[17,29,85,126]. For example, bevacizumab and trastuzumab diluted in 5% dextrose were shown to undergo rapid aggregation when mixed in-vitro to human plasma. However, the aggregation mechanisms involved seemed to be linked to pH-dependent precipitation of plasma proteins, secondarily causing antibody aggregation[127].

Preservatives can induce aggregation, through an incompletely understood mechanism. However, it has been observed that addition of benzyl alcohol perturbed the tertiary structure of some proteins without affecting their secondary structure, and the rate of protein aggregation increased as the molar ratio of benzyl alcohol to protein increased[47]. Recently, Arora *et al.* studied four phenolic preservatives and showed them to promote thermal instability, aggregation and flexibility alteration[128].

Surfactants are generally added in mAbs formulations in order to reduce the exposure of hydrophobic regions and so decreasing protein-protein interactions and interface-induced aggregation, also prevented by competition for adsorption sites[129,130]. Frequently used nonionic surfactants in mAb drug formulation are polysorbate 20 and polysorbate 80[17,83], with poloxamers being a potential alternative[131]. Indeed, polysorbate 80 seems to be one of the most protecting surfactant against mechanical stress induced aggregation when compared to some other nonionic and anionic surfactants at the same concentration[130], and also seems to be less perturbing towards mAb higher structure stability when compared to polysorbate 20[132]. This protection also depends of the protein to surfactant ratio[29,93,130]. In addition, surfactants have been shown to act as chemical chaperones, increasing rates of protein refolding and thus reducing aggregation[46,47]. For example, Gerhardt *et al.* reported that addition of polysorbate 20 to a solution containing various concentrations of a humanized IgG1 antibody reduced particle formation during incubation or agitation with siliconized glass syringes, but noted a lack of correlation between the critical micelle concentration of the surfactant and its protective effect against aggregation. The authors therefore speculated that polysorbate 20 binding to the protein interferes with protein-protein interactions required for protein gelation at the silicone oil-water interface. An application in drug formulation is the presence of a coating solution of polysorbate 80 in Blincyto® (Blinatumomab, a bispecific antibody) for the infusion bag before drug dilution, in order to avoid unacceptable protein adsorption. However, surfactants can in some cases reduce mAb stability, for example by binding preferentially to the unfolded state, resulting in a decrease in the native protein state stability[47]. Also, a decrease in chemical and thermal photostability of an IgG1 with polysorbate 80 when exposed to intense light (1,200,000 lx-h and 200 W/h of near UV light) has been shown by Agarkhed *et al.*, probably due to high amounts of peroxides resulting from the autooxidation of polysorbate (which would oxidize the methionine and tryptophan residues, but without modifying the tertiary structure). But this was only observed at a polysorbate 80 concentration of 1% w/v, *i.e.* at least 10 folds over usual concentrations[133]. Another issue with polysorbates comes from potential contamination of final formulations with residual host cell proteins, especially lipases, due to a bad

separation from therapeutic proteins during purification steps[134]. Lipases, like lipoprotein lipase, have been shown to hydrolyze polysorbates, resulting in a decrease of polysorbate concentration, but also potentially in formation of free fatty acids particles from the polysorbate itself[135,136]. As a lead for formulation improvements, Yarbrough *et al.* exhibited a potential protection of edetate disodium towards polysorbate oxidation, hypothetically by chelating calcium and then inhibiting the activity of lipase remaining from mAb production[137]. Singh *et al.* demonstrated that polysorbates provoke an inversion in aggregation propensity between Fab and Fc regions. Indeed, they showed that without surfactants, the Fc region is more prone to aggregation than the Fab region, but, as polysorbates preferentially bind to the Fab region, they introduce a denaturation risk that counterbalances their protective effect, whilst protection toward the Fc region is almost total. Overall protection is however still increased[132].

Close to surfactants, cyclodextrins may be an alternative as they could present a good toxicological profile, no peroxide generation and be less disturb towards proteins[138]. In 1992, Ressing *et al.* showed that hydroxypropyl-β-cyclodextrin can protect mAbs from aggregation due to lyophilization[139]. It also showed potential to suppress agitation-induced aggregation of an IgG in aqueous solution, even at relatively low concentrations, probably by efficiently competing with the mAb for the air-water interface[140], but with a different mechanism than non-ionic surfactants[138]. Callahan *et al.* observed a decrease of aggregates count with 10 mM of hydroxypropyl-β-cyclodextrin, but to a lesser extent than 0,0016% polysorbate 80[141].

Sugars and polyols can also be used as excipients, as they interact with proteins, being preferentially exclude from their surface, what increases the protein-water potential, which may increase protein thermodynamic stability against unfolding and aggregation[142]. They also have a role in the stabilization of lyophilized protein formulations[143], as they replace water molecules as a shell surrounding proteins when dried, involving hydrogen bonds[144]. Sucrose is often used, but it may be hydrolyzed into glucose and fructose (*e.g.* at low pH), which can lead to glycation of proteins[38]. Non reducing sugars, such as trehalose, are preferred as they don't participate in Maillard reaction with proteins. The effectiveness of sugars as lyoprotectants requires them to stay in an amorphous state all along the process of freeze-drying. Trehalose crystallization, especially, has been observed and studied, and is highly influenced by co-solute crystallization potential[145,146], while sucrose doesn't crystallize during this process[147]. Formulations containing melibiose (a reducing disaccharide) seem to be more protected against aggregation. Indeed, formulations of rituximab containing melibiose suffered less pronounced changes than formulation without (secondary structure alteration and non-covalent aggregation), when stored in atmospheres with a residual humidity ranging from 5% to 23%, supposedly due to a lower molecular mobility in melibiose/water mixtures. However, this disaccharide has yet to be registered as a useable excipient before being actually used in human drugs[148]. Among polyols, mannitol is used as a bulking agent for the stabilization of freeze-

dried formulations. It has a crystallization potential that may either be desired or unwanted. Bulking agents in crystalline state are required for an adequate structure of the lyophilized form[145,146,149]. However, mannitol then loses its protection capacity towards protein aggregation and may induce other co-solute crystallization (especially trehalose)[150].

Added as excipients, some amino acids have also been reported to influence mAbs stability. Arginine is often used as an aggregation protecting excipient, as it is known to have several benefits in mAbs formulations. It increases protein solubility and could protect it from photo-induced and thermal-induced aggregation, as well as preventing unfolding[151]. The mechanism behind arginine stabilizing effect could be its strong tendency to bind to specific mAb surfaces (like carboxylate groups and cation-Π interactions with aromatic side chains) and strengthen the electrostatic repulsion between the positive charged antibody molecules. However, Zhang *et al.* showed that its effect on thermal stability was strongly anion dependent, and that whilst being generally better than sodium salts, acetate and glutamate arginine salts improved stability of an IgG1 mAb and reduced aggregation kinetics, whilst chloride and sodium sulfate arginine salts reduced its stability and increased aggregation kinetics[152]. Furthermore, Toth *et al.* described arginine monohydrochloride as being able to reduce thermal stability while decreasing stir stress aggregation[153]. Also, Shah *et al.* exhibited that depending on the binding site on protein, arginine may as well induce aggregation[154]. Histidine is often used as buffer for mAbs formulations. Baek *et al.* studied its effect and tried to bring an explanation to contradictory results, as some studies have shown that it could possess protective properties against aggregation, while other studies exhibited an increase in aggregation imputed to histidine. They showed that histidine concentration modifies the mAbs' hydrodynamic diameter in an unproportioned way, potentially explaining differences between results, destabilization being observed with a histidine concentration near the ones inducing a maximum hydrodynamic radius[155]. Proline is a cyclic amino acid that is also claimed to enhance mAb solubilization, by binding to aromatic residues and exposed hydrophobic regions and thus decreasing protein-protein interactions and solution viscosity, reducing aggregation at pH near their pI[156,157]. Glycine is used as a bulking agent in freeze-dried formulations[147]. Like mannitol, even if used for mechanical properties of the lyophilized formulations, it may also possess stabilization properties towards protein aggregation if remaining in amorphous state, being then more protective[158]. Shah *et al.* showed that methionine may protect mAbs from photodegradation, while tryptophan is itself subject to photodegradation, generating reactive oxygen species that may induce photodegradation[107].

Metallic ions have also shown to be a possible cause of mAb fragmentation and free radicals formation. They can react directly with proteins to produce radicals or with oxygen to produce reactive oxygen species that may cleave proteins[159]. Ouellette *et al.* studied the effect of several metal ions on fragmentation and found copper and iron ions to be the most deleterious for the mAb used, while no difference from control was observed with zinc, magnesium, nickel, cobalt, and manganese ions. They also observed fragmentation only when the light chain was of

lambda subtype and not with kappa[159]. Site specific hinge fragmentation in presence of copper ions binding to a specific pocket of a IgG1 mAb was observed several times[159–161]. The observed fragmentation was increased for higher copper ions concentrations, but was reduced when the solvent was histidine acetate compared to sodium acetate[160]. Copper is a redox active transition metal that can mediate hydrolytic cleavage, when binding conditions are present. Also, some studies have described that tungsten residues from syringes manufacture as being a cause of protein (mAbs or not) unfolding and aggregation[162,163]. Protection from metal induced instability may come from the addition of chelators (*e.g.* ethylenediaminetetraacetic acid) to the formulation.

In addition to the nature of the excipients, their quality and potential impurities have a major impact on drug product stability. Impurities and contaminants may result from manufacturing process, excipient degradation and/or drug storage[164]. For example, traces of reducing sugars may be found in mannitol or sucrose, organic acids may be found in polysorbates, metals may contaminate any substance during its production[165]. Peroxides especially may arise from a variety of excipients[166]. Excipient packaging may also be more or less protective and a potential source of contamination, for example through oxygen entrapment or container-content interactions (leaching and permeation)[164,165]. The subject of excipients quality and contaminations[165], and protein drugs contaminations in general[164], have been extensively reviewed and we refer readers to these reviews for more details.

3.7 Mechanical stress

During its life, a mAb drug can be subjected to significant mechanical stress during transport (shipping from manufacturer to user, diluted IV-bags sent through pneumatic tube systems) and manipulations (IV-bag preparation, mishandling)[46]. The two main mechanical stresses described here are agitation (or shaking) and shearing.

Agitation is used to ensure mAb solution homogeneity but leads to cavitation (creation of void and bubbles increasing streams and pressure modifications) thus creating and constantly renewing air-water interfaces[29]. Jayaraman *et al.* investigated the mechanisms of agitation-induced particle formation in protein solutions[167]. The analysis of agitated samples showed a rapid (in less than 4 hours) and clear increase in the number of sub-visible particles, with sizes ranging from 1.5 to 80 µm, with the most abundant particles being the smaller ones (size 1.5 and 2 µm). The proposed mechanism explaining the increased particle formation was the increase in exposed hydrophobic surfaces as a result of the agitation stress. This idea is reinforced by Dobson *et al.* who found an increase in exposed sulfhydryl residues after being exposed to an extensional flow[168]. It has also been demonstrated that an exponential relationship links the aggregation level to the air-liquid interfacial surface, as a resultant of agitation by stirring cetuximab solutions for up to 24 hours[46]. Cavitation bubbles have been shown to form within 30 µs

after contact after a 1 m drop of a 1 mg/ml mAb formulation in glass vials, thus raising questions about the mAb's stability after such accidents. These shock induced cavities are associated with extreme and very localized increases in temperature and pressure, as well as free radicals formations, all factors potentially leading to mAb instability[169]. The impact of the formulation (absence or presence of polysorbate 80) also influenced the results after stirring, as the presences of the surfactant reduced particle formation in size and quantity. However, Kiese *et al.* also showed that certain concentrations of surfactants may lead to even higher aggregation rates[170]. Koepf *et al.* studied the formation of an IgG film at the air-liquid interface, and found it to be inhomogeneous in thickness but mainly composed of native structures of the mAbs[171], thickness and particle leaching depending on the pH value of the solution[172]. Removing the headspace from vials may prevent shaking-related aggregation[170].

For mAbs formulations stored in solid state, agitation can induce in-vial friction between powder and walls, leading in worst cases to localized heating and therefore thermal stress, which can be another explanation of agitation-induced aggregation. This may be why it has been shown that lyophilized powders may be more sensitive to mechanical stress than liquid states of mAb formulations, with an increased number of subvisible particles (size over 1 µm)[82,173].

Shearing occurs from the differences of fluid velocity (velocity gradients) in moving liquids, for example between solution and surface (*e.g.* solution going through syringes and infusion tubes). It has been demonstrated that shearing does not lead to aggregation by itself (even though believed otherwise before), but rather through shear-related effects[174–176]. Shearing does promote interfacial interactions, for solid-liquid (adsorption and leaching), gas-liquid (air-bubble entrainment, cavitation) and oil-water (silicon oil as lubricant)[174,177], which were discussed earlier in this review (section 3.4). Several production processes may expose mAbs to shearing. For example, stirring results in shearing, but aggregation due to solution stirring can also result from friction of the stirring bar against the container leading to heat production, forming small particles (<2 µm), even when an optimal surfactant formulation is used[28,170,178]. Pabari *et al.* found that sonication did not have a significant influence on mAbs aggregation[56]. They also studied the effect of spray-drying, exploring the possibility of mAb nanoparticle encapsulation, and came to the same conclusion, despite the high operating temperature. During spray-drying, Batens *et al.* showed that especially polysorbate 20, but also basic amino acids, reduced even more this low risk[179]. Pumping provokes shearing and cavitation in tubing and has been shown to decrease bioactivity and increase particle count[177,180,181]. Filtration can also be associated to shearing. Shear-thinning behavior (decrease of viscosity under shear strain) has been reported for high concentration mAb solutions, perhaps linked to the dissociation of self-associating of mAb clusters[182], but it is unclear if shear forces during by filtration induced other modifications. In a study mimicking an IV administration setup, Pardeshi *et al.* showed that in-line filtering (1.2 and 0.2 µm filter) considerably reduced the number of subvisible particles, but that even after the filter the amount of particles of all sizes was 2 to 10 times greater

than with a blank saline solution, the particles being possibly generated downstream of the filter[183]. It is therefore difficult to conclude on the effect of any specific filters like the ones used for the intraocular administration of certain antibodies on mAb stability.

4 Methods for investigating mAb stability

MAb drugs stability is a more complex subject than for small molecules drugs and cannot be defined solely on the basis of a stable concentration or absence of degradation products, and must include a complete evaluation of instability critical points[184]. Three main axes should be developed: an evaluation of the physical stability study should investigate aggregation and fragmentation, as well as mAb structure, a chemical stability study should look for chemical degradations (as described before) and a biological stability study should corroborate the conservation of mAb's activity towards its target with its physicochemical stability. It is a challenging venture, especially as different methods may lead to inconsistent results, as it can sometimes happen for example for aggregation determination, notably due to the different ranges of measures of the different techniques[29].

The ICH guidelines on stability assessment of biotechnological products include assays on biological activity, analysis of the molecular entity and purity (with quantitative detection of degradation products). Other parameters should also be monitored (*e.g.* visual appearance, pH…). Drug product evaluation should be performed on at least 3 different batches[185]. An European consensus has been published about stability studies of anticancer drugs, with a particular point about protein pharmaceuticals, including mAbs[186]. To study physical instability, several orthogonal techniques should be used, including at least turbidimetry and SEC, but other techniques, as for example circular dichroism, are also recommended. To study chemical instability, at least three separative techniques should be used, with ion-exchange chromatography (IEC), SEC and peptide mapping being recommended. Biological stability should be assessed, using be immunological or cytotoxic assays, but only as a complementary test and the results should never be considered as a self-sufficient proof for overall stability, as a conserved biological activity does not imply that the whole structure is unchanged[186]. However, this consensus is an improvement in the analytic rigor of studies, but the meaningful acceptance criteria are often undefined and reference standards absent, making method validation a challenge[85]. In complement to this consensus, British recommendations indicate that one should at least assess visual aspect, pH, particulates, physical stability, chemical stability, and biological activity. Freshly reconstituted drug formulation should be used as the reference material, and set acceptance criteria as a 5% maximum loss in active protein and a maximum increase of secondary species of 2%[187].

Analysis of physicochemical stability during simulated administration is also warranted, given that additional surfaces come into contact with the protein, the drug product formulation may be diluted, including dilution of critical excipients required for protein stability, and environmental stressors may be present during use[85].

U.S. Pharmacopeia described a series of analytical techniques to be used in the analysis of monoclonal antibodies, including SEC, CE-SDS and HPLC with fluorescence detection[188]. A wide panel of techniques used in physicochemical stability studies is presented in Table 1. The following analytical methods and techniques are organized regarding their potential use in mAb stability studies, along with the advantages and disadvantages one could encounter when using them. We did not evaluate the cost of each technique, neither in terms of base equipment nor in terms of laboratory supplies, but this parameter shouldn't be sidestepped when considering the execution of a mAb stability study.

5 Current monoclonal antibodies' physicochemical stability data

Mab drug development is a procedure where manufacturers have to identify and prevent any factor susceptible to lower the quality of the final product. The quality by design approach has been promoted by FDA and ICH and includes the understanding and management of risks and the development of a control strategy to ensure process performance and product quality[250]. For marketing approval, manufacturers perform stability studies in order to set an expiry date that ensures a safe use for patients. However, a prolongation of this stability limit or the assessment of stability in other situations than those specified in the summary of product characteristics may be interesting for both manufacturers and users. In practice, those stability studies may be performed by university and/or hospital researchers, on request of manufacturers or not, and should follow at least the same degree of exigency than those initially performed. Table 2 summarizes the data from different mAb stability studies that have been published about commercially available diluted mAb solutions and proposes an evaluation of them. Three parameters were taken into account. First, and most importantly, criterion I represents the compliance with the European consensus published by Bardin *et al.*[186], the scoring algorithm being presented in Table 3. Criterion II is the meaning of the established limit of stability, *i.e.* does this limit derive from an observed instability at the following analytical time, or from the absence of further data, in what case one can wonder if instability was in fact absent or was missed. Finally, criterion III tags the independence of the study from the manufacturer of the tested drug, even though this fact doesn't necessarily impact the quality of a study. This evaluation remains the authors' interpretation of criteria required by recommendations. Readers are invited to reach for the original publications for personal interpretation. It is also to be noted that microbiological evaluation, although a major concern, wasn't taken into account here nor discussed.

Out of the 25 studies referenced, 11 have a C or a D for criterion I, suggesting a potential lack in performed assays and a potential failure to evaluate all aspects of mAbs stability. However, it is to be noted that a satisfactory result to this score doesn't guarantee the quality of assays, which was not evaluated here. Also, an issue in mAbs stability studies is the difficulty of method validation, especially the analytical variability and significance threshold. A solution would be the evaluation of inter-batch variability to estimate these parameters, but this implies an increase of mAb consumption, so of overall cost.

In any case, the remaining data is of sufficient quality to suggest that most of the studied antibody medications are a lot more stable once diluted that what is generally noted in the summary of product characteristics. However, more studies are still needed to evaluated stability of the drugs in some specific conditions, for example after accidental freezing or during pneumatic tube transport.

6 Conclusion

Many stability studies about commercially available mAbs have been published, independently or requested by the manufacturers. Those studies tend to show a prolonged physicochemical stability when compared to manufacturer recommendations, but many of them are limited in the performed assays. MAbs' physicochemical stability is linked to numerous factors such as formulation, environment, manipulations as well as their own structure. It must be thoroughly investigated using several complementary analytical assays, each of which allowing specific characterization information to be harvested, including but not limited to biologic potency assays, as inefficacy is not the only consequence of aggregation and degradation. Biological stability (preservation of the mAb's efficacy) should be assessed (protein activity assay), even though by nature a higher variability of response is to be expected, questioning its relevance for the detection of small alterations or sub-visible aggregates formation that are possibly responsible of increased immunogenicity. In parallel to potential physicochemical limitations, other long-term stability limitations must be analysed. Microbiological stability (linked to container systems) and contamination risks should be assessed before usage.

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References

1. Fekete S, Veuthey J-L, Guillarme D. New trends in reversed-phase liquid chromatographic separations of therapeutic peptides and proteins: Theory and applications. *J Pharm Biomed Anal*. 2012;69:9-27. doi:10.1016/j.jpba.2012.03.024

2. Anticorpora monoclonalia ad usum humanum. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:795-797.

3. Lee CC, Perchiacca JM, Tessier PM. Toward aggregation-resistant antibodies by design. *Trends Biotechnol*. 2013;31(11):612-620. doi:10.1016/j.tibtech.2013.07.002

4. Hernandez-Alba O, Wagner-Rousset E, Beck A, Cianférani S. Native Mass Spectrometry, Ion Mobility, and Collision-Induced Unfolding for Conformational Characterization of IgG4 Monoclonal Antibodies. *Anal Chem*. 2018;90(15):8865-8872. doi:10.1021/acs.analchem.8b00912

5. van der Neut Kolfschoten M, Schuurman J, Losen M, et al. Anti-Inflammatory Activity of Human IgG4 Antibodies by Dynamic Fab Arm Exchange. *Science*. 2007;317(5844):1554-1557. doi:10.1126/science.1144603

6. European medicines agency - Biosimilar medicines: Overview. https://www.ema.europa.eu/en/human-regulatory/overview/biosimilar-medicines-overview. Accessed June 25, 2019.

7. U.S. Food and Drug Administration - Biosimilars. https://www.fda.gov/drugs/therapeutic-biologics-applications-bla/biosimilars. Accessed June 25, 2019.

8. *Guideline on Similar Biological Medicinal Products Containing Monoclonal Antibodies – Non-Clinical and Clinical Issues*. European Medicines Agengy; 2012.

9. Curigliano G, O'Connor DP, Rosenberg JA, Jacobs I. Biosimilars: Extrapolation for oncology. *Crit Rev Oncol Hematol*. 2016;104:131-137. doi:10.1016/j.critrevonc.2016.06.002

10. Kálmán-Szekeres Z, Olajos M, Ganzler K. Analytical aspects of biosimilarity issues of protein drugs. *J Pharm Biomed Anal*. 2012;69:185-195. doi:10.1016/j.jpba.2012.04.037

11. Corrêa AL, Senna JPM, de Sousa ÁPB. Effects of passage number on growth and productivity of hybridoma secreting MRSA anti-PBP2a monoclonal antibodies. *Cytotechnology*. 2016;68(3):419-427. doi:10.1007/s10616-014-9794-0

12. Hmiel LK, Brorson KA, Boyne MT. Post-translational structural modifications of immunoglobulin G and their effect on biological activity. *Anal Bioanal Chem*. 2015;407(1):79-94. doi:10.1007/s00216-014-8108-x

13. Chung S, Quarmby V, Gao X, et al. Quantitative evaluation of fucose reducing effects in a humanized antibody on Fcγ receptor binding and antibody-dependent cell-mediated cytotoxicity activities. *mAbs*. 2012;4(3):326-340. doi:10.4161/mabs.19941

14. Zheng K, Yarmarkovich M, Bantog C, Bayer R, Patapoff TW. Influence of glycosylation pattern on the molecular properties of monoclonal antibodies. *mAbs*. 2014;6(3):649-658. doi:10.4161/mabs.28588

15. Mimura Y, Church S, Ghirlando R, et al. The influence of glycosylation on the thermal stability and effector function expression of human IgG1-Fc: properties of a series of truncated glycoforms. *Mol Immunol*. 2000;37(12-13):697-706. doi:10.1016/S0161-5890(00)00105-X

16. Gaza-Bulseco G, Liu H. Fragmentation of a Recombinant Monoclonal Antibody at Various pH. *Pharm Res*. 2008;25(8):1881-1890. doi:10.1007/s11095-008-9606-3

17. Sreedhara A, Glover ZK, Piros N, Xiao N, Patel A, Kabakoff B. Stability of IgG1 monoclonal antibodies in intravenous infusion bags under clinical in-use conditions. *J Pharm Sci*. 2012;101(1):21-30. doi:10.1002/jps.22739

18. Yin L, Chen X, Vicini P, Rup B, Hickling TP. Therapeutic outcomes, assessments, risk factors and mitigation efforts of immunogenicity of therapeutic protein products. *Cell Immunol*. 2015;295(2):118-126. doi:10.1016/j.cellimm.2015.03.002

19. Ahmadi M, Bryson CJ, Cloake EA, et al. Small amounts of sub-visible aggregates enhance the immunogenic potential of monoclonal antibody therapeutics. *Pharm Res*. 2015;32(4):1383-1394. doi:10.1007/s11095-014-1541-x

20. Joubert MK, Hokom M, Eakin C, et al. Highly Aggregated Antibody Therapeutics Can Enhance the *in Vitro* Innate and Late-stage T-cell Immune Responses. *J Biol Chem*. 2012;287(30):25266-25279. doi:10.1074/jbc.M111.330902

21. Rombach-Riegraf V, Karle AC, Wolf B, et al. Aggregation of Human Recombinant Monoclonal Antibodies Influences the Capacity of Dendritic Cells to Stimulate Adaptive T-Cell Responses In Vitro. Gangopadhyay N, ed. *PLoS ONE*. 2014;9(1):e86322. doi:10.1371/journal.pone.0086322

22. Filipe V, Jiskoot W, Basmeleh AH, Halim A, Schellekens H, Brinks V. Immunogenicity of different stressed IgG monoclonal antibody formulations in immune tolerant transgenic mice. *mAbs*. 2012;4(6):740-752. doi:10.4161/mabs.22066

23. Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. *Int J Pharm*. 1999;185(2):129-188. doi:10.1016/S0378-5173(99)00152-0

24. Wang W. Protein aggregation and its inhibition in biopharmaceutics. *Int J Pharm*. 2005;289(1-2):1-30. doi:10.1016/j.ijpharm.2004.11.014

25. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of protein pharmaceuticals: an update. *Pharm Res*. 2010;27(4):544-575. doi:10.1007/s11095-009-0045-6

26. Wang W, Singh S, Zeng DL, King K, Nema S. Antibody structure, instability, and formulation. *J Pharm Sci*. 2007;96(1):1-26. doi:10.1002/jps.20727

27. Uchiyama S. Liquid formulation for antibody drugs. *Biochim Biophys Acta BBA - Proteins Proteomics*. 2014;1844(11):2041-2052. doi:10.1016/j.bbapap.2014.07.016

28. Shire SJ. Stability of monoclonal antibodies (mAbs). In: *Monoclonal Antibodies*. Elsevier; 2015:45-92. doi:10.1016/B978-0-08-100296-4.00003-8

29. Mahler H-C, Friess W, Grauschopf U, Kiese S. Protein aggregation: pathways, induction factors and analysis. *J Pharm Sci*. 2009;98(9):2909-2934. doi:10.1002/jps.21566

30. Luo Q, Joubert MK, Stevenson R, Ketchem RR, Narhi LO, Wypych J. Chemical Modifications in Therapeutic Protein Aggregates Generated under Different Stress Conditions. *J Biol Chem*. 2011;286(28):25134-25144. doi:10.1074/jbc.M110.160440

31. Berrill A, Biddlecombe J, Bracewell D. Product Quality During Manufacture and Supply. In: *Peptide and Protein Delivery*. Elsevier; 2011:313-339. doi:10.1016/B978-0-12-384935-9.10013-6

32. Paul M, Vieillard V, Jaccoulet E, Astier A. Long-term stability of diluted solutions of the monoclonal antibody rituximab. *Int J Pharm*. 2012;436(1-2):282-290. doi:10.1016/j.ijpharm.2012.06.063

33. Liu H, Gaza-Bulseco G, Sun J. Characterization of the stability of a fully human monoclonal IgG after prolonged incubation at elevated temperature. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2006;837(1-2):35-43. doi:10.1016/j.jchromb.2006.03.053

34. Chelius D, Rehder DS, Bondarenko PV. Identification and Characterization of Deamidation Sites in the Conserved Regions of Human Immunoglobulin Gamma Antibodies. *Anal Chem*. 2005;77(18):6004-6011. doi:10.1021/ac050672d

35. Cordoba AJ, Shyong B-J, Breen D, Harris RJ. Non-enzymatic hinge region fragmentation of antibodies in solution. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2005;818(2):115-121. doi:10.1016/j.jchromb.2004.12.033

36. Leblanc Y, Bihoreau N, Jube M, Andre M-H, Tellier Z, Chevreux G. Glycation of polyclonal IgGs: Effect of sugar excipients during stability studies. *Eur J Pharm Biopharm*. 2016;102:185-190. doi:10.1016/j.ejpb.2016.03.016

37. Quan C, Alcala E, Petkovska I, et al. A study in glycation of a therapeutic recombinant humanized monoclonal antibody: Where it is, how it got there, and how it affects charge-based behavior. *Anal Biochem*. 2008;373(2):179-191. doi:10.1016/j.ab.2007.09.027

38. Fischer S, Hoernschemeyer J, Mahler H-C. Glycation during storage and administration of monoclonal antibody formulations. *Eur J Pharm Biopharm Off J Arbeitsgemeinschaft Für Pharm Verfahrenstechnik EV*. 2008;70(1):42-50. doi:10.1016/j.ejpb.2008.04.021

39. Du Y, Walsh A, Ehrick R, Xu W, May K, Liu H. Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies. *mAbs*. 2012;4(5):578-585. doi:10.4161/mabs.21328

40. Yang R, Jain T, Lynaugh H, et al. Rapid assessment of oxidation via middle-down LCMS correlates with methionine side-chain solvent-accessible surface area for 121 clinical stage monoclonal antibodies. *mAbs*. 2017;9(4):646-653. doi:10.1080/19420862.2017.1290753

41. Li S, Nguyen TH, Schoneich C, Borchardt RT. Aggregation and Precipitation of Human Relaxin Induced by Metal-Catalyzed Oxidation. *Biochemistry*. 1995;34(17):5762-5772. doi:10.1021/bi00017a008

42. Burkitt W, Domann P, O'Connor G. Conformational changes in oxidatively stressed monoclonal antibodies studied by hydrogen exchange mass spectrometry. *Protein Sci*. 2010;19(4):826-835. doi:10.1002/pro.362

43. Khawli LA, Goswami S, Hutchinson R, et al. Charge variants in IgG1: Isolation, characterization, in vitro binding properties and pharmacokinetics in rats. *mAbs*. 2010;2(6):613-624. doi:10.4161/mabs.2.6.13333

44. Zheng Y, Tesar DB, Benincosa L, et al. Minipig as a potential translatable model for monoclonal antibody pharmacokinetics after intravenous and subcutaneous administration. *mAbs*. 2012;4(2):243-255. doi:10.4161/mabs.4.2.19387

45. Boswell CA, Tesar DB, Mukhyala K, Theil F-P, Fielder PJ, Khawli LA. Effects of Charge on Antibody Tissue Distribution and Pharmacokinetics. *Bioconjug Chem*. 2010;21(12):2153-2163. doi:10.1021/bc100261d

46. Lahlou A, Blanchet B, Carvalho M, Paul M, Astier A. Mechanically-induced aggregation of the monoclonal antibody cetuximab. *Ann Pharm Fr*. 2009;67(5):340-352. doi:10.1016/j.pharma.2009.05.008

47. 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(9):1325-1336. doi:10.1023/A:1025771421906

48. Wang M, Li Y, Srinivasan P, et al. Interactions Between Biological Products and Product Packaging and Potential Approaches to Overcome Them. *AAPS PharmSciTech*. 2018;19(8):3681-3686. doi:10.1208/s12249-018-1184-z

49. Hawe A, Kasper JC, Friess W, Jiskoot W. Structural properties of monoclonal antibody aggregates induced by freeze-thawing and thermal stress. *Eur J Pharm Sci Off J Eur Fed Pharm Sci*. 2009;38(2):79-87. doi:10.1016/j.ejps.2009.06.001

50. Singh SK. Impact of Product-Related Factors on Immunogenicity of Biotherapeutics. *J Pharm Sci*. 2011;100(2):354-387. doi:10.1002/jps.22276

51. Kumar S, Singh SK, Wang X, Rup B, Gill D. Coupling of Aggregation and Immunogenicity in Biotherapeutics: T- and B-Cell Immune Epitopes May Contain Aggregation-Prone Regions. *Pharm Res*. 2011;28(5):949-961. doi:10.1007/s11095-011-0414-9

52. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. *AAPS J*. 2006;8(3):E501-507. doi:10.1208/aapsj080359

53. Kraus T, Winter G, Engert J. Test models for the evaluation of immunogenicity of protein aggregates. *Int J Pharm*. 2019;559:192-200. doi:10.1016/j.ijpharm.2019.01.015

54. Parenteral preparations. In: *European Pharmacopeia*. Vol 8.4. 8th edition. ; 2015:5013-5015.

55. Particulate contamination- sub-visible particles. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2011:342-344.

56. Pabari RM, Ryan B, McCarthy C, Ramtoola Z. Effect of microencapsulation shear stress on the structural integrity and biological activity of a model monoclonal antibody, trastuzumab. *Pharmaceutics*. 2011;3(3):510-524. doi:10.3390/pharmaceutics3030510

57. Fukuda J, Iwura T, Yanagihara S, Kano K. Factors to Govern Soluble and Insoluble Aggregate-formation in Monoclonal Antibodies. *Anal Sci Int J Jpn Soc Anal Chem*. 2015;31(12):1233-1240. doi:10.2116/analsci.31.1233

58. Pisupati K, Benet A, Tian Y, et al. Biosimilarity under stress: A forced degradation study of Remicade® and Remsima™. *mAbs*. 2017;9(7):1197-1209. doi:10.1080/19420862.2017.1347741

59. Skamris T, Tian X, Thorolfsson M, et al. Monoclonal Antibodies Follow Distinct Aggregation Pathways During Production-Relevant Acidic Incubation and Neutralization. *Pharm Res*. 2016;33(3):716-728. doi:10.1007/s11095-015-1821-0

60. Ito T, Tsumoto K. Effects of subclass change on the structural stability of chimeric, humanized, and human antibodies under thermal stress. *Protein Sci Publ Protein Soc*. 2013;22(11):1542-1551. doi:10.1002/pro.2340

61. Nakamura H, Oda-Ueda N, Ueda T, Ohkuri T. Introduction of a glycosylation site in the constant region decreases the aggregation of adalimumab Fab. *Biochem Biophys Res Commun*. 2018;503(2):752-756. doi:10.1016/j.bbrc.2018.06.071

62. Schermeyer M-T, Wöll AK, Kokke B, Eppink M, Hubbuch J. Characterization of highly concentrated antibody solution - A toolbox for the description of protein long-term solution stability. *mAbs*. 2017;9(7):1169-1185. doi:10.1080/19420862.2017.1338222

63. Hauptmann A, Podgoršek K, Kuzman D, Srčič S, Hoelzl G, Loerting T. Impact of Buffer, Protein Concentration and Sucrose Addition on the Aggregation and Particle Formation during Freezing and Thawing. *Pharm Res*. 2018;35(5). doi:10.1007/s11095-018-2378-5

64. Nicoud L, Jagielski J, Pfister D, et al. Kinetics of Monoclonal Antibody Aggregation from Dilute toward Concentrated Conditions. *J Phys Chem B*. 2016;120(13):3267-3280. doi:10.1021/acs.jpcb.5b11791

65. Harn N, Allan C, Oliver C, Middaugh CR. Highly concentrated monoclonal antibody solutions: direct analysis of physical structure and thermal stability. *J Pharm Sci*. 2007;96(3):532-546. doi:10.1002/jps.20753

66. Kalonia C, Toprani V, Toth R, et al. Effects of Protein Conformation, Apparent Solubility, and Protein–Protein Interactions on the Rates and Mechanisms of Aggregation for an IgG1Monoclonal Antibody. *J Phys Chem B*. 2016;120(29):7062-7075. doi:10.1021/acs.jpcb.6b03878

67. Liu J, Nguyen MDH, Andya JD, Shire SJ. Reversible Self-Association Increases the Viscosity of a Concentrated Monoclonal Antibody in Aqueous Solution. *J Pharm Sci*. 2005;94(9):1928-1940. doi:10.1002/jps.20347

68. Kanai S, Liu J, Patapoff TW, Shire SJ. Reversible Self-Association of a Concentrated Monoclonal Antibody Solution Mediated by Fab–Fab Interaction That Impacts Solution Viscosity. *J Pharm Sci*. 2008;97(10):4219-4227. doi:10.1002/jps.21322

69. Yadav S, Shire SJ, Kalonia DS. Viscosity Behavior of High-Concentration Monoclonal Antibody Solutions: Correlation with Interaction Parameter and Electroviscous Effects. *J Pharm Sci*. 2012;101(3):998-1011. doi:10.1002/jps.22831

70. Connolly BD, Petry C, Yadav S, et al. Weak Interactions Govern the Viscosity of Concentrated Antibody Solutions: High-Throughput Analysis Using the Diffusion Interaction Parameter. *Biophys J*. 2012;103(1):69-78. doi:10.1016/j.bpj.2012.04.047

71. Alford JR, Kendrick BS, Carpenter JF, Randolph TW. Measurement of the second osmotic virial coefficient for protein solutions exhibiting monomer–dimer equilibrium. *Anal Biochem*. 2008;377(2):128-133. doi:10.1016/j.ab.2008.03.032

72. Hedberg SHM, Lee D, Mishra Y, Haigh JM, Williams DR. Mapping the mAb Aggregation Propensity Using Self-Interaction Chromatography as a Screening Tool. *Anal Chem*. 2018;90(6):3878-3885. doi:10.1021/acs.analchem.7b04605

73. Liu L, Braun LJ, Wang W, Randolph TW, Carpenter JF. Freezing-induced perturbation of tertiary structure of a monoclonal antibody. *J Pharm Sci*. 2014;103(7):1979-1986. doi:10.1002/jps.24013

74. Horn J, Schanda J, Friess W. Impact of fast and conservative freeze-drying on product quality of protein-mannitol-sucrose-glycerol lyophilizates. *Eur J Pharm Biopharm*. 2018;127:342-354. doi:10.1016/j.ejpb.2018.03.003

75. Kolhe P, Badkar A. Protein and solute distribution in drug substance containers during frozen storage and post-thawing: A tool to understand and define freezing-thawing parameters in biotechnology process development. *Biotechnol Prog*. 2011;27(2):494-504. doi:10.1002/btpr.530

76. Zhang A, Singh SK, Shirts MR, Kumar S, Fernandez EJ. Distinct aggregation mechanisms of monoclonal antibody under thermal and freeze-thaw stresses revealed by hydrogen exchange. *Pharm Res*. 2012;29(1):236-250. doi:10.1007/s11095-011-0538-y

77. Horn J, Jena S, Aksan A, Friess W. Freeze/thaw of IGG solutions. *Eur J Pharm Biopharm*. 2019;134:185-189. doi:10.1016/j.ejpb.2018.12.001

78. Telikepalli SN, Kumru OS, Kalonia C, et al. Structural characterization of IgG1 mAb aggregates and particles generated under various stress conditions. *J Pharm Sci*. 2014;103(3):796-809. doi:10.1002/jps.23839

79. Barnard JG, Singh S, Randolph TW, Carpenter JF. Subvisible Particle Counting Provides a Sensitive Method of Detecting and Quantifying Aggregation of Monoclonal Antibody Caused by Freeze-Thawing: Insights Into the Roles of Particles in the Protein Aggregation Pathway. *J Pharm Sci*. 2011;100(2):492-503. doi:10.1002/jps.22305

80. Rayfield WJ, Kandula S, Khan H, Tugcu N. Impact of Freeze/Thaw Process on Drug Substance Storage of Therapeutics. *J Pharm Sci*. 2017;106(8):1944-1951. doi:10.1016/j.xphs.2017.03.019

81. Kueltzo LA, Wang W, Randolph TW, Carpenter JF. Effects of solution conditions, processing parameters, and container materials on aggregation of a monoclonal antibody during freeze-thawing. *J Pharm Sci*. 2008;97(5):1801-1812. doi:10.1002/jps.21110

82. Singh SN, Kumar S, Bondar V, et al. Unexplored benefits of controlled ice nucleation: Lyophilization of a highly concentrated monoclonal antibody solution. *Int J Pharm*. 2018;552(1-2):171-179. doi:10.1016/j.ijpharm.2018.09.057

83. Mehta SB, Lewus R, Bee JS, Randolph TW, Carpenter JF. Gelation of a Monoclonal Antibody at the Silicone Oil–Water Interface and Subsequent Rupture of the Interfacial Gel Results in Aggregation and Particle Formation. *J Pharm Sci*. 2015;104(4):1282-1290. doi:10.1002/jps.24358

84. Wei Q, Becherer T, Angioletti-Uberti S, et al. Protein Interactions with Polymer Coatings and Biomaterials. *Angew Chem Int Ed*. 2014;53(31):8004-8031. doi:10.1002/anie.201400546

85. Ricci MS, Frazier M, Moore J, et al. In-use physicochemical and microbiological stability of biological parenteral products. *Am J Health Syst Pharm*. 2015;72(5):396-407. doi:10.2146/ajhp140098

86. Perevozchikova T, Nanda H, Nesta DP, Roberts CJ. Protein adsorption, desorption, and aggregation mediated by solid-liquid interfaces. *J Pharm Sci*. 2015;104(6):1946-1959. doi:10.1002/jps.24429

87. Nakanishi K, Sakiyama T, Imamura K. On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon. *J Biosci Bioeng*. 2001;91(3):233-244. doi:10.1016/S1389-1723(01)80127-4

88. Couston RG, Lamprou DA, Uddin S, van der Walle CF. Interaction and destabilization of a monoclonal antibody and albumin to surfaces of varying functionality and hydrophobicity. *Int J Pharm*. 2012;438(1-2):71-80. doi:10.1016/j.ijpharm.2012.09.002

89. Hoehne M, Samuel F, Dong A, et al. Adsorption of monoclonal antibodies to glass microparticles. *J Pharm Sci*. 2011;100(1):123-132. doi:10.1002/jps.22275

90. Bee JS, Chiu D, Sawicki S, et al. Monoclonal antibody interactions with micro- and nanoparticles: adsorption, aggregation, and accelerated stress studies. *J Pharm Sci*. 2009;98(9):3218-3238. doi:10.1002/jps.21768

91. Ruiz L, Reyes N, Aroche K, et al. Influence of packaging material on the liquid stability of interferon-alpha2b. *J Pharm Pharm Sci Publ Can Soc Pharm Sci Soc Can Sci Pharm*. 2005;8(2):207-216.

92. Rodriguez-Aller M, Cusumano A, Alain B, Guillarme D, Fekete S. Importance of vial shape and type on the reproducibility of size exclusion chromatography measurement of monoclonal antibodies. *J Chromatogr B*. 2016;1032:131-138. doi:10.1016/j.jchromb.2016.04.032

93. Kumru OS, Liu J, Ji JA, et al. Compatibility, physical stability, and characterization of an IgG4 monoclonal antibody after dilution into different intravenous administration bags. *J Pharm Sci*. 2012;101(10):3636-3650. doi:10.1002/jps.23224

94. Bee JS, Randolph TW, Carpenter JF, Bishop SM, Dimitrova MN. Effects of Surfaces and Leachables on the Stability of Biopharmaceuticals. *J Pharm Sci*. 2011;100(10):4158-4170. doi:10.1002/jps.22597

95. Kahook MY, Liu L, Ruzycki P, et al. High-molecular-weight aggregates in repackaged bevacizumab. *Retina Phila Pa*. 2010;30(6):887-892. doi:10.1097/IAE.0b013e3181d50cea

96. Zhang Z, Orski S, Woys AM, et al. Adsorption of polysorbate 20 and proteins on hydrophobic polystyrene surfaces studied by neutron reflectometry. *Colloids Surf B Biointerfaces*. 2018;168:94-102. doi:10.1016/j.colsurfb.2018.04.036

97. Krayukhina E, Tsumoto K, Uchiyama S, Fukui K. Effects of Syringe Material and Silicone Oil Lubrication on the Stability of Pharmaceutical Proteins. *J Pharm Sci*. 2015;104(2):527-535. doi:10.1002/jps.24184

98. Thirumangalathu R, Krishnan S, Ricci MS, Brems DN, Randolph TW, Carpenter JF. Silicone oil- and agitation-induced aggregation of a monoclonal antibody in aqueous solution. *J Pharm Sci*. 2009;98(9):3167-3181. doi:10.1002/jps.21719

99. Lubiniecki A, Volkin DB, Federici M, et al. Comparability assessments of process and product changes made during development of two different monoclonal antibodies. *Biologicals*. 2011;39(1):9-22. doi:10.1016/j.biologicals.2010.08.004

100. Eu B, Cairns A, Ding G, Cao X, Wen Z-Q. Direct visualization of protein adsorption to primary containers by gold nanoparticles. *J Pharm Sci*. 2011;100(5):1663-1670. doi:10.1002/jps.22410

101. Werner BP, Winter G. Particle contamination of parenteralia and in-line filtration of proteinaceous drugs. *Int J Pharm*. 2015;496(2):250-267. doi:10.1016/j.ijpharm.2015.10.082

102. Besheer A. Protein Adsorption to In-Line Filters of Intravenous Administration Sets. *J Pharm Sci*. 2017;106(10):2959-2965. doi:10.1016/j.xphs.2017.05.028

103. Chen H, Yuan L, Song W, Wu Z, Li D. Biocompatible polymer materials: Role of protein–surface interactions. *Prog Polym Sci*. 2008;33(11):1059-1087. doi:10.1016/j.progpolymsci.2008.07.006

104. Chapman RG, Ostuni E, Takayama S, Holmlin RE, Yan L, Whitesides GM. Surveying for Surfaces that Resist the Adsorption of Proteins. *J Am Chem Soc*. 2000;122(34):8303-8304. doi:10.1021/ja000774f

105. Ostuni E, Chapman RG, Holmlin RE, Takayama S, Whitesides GM. A Survey of Structure−Property Relationships of Surfaces that Resist the Adsorption of Protein. *Langmuir*. 2001;17(18):5605-5620. doi:10.1021/la010384m

106. Lei M, Quan C, Wang YJ, Kao Y-H, Schöneich C. Light-Induced Covalent Buffer Adducts to Histidine in a Model Protein. *Pharm Res*. 2018;35(3). doi:10.1007/s11095-017-2339-4

107. Shah DD, Zhang J, Maity H, Mallela KMG. Effect of photo-degradation on the structure, stability, aggregation, and function of an IgG1 monoclonal antibody. *Int J Pharm*. 2018;547(1-2):438-449. doi:10.1016/j.ijpharm.2018.06.007

108. Kerwin BA, Remmele RL. Protect from light: photodegradation and protein biologics. *J Pharm Sci*. 2007;96(6):1468-1479. doi:10.1002/jps.20815

109. Qi P, Volkin DB, Zhao H, et al. Characterization of the photodegradation of a human IgG1 monoclonal antibody formulated as a high-concentration liquid dosage form. *J Pharm Sci*. 2009;98(9):3117-3130. doi:10.1002/jps.21617

110. Hawe A, Wiggenhorn M, van de Weert M, Garbe JHO, Mahler H-C, Jiskoot W. Forced degradation of therapeutic proteins. *J Pharm Sci*. 2012;101(3):895-913. doi:10.1002/jps.22812

111. Sreedhara A, Yin J, Joyce M, et al. Effect of ambient light on IgG1 monoclonal antibodies during drug product processing and development. *Eur J Pharm Biopharm Off J Arbeitsgemeinschaft Pharm Verfahrenstechnik EV*. 2016;100:38-46. doi:10.1016/j.ejpb.2015.12.003

112. Luis LM, Hu Y, Zamiri C, Sreedhara A. Determination of the Acceptable Ambient Light Exposure during Drug Product Manufacturing for Long-Term Stability of Monoclonal Antibodies. *PDA J Pharm Sci Technol*. 2018;72(4):393-403. doi:10.5731/pdajpst.2018.008581

113. Singh SR, Zhang J, O'Dell C, et al. Effect of polysorbate 80 quality on photostability of a monoclonal antibody. *AAPS PharmSciTech*. 2012;13(2):422-430. doi:10.1208/s12249-012-9759-6

114. Kishore RSK, Pappenberger A, Dauphin IB, et al. Degradation of Polysorbates 20 and 80: Studies on Thermal Autoxidation and Hydrolysis. *J Pharm Sci*. 2011;100(2):721-731. doi:10.1002/jps.22290

115. Borisov OV, Ji JA, John Wang Y. Oxidative Degradation of Polysorbate Surfactants Studied by Liquid Chromatography–Mass Spectrometry. *J Pharm Sci*. 2015;104(3):1005-1018. doi:10.1002/jps.24314

116. Allain LR, Pierce BC, Wuelfing WP, Templeton AC, Helmy R. In-Use Photostability Practice and Regulatory Evaluation for Pharmaceutical Products in an Age of Light-Emitting Diode Light Sources. *J Pharm Sci*. 2019;108(3):1172-1176. doi:10.1016/j.xphs.2018.10.003

117. Usami A, Ohtsu A, Takahama S, Fujii T. The effect of pH, hydrogen peroxide and temperature on the stability of human monoclonal antibody. *J Pharm Biomed Anal*. 1996;14(8-10):1133-1140. doi:10.1016/S0731-7085(96)01721-9

118. Lu X, Nobrega RP, Lynaugh H, et al. Deamidation and isomerization liability analysis of 131 clinical-stage antibodies. *mAbs*. 2019;11(1):45-57. doi:10.1080/19420862.2018.1548233

119. Brummitt RK, Nesta DP, Chang L, Kroetsch AM, Roberts CJ. Nonnative Aggregation of an IgG1 Antibody in Acidic Conditions, Part 2: Nucleation and Growth Kinetics with Competing Growth Mechanisms. *J Pharm Sci*. 2011;100(6):2104-2119. doi:10.1002/jps.22447

120. Vlasak J, Ionescu R. Fragmentation of monoclonal antibodies. *mAbs*. 2011;3(3):253-263. doi:10.4161/mabs.3.3.15608

121. Rowe JB, Flynn RP, Wooten HR, et al. Submicron Aggregation of Chemically Denatured Monoclonal Antibody. *Mol Pharm*. 2018;15(10):4710-4721. doi:10.1021/acs.molpharmaceut.8b00690

122. Laber JR, Dear BJ, Martins ML, et al. Charge Shielding Prevents Aggregation of Supercharged GFP Variants at High Protein Concentration. *Mol Pharm*. 2017;14(10):3269-3280. doi:10.1021/acs.molpharmaceut.7b00322

123. 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 Off J Arbeitsgemeinschaft Pharm Verfahrenstechnik EV*. 2016;107:310-320. doi:10.1016/j.ejpb.2016.07.020

124. Roberts D, Keeling R, Tracka M, et al. Specific Ion and Buffer Effects on Protein–Protein Interactions of a Monoclonal Antibody. *Mol Pharm*. 2015;12(1):179-193. doi:10.1021/mp500533c

125. Arosio P, Jaquet B, Wu H, Morbidelli M. On the role of salt type and concentration on the stability behavior of a monoclonal antibody solution. *Biophys Chem*. 2012;168-169:19-27. doi:10.1016/j.bpc.2012.05.004

126. Demeule B, Palais C, Machaidze G, Gurny R, Arvinte T. New methods allowing the detection of protein aggregates: a case study on trastuzumab. *mAbs*. 2009;1(2):142-150. doi:10.4161/mabs.1.2.7632

127. Luo S, Zhang B. Dextrose-mediated aggregation of therapeutic monoclonal antibodies in human plasma: Implication of isoelectric precipitation of complement proteins. *mAbs*. 2015;7(6):1094-1103. doi:10.1080/19420862.2015.1087636

128. Arora J, Joshi SB, Middaugh CR, Weis DD, Volkin DB. Correlating the Effects of Antimicrobial Preservatives on Conformational Stability, Aggregation Propensity, and Backbone Flexibility of an IgG1 mAb. *J Pharm Sci*. 2017;106(6):1508-1518. doi:10.1016/j.xphs.2017.02.007

129. Kapp SJ, Larsson I, Van De Weert M, Ćardenas M, Jorgensen L. Kapp SJ, Larsson I, Van De Weert M, Ćardenas M, Jorgensen L. 2015. Competitive Adsorption of Monoclonal Antibodies and Nonionic Surfactants at Solid Hydrophobic Surfaces. 2015. J Pharm Sci 104:593-601. *J Pharm Sci*. 2015;104(10):3587. doi:10.1002/jps.24542

130. Agarkhed M, O'Dell C, Hsieh M-C, Zhang J, Goldstein J, Srivastava A. Effect of Surfactants on Mechanical, Thermal, and Photostability of a Monoclonal Antibody. *AAPS PharmSciTech*. 2018;19(1):79-92. doi:10.1208/s12249-017-0845-7

131. Kannan A, Shieh IC, Fuller GG. Linking aggregation and interfacial properties in monoclonal antibody-surfactant formulations. *J Colloid Interface Sci*. 2019;550:128-138. doi:10.1016/j.jcis.2019.04.060

132. Singh SM, Bandi S, Jones DNM, Mallela KMG. Effect of Polysorbate 20 and Polysorbate 80 on the Higher-Order Structure of a Monoclonal Antibody and Its Fab and Fc Fragments Probed Using 2D Nuclear Magnetic Resonance Spectroscopy. *J Pharm Sci*. 2017;106(12):3486-3498. doi:10.1016/j.xphs.2017.08.011

133. Agarkhed M, O'Dell C, Hsieh M-C, Zhang J, Goldstein J, Srivastava A. Effect of polysorbate 80 concentration on thermal and photostability of a monoclonal antibody. *AAPS PharmSciTech*. 2013;14(1):1-9. doi:10.1208/s12249-012-9878-0

134. Chiu J, Valente KN, Levy NE, Min L, Lenhoff AM, Lee KH. Knockout of a difficult-to-remove CHO host cell protein, lipoprotein lipase, for improved polysorbate stability in monoclonal antibody formulations: CHO LPL Knockout and Polysorbate Degradation. *Biotechnol Bioeng*. 2017;114(5):1006-1015. doi:10.1002/bit.26237

135. Honemann MN, Wendler J, Graf T, Bathke A, Bell CH. Monitoring polysorbate hydrolysis in biopharmaceuticals using a QC-ready free fatty acid quantification method. *J Chromatogr B*. 2019;1116:1-8. doi:10.1016/j.jchromb.2019.03.030

136. Siska CC, Pierini CJ, Lau HR, Latypov RF, Matthew Fesinmeyer R, Litowsk JR. Free Fatty Acid Particles in Protein Formulations, Part 2: Contribution of Polysorbate Raw Material. *J Pharm Sci*. 2015;104(2):447-456. doi:10.1002/jps.24144

137. Yarbrough M, Hodge T, Menard D, et al. Edetate Disodium (EDTA) as a Polysorbate Degradation and Monoclonal Antibody Oxidation Stabilizer. *J Pharm Sci*. November 2018. doi:10.1016/j.xphs.2018.11.031

138. Serno T, Härtl E, Besheer A, Miller R, Winter G. The role of polysorbate 80 and HPβCD at the air-water interface of IgG solutions. *Pharm Res*. 2013;30(1):117-130. doi:10.1007/s11095-012-0854-x

139. Ressing ME, Jiskoot W, Talsma H, van Ingen CW, Beuvery EC, Crommelin DJ. The influence of sucrose, dextran, and hydroxypropyl-beta-cyclodextrin as lyoprotectants for a freeze-dried mouse IgG2a monoclonal antibody (MN12). *Pharm Res*. 1992;9(2):266-270. doi:https://doi.org/10.1023/A:1018905927544

140. Serno T, Carpenter JF, Randolph TW, Winter G. Inhibition of agitation-induced aggregation of an IgG-antibody by hydroxypropyl-beta-cyclodextrin. *J Pharm Sci*. 2010;99(3):1193-1206. doi:10.1002/jps.21931

141. Callahan DJ, Stanley B, Li Y. Control of Protein Particle Formation During Ultrafiltration/Diafiltration Through Interfacial Protection. *J Pharm Sci*. 2014;103(3):862-869. doi:10.1002/jps.23861

142. Sudrik CM, Cloutier T, Mody N, Sathish HA, Trout BL. Understanding the Role of Preferential Exclusion of Sugars and Polyols from Native State IgG1 Monoclonal Antibodies and its Effect on Aggregation and Reversible Self-Association. *Pharm Res*. 2019;36(8). doi:10.1007/s11095-019-2642-3

143. Sane SU, Wong R, Hsu CC. Raman spectroscopic characterization of drying-induced structural changes in a therapeutic antibody: correlating structural changes with long-term stability. *J Pharm Sci*. 2004;93(4):1005-1018. doi:10.1002/jps.20014

144. Carpenter JF, Crowe JH. An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry*. 1989;28(9):3916-3922. doi:10.1021/bi00435a044

145. Jena S, Horn J, Suryanarayanan R, Friess W, Aksan A. Effects of Excipient Interactions on the State of the Freeze-Concentrate and Protein Stability. *Pharm Res*. 2017;34(2):462-478. doi:10.1007/s11095-016-2078-y

146. Jena S, Suryanarayanan R, Aksan A. Mutual Influence of Mannitol and Trehalose on Crystallization Behavior in Frozen Solutions. *Pharm Res*. 2016;33(6):1413-1425. doi:10.1007/s11095-016-1883-7

147. Li X, Nail SL. Kinetics of glycine crystallization during freezing of sucrose/glycine excipient systems. *J Pharm Sci*. 2005;94(3):625-631. doi:10.1002/jps.20286

148. Heljo VP, Filipe V, Romeijn S, Jiskoot W, Juppo AM. Stability of rituximab in freeze-dried formulations containing trehalose or melibiose under different relative humidity atmospheres. *J Pharm Sci*. 2013;102(2):401-414. doi:10.1002/jps.23392

149. Carpenter JF, Pikal MJ, Chang BS, Randolph TW. Rational Design of Stable Lyophilized Protein Formulations: Some Practical Advice. *Pharm Res*. 1997;14(8):969-975. doi:10.1023/A:1012180707283

150. Cleland JL, Lam X, Kendrick B, et al. A specific molar ratio of stabilizer to protein is required for storage stability of a lyophilized monoclonal antibody. *J Pharm Sci*. 2001;90(3):310-321.

151. Maity H, O'Dell C, Srivastava A, Goldstein J. Effects of arginine on photostability and thermal stability of IgG1 monoclonal antibodies. *Curr Pharm Biotechnol*. 2009;10(8):761-766. doi:10.2174/138920109789978711

152. Zhang J, Frey V, Corcoran M, Zhang-van Enk J, Subramony JA. Influence of Arginine Salts on the Thermal Stability and Aggregation Kinetics of Monoclonal Antibody: Dominant Role of Anions. *Mol Pharm*. 2016;13(10):3362-3369. doi:10.1021/acs.molpharmaceut.6b00255

153. Toth RT, Pace SE, Mills BJ, et al. Evaluation of Hydrogen Exchange Mass Spectrometry as a Stability-Indicating Method for Formulation Excipient Screening for an IgG4 Monoclonal Antibody. *J Pharm Sci*. 2018;107(4):1009-1019. doi:10.1016/j.xphs.2017.12.009

154. Shah D, Shaikh AR, Peng X, Rajagopalan R. Effects of arginine on heat-induced aggregation of concentrated protein solutions. *Biotechnol Prog*. 2011;27(2):513-520. doi:10.1002/btpr.563

155. Baek Y, Singh N, Arunkumar A, Zydney AL. Effects of Histidine and Sucrose on the Biophysical Properties of a Monoclonal Antibody. *Pharm Res*. 2017;34(3):629-639. doi:10.1007/s11095-016-2092-0

156. Hung JJ, Dear BJ, Dinin AK, et al. Improving Viscosity and Stability of a Highly Concentrated Monoclonal Antibody Solution with Concentrated Proline. *Pharm Res*. 2018;35(7):133-146. doi:10.1007/s11095-018-2398-1

157. Hagan JB, Wasserman RL, Baggish JS, et al. Safety of L-proline as a stabilizer for immunoglobulin products. *Expert Rev Clin Immunol*. 2012;8(2):169-178. doi:10.1586/eci.11.97

158. Meyer JD, Nayar R, Manning MC. Impact of bulking agents on the stability of a lyophilized monoclonal antibody. *Eur J Pharm Sci Off J Eur Fed Pharm Sci*. 2009;38(1):29-38. doi:10.1016/j.ejps.2009.05.008

159. Ouellette D, Alessandri L, Piparia R, et al. Elevated cleavage of human immunoglobulin gamma molecules containing a lambda light chain mediated by iron and histidine. *Anal Biochem*. 2009;389(2):107-117. doi:10.1016/j.ab.2009.03.027

160. Glover ZK, Basa L, Moore B, Laurence JS, Sreedhara A. Metal ion interactions with mAbs: Part 1. *mAbs*. 2015;7(5):901-911. doi:10.1080/19420862.2015.1062193

161. Smith MA, Easton M, Everett P, et al. Specific cleavage of immunoglobulin G by copper ions. *Int J Pept Protein Res*. 1996;48(1):48-55. doi:10.1111/j.1399-3011.1996.tb01105.x

162. Seidl A, Hainzl O, Richter M, et al. Tungsten-Induced Denaturation and Aggregation of Epoetin Alfa During Primary Packaging as a Cause of Immunogenicity. *Pharm Res*. 2012;29(6):1454-1467. doi:10.1007/s11095-011-0621-4

163. Bee JS, Nelson SA, Freund E, Carpenter JF, Randolph TW. Precipitation of a Monoclonal Antibody by Soluble Tungsten. *J Pharm Sci*. 2009;98(9):3290-3301. doi:10.1002/jps.21707

164. Wang W, Ignatius AA, Thakkar SV. Impact of Residual Impurities and Contaminants on Protein Stability. *J Pharm Sci*. 2014;103(5):1315-1330. doi:10.1002/jps.23931

165. Wu Y, Levons J, Narang AS, Raghavan K, Rao VM. Reactive Impurities in Excipients: Profiling, Identification and Mitigation of Drug–Excipient Incompatibility. *AAPS PharmSciTech*. 2011;12(4):1248-1263. doi:10.1208/s12249-011-9677-z

166. Wasylaschuk WR, Harmon PA, Wagner G, et al. Evaluation of Hydroperoxides in Common Pharmaceutical Excipients. *J Pharm Sci*. 2007;96(1):106-116. doi:10.1002/jps.20726

167. Jayaraman M, Buck PM, Alphonse Ignatius A, King KR, Wang W. Agitation-induced aggregation and subvisible particulate formation in model proteins. *Eur J Pharm Biopharm*. 2014;87(2):299-309. doi:10.1016/j.ejpb.2014.01.004

168. Dobson J, Kumar A, Willis LF, et al. Inducing protein aggregation by extensional flow. *Proc Natl Acad Sci*. 2017;114(18):4673-4678. doi:10.1073/pnas.1702724114

169. Randolph TW, Schiltz E, Sederstrom D, et al. Do not drop: mechanical shock in vials causes cavitation, protein aggregation, and particle formation. *J Pharm Sci*. 2015;104(2):602-611. doi:10.1002/jps.24259

170. Kiese S, Papppenberger A, Friess W, Mahler H-C. Shaken, not stirred: mechanical stress testing of an IgG1 antibody. *J Pharm Sci*. 2008;97(10):4347-4366. doi:10.1002/jps.21328

171. Koepf E, Eisele S, Schroeder R, Brezesinski G, Friess W. Notorious but not understood: How liquid-air interfacial stress triggers protein aggregation. *Int J Pharm*. 2018;537(1-2):202-212. doi:10.1016/j.ijpharm.2017.12.043

172. Koepf E, Richert M, Braunschweig B, Schroeder R, Brezesinski G, Friess W. Impact of formulation pH on physicochemical protein characteristics at the liquid-air interface. *Int J Pharm*. 2018;541(1-2):234-245. doi:10.1016/j.ijpharm.2018.02.009

173. Telikepalli S, Kumru OS, Kim JH, et al. Characterization of the Physical Stability of a Lyophilized IgG1 mAb after Accelerated Shipping-Like Stress. *J Pharm Sci*. 2015;104(2):495-507. doi:10.1002/jps.24242

174. Thomas CR, Geer D. Effects of shear on proteins in solution. *Biotechnol Lett*. 2011;33(3):443-456. doi:10.1007/s10529-010-0469-4

175. Biddlecombe JG, Smith G, Uddin S, et al. Factors influencing antibody stability at solid-liquid interfaces in a high shear environment. *Biotechnol Prog*. 2009;25(5):1499-1507. doi:10.1002/btpr.211

176. Tavakoli-Keshe R, Phillips JJ, Turner R, Bracewell DG. Understanding the relationship between biotherapeutic protein stability and solid-liquid interfacial shear in constant region mutants of IgG1 and IgG4. *J Pharm Sci*. 2014;103(2):437-444. doi:10.1002/jps.23822

177. Bee JS, Stevenson JL, Mehta B, et al. Response of a concentrated monoclonal antibody formulation to high shear. *Biotechnol Bioeng*. 2009;103(5):936-943. doi:10.1002/bit.22336

178. Gikanga B, Eisner DR, Ovadia R, Day ES, Stauch OB, Maa Y-F. Processing Impact on Monoclonal Antibody Drug Products: Protein Subvisible Particulate Formation Induced by Grinding Stress. *PDA J Pharm Sci Technol*. 2017;71(3):172-188. doi:10.5731/pdajpst.2016.006726

179. Batens M, Massant J, Teodorescu B, Van den Mooter G. Formulating monoclonal antibodies as powders for reconstitution at high concentration using spray drying: Models and pitfalls. *Eur J Pharm Biopharm*. 2018;127:407-422. doi:10.1016/j.ejpb.2018.02.002

180. Saller V, Hediger C, Matilainen J, et al. Influence of particle shedding from silicone tubing on antibody stability. *J Pharm Pharmacol*. 2018;70(5):675-685. doi:10.1111/jphp.12603

181. Her C, Carpenter JF. Effects of Tubing Type, Formulation, and Postpumping Agitation on Nanoparticle and Microparticle Formation in Intravenous Immunoglobulin Solutions Processed With a Peristaltic Filling Pump. *J Pharm Sci*. May 2019. doi:10.1016/j.xphs.2019.05.013

182. Allmendinger A, Mueller R, Huwyler J, Mahler H-C, Fischer S. Sterile Filtration of Highly Concentrated Protein Formulations: Impact of Protein Concentration, Formulation Composition, and Filter Material. *J Pharm Sci*. 2015;104(10):3319-3329. doi:10.1002/jps.24561

183. Pardeshi NN, Qi W, Dahl K, Caplan L, Carpenter JF. Microparticles and Nanoparticles Delivered in Intravenous Saline and in an Intravenous Solution of a Therapeutic Antibody Product. *J Pharm Sci*. 2017;106(2):511-520. doi:10.1016/j.xphs.2016.09.028

184. Ikeda R, Vermeulen LC, Lau E, et al. Stability of infliximab in polyvinyl chloride bags. *Am J Health Syst Pharm*. 2012;69(17):1509-1512. doi:10.2146/ajhp100116

185. *Quality of Biotechnological Products: Stability Testing of Biotechnological/Biological Products (Q5C)*. International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; 1995. https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5C/Step4/Q5C_Guideline.pdf.

186. Bardin C, Astier A, Vulto A, et al. Guidelines for the practical stability studies of anticancer drugs: a European consensus conference. *Ann Pharm Fr*. 2011;69(4):221-231. doi:10.1016/j.pharma.2011.07.002

187. *A Standard Protocol for Deriving and Assessment of Stability - Part 2: Aseptic Preparations (Biopharmaceuticals) - Edition 3*. National Health Service - Pharmaceutical Quality Assurance Committee; 2017. https://www.sps.nhs.uk/articles/standard-protocol-for-deriving-and-assessment-of-stability-part-2-aseptic-preparations-biopharmaceuticals-edition-2-2015-yellow-cover/.

188. USP 129 Analytical Procedures of Recombinant Therapeutic Monoclonal Antibodies. In: *The United States Pharmacopeia - National Formulary*. USP 40 – NF 35. ; 2017.

189. Clarity and degree of opalescence of liquids. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:21-22.

190. Particulate contamination- visible particles. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:344.

191. Lee H, Kirchmeier M, Mach H. Monoclonal antibody aggregation intermediates visualized by atomic force microscopy. *J Pharm Sci*. 2011;100(2):416-423. doi:10.1002/jps.22279

192. Sharma DK, King D, Oma P, Merchant C. Micro-Flow Imaging: Flow Microscopy Applied to Sub-visible Particulate Analysis in Protein Formulations. *AAPS J*. 2010;12(3):455-464. doi:10.1208/s12248-010-9205-1

193. Kiyoshi M, Shibata H, Harazono A, et al. Collaborative Study for Analysis of Subvisible Particles Using Flow Imaging and Light Obscuration: Experiences in Japanese Biopharmaceutical Consortium. *J Pharm Sci*. 2019;108(2):832-841. doi:10.1016/j.xphs.2018.08.006

194. Panchal J, Kotarek J, Marszal E, Topp EM. Analyzing Subvisible Particles in Protein Drug Products: a Comparison of Dynamic Light Scattering (DLS) and Resonant Mass Measurement (RMM). *AAPS J*. 2014;16(3):440-451. doi:10.1208/s12248-014-9579-6

195. Stetefeld J, McKenna SA, Patel TR. Dynamic light scattering: a practical guide and applications in biomedical sciences. *Biophys Rev*. 2016;8(4):409-427. doi:10.1007/s12551-016-0218-6

196. Vasudev R, Mathew S, Afonina N. Characterization of Submicron (0.1–1 µm) Particles in Therapeutic Proteins by Nanoparticle Tracking Analysis. *J Pharm Sci*. 2015;104(5):1622-1631. doi:10.1002/jps.24411

197. Filipe V, Hawe A, Jiskoot W. Critical Evaluation of Nanoparticle Tracking Analysis (NTA) by NanoSight for the Measurement of Nanoparticles and Protein Aggregates. *Pharm Res*. 2010;27(5):796-810. doi:10.1007/s11095-010-0073-2

198. Size-exclusion chromatography. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:48.

199. Fekete S, Beck A, Veuthey J-L, Guillarme D. Theory and practice of size exclusion chromatography for the analysis of protein aggregates. *J Pharm Biomed Anal*. 2014;101:161-173. doi:10.1016/j.jpba.2014.04.011

200. Berkowitz SA. Role of analytical ultracentrifugation in assessing the aggregation of protein biopharmaceuticals. *AAPS J*. 2006;8(3):E590-E605. doi:10.1208/aapsj080368

201. Guttman A, Nolan J. Comparison of the Separation of Proteins by Sodium Dodecyl Sulfate-Slab Gel Electrophoresis and Capillary Sodium Dodecyl Sulfate-Gel Electrophoresis. *Anal Biochem*. 1994;221(2):285-289. doi:10.1006/abio.1994.1413

202. Patro SY, Przybycien TM. Self-interaction chromatography: A tool for the study of protein-protein interactions in bioprocessing environments. *Biotechnol Bioeng*. 2000;52(2):193-203. doi:10.1002/(SICI)1097-0290(19961020)52:2<193::AID-BIT2>3.0.CO;2-L

203. Hedberg SHM, Rapley J, Haigh JM, Williams DR. Cross-interaction chromatography as a rapid screening technique to identify the stability of new antibody therapeutics. *Eur J Pharm Biopharm*. 2018;133:131-137. doi:10.1016/j.ejpb.2018.10.009

204. Clarkson BR, Chaudhuri R, Schön A, Cooper JW, Kueltzo L, Freire E. Long term stability of a HIV-1 neutralizing monoclonal antibody using isothermal calorimetry. *Anal Biochem*. 2018;554:61-69. doi:10.1016/j.ab.2018.05.008

205. Pierce MM, Raman CS, Nall BT. Isothermal Titration Calorimetry of Protein–Protein Interactions. *Methods*. 1999;19(2):213-221. doi:10.1006/meth.1999.0852

206. Dick LW, Mahon D, Qiu D, Cheng K-C. Peptide mapping of therapeutic monoclonal antibodies: improvements for increased speed and fewer artifacts. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877(3):230-236. doi:10.1016/j.jchromb.2008.12.009

207. Peptide mapping. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:92-96.

208. Absorption spectrophotometry infrared. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:39‑41.

209. Kong J, Yu S. Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochim Biophys Sin*. 2007;39(8):549-559. doi:10.1111/j.1745-7270.2007.00320.x

210. Doglia SM, Ami D, Natalello A, Gatti-Lafranconi P, Lotti M. Fourier transform infrared spectroscopy analysis of the conformational quality of recombinant proteins within inclusion bodies. *Biotechnol J*. 2008;3(2):193-201. doi:10.1002/biot.200700238

211. Barth A. Infrared spectroscopy of proteins. *Biochim Biophys Acta BBA - Bioenerg*. 2007;1767(9):1073-1101. doi:10.1016/j.bbabio.2007.06.004

212. Dong A, Kendrick B, Kreilgård L, Matsuura J, Manning MC, Carpenter JF. Spectroscopic study of secondary structure and thermal denaturation of recombinant human factor XIII in aqueous solution. *Arch Biochem Biophys*. 1997;347(2):213-220. doi:10.1006/abbi.1997.0349

213. Jackson M, Mantsch HH. The Use and Misuse of FTIR Spectroscopy in the Determination of Protein Structure. *Crit Rev Biochem Mol Biol*. 1995;30(2):95-120. doi:10.3109/10409239509085140

214. Lin JC, Glover ZK, Sreedhara A. Assessing the Utility of Circular Dichroism and FTIR Spectroscopy in Monoclonal-Antibody Comparability Studies. *J Pharm Sci*. 2015;104(12):4459-4466. doi:10.1002/jps.24683

215. Circular Dichroism. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:70-71.

216. Joshi V, Shivach T, Yadav N, Rathore AS. Circular Dichroism Spectroscopy as a Tool for Monitoring Aggregation in Monoclonal Antibody Therapeutics. *Anal Chem*. 2014;86(23):11606-11613. doi:10.1021/ac503140j

217. Picquart M, Haro-Poniatowski E, Morhange JF, Jouanne M, Kanehisa M. Low frequency vibrations and structural characterization of a murine IgG2a monoclonal antibody studied by Raman and IR spectroscopies. *Biopolymers*. 2000;53(4):342-349. doi:10.1002/(SICI)1097-0282(20000405)53:4<342::AID-BIP6>3.0.CO;2-M

218. Raman spectroscopy. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:88-90.

219. Absorption spectrophotometry ultraviolet and visible. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:41-43.

220. Mach H, Middaugh CR. Ultraviolet Spectroscopy as a Tool in Therapeutic Protein Development. *J Pharm Sci*. 2011;100(4):1214-1227. doi:10.1002/jps.22385

221. Paul M, Vieillard V, Da Silva Lemos R, Escalup L, Astier A. Long-term physico-chemical stability of diluted trastuzumab. *Int J Pharm*. 2013;448(1):101-104. doi:10.1016/j.ijpharm.2013.02.039

222. Niedziela-Majka A, Kan E, Weissburg P, Mehra U, Sellers S, Sakowicz R. High-throughput screening of formulations to optimize the thermal stability of a therapeutic monoclonal antibody. *J Biomol Screen*. 2015;20(4):552-559. doi:10.1177/1087057114557781

223. Johnson CM. Differential scanning calorimetry as a tool for protein folding and stability. *Arch Biochem Biophys*. 2013;531(1):100-109. doi:10.1016/j.abb.2012.09.008

224. Niesen FH, Berglund H, Vedadi M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. *Nat Protoc*. 2007;2(9):2212-2221. doi:10.1038/nprot.2007.321

225. Svilenov H, Markoja U, Winter G. Isothermal chemical denaturation as a complementary tool to overcome limitations of thermal differential scanning fluorimetry in predicting physical stability of protein formulations. *Eur J Pharm Biopharm*. 2018;125:106-113. doi:10.1016/j.ejpb.2018.01.004

226. Svilenov H, Gentiluomo L, Friess W, Roessner D, Winter G. A New Approach to Study the Physical Stability of Monoclonal Antibody Formulations—Dilution From a Denaturant. *J Pharm Sci*. 2018;107(12):3007-3013. doi:10.1016/j.xphs.2018.08.004

227. Navas N, Herrera A, Martínez-Ortega A, Salmerón-García A, Cabeza J, Cuadros-Rodríguez L. Quantification of an intact monoclonal antibody, rituximab, by (RP)HPLC/DAD in compliance with ICH guidelines. *Anal Bioanal Chem*. 2013;405(29):9351-9363. doi:10.1007/s00216-013-7368-1

228. Martínez-Ortega A, Herrera A, Salmerón-García A, Cabeza J, Cuadros-Rodríguez L, Navas N. Validated reverse phase HPLC diode array method for the quantification of intact bevacizumab, infliximab and trastuzumab for long-term stability study. *Int J Biol Macromol*. 2018;116:993-1003. doi:10.1016/j.ijbiomac.2018.05.142

229. Yamamoto S, Nakanishi K, Matsuno R. *Ion-Exchange Chromatography of Proteins*.; 1988. https://www.taylorfrancis.com/books/9781482231670. Accessed February 20, 2019.

230. Song Y, Yu D, Mayani M, Mussa N, Li ZJ. Monoclonal antibody higher order structure analysis by high throughput protein conformational array. *mAbs*. 2018;10(3):397-405. doi:10.1080/19420862.2017.1421880

231. Guo J, Carta G. Unfolding and aggregation of monoclonal antibodies on cation exchange columns: Effects of resin type, load buffer, and protein stability. *J Chromatogr A*. 2015;1388:184-194. doi:10.1016/j.chroma.2015.02.047

232. Moorthy BS, Zarraga IE, Kumar L, et al. Solid-State Hydrogen–Deuterium Exchange Mass Spectrometry: Correlation of Deuterium Uptake and Long-Term Stability of Lyophilized Monoclonal Antibody Formulations. *Mol Pharm*. 2018;15(1):1-11. doi:10.1021/acs.molpharmaceut.7b00504

233. Zhang A, Hu P, MacGregor P, et al. Understanding the Conformational Impact of Chemical Modifications on Monoclonal Antibodies with Diverse Sequence Variation Using Hydrogen/Deuterium Exchange Mass Spectrometry and Structural Modeling. *Anal Chem*. 2014;86(7):3468-3475. doi:10.1021/ac404130a

234. Chen K, Long DS, Lute SC, Levy MJ, Brorson KA, Keire DA. Simple NMR methods for evaluating higher order structures of monoclonal antibody therapeutics with quinary structure. *J Pharm Biomed Anal*. 2016;128:398-407. doi:10.1016/j.jpba.2016.06.007

235. Jenkins N, Murphy L, Tyther R. Post-translational modifications of recombinant proteins: significance for biopharmaceuticals. *Mol Biotechnol*. 2008;39(2):113-118. doi:10.1007/s12033-008-9049-4

236. Fekete S, Beck A, Veuthey J-L, Guillarme D. Ion-exchange chromatography for the characterization of biopharmaceuticals. *J Pharm Biomed Anal*. 2015;113:43-55. doi:10.1016/j.jpba.2015.02.037

237. Sankaran PK, Kabadi PG, Honnappa CG, et al. Identification and quantification of product-related quality attributes in bio-therapeutic monoclonal antibody via a simple, and robust cation-exchange HPLC method compatible with direct online detection of UV and native ESI-QTOF-MS analysis. *J Chromatogr B*. 2018;1102-1103:83-95. doi:10.1016/j.jchromb.2018.10.019

238. Demirdirek B, Lan W, Qiu D, et al. Comparison of imaged capillary isoelectric focusing and cation exchange chromatography for monitoring dextrose-mediated glycation of monoclonal antibodies in infusion solutions. *J Chromatogr B*. 2019;1105:156-163. doi:10.1016/j.jchromb.2018.12.021

239. Leblanc Y, Ramon C, Bihoreau N, Chevreux G. Charge variants characterization of a monoclonal antibody by ion exchange chromatography coupled on-line to native mass spectrometry: Case study after a long-term storage at +5°C. *J Chromatogr B*. 2017;1048:130-139. doi:10.1016/j.jchromb.2017.02.017

240. Stoll DR, Harmes DC, Danforth J, et al. Direct Identification of Rituximab Main Isoforms and Subunit Analysis by Online Selective Comprehensive Two-Dimensional Liquid Chromatography–Mass Spectrometry. *Anal Chem*. 2015;87(16):8307-8315. doi:10.1021/acs.analchem.5b01578

241. Štěpánová S, Kašička V. Recent applications of capillary electromigration methods to separation and analysis of proteins. *Anal Chim Acta*. 2016;933:23-42. doi:10.1016/j.aca.2016.06.006

242. Zhao SS, Chen DDY. Applications of capillary electrophoresis in characterizing recombinant protein therapeutics: CE and CEC. *ELECTROPHORESIS*. 2014;35(1):96-108. doi:10.1002/elps.201300372

243. Shen Y, Berger SJ, Anderson GA, Smith RD. High-Efficiency Capillary Isoelectric Focusing of Peptides. *Anal Chem*. 2000;72(9):2154-2159. doi:10.1021/ac991367t

244. Wehr T, Zhu M, Rodriguez-Diaz R. [16] Capillary isoelectric focusing. In: *Methods in Enzymology*. Vol 270. Elsevier; 1996:358-374. doi:10.1016/S0076-6879(96)70018-6

245. Strong RA, Liu H, Krull IS, Cho B-Y, Cohen SA. Advantages and limitations of derivatization of peptides for imported performance and detectability in capillary isoelectric focusing (cIEF). *J Liq Chromatogr Relat Technol*. 2000;23(12):1775-1807. doi:10.1081/JLC-100100451

246. Bommana R, Mozziconacci O, John Wang Y, Schöneich C. An Efficient and Rapid Method to Monitor the Oxidative Degradation of Protein Pharmaceuticals: Probing Tyrosine Oxidation with Fluorogenic Derivatization. *Pharm Res*. 2017;34(7):1428-1443. doi:10.1007/s11095-017-2159-6

247. Total protein. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:176-179.

248. Katayama DS, Nayar R, Chou DK, et al. Solution behavior of a novel type 1 interferon, interferon-τ. *J Pharm Sci*. 2005;94(12):2703-2715. doi:10.1002/jps.20461

249. Potentiometric determination of pH. In: *European Pharmacopeia*. Vol 8.0. 8th Edition. ; 2008:24-25.

250. Finkler C, Krummen L. Introduction to the application of QbD principles for the development of monoclonal antibodies. *Biologicals*. 2016;44(5):282-290. doi:10.1016/j.biologicals.2016.07.004

251. Kupfer M, Scriba G, Hartmann M. Stability of alemtuzumab in infusion-bags. *Pharm*. 2009;64(9):622-623.

252. Bakri SJ, Snyder MR, Pulido JS, McCannel CA, Weiss WT, Singh RJ. Six-month stability of bevacizumab (Avastin) binding to vascular endothelial growth factor after withdrawal into a syringe and refrigeration or freezing. *Retina Phila Pa*. 2006;26(5):519-522. doi:10.1097/01.iae.0000225354.92444.7a

253. Paul M, Vieillard V, Roumi E, et al. Long-term stability of bevacizumab repackaged in 1mL polypropylene syringes for intravitreal administration. *Ann Pharm Fr*. 2012;70(3):139-154. doi:10.1016/j.pharma.2012.03.006

254. Khalili H, Sharma G, Froome A, Khaw PT, Brocchini S. Storage stability of bevacizumab in polycarbonate and polypropylene syringes. *Eye Lond Engl*. 2015;29(6):820-827. doi:10.1038/eye.2015.28

255. Pereboom M, Becker ML, Amenchar M, Verweij SL, van der Hoeven RTM, Mulder IJ. Stability assessment of repackaged bevacizumab for intravitreal administration. *Int J Pharm Compd*. 2015;19(1):70-72.

256. Ikesue H, Vermeulen LC, Hoke R, Kolesar JM. Stability of cetuximab and panitumumab in glass vials and polyvinyl chloride bags. *Am J Health-Syst Pharm AJHP Off J Am Soc Health-Syst Pharm*. 2010;67(3):223-226. doi:10.2146/ajhp090031

257. Beer PM, Wong SJ, Schartman JP, et al. Infliximab stability after reconstitution, dilution, and storage under refrigeration. *Retina Phila Pa*. 2010;30(1):81-84. doi:10.1097/IAE.0b013e3181b48fb4

258. Robert M-C, Spurr-Michaud S, Frenette M, Young D, Gipson IK, Dohlman CH. Stability and in vitro toxicity of an infliximab eye drop formulation. *Int J Pharm Compd*. 2014;18(5):418-426.

259. Young BL, Khan MA, Chapman TJ, Parry R, Connolly MA, Watts AG. Evaluation of the physicochemical and functional stability of diluted REMSIMA® upon extended storage—A study compliant with NHS (UK) guidance. *Int J Pharm*. 2015;496(2):421-431. doi:10.1016/j.ijpharm.2015.10.016

260. Vieillard V, Astier A, Sauzay C, Paul M. One-month stability study of a biosimilar of infliximab (Remsima®) after dilution and storage at 4 °C and 25 °C. *Ann Pharm Fr*. 2017;75(1):17-29. doi:10.1016/j.pharma.2016.08.002

261. Tokhadze N, Chennell P, Le Basle Y, Sautou V. Stability of infliximab solutions in different temperature and dilution conditions. *J Pharm Biomed Anal*. 2018;150:386-395. doi:10.1016/j.jpba.2017.12.012

262. Kim J, Chung J, Park S, Jung S, Kang D. Evaluation of the physicochemical and biological stability of reconstituted and diluted SB2 (infliximab). *Eur J Hosp Pharm*. 2018;25(3):157-164. doi:10.1136/ejhpharm-2016-001085

263. Bardo-Brouard P, Vieillard V, Shekarian T, Marabelle A, Astier A, Paul M. Stability of ipilimumab in its original vial after opening allows its use for at least 4 weeks and facilitates pooling of residues. *Eur J Cancer*. 2016;58:8-16. doi:10.1016/j.ejca.2016.01.008

264. Glover ZWK, Gennaro L, Yadav S, Demeule B, Wong PY, Sreedhara A. Compatibility and stability of pertuzumab and trastuzumab admixtures in i.v. infusion bags for coadministration. *J Pharm Sci*. 2013;102(3):794-812. doi:10.1002/jps.23403

265. Mueller C, Dietel E, Heynen SR, et al. Physico-chemical stability of MabThera drug-product solution for subcutaneous injection under in-use conditions with different administration materials. *Int J Pharm Compd*. 2015;19(3):261-267.

266. Kim SJ, Kim KW, Shin YK, et al. In-Use Stability of the Rituximab Biosimilar CT-P10 (Truxima®) Following Preparation for Intravenous Infusion and Storage. *BioDrugs*. February 2019. doi:10.1007/s40259-019-00336-7

267. Kaiser J, Kramer I. Physiochemical Stability of Diluted Trastuzumab Infusion Solutions in Polypropylene Infusion Bags. *Int J Pharm Compd*. 2011;15(6):515-520.

268. Pabari RM, Ryan B, Ahmad W, Ramtoola Z. Physical and structural stability of the monoclonal antibody, trastuzumab (Herceptin®), intravenous solutions. *Curr Pharm Biotechnol*. 2013;14(2):220-225. doi:10.2174/1389201011314020012

269. Nalenz H, Köpf E, Dietel E. Prolonged In-use Stability of Reconstituted Herceptin in Commercial Intravenous Bags. *Int J Pharm Compd*. 2018;22(5):417-423.

270. Kim SJ, Lee JW, Kang HY, et al. In-Use Physicochemical and Biological Stability of the Trastuzumab Biosimilar CT-P6 Upon Preparation for Intravenous Infusion. *BioDrugs*. 2018;32(6):619-625. doi:10.1007/s40259-018-0314-1

271. Vieillard V, Astier A, Paul M. Extended stability of a biosimilar of trastuzumab (CT-P6) after reconstitution in vials, dilution in polyolefin bags and storage at various temperatures. *Generics Biosimilars Initiat J*. 2018;7(3):101-110. doi:10.5639/gabij.2018.0703.022

Figure 1: Antibody general structure (CDR = complementary determining region; Fc = crystallisable fragment; Fab = antigen binding fragment; V = variable domain ; C = constant domain ; L = light chain ; H = heavy chain ; S-S = dissulfide bond)

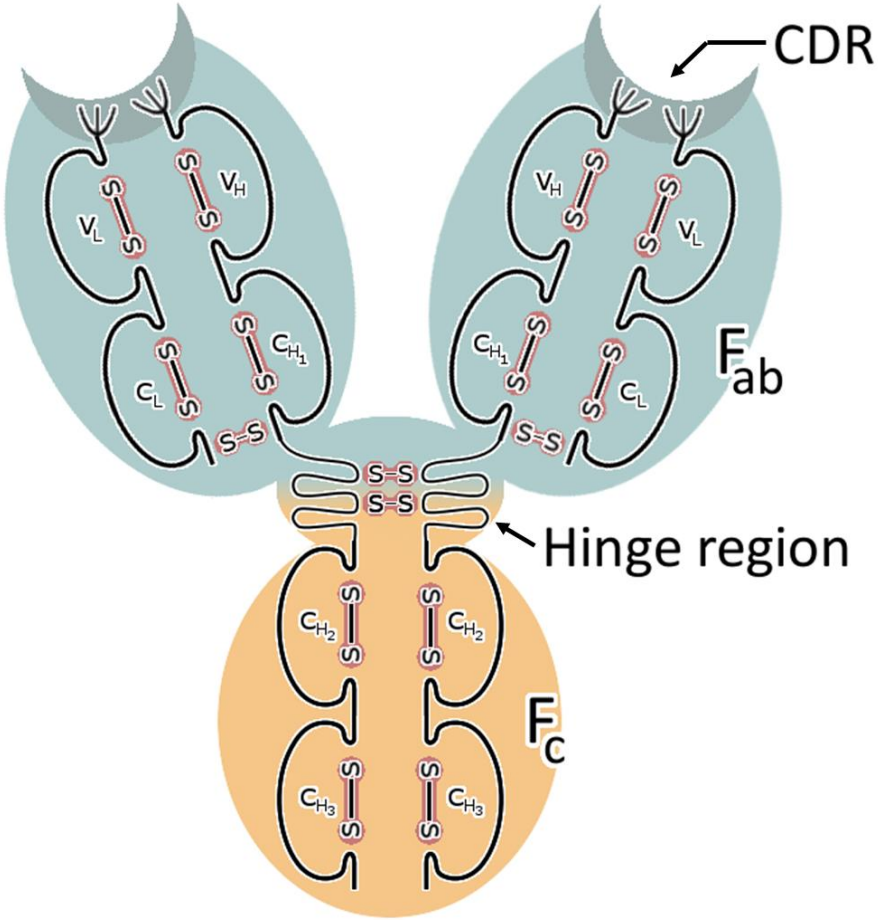

Table 1: Analytical techniques used in mAbs stability studies

Technique	Analytical procedure	Advantages	Disadvantages	References
Physical stability assays				
Opalescence measurements				
Nephelometry	Measure of the perpendicularly (compared to incident beam) diffused light (Tyndall effect)	Simple to implement Especially reliable for low turbidity measures	Only suitable for slightly opalescent solutions Need for a calibration curve for higher turbidity levels Case-by-case analysis, as many other factors may influence opalescence (should only be used for comparative measurements)	29,189
Turbidimetry	Measure of transmitted light	Simple to implement	Identical to nephelometry	29,189
Size-related variants: Visible particles detection				
Visual method	Observation of agitated solution in front of 2 panels (black and white)	Simple to implement	Dependent on operator's skills and experience Needs a comparator.	190
Size-related variants: Sub-visible and sub-micron particles detection				
Microscopic methods	Eg. Optical microscopy, Electronic microscopy or Atomic force microscopy (AFM) Automatic or manual count of particles seen through a microscope after filtration	Visual characterization of aggregates AFM allows examining the morphology of mAb aggregates and its intermediates	Analysis only of small samples (few microliters)	29,55,191
Light obscuration	Measure of the amount of light blocked when a particle gets in front of the beam, as particles pass as a single file through the detector Range: 1 – 150 µm	Allows determination in large range of particle size	Bad resolution of particles of refractive index close to solution's Pertinence of the method if an in-line 0.22 µm filter is used May need a relatively high volume of solution Sensitive to contaminations	29,55
Micro-flow imaging	Detection of particles in a sample stream from successive frames with real-time software analysis Range: larger than 1 µm	More accurate than light obscuration Detects translucid particles Distinction between particles sub-populations (proteins and contaminants) Small sample required	Pertinence of the method if an in-line 0.22 µm filter is used Samples submitted to shear forces	83,192,193

Dynamic light scattering (DLS)	Measure of the size of particles using the Stockes-Einstein equation to infer particle movement induced scattered light intensity fluctuations into size Range: 0.3 nm – 10 µm	Large range of particle size and concentration Low volume required Non destructive Can be used as detector coupled to SEC-HPLC Fast Allows to measure mAb's melting temperature	Important sensitivity to temperature and viscosity Matrix refractive index often unknown, but required for accurate measurements Bad resolution of particles of close size Underestimation at high particle concentrations Overestimation at low particle concentrations Interference of large particles	[29,32,194,195]
Nanoparticle tracking analysis	Measure of the size of particles from their Brownian movements using the Stockes-Einstein equation Range: 30 nm – 1 µm	Compared to DLS, better peak resolution and no interference of larger particles Gives an approximate particle counts Possibility to heat samples and follow subsequent variations	Mandatory dilutions to a narrow range of particles concentration => only semiquantitative and lengthened process (up to 1h per sample) May not detect monomers and smaller size molecules	[196,197]
Resonant mass measurement	Measure of the shift in specific resonating frequency when a particle goes through a resonator, dependent of its size Range: 50 nm – 5 µm	Better resolution of particles of close size than DLS	Of 2 particles entering the resonator at the same time, only the bigger one shall be detected	[194]
Size-related variants: Aggregates detection				
Size-exclusion chromatography	Species separation according to their molecular weight, high molecular weight species being eluting first	Separative method, can be mAb specific Detection of small high and low molecular weight species Compatible with numbers of detectors	Not suitable for large aggregates, usually filtered before Lack of accuracy for non-spherical particles (function of the detector(s) Possible analytical aggregation	[24,29,32,198–200]
Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis Sodium Dodecyl Sulfate Capillary Gel Electrophoresis	Molecules are separated according to the length of the polypeptide chain and its charge. SDS denatures proteins and gives them an overall similar negative charge, so separation is mostly due to size differences.	Separative method Identification, size measurement and purity assessment Bond strength in aggregates Better separation with SDS-CGE than with SDS-PAGE	SDS may dissociate aggregates SDS-PAGE: time consuming preparation	[1,24,29,49,201]

Analytical ultracentrifugation	Direct measurement of the protein aggregates through sedimentation characteristics, especially sedimentation velocity	Reduced sample preparation Decreased contact surface during the analysis Important size range detection by variating the operating speed	Lower sensitivity, precision and reproducibility than with SEC	[29,200]
Asymmetrical flow field flow fractionation	Use of a parabolic flow associated with an asymmetrical crossfield flow created by a semi-permeable membrane to separate proteins regarding their size but regardless of their density	Separative method Quantitative determination of each form in solution	Quality of the separation strongly linked to specific formulations of the running buffer Adsorption hazard to the channel membrane	[29,126]
Size-related variants: Aggregation prediction				
Self-interaction chromatography	Determination of the propensity of proteins to form oligomers, through the preparation of a stationary phase composed of the protein to study	Separative method Fast (1 to 2 days for a complete study) Requires low quantity of protein	Only predictive Contradictory studies Need to immobilize every protein of interest	[72,202]
Cross-interaction chromatography	Retention based on the interactions of mAbs with a stationary phase composed of a polyclonal antibody	Same as self-interaction chromatography No need to immobilize every protein of interest	Only predictive Indirect estimation of second virial coefficient B22 from B23	[203]
Isothermal calorimetry	Measure of the rate of heat variations caused by a spontaneous process at a given temperature	Direct observation of processes May be faster than SEC	Sensitivity Only predictive	[204,205]
Structural variants: Primary structure characterization				
Peptide mapping	Enzymatic clivage of biomolecules into specific peptides then separated by liquid chromatography	Separative method Can be coupled with MS (then called Peptide Mass Fingerprinting) Monitors oxidation and deamidation	Very sensible to environmental variations Spectrum complexity for protein mixtures Analytical length (preparation and analysis itself)	[206,207]
Structural variants: Secondary structure characterization				
Fourier transform infrared spectroscopy	Explores bonds vibrations in order to provide a second derivative spectrum inside the amide I region, then permitting identification and quantification of secondary structures in proteins	Fast No limitation in molecular weight	Water interference, implying working concentrations higher than usually studied Spectrum complexity Lack of sensitivity when compared to chromatographic techniques for stability studies	[208–214]

Circular dichroism	Based on the property of optically active molecules to absorb differently left circular polarized light and right circular polarized light at a given wavelength Far-UV CD (180-240 nm) is the reflection of peptide bond absorption, thus of secondary structure, while near-UV CD (260-320 nm) corresponds to side chains, giving indications about the tertiary structure	Fast Study of both secondary and tertiary structures depending on wavelength range studied	Lack of sensitivity when compared to chromatographic techniques for stability studies	[49,210,214–216]
Raman spectroscopy	As FT-IR, detects molecular vibrations but is more sensitive to non-polar bindings. Also use the amide I region. Doesn't follow Lambert-Beer law	Less interference with water than FT-IR Can be used with solid, liquid or gaseous samples	Spectrum complexity Less precise than FT-IR	[143,217,218]
Structural variants: Tertiary structure characterization				
Second derivative UV spectroscopy	Use UV spectroscopy in the interval of 250 – 320 nm to obtain a second derivative spectrum	Easy to perform Non-destructive technique Sensitive	Interference of excipients May be limited by higher concentrations, due to excessive light absorption	[32,65,81,219,220]
Fluorimetry	Use of noncovalent extrinsic fluorescent dyes that can interact with hydrophobic sites of proteins formed from partial unfolding or aggregation	May exhibit specific instabilities (eg. amyloid-like structures, modification of tertiary structure)	Interference of excipients (surfactants)	[29,49,126]
	Measure of intrinsic fluorescence due to aromatic amino acids	May exhibit unhidden residues from unfolding Can be used as a detection coupled with other techniques	Interference of excipients (surfactants)	[65,86]
Differencial scanning calorimetry	Determination of the melting point of the mAb, shifts being the reflection of modifications of intra- or intermolecular interactions as well as alteration of structural integrity Based on measuring the enthalpy caused by thermally induced processes	Universal method for studying thermal denaturation No reliance on changes in spectroscopic signal More capable of resolving multiple overlapping processes	Time per sample analysis around 90 min Influence of excipients (properties modification)	[221–225]
Differencial scanning fluorimetry	Determination of the melting point of the mAb Based on measuring the increase of extrinsic or intrinsic fluorescence caused by unfolding	Wide range of temperatures for scanning Fast Low quantity of mAb required	Dependent on the affinity for the fluorescent dye or the number of aromatic residues Influence of excipients (properties	[224,225]

	(revealing hydrophobic sites and aromatic residues)		modification)	
Isothermal chemical denaturation	Determination of the concentration of a given denaturant for which 50% of proteins are unfolded and of Gibbs free energy of unfolding	Not based on temperature Reversible denaturation	High sample consumption Slow (sufficient incubation time needed) Need to know the unfolding process of the studied mAb	[121,225,226]
Reverse-phase liquid chromatography	Separation according to hydrophobicity	Separative method, can be mAb specific Suitable for quantification	Use at high temperature => unpredictably cause the denaturation of proteins The use of organic solvents as mobile phase may denature proteins	[24,227–229]
Protein conformational array	Detection of accessible residues due to unfolding based on immunologic reaction (ELISA method or other)	Provides specific regional structure information No need of protein purification	Low throughput unless combined with other preparation methods	[230]
Structural variants: Higher order structure characterization				
Hydrogen-deuterium exchange	Based on the property of solvent-exposed amide groups to exchange their hydrogen atom with the solvent at a higher rate than when hidden	Fast Evaluation of local flexibility Evaluation of consequences of chemical alterations	Results depend on the degradation pathway => may be too specific for a stability study Only predictive	[153,231–233]
Quinary structure analysis	High order species characterization using dilution-injection SEC fast protein liquid chromatography and one dimensional 1H nuclear magnetic resonance spectroscopy treated with multivariate analysis	Differenciation of mAbs of close Fc structures	Presence of excipients can render the analyses more complicated to interpret	[234]
Chemical stability assays				
Ion exchange chromatography	Detects different charge-related variants of mAbs. Species are eluted according to their apparent pI, as acidic (lower apparent pI than main peak) or basic (higher apparent pI) species. Mostly cationic exchange chromatography (acidic eluted first)	Separative method Separation and identification of charge variants Non denaturating technique	Lack of compatibility with mass spectrometry (may be solved by using 2-dimensionnal liquid chromatography) Highly diluted samples in mobile phase On-line buffer exchange	[17,39,229,235–240]
Capillary zone electrophoresis	Separates molecules based on the difference of mobility of the analytes in an electric field which result from the different ratios of charge and hydrodynamic radius	Separative method High separation efficiency	Limited sample injection volume, implying very high concentrations	[241–243]

Isoelectric focusing	Separation according to pI	Separative method Isolate fragments from intact form and from each other	May not be suitable for large (over 150 kDa) or hydrophobic proteins Longer and slightly less sensitive than cIEF Close reactivity of different peptides with detection methods	[117,243,244]
Capillary isoelectric focusing	Separates molecules based on their pIs in a capillary format by formation of a pH gradient	Separative method Isolate fragments from intact form and from each other Samples less diluted than in IEX Protein initial environment partially conserved	May not be suitable for large (over 150 kDa) or hydrophobic proteins With UV detection, concentration may need to be over 1 mg/ml	[238,241,242,245]
Fluorogenic derivatization	Derivatization of chemically modified residues to gain fluorescence	Fast and possibility to evaluate multiple samples at the same time	Too specific of a certain chemical degradation for a stability study Non separative method	[246]
Quantitative assays				
UV spectroscopy	Measure of the absorbance at 280 nm	Polyvalent and time saving	Possible overestimation due to aggregates	[247]
Chromatographic techniques	Size exclusion chromatography Reverse phase liquid chromatography	Can be mAb specific Polyvalent and time saving		
Other analytical techniques				
UV spectroscopy	Attenuation of incident beam due to light scattering by suspended particles Measure of optical density at 280 nm and 350 nm in order to get the aggregation index	Simple Non destructive Overall view	Potential interference of excipients	[29,46,49,248]
pH measurement	Potential difference between 2 electrodes, generally a glass electrode and a calomel electrode, measured with a voltmeter graduated in pH units	Easy to perform Non-destructive technique		[249]

Table 2: Summary of different published mAbs stability studies (I: Compliance with European consensus (A=6 points, B=4-5 points, C=2-3 points, D=0-1 points, see Table 3 for scoring; concerned assays in bold letters); II: Stability limit because of A: Instability detected on later samples, or B: Lack of further data; III: Link to the manufacturer (A: No, B: Yes or undetermined))

Drug	Pharmaceutical form	Container	Tested Concentration(s)	Assays	Conclusion of the stability study	Evaluation of the study			Reference
						I	II	III	
Alemtuzumab (MabCampath®)	Intravenous solution	Polypropylene bags	0.28 mg/mL	**Size Exclusion Chromatography**	14 days at 6°C or 25°C	D	B	B	[251]
Bevacizumab (Avastin®)		Latex-free Syringes	25 mg/mL	**Immunoassay**	6 months storage at 4°C with a maximum activity loss of 15,9%	D	B	A	[252]
Bevacizumab (Avastin®)	Intravitreal solution	1 mL polypropylene syringes	25 mg/mL	**Turbidity** Second derivative UV spectroscopy **Dynamic Light Scattering** Derivative FTIR spectroscopy **Cation Exchange Chromatography** **Size Exclusion Chromatography** **Peptide mapping** Thermal aggregation curves Microscopic examination	3 months at 4°C	B	B	B	[253]
Bevacizumab (Avastin®)	Intravitreal solution	1 mL polypropylene syringes and 1 mL polycarbonate syringes	25 mg/mL	**SDS-PAGE** **Size Exclusion Chromatography** **Dynamic Light Scattering** Surface Plasmon Resonnance	6 months at 4°C	C	B	B	[254]
Bevacizumab (Avastin®)	Intravitreal solution	1 mL polypropylene syringes	3.75 and 25 mg/mL	pH Osmolarity mAb concentration (Cation Exchange Chromatography) RP-HPLC **Light Obscuration**	3 days at 4°C	D	A	B	[255]
Cetuximab (Erbitux®)	Intravenous solution	Original vial Polyvinylchloride bags	2 mg/mL	**ELISA**	14 days at 4°C	D	B	A	[256]

Infliximab (Remicade®)	Intravitreal solution	Original vial	Stock solution: 50 mg/mL at 2-8°C Assayed solutions: 69-50000 pg/mL	**Microsphere immunoassay**	6 week with stock solution stored at 2-8°C	D	B	A	[257]
Infliximab (Remicade®)	Intravenous solution	Polyvinylchloride bags	0.4 mg/mL	**ELISA**	14 days at 4°C	D	B	A	[184]
Infliximab (Remicade®)	Eye drop	Eye drop bottles	10 mg/mL	Visual observation pH **Turbidity** **Light scattering (fluorimetry)** **Gel electrophoresis** **ELISA** Cytotoxicity assay (safety)	After preparation: 9 days at 4°C and 45 days at -20°C	B	B	A	[258]
Infliximab (Remsima®)	Intravenous solution	Polyolefin bags	0.6, 0.84 and 1.88 mg/mL	Visual observation Particle imaging **Size Exclusion Chromatography** **Gel electrophoresis** **Dynamic Light Scattering** **Circular Dichroism** **HPLC-MS** **Cytotoxicity assay**	7 days at 2-8°C or 25°C	C	B	A	[259]
Infliximab (Remsima®)	Intravenous solution	Polypropylene bags	0.7 mg/mL 1.6 mg/mL	**Turbidity** Second derivative UV spectroscopy **Dynamic Light Scattering** Thermal aggregation curve **Size Exclusion Chromatography** **Cation Exchange Chromatography** Optical microscopy	7 days at 4°C or 25°C	B	A	B	[260]
Infliximab (Inflectra®)	Intravenous solution	Original vial Polypropylene/SIS-polypropylene /Styrene-ethylene-butadiene bags	10 mg/mL 0.4 mg/mL 2 mg/mL	Visual inspection **Turbidity** **Light obscuration** **Dynamic Light Scattering** **Size Exclusion Chromatography** **Cation Exchange Chromatography** **Peptide mapping** Derivative FTIR spectroscopy Second derivative UV spectroscopy MAb concentration (UV	Vials : 7 days at 25°C or 14 days at 5°C Bags : 30 days at 25°C 90 days at 5°C After 60h at -20°C : instability exhibited	B	A	B	[261]

				spectroscopy) pH Osmolality					
Infliximab (Flixabi®)	Intravenous solution	Original vials Polyethylene bags		Visual inspection **Light obscuration** **Size Exclusion Chromatography** **SDS-Capillary electrophoresis** **Image capillary isoelectrofocusing** MAb concentration (UV spectroscopy) pH Osmolality **Biological activity assays**	Vials : 7 days at 25°C 60 days at 5°C Bags : 7 days at 5°C or 25°C	B	B	B	[262]
Ipilimumab (Yervoy®)	Intravenous solution	Original vial	5 mg/mL	**Turbidity** Second derivative UV spectroscopy **Dynamic Light Scattering** Thermal aggregation curve **Cation Exchange Chromatography** **Size Exclusion Chromatography** **Immunoassay**	4 weeks at 4°C or 25°C	B	B	A	[263]
Panitumumab (Vectibix®)	Intravenous solution	Original vial Polyvinylchloride bags	20 mg/mL 2.5 mg/mL	**ELISA**	14 days at 4°C	D	B	A	[256]
Pertuzumab (Perjeta®) / Trastuzumab (Herceptin®)	Intravenous solution	Polyvinylchloride bags Polyolefin bags	3 mg/mL each 5 mg/mL each	Visual observation MAb concentration (UV spectroscopy) **Turbidity** **Light obscuration** **Size Exclusion Chromatography** **SDS-Capillary electrophoresis** **Analytical ultracentrifugation** **Cation Exchange Chromatography** **Capillary zone electrophoresis** **Image capillary isoelectrofocusing** **Biological assay**	24 hours at 5°C or 30°C	B	B	B	[264]

Rituximab (Mabthera®)	Intravenous solution	Polyolefin bags	1 mg/mL	**Turbidity** Second derivative UV spectroscopy **Dynamic Light Scattering** Derivative FTIR spectroscopy **Cation Exchange Chromatography** **Size Exclusion Chromatography** **Peptide mapping** **Direct cytotoxicity assay (activity)** Thermal aggregation curves	6 months at 4°C after dilution	A	B	B	[32]
Rituximab (Mabthera®)	Subcutaneous solution	Polypropylene syringes	120 mg/mL	Visible particles **Turbidity** pH **Light Obscuration** Color Osmolality mAb content **Cytotoxicity assay (activity)** Hyaluronidase activity assay **Size Exclusion Chromatography** **Cation Exchange Chromatography**	24 hours at 30°C after a 4-week storage at 2-8°C	B	B	B	[265]
Rituximab (Truxima®)	Intravenous solution	Polyvinylchloride bags Polyethylene bags	1 and 4 mg/mL	Visible particles UV spectroscopy (total protein quantification) **Micro-Flow Imaging** **Size Exclusion Chromatography** **Ion Exchange Chromatography** **Capillary Electrophoresis-SDS** pH Osmolality **Complement ELISA** **Cytotoxicity assay**	6 weeks at 2-8°C + 1 day at 25±2°C, away from light Bags reconstituted from vials in their 36th month of conservation	B	B	B	[266]

Trastuzumab	Intravenous solution	Polypropylene bags	0.4 mg/mL 4 mg/mL	Visual examination pH **Size Exclusion Chromatography** **SDS-PAGE** UV spectroscopy (total protein quantification)	28 days at 2-8°C or 25°C, with or without light protection	C	B	B	[267]
Trastuzumab (Herceptin®)	Intravenous solution	As specified by manufacturer's instructions (using a 0.9% saline solution)	0.4-4 mg/mL	Visual examination pH **Dynamic Light Scattering** **Size Exclusion Chromatography** **SDS-PAGE** **Circular dichroism**	28 days at 2-8°C	C	B	A	[268]
Trastuzumab (Herceptin®)	Intravenous solution	Polypropylene bags	0.8 and 2.4 mg/mL	**Size Exclusion Chromatography** **Cation Exchange Chromatography** **Peptide mapping** **Dynamic Light Scattering** **Turbidity at 350 nm** Second derivative UV spectroscopy Second derivative FTIR spectroscopy Thermal aggregation curves	6 months at 2-8°C or 20°C	B	B	B	[221]
Trastuzumab (Herceptin®)	Intravenous solution	Polyvinylchloride bags Polyolefin/Poly-ethylene/Poly-propylene bags	0.24 and 3.84 mg/mL	Visual examination **Turbidity** **Light Obscuration** **Size Exclusion Chromatography** **Ion Exchange Chromatography** UV spectroscopy (total protein quantification) pH Osmolality **Biological assay**	Bags from a 2 day-old reconstituted vial stored at 2-8°C Stable after 7 days at 5°C then 1 day at 30°C and then simulated administration	B	B	B	[269]

Trastuzumab (Herzuma®)	Intravenous solution	Polypropylene bags Polyvinylchloride bags	0.4, 1.0 and 4.0 mg/mL	Visual examination UV spectroscopy (total protein quantification) **Light Obscuration** **Micro-Flow Imaging** **Size Exclusion Chromatography** **SDS-Capillary electrophoresis** **Peptide mapping** pH **Biological assay**	1 month at 2-8°C + 1 day at 25°C	B	B	B	[270]
Trastuzumab (Herzuma®)	Intravenous solution	Original vial Polyolefin bags	Vial: 21 mg/mL Bags: 0.8 and 2.4 mg/mL	**Turbidity** Second derivative UV spectroscopy **Fluorescence spectroscopy** **Cation Exchange Chromatography** **Size Exclusion Chromatography** **Dynamic Light Scattering** Thermal aggregation curves Optical microscopy pH Osmolarity	90 days at 4°C (bags and vials, both concentrations)	B	A	B	[271]

1 **Table 3: Scoring for the I criterion of Table 2, *i.e.* compliance with European consensus (*: only counted once)**

Stability evaluated	Technique	Points
Opalescence	Turbidimetry	1
Soluble aggregates	Size Exclusion Chromatography	1
Particles	Supplementary technique(s) (eg. Dynamic Light Scattering, Light obscuration,…)	1
Chemical stability	Ion-exchange chromatography or Capillary electrophoresis	1
	Peptide mapping	1
Biological stability	Immunological of Cytotoxic evaluation	1*

2

3